Predicted Indirectly ReCognizable HLA Epitopes
A novel approach to identify permissible HLA mismatches

Kirsten Thus

© Kirsten Thus, 2015
All rights reserved. No part of this thesis may be reproduced or transmitted in any form or by any means, without prior permission of the author.

ISBN: 978-94-6295-128-0
Printed by: Proefschriftmaken.nl || Uitgeverij BOXPress
Published by: Uitgeverij BOXPress, 's-Hertogenbosch
Cover design by: Mark de Groot

Printing of this thesis was financially supported by: Infection & Immunity Utrecht; Cytolon; and the DLA science day award

Predicted Indirectly ReCognizable HLA Epitopes
A novel approach to identify permissible HLA mismatches

Voorspelde Indirect Herkenbare HLA Epitopen
Een nieuwe methode om tolerante HLA mismatches te identificeren

(met een samenvatting in het Nederlands)

Proefschrift

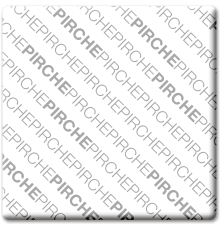
ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de rector magnificus, prof.dr. G.J. van der Zwaan, ingevolge het
besluit van het college voor promoties in het openbaar te verdedigen op
dinsdag 19 mei 2015 des middags te 2.30 uur

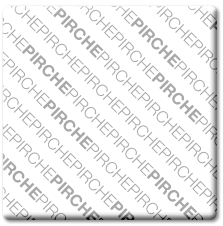
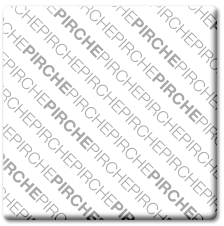
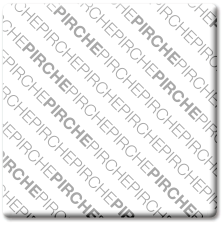
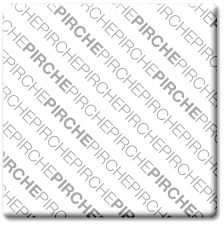
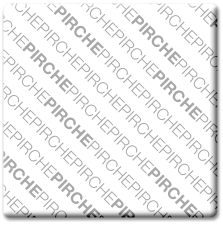
door

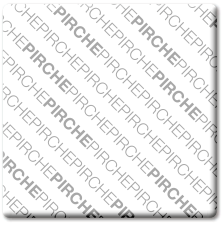
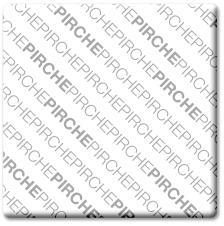
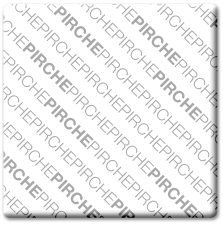
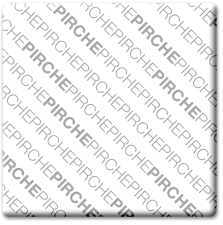
Kirsten Anne Thus
geboren op 11 januari 1987 te 's Graveland

Promotoren
Prof.dr. C. E. Hack
Prof.dr. J. Kuball
Copromotor
Dr. E. Spierings

Contents



1






Chapter 1

General Introduction

Allogeneic hematopoietic cell transplantation

Hematopoietic cell transplantation (HCT) can be curative for patients with malignant and non-malignant bone marrow (BM) diseases, as well as for patients with enzymatic deficiencies. These hematopoietic cells (HC) are harvested from the patient itself (autologous) or from a donor (allogeneic). Allogeneic HCT (allo-HCT) is preferred in many underlying diseases, because of an additive therapeutic effect (1): the alloreactive donor-derived immune system recognizes the patient's cells as foreign. This graft-versus-host directed alloreactivity potentially results in the beneficial graft-versus-tumor effect and in the pathological graft-versus-host disease (GVHD). Both these effects will be described in detail below. In general, the success of allo-HCT is determined by a delicate balance of the GVH-directed alloreactivity, ultimately resulting in rapid engraftment of the donor HC, low toxicity and good anti-tumor effects.

Conditioning

Before patients receive allo-HCT, the patient's autologous HC have to be eradicated, as they will prevent the donor HC from engrafting. Furthermore, when patients with a malignancy are transplanted, the primary malignancy can repopulate from the residual patient-HC, resulting in a relapse. Hence, to remove autologous HC prior to HCT, patients receive so-called conditioning regimens.

Historically, conditioning regimens were aimed at complete eradication of the autologous HC, targeting both the immune system, with a focus on T cells, as well as residual malignant cells. These so-called myeloablative (MA) conditioning regimens promote engraftment of donor HC and prevent tumor relapse. Despite the positive effects, MA conditioning regimens cause severe tissue damage leading to serious complications. The tissue damage results in immune activation, a cytokine storm, and subsequent triggering of T cells that leads to GVHD (reviewed in (2)). So, even though MA conditioning regimens play a large role in the success of allo-HCT, their application is hampered by the damage that is caused.

To reduce the toxicity, non-myeloablative (NMA) and reduced intensity conditioning (RIC) regimens were introduced. NMA conditioning regimens are defined as conditioning regimens that do not eradicate autologous hematopoiesis, leading to the presence of both patient and donor cells upon engraftment, and the immediate recovery of autologous cells after graft failure (3). RIC regimens are just less intensive than MA conditioning regimens, leading to, in contrast to NMA regimens, prolonged aplasia upon graft rejection (reviewed in (4)). In comparison to MA conditioning regimens, NMA conditioning and RIC regimens result in less tissue damage, better protection for (opportunistic) infections, require less platelet and erythrocyte infusions and are associated with reduced GVHD risk, at the cost of diminished anti-tumor effects (reviewed in (4, 5)). The choice between MA and NMA/RIC conditioning regimens is therefore weighted carefully for each patient, based on the underlying disease, the risks of relapse and toxicity and the patient's clinical condition.

Immunosuppressive drugs are administered to prevent GVHD. Because GVHD clearly involves T-cell activation (reviewed in (2)), prevention of GVHD is largely based on T-cell depletion. Anti-thymocyte globulin (ATG) is the best established method of *in vivo* T-cell depletion. Usage of ATG greatly reduces the chance of GVHD (6), however, it comes with severe side effects, as the T cells that are depleted are also essential for anti-viral as well as anti-tumor responses. Depletion or earlier administration of ATG pre-HCT significantly improves T-cell reconstitution and reduces the risk of viral reactivations after HCT, and it is

therefore currently suggested to omit or reduce the dosage of ATG prior to HCT (7).

Engraftment and chimerism

The outcome of allo-HCT is dependent on donor engraftment. Successful engraftment is usually defined as three consecutive days of more than $1x10^9$ white blood cells/liter (leukocyte engraftment); more than $0.5x10^9$ neutrophils/liter (neutrophil engraftment); or more than $20x10^9$ or $50x10^9$ platelets/liter (platelet engraftment) (8). Engraftment is thus established by the measurement of absolute blood cell count and not based on the origin of the cells measured. Without successful engraftment, patients will encounter a period of cell aplasia, leading to a high risk of infections and bleeding.

The descent (donor or patient) of peripheral blood and BM cells can be investigated during chimerism analyses. The percentage of cells originating from donor or patient, contributing to the total HC population, is determined based on known genetic differences between donor and patient. This measurement is of great prognostic value as complete donor chimerism (>95% of donor origin) is associated with increased leukemia-free survival and reduced relapse risk after MA conditioning regimens (9), and is associated with acute GVHD development after NMA conditioning regimens (reviewed in (10)).

Graft-versus-host disease, infections and graft-versus-tumor effect

After allo-HCT, patients face three major risks: GVHD, infections and relapse. GVHD develops when the allogeneic immune system of the donor recognizes the patient tissue as foreign, and is thus a result of an (over-) activated immune system. Infections occur because the patient's new immune system still has to develop, and is thus the consequence of an absent/incomplete immune system. A relapse is prevented when the alloreactive donor cells recognize the patient's tumor, which only occurs in presence of a well-functioning immune system. Thus, the success of allo-HCT associated with these three risks is a delicate balance of both the pathological and beneficial donor immune response.

GVHD remains the major complication of allo-HCT (2), and has two main clinical phenotypes. Acute GVHD occurs early (usually within a 100 days) after HCT and mainly affects the skin, intestine and liver (11). Chronic GVHD develops later after transplantation and mimics auto-immune diseases like Systemic Sclerosis (12). Both forms of GVHD are potentially life-threatening complications and lead to severe illness and impaired quality of life. Acute GVHD occurs in up to 90% of the transplantations, depending on various risk factors (13); and chronic GVHD occurs in up to 60% (14), being the most invalidating long-term complication of allo-HCT. Preventing GVHD would greatly enhance the success and broaden the application of allo-HCT.

In the immunocompromised period early post-HCT, patients have an increased risk of opportunistic infections (reviewed in (15)). This infection risk may be due to, amongst non-T cell causes: absolute low T-cell numbers (16), a different composition of T cells, and hampered T-cell function. Whereas healthy individuals have relatively more CD4+ than CD8+ T cells, the early post-HCT period is marked by the presence of relatively more CD8+ than CD4+ T cells. Next to low absolute T-cell numbers and the inverted CD4+/CD8+ T-cell ratio, T-cell function can be impaired. There is an important difference in T-cell function after MA and NMA conditioning regimens: after MA conditioning regimens, the T-cell response is abrogated up to 12 months post-HCT, whereas after NMA conditioning regimens the repopulating T cells respond normal to mitogenic stimuli already at 1 month post-HCT (16). This

difference may result in reduced T-cell responses to pathogens after MA compared to NMA conditioning regimens, thereby leading to a higher risk of infections. Treatment or prevention of infections post allo-HCT is mostly based on improving immune reconstitution, for example by reducing the usage of ATG or by favoring NMA over MA conditioning regimens.

In patients transplanted because of a malignancy, the ultimate goal of the allo-HCT is that the alloreactivity will be directed against the tumor. This beneficial alloreactivity is designated as the graft-versus-tumor (GVT) effect. Donor T cells play a key role in the GVT effect, as is illustrated by the sustained remissions that are achieved by donor lymphocyte infusions (DLI) (17-19). Procedures that enhance the antitumor effect, like MA conditioning regimens and DLI, often come with the downside of an increased risk of GVHD (17). The major challenge of improving HCT outcome lies in the dissection of the GVT effect from the GVHD risk.

Donor selection

Optimal donor selection is critical to improve the engraftment rate, reduce the GVHD and infection risks and increase the GVT effect. Donors are selected based on multiple factors, of which HLA matching between patient and donor has the highest priority (20). However, non-HLA factors are frequently considered during donor selection as well; such factors include cell dose, CMV concordance, sex (avoiding female donors for male patients), age, blood group and gestational status (number of pregnancies of female donors) (20). Besides these selection criteria, there are also different choices with respect to HC source. Both HLA- and HC-source-related considerations will be described in detail below.

Hematopoietic cell sources

HC can be derived from BM, harvested from peripheral blood (PBSC) after growth-factor induction, and from umbilical cord blood (CB). BM and PBSC are freshly harvested shortly before the HCT, whereas CB has the advantage of being harvested upon delivery of healthy babies and is subsequently stored in freezers. While BM or PBSC donors first have to be admitted to a hospital for HC collection after proper clinical examination, which can be time-consuming, the storage of CB leads to immediate availability of the CB HC upon request.

The clinical outcomes, with respect to engraftment, infections, GVHD, and relapse are different depending on the HC source (reviewed in (21-23)). Important in this aspect is that BM-HCT is in principle only possible after MA conditioning, as there are unacceptable high rates of graft rejection after NMA conditioning (23). Relative to BM-HCT, PBSC-HCT is characterized by rapid engraftment, a reduced infection risk, a comparable acute GVHD risk but a markedly increased chronic GVHD risk, and a comparable relapse risk (Table 1). In contrast, CB-HCT is characterized by slower engraftment leading to a high infection risk, but decreased acute and chronic GVHD risks and according to some studies a reduced relapse risk (Table 1) (24). In short, the major challenge of BM- and PBSC-HCT is the GVHD risk, whereas after CB-HCT delayed engraftment is the main issue.

The different cellular characteristics of the donor sources explain the specific risks related to each HC source described above. For example, BM is a major reservoir of antigen-experienced memory-T cells while PBSC grafts typically contain a higher absolute number of T cells than BM grafts (25). Contrarily, CB grafts are characterized by a very low frequency of memory-T cells (26) and the rapid development of regulatory-T cells upon stimulation (27). In addition, it has been shown *in vitro* that T-cell differentiation of BM cells is slower

Outcome	BM	PBSC	CB
Engraftment	+	++	-
Infections	+	±	++
Acute GVHD	+	±+	±
Chronic GVHD	+	++	±
Relapse	+	+	±+

Table 1: Comparison of clinical outcomes related to cell source. The probability of clinical outcomes after peripheral blood stem cell (PBSC) or cord blood (CB) transplantation relative to bone marrow (BM) transplantation, adapted from (23).

than that of CB cells (28). The higher number of T cells in BM and PBSC likely results in faster engraftment and higher GVHD rates, whereas the regulatory-T cells of CB may reduce the GVHD risk.

For CB-HCT, the amount of nucleated cells infused per kilogram bodyweight of the patient, has a major influence on clinical outcome. There is a critical requirement of $2.0x10^7$ cells per kilogram for effective engraftment after CB-HCT (29). For adults and larger children, the number of nucleated cells from one donor of CB is generally not sufficient. Therefore, these patients are often transplanted with HC from two CB donors. Interestingly, around 100 days after this double CB-HCT, in up to 100% of the cases only one of the CB donors contributes to the hematopoietic reconstitution (30). The exact mechanisms responsible for this single donor dominance have not been established yet (30).

To summarize, the three sources of HC (BM, PBSC and CB), all have their own distinct features, resulting in different clinical outcomes and donor selection criteria. The donor-selection process is determined both by these distinct features as well as the HLA match status, as is described below.

HLA

HLA is the human counterpart of major histocompatibility complex (MHC). HLA is a group of proteins that is expressed on the surface of cells. The main function of HLA is to present peptides to T cells. HLA is encoded on chromosome 6 (reviewed in detail in (31)), and displays the most polymorphisms of the human genetic system, with almost 12,000 different alleles encoding for more than 8,500 different proteins ((32) accessed September 2014), as not all alleles encode for a different protein. The high level of polymorphism in HLA is likely an evolutionary result of providing maximal herd protection against pathogens; the different HLA proteins can present different peptide repertoires to the T cells, thereby magnifying the chance of encountering a T cell specific for each pathogen (33).

Classical HLA is divided into two subclasses: HLA class I and II (both reviewed in detail in (31)). HLA class I (HLA-A, -B, -C) consists of one polymorphic heavy chain bound to the non-polymorphic β2-microglobulin (Figure 1). HLA class I presents mostly endogenous peptides (*i.e.* peptides from within the cell) with a preferential length of 8-10 amino acids, to CD8+ T cells (31). Endogenous proteins are cleaved into peptides by the proteasome, transported into the endoplasmatic reticulum by the transporter associated with antigen processing (TAP) protein to subsequently be loaded on HLA class I (Figure 2). HLA class I is expressed on all nucleated cells.

HLA class II (HLA-DR, -DQ, -DP) consists of a polymorphic α- and β-chain (Figure 1). As every individual has two allelic variants of the α- and β-chains (a maternal and paternal allele), and both of the α-chains can associate with both of the β-chains, the two allelic variants of each chain lead to four potential different proteins. In contrast to HLA class I,

HLA class II presents in general exogenous peptides (*i.e.* peptides derived from outside the cell) to CD4+ T cells. HLA class II can present peptides with a more variable length, varying mostly between 13-25 amino acids, due to a more open structure (31). Exogenous proteins are taken up into endosomes, subsequently processed by hydrolytic enzymes into peptides and thereafter loaded on HLA class II (Figure 2). HLA class II is generally present on professional antigen presenting cells (APC), but can be up regulated on non-professional APC upon stimulation as well (34).

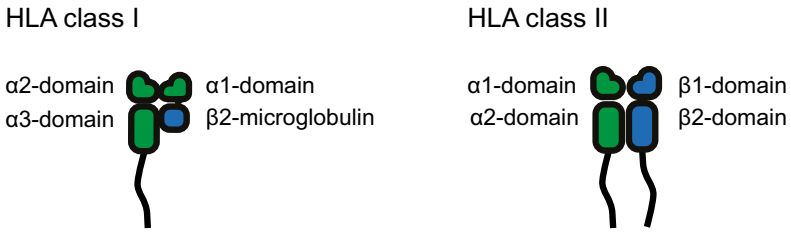


Figure 1: HLA class I and II structure. HLA class I (left panel) consists of one polymorphic heavy chain (green), formed by the α1-3-domains, bound to non-polymorphic β2-microglobulin (blue). The α1- and 2-domains form the peptide-binding groove. HLA class II (right panel) consists of two polymorphic chains, the α-chain consisting of two α-domains (green), and the β-chain consisting of two β-domains (blue). The α1- and β1-domains form the peptide-binding groove.

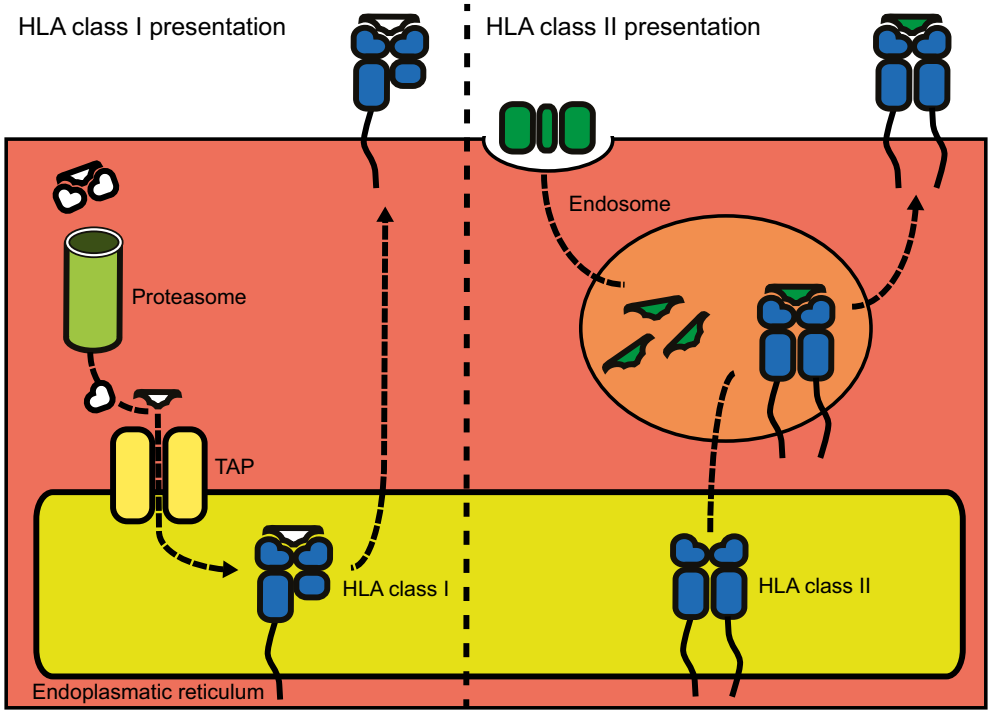


Figure 2: HLA class I and II classical peptide presentation pathways. Endogenous proteins (left panel) are processed into peptides by the proteasome and transported into the endoplasmatic reticulum by the transporter associated with antigen processing (TAP) protein, and then loaded onto HLA class I. HLA class I is expressed on all nucleated cells. Exogenous proteins (right panel) are taken up into the cell via endocytosis. These endocytozed proteins are cleaved by lysosomal enzymes into peptides, and are loaded in the endosomal compartment onto HLA class II. HLA class II is mainly expressed on professional antigen-presenting cells. Figure adapted from (36).

HLA matching

HLA matching between donor and patient is important for the success of allo-HCT (36, 37), as HLA mismatching leads to a high risk of alloreactivity (37-42). In principle, patients and donors are preferably matched for the loci HLA-A, -B, -C, -DRB1, -DQB1; as individuals inherit both a maternal as well as paternal HLA-haplotype, and consequently exhibit two alleles of each locus, patients and donors are preferably a 10/10 match. Due to the enormous polymorphism of the HLA system, finding an HLA-matched donor is challenging.

Diagnostic HLA typing to assist in donor selection can be performed on different levels: low-resolution or serological typing based on cell-surface expression, for example HLA-A2; intermediate and high-resolution or allele-level typing based on the DNA sequence, for example HLA-A*02:AB or HLA-A*02:01:01:01, which are subtypes of HLA-A2. These different resolution levels are important for donor selection.

HLA match criteria for allo-HCT differ depending on the donor source. An HLA-identical related donor (IRD) is usually regarded the best option. In absence of an IRD, most centers preferentially select a high-resolution 10/10-matched unrelated donor (URD). Guidelines for HLA matchgrade are similar for BM as well as PBSC as a HC source, even though matchgrade is less well studied in PBSC-HCT (43). For URDs, HLA matchgrades below 7/10 or 8/10 are usually not accepted, although acceptance criteria differ depending on local guidelines (1, 43). For CB-HCT, donor-patient match criteria are less stringent (44): CBs are usually matched for HLA-A, -B on a serological level and HLA-DRB1 on a high-resolution level, and are considered a sufficient match when they are a 4/6 to 6/6 match. Despite this general lower matchgrade acceptance when selecting CB cells, higher-resolution level matching on more loci also reduces the risk of transplant-related mortality after CB-HCT (45, 46).

Permissible HLA mismatches

T-cell recognition is clearly involved in mismatched-HLA induced alloreactivity (2), as evidenced by a reduced GVHD risk when T cells are depleted from the grafts (47) and a risk of GVHD development after DLI (48). However, the *in vivo* severity of the T-cell mediated immune responses is highly variable: some HLA mismatches are better tolerated than others (40, 49). Poorly tolerated mismatches are designated non-permissive (40), taboo (49), or non-permissible mismatches (50). An example of a poorly understood non-permissible mismatch is the HLA-B*44:02 and -B*44:03 mismatch, leading to severe alloreactivity (51) and graft rejection (52), despite the fact that these alleles are closely related and present similar peptide repertoires (53). On the other hand, highly divergent mismatches seem to have a better outcome in some transplant settings (54), suggesting that highly diverse HLA molecules lack T-cell receptor contact residues for sufficient affinity. The underlying mechanisms that lead to differential permissibility of HLA mismatches are, however, still poorly understood.

Better definition of permissible HLA mismatches can aid in donor selection. Epidemiological studies using very large cohorts have identified specific non-permissible mismatch combinations (40), and combinations leading to a better clinical outcome (39, 55). In addition, permissibility of mismatches was determined *in vitro* (56-58). However, identifying permissible/non-permissible HLA mismatches for all potential mismatch combinations, either epidemiologically or by *in vitro* studies, is a laborious, extremely costly and hardly possible task, due to the enormous diversity of the HLA system. Therefore, using the knowledge regarding HLA-induced alloreactivity for the development of *in silico* prediction methods,

may generate universally applicable tools that help prevent alloreactivity and thereby improve transplant outcome.

Outline of this thesis

The aim of this thesis was to develop a method that can predict permissible HLA-mismatches prior to HCT. As T cells play a key role in both GVHD and GVT development, we focused on the knowledge of T-cell recognition of mismatched HLA. Theoretically, donor T cells can recognize mismatched HLA via either direct or indirect recognition. During direct recognition, the donor T cell recognizes the complete HLA protein on the cell surface of a patient APC. The T cell interacts with the mismatched HLA presenting a certain peptide, potentially resembling a self-HLA presenting a pathogenic peptide, consequently leading to T-cell activation (Figure 3) (59, 60). During indirect recognition, the mismatched HLA is, just like any other protein, processed into peptides and presented as a peptide on HLA to the donor T cell (Figure 3, 4). The donor T cell then recognizes the peptide as foreign, similar as during a "normal" pathogen-induced response. In short, during direct recognition the donor T cell recognizes the mismatched HLA protein, whereas during indirect recognition a peptide derived from the mismatched HLA is recognized. The methods to predict both direct and indirect recognition will be reviewed in detail in Chapter 2.

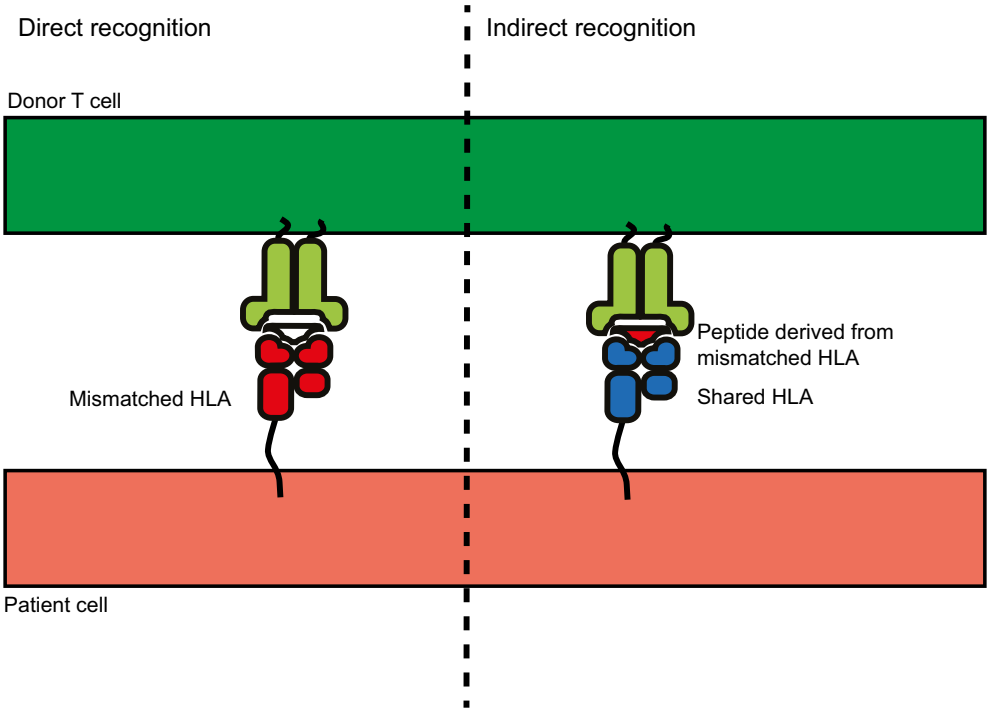


Figure 3: Direct versus indirect recognition. During direct recognition (left panel) the donor T cell recognizes the mismatched HLA as an intact protein on the cell surface of a patient cell. During indirect recognition (right panel) the donor T cell recognizes a peptide derived from the mismatched HLA presented on a shared HLA molecule.

This thesis describes Predicted Indirectly ReCognizable HLA Epitopes (PIRCHE) as a novel method to predict the probability of indirect T-cell recognition. The PIRCHE method (Figure 4) determines *in silico* which unique mismatched-HLA derived peptides can be presented on shared HLA to the donor T cells. From each HLA protein the proteasomal cleavage sites are located, to identify which potential peptides derived from the protein can be generated. From these potential peptides, the level of binding to shared HLA class I is subsequently predicted. For HLA class-II presentation, only binding to shared HLA class II is predicted, as HLA class II-processing routes are not reliably predictable yet. For all peptides that are predicted to bind to shared HLA, is determined whether they are specific to the mismatched HLA (Figure 4B). When peptides derived from the mismatched HLA are similar to peptides derived from self HLA, they should not lead to alloreactivity, as T cells are educated not to respond to self peptides. This strategy then results in a number of unique mismatched-HLA derived peptides presented by shared HLA class I and II (PIRCHE-I and -II, respectively). In theory, PIRCHE-I predict the likelihood of CD8+ T-cell responses and PIRCHE-II CD4+ T-cell responses.

Chapter 2 of this thesis reviews the available knowledge regarding methods that can predict permissible HLA mismatches, including the PIRCHE concept. Using the PIRCHE model, the effect of indirect T-cell recognition on clinical outcomes was studied in adult URD-HCT (Chapter 3-5) and CB-HCT (Chapter 6-7).

In **Chapter 3** the effect of PIRCHE after single HLA-mismatched HCT (9/10) was compared with HLA-matched HCT (10/10). Patients with low PIRCHE-I and -II had similar overall survival as patients after a 10/10-matched HCT. A well-established model exists to predict direct recognition of HLA-DP mismatches. The presence of this model allowed to study the correlation between a direct and indirect recognition model in **Chapter 4**. Both models were highly correlated, although the PIRCHE model had a better predictive capability for acute GVHD. HLA-C mismatches lead to alloreactivity, besides their low expression level on the cell surface. We hypothesized that indirect recognition of HLA-C mismatches may explain this. Indeed, **Chapter 5** shows that HLA-C mismatches led to high numbers of PIRCHE. We speculate that, due to these high numbers of PIRCHE, it may sometimes be preferable to select an HLA-B mismatch rather than an HLA-C mismatch.

The results of Chapter 3 urged us to investigate the role of PIRCHE in single (**Chapter 6**) and double (**Chapter 7**) CB-HCT. Strikingly, after CB-HCT, PIRCHE-I strongly predicted the GVT effect, leading to superior survival in case of high PIRCHE-I numbers, and these chapters therefore indicate specific PIRCHE-based donor-selection criteria, depending on the HC source.

In **Chapter 8**, we studied the influence of patient-donor chimeric status on the effect of PIRCHE. The PIRCHE-induced effects on acute GVHD were only detectable in patients with complete donor chimerism, indicating that donor T cells are required for the PIRCHE effect. In **Chapter 9**, the numbers of CD4+ and CD8+ T cells in skin tissue sections of pediatric patients with GVHD were quantified and correlated to donor characteristics. Patients with PIRCHE-I or -II had higher numbers of T cells in their cutaneous infiltrates.

Chapter 10, the discussion and conclusions chapter, summarizes all the data and puts them in a broader perspective, especially with respect to the usage of PIRCHE in clinical practice and potential improvements.

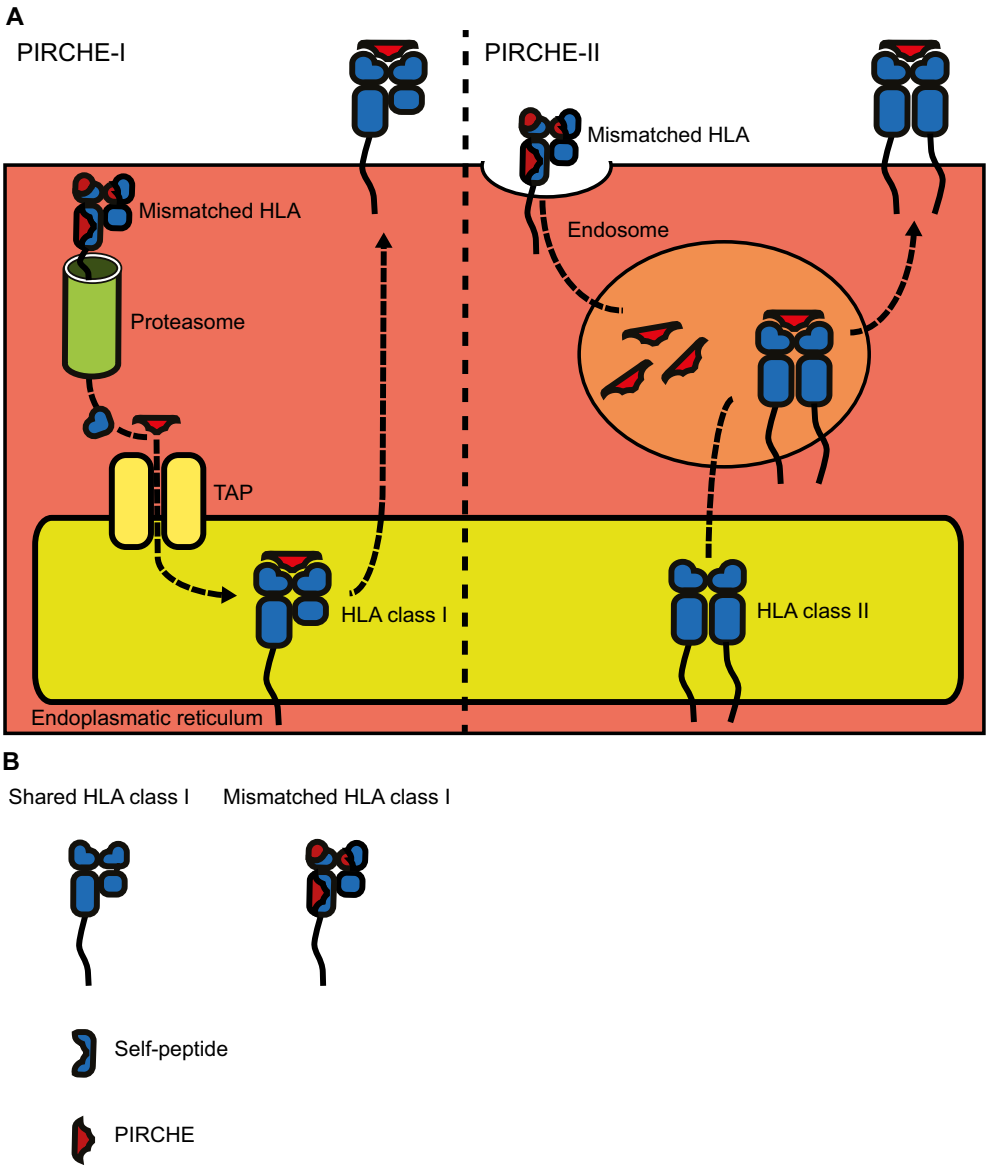


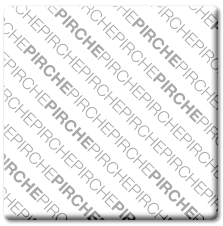
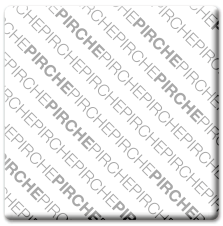
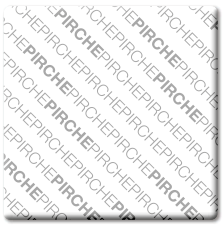
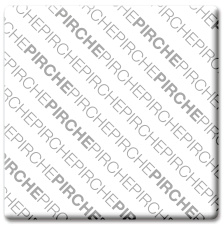
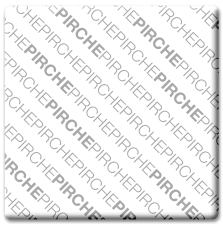
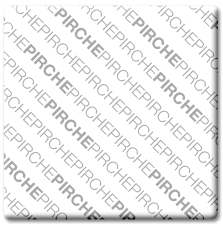
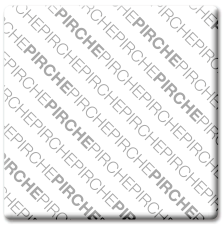
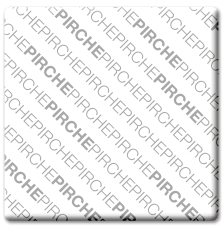
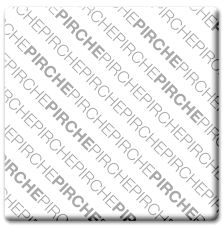
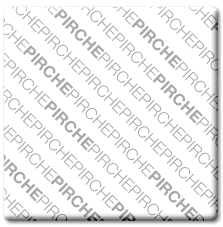
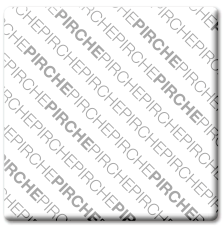
Figure 4: PIRCHE prediction. (A) For PIRCHE-I proteasomal cleavage sites are first determined to establish which potential peptides derived from the mismatched HLA can be presented. Subsequently binding affinity to shared HLA class I is used to select binders. For PIRCHE-II (right panel) only binding to shared HLA class II is predictable. (B) Only presented peptides that are different (the red peptide) between shared and mismatched HLA are counted as PIRCHE.

References

1. Indicaties allogene hematopoïetische stamceltransplantatie bij volwassenen. HOVON. 23-6-2013
2. Ferrara JL, Levine JE, Reddy P, et al. Graft-versus-host disease. Lancet 2009; 373(9674): 1550-1561.
3. Storb RF, Champlin R, Riddell SR, et al. Non-myeloablative transplants for malignant disease. Hematology.Am.Soc.Hematol.Educ.Program. 2001; 375-391.
4. Schattenberg AV and Levenga TH. Differences between the different conditioning regimens for allogeneic stem cell transplantation. Curr.Opin.Oncol. 2006; 18(6): 667-670.
5. Baron F and Storb R. Allogeneic hematopoietic cell transplantation following nonmyeloablative conditioning as treatment for hematologic malignancies and inherited blood disorders. Mol.Ther. 2006; 13(1): 26-41.
6. Theurich S, Fischmann H, Shimabukuro-Vornhagen A, et al. Polyclonal anti-thymocyte globulins for the prophylaxis of graft-versus-host disease after allogeneic stem cell or bone marrow transplantation in adults. Cochrane.Database.Syst.Rev. 2012; 9: CD009159.
7. Lindemans CA, Chiesa R, Amrolia PJ, et al. Impact of thymoglobulin prior to pediatric unrelated umbilical cord blood transplantation on immune reconstitution and clinical outcome. Blood. 2014; 123(1): 126-132.
8. Statistical guidelines for EBMT. EBMT 2014.
9. Huisman C, de Weger RA, de VL, et al. Chimerism analysis within 6 months of allogeneic stem cell transplantation predicts relapse in acute myeloid leukemia. Bone Marrow Transplant. 2007; 39(5): 285-291.
10. Baron F and Sandmaier BM. Chimerism and outcomes after allogeneic hematopoietic cell transplantation following nonmyeloablative conditioning. Leukemia. 2006; 20(10): 1690-1700.
11. Glucksberg H, Storb R, Fefer A, et al. Clinical manifestations of graft-versus-host disease in human recipients of marrow from HL-A-matched sibling donors. Transplantation 1974; 18(4): 295-304.
12. Shulman HM, Sullivan KM, Weiden PL, et al. Chronic graft-versus-host syndrome in man. A long-term clinicopathologic study of 20 Seattle patients. Am.J.Med. 1980; 69(2): 204-217.
13. Murphy WJ and Blazar BR. New strategies for preventing graft-versus-host disease. Curr.Opin.Immunol. 1999; 11(5): 509-515.
14. Wingard JR, Vogelsang GB, and Deeg HJ. Stem cell transplantation: supportive care and long-term complications. Hematology.Am.Soc.Hematol.Educ.Program. 2002; 422-444.
15. Lin R and Liu Q. Diagnosis and treatment of viral diseases in recipients of allogeneic hematopoietic stem cell transplantation. J.Hematol.Oncol. 2013; 6: 94-96.
16. Morecki S, Gelfand Y, Nagler A, et al. Immune reconstitution following allogeneic stem cell transplantation in recipients conditioned by low intensity vs myeloablative regimen. Bone Marrow Transplant. 2001; 28(3): 243-249.
17. Lokhorst HM, Wu K, Verdonck LF, et al. The occurrence of graft-versus-host disease is the major predictive factor for response to donor lymphocyte infusions in multiple myeloma. Blood. 2004; 103(11): 4362-4364.
18. Krishnamurthy P, Potter VT, Barber LD, et al. Outcome of donor lymphocyte infusion after T cell-depleted allogeneic hematopoietic stem cell transplantation for acute myelogenous leukemia and myelodysplastic syndromes. Biol.Blood Marrow Transplant. 2013; 19(4): 562-568.
19. Rutten CE, van Luxemburg-Heijs SA, Halkes CJ, et al. Patient HLA-DP-Specific CD4(+) T Cells from HLA-DPB1-Mismatched Donor Lymphocyte Infusion Can Induce Graft-versus-Leukemia Reactivity in the Presence or Absence of Graft-versus-Host Disease. Biol.Blood Marrow Transplant. 2013; 19(1): 40-48.
20. Spellman SR, Eapen M, Logan BR, et al. A perspective on the selection of unrelated donors and cord blood units for transplantation. Blood. 2012; 120(2): 259-265.
21. Peters C, Cornish JM, Parikh SH, et al. Stem cell source and outcome after hematopoietic stem cell transplantation (HSCT) in children and adolescents with acute leukemia. Pediatr.Clin.North Am. 2010; 57(1): 27-46.
22. Urbano-Ispizua A. Risk assessment in haematopoietic stem cell transplantation: stem cell source. Best. Pract.Res.Clin.Haematol. 2007; 20(2): 265-280.
23. Bensinger WI. Allogeneic transplantation: peripheral blood vs. bone marrow. Curr.Opin.Oncol. 2012; 24(2): 191-196.
24. Brunstein CG, Gutman JA, Weisdorf DJ, et al. Allogeneic hematopoietic cell transplantation for hematologic malignancy: relative risks and benefits of double umbilical cord blood. Blood 2010; 116(22): 4693-4699.
25. Korbling M and Anderlini P. Peripheral blood stem cell versus bone marrow allotransplantation: does the source of hematopoietic stem cells matter? Blood 2001; 98(10): 2900-2908.
26. Szabolcs P, Park KD, Reese M, et al. Coexistent naive phenotype and higher cycling rate of cord blood T cells as compared to adult peripheral blood. Exp.Hematol. 2003; 31(8): 708-714.
27. de Roock S, Hoeks SB, Meurs L, et al. Critical role for programmed death 1 signaling and protein kinase B in augmented regulatory T-cell induction in cord blood. J.Allergy Clin.Immunol. 2011; 128(6): 1369-1371.
28. De Smedt M, Leclercq G, Vandekerckhove B, et al. T-lymphoid differentiation potential measured in vitro is higher in CD34+CD38-/lo hematopoietic stem cells from umbilical cord blood than from bone marrow and is an intrinsic property of the cells. Haematologica 2011; 96(5): 646-654.
29. Barker JN, Byam C, and Scaradavou A. How I treat: the selection and acquisition of unrelated cord blood grafts. Blood 2011; 117(8): 2332-2339.

30. Majhail NS, Brunstein CG, and Wagner JE. Double umbilical cord blood transplantation. Curr.Opin.Immunol. 2006; 18(5): 571-575.
31. Williams TM. Human leukocyte antigen gene polymorphism and the histocompatibility laboratory. J.Mol.Diagn. 2001; 3(3): 98-104.
32. IMGT sequence alignment tool. IMGT 2013
33. Trowsdale J and Knight JC. Major histocompatibility complex genomics and human disease. Annu.Rev. Genomics Hum.Genet. 2013; 14: 301-323.
34. Collins T, Korman AJ, Wake CT, et al. Immune interferon activates multiple class II major histocompatibility complex genes and the associated invariant chain gene in human endothelial cells and dermal fibroblasts. Proc.Natl.Acad.Sci.U.S.A 1984; 81(15): 4917-4921.
35. Villadangos JA and Schnorrer P. Intrinsic and cooperative antigen-presenting functions of dendritic-cell subsets in vivo. Nat.Rev.Immunol. 2007; 7(7): 543-555.
36. Lee SJ, Klein J, Haagenson M, et al. High-resolution donor-recipient HLA matching contributes to the success of unrelated donor marrow transplantation. Blood 2007; 110(13): 4576-4583.
37. Flomenberg N, Baxter-Lowe LA, Confer D, et al. Impact of HLA class I and class II high-resolution matching on outcomes of unrelated donor bone marrow transplantation: HLA-C mismatching is associated with a strong adverse effect on transplantation outcome. Blood 2004; 104(7): 1923-1930.
38. Crocchiolo R, Zino E, Vago L, et al. Nonpermissive HLA-DPB1 disparity is a significant independent risk factor for mortality after unrelated hematopoietic stem cell transplantation. Blood 2009; 114(7): 1437-1444.
39. Kawase T, Matsuo K, Kashiwase K, et al. HLA mismatch combinations associated with decreased risk of relapse: implications for the molecular mechanism. Blood 2009; 113(12): 2851-2858.
40. Kawase T, Morishima Y, Matsuo K, et al. High-risk HLA allele mismatch combinations responsible for severe acute graft-versus-host disease and implication for its molecular mechanism. Blood 2007; 110(7): 2235-2241.
41. Morishima Y, Sasazuki T, Inoko H, et al. The clinical significance of human leukocyte antigen (HLA) allele compatibility in patients receiving a marrow transplant from serologically HLA-A, HLA-B, and HLA-DR matched unrelated donors. Blood 2002; 99(11): 4200-4206.
42. Mosaad YM and Kamel H. HLA-DPB1 mismatch and acute graft-versus host disease in HLA-identical sibling donors. Egypt.J.Immunol. 2005; 12(2): 21-28.
43. Bray RA, Hurley CK, Kamani NR, et al. National marrow donor program HLA matching guidelines for unrelated adult donor hematopoietic cell transplants. Biol.Blood Marrow Transplant. 2008; 14(9 Suppl): 45-53.
44. Spellman SR, Eapen M, Logan BR, et al. A perspective on the selection of unrelated donors and cord blood units for transplantation. Blood 2012; 120(2): 259-265.
45. Eapen M, Klein JP, Sanz GF, et al. Effect of donor-recipient HLA matching at HLA A, B, C, and DRB1 on outcomes after umbilical-cord blood transplantation for leukaemia and myelodysplastic syndrome: a retrospective analysis. Lancet Oncol. 2011; 12(13): 1214-1221.
46. Eapen M, Klein JP, Ruggeri A, et al. Impact of allele-level HLA matching on outcomes after myeloablative single unit umbilical cord blood transplantation for hematologic malignancy. Blood 2014; 123(1): 133-140.
47. Perez L, Anasetti C, and Pidala J. Have we improved in preventing and treating acute graft-versus-host disease? Curr.Opin.Hematol. 2011; 18(6): 408-413.
48. Lokhorst HM, Wu K, Verdonck LF, et al. The occurrence of graft-versus-host disease is the major predictive factor for response to donor lymphocyte infusions in multiple myeloma. Blood. 2004; 103(11): 4362-4364.
49. Doxiadis II, Smits JM, Schreuder GM, et al. Association between specific HLA combinations and probability of kidney allograft loss: the taboo concept. Lancet 1996; 348(9031): 850-853.
50. van der Meer A, Joosten I, Schattenberg AV, et al. Cytotoxic T-lymphocyte precursor frequency (CTLp-f) as a tool for distinguishing permissible from non-permissible class I mismatches in T-cell-depleted allogeneic bone marrow transplantation. Br.J.Haematol. 2000; 111(2): 685-694.
51. Mifsud NA, Purcell AW, Chen W, et al. Immunodominance hierarchies and gender bias in direct T(CD8)-cell alloreactivity. Am.J.Transplant. 2008; 8(1): 121-132.
52. Fleischhauer K, Kernan NA, O'Reilly RJ, et al. Bone marrow-allograft rejection by T lymphocytes recognizing a single amino acid difference in HLA-B44. N.Engl.J.Med. 1990; 323(26): 1818-1822.
53. Macdonald WA, Purcell AW, Mifsud NA, et al. A naturally selected dimorphism within the HLA-B44 supertype alters class I structure, peptide repertoire, and T cell recognition. J.Exp.Med. 2003; 198(5): 679-691.
54. Heemskerk MB, Cornelissen JJ, Roelen DL, et al. Highly diverged MHC class I mismatches are acceptable for haematopoietic stem cell transplantation. Bone Marrow Transplant. 2007; 40(3): 193-200.
55. Fernandez-Vina MA, Wang T, Lee SJ, et al. Identification of a permissible HLA mismatch in hematopoietic stem cell transplantation. Blood 2014.
56. Bettens F, Schanz U, and Tiercy JM. Lack of recognition of HLA class I mismatches outside alpha1/alpha2 domains by CD8+ alloreactive T lymphocytes: the HLA-B44 paradigm. Tissue Antigens 2013; 81(6): 414-418.
57. Zino E, Frumento G, Marktel S, et al. A T-cell epitope encoded by a subset of HLA-DPB1 alleles determines nonpermissive mismatches for hematologic stem cell transplantation. Blood 2004; 103(4): 1417-1424.
58. Roelen DL, Stobbe I, Young NT, et al. Permissible and immunogenic HLA-A mismatches: cytotoxic T-cell

precursor frequencies reflect graft survival data. Hum. Immunol. 2001; 62(7): 661-667.
59. Amir AL, D'Orsogna LJ, Roelen DL, et al. Allo-HLA reactivity of virus-specific memory T cells is common. Blood 2010; 115(15): 3146-3157.
60. Amir AL, van der Steen DM, Hagedoorn RS, et al. Allo-HLA-reactive T cells inducing graft-versus-host disease are single peptide specific. Blood 2011; 118(26): 6733-6742.



2










Chapter 2

Predicting alloreactivity in transplantation

Kirsten Geneugelijk,[1,*] Kirsten A. Thus,[1,*] and Eric Spierings[1]

[*]authors contributed equally
[1]Laboratory of Translational Immunology,
University Medical Center Utrecht, Utrecht, the Netherlands

Journal of Immunology Research 2014; 2014: 159479

Abstract

Human Leukocyte Antigen (HLA) mismatching leads to severe complications after solid-organ transplantation and hematopoietic stem-cell transplantation. The alloreactive responses underlying the post-transplantation complications include both direct recognition of allogeneic HLA by HLA-specific alloantibodies and T cells, and indirect T-cell recognition. However, the immunogenicity of HLA mismatches is highly variable; some HLA mismatches lead to severe clinical B cell- and T cell-mediated alloreactivity, whereas others are well-tolerated. Definition of the permissibility of HLA mismatches prior to transplantation allows selection of donor-recipient combinations that will have a reduced chance to develop deleterious host-versus-graft responses after solid-organ transplantation and graft-versus-host responses after hematopoietic stem-cell transplantation. Therefore, several methods have been developed to predict permissible HLA-mismatch combinations. In this review we aim to give a comprehensive overview about the current knowledge regarding HLA-directed alloreactivity and several developed *in vitro* and *in silico* tools that aim to predict direct and indirect alloreactivity.

Introduction

Human Leukocyte Antigen (HLA) matching significantly reduces the risk of graft rejection and graft failure after solid-organ transplantation (1-3) and graft-versus-host disease (GVHD) after hematopoietic stem-cell transplantation (HSCT) (4-9). These pathological conditions evolve due to an alloreactive immune response that is initiated through interaction of allogeneic HLA with antibodies or the T-cell receptor (TCR). The subsequent immune response directed against allogeneic HLA impairs transplant outcome, emphasizing the need to avoid alloreactive responses after transplantation.

The highly polymorphic HLA system can subdivided into two major classical classes: HLA class I and HLA class II. In general, HLA class-I molecules (HLA-A, -B, -C) present endogenous peptides of 8-11 amino acids in length that can be recognized by CD8+ T cells, while HLA class-II molecules (HLA-DR, -DQ, -DP) present exogenous peptides of 13-18 amino acids in length that can be recognized by CD4+ T cells. HLA class-I molecules consist of a polymorphic alpha chain and a non-polymorphic beta-2-microglobulin, and have a rather closed peptide-binding groove. On the other hand, HLA class-II molecules consist of a polymorphic alpha and beta chain, and have a more open structure.

Acquiring HLA-matched donors for transplantation is very challenging, due to the high level of polymorphisms in the HLA system. HLA-incompatible transplantations can therefore not be avoided for a large number of patients. In those cases where a fully HLA-matched donor is not available, there is a clinical need to predict whether a certain HLA mismatch will elicit severe B-cell and T-cell mediated alloreactive responses or not. There is cumulating evidence that these high-risk HLA mismatches (so-called non-permissible mismatches / unacceptable mismatches) and well-tolerated HLA mismatches (so-called permissible mismatches / acceptable mismatches) exist, as epidemiological studies have shown that permissibility of HLA-mismatched combinations is highly variable (6, 7, 10). For example, HLA-B*44:02 and HLA-B*44:03 mismatching leads to the induction of allospecific CD8+ T cells *in vitro* (11) and bone marrow-allograft rejection *in vivo* (12). The amino-acid sequences of HLA-B*44:02 and HLA-B*44:03 differ only in one amino acid (13), indicating that even small amino-acid changes between HLA molecules can result in major alloreactive immune responses after transplantation. On the other hand, HLA class-I mismatches that are highly diverse may well be tolerated in HSCT (14). Differences in permissibility between HLA-mismatched combinations may be explained by a different impact of amino-acid polymorphisms on peptide-binding features. Some amino-acid sequence polymorphisms will alter peptide-binding motifs and peptide-HLA complex conformation, thereby potentially inducing alloreactive immune responses, while others will not alter peptide-HLA landscapes.

Characterizing the permissibility of HLA mismatches prior to transplantation allows selection of the most optimal donor-recipient match, and thereby will help to diminish the risk of post-transplantation complications after HLA-incompatible transplantations. However, epidemiological studies do not provide a universal tool for defining permissibility for every HLA-mismatched combination, as these data are limited to the specific HLA-mismatched combinations studied; very large study populations would be required to study all potential combinations. Several approaches have therefore been developed to define permissibility of HLA-mismatched combinations; some of these approaches are very useful in predicting alloreactivity. We here review the current knowledge regarding HLA-directed alloreactivity and the various *in vitro* and *in silico* methods that can be used to predict this alloreactivity.

Pathways of allorecognition

HLA alloreactivity in transplantation involves both B cell- and T cell-mediated responses. Three mechanisms of alloreactivity directed towards allogeneic HLA have been described: direct, indirect, and semi-direct allorecognition.

IgG HLA alloantibodies directly recognize intact allogeneic HLA molecules that are present on the cell surface. These antibodies play a pivotal role in solid-organ transplantation and probably also have a role in HSCT (15-18). The humoral response directed against allogeneic HLA can be established upon exposure to allogeneic HLA during pregnancies, blood transfusions, or (previous) transplantations. During this response, allogeneic HLA antigens are internalized by B cells and processed into peptides. These peptides can subsequently be presented on HLA class-II molecules that are present on the cell surface. Recognition of these HLA class-II presented HLA-derived epitopes by CD4+ T cells results in B-cell activation and IgM to IgG isotype switching (19). Donor-specific IgG HLA antibodies (DSA) that are subsequently produced can bind directly to small polymorphic amino-acid residue patches that are present on the molecular surface of HLA antigens (20-22), thereby inducing rejection of graft tissue/cells, designated as antibody-mediated rejection.

In addition to alloantibodies, alloreactive T cells can also directly recognize intact allogeneic HLA molecules (23). There is compelling evidence that cross-reactive T cells are involved in direct T-cell recognition (24-31). These cross-reactive T cells initially react towards a foreign peptide, for instance a viral peptide, presented by self HLA. However, these T cells can also respond to allogeneic HLA presenting a self or viral peptide (24-31). Although these cross-reactive T cells can persist over time, direct T-cell recognition is predominantly involved in the acute stage of alloreactivity (32). Intact HLA molecules present on resident donor-derived antigen-presenting dendritic cells are considered to be the driving force behind direct recognition in solid-organ transplantation, since parenchymal cells within transplanted tissues are unable to induce direct T-cell recognition (reviewed in (33)). Because these dendritic cells are depleted over time, the contribution of direct recognition in chronic graft rejection after solid-organ transplantation is limited (32,33).

In contrast to direct T-cell recognition, indirect T-cell recognition is considered to be mainly involved in later stages of alloreactivity (34). During indirect recognition, T cells recognize processed epitopes derived from allogeneic HLA that are presented by HLA molecules that are likely shared between donor and recipient (35, 36), as T cells are restricted to self HLA. Indirect T-cell recognition is also involved in the formation of HLA alloantibodies, since T-cell recognition of B-cell presented HLA epitopes is required in this process (19, 37). Thus, indirect T-cell recognition may also partly contribute to early alloreactivity, as indirect recognition can amplify the direct recognition response.

In semi-direct allorecognition, allogeneic HLA:peptide complexes are transferred from allogeneic cells to autologous dendritic cells, resulting in a chimeric antigen-presenting cell (38). Transfer of allogeneic HLA:peptide complexes can be achieved through secretion of endosomes containing HLA:peptide complexes (39) or through cell-to-cell contact between donor and recipient dendritic cells (40). Antigen-presenting cells that acquire intact allogeneic HLA:peptide complexes on their cell surface may elicit both direct and indirect alloreactive T-cell responses. Although *in vivo* evidence for the role of the semi-direct allorecognition pathway in graft rejection and GVHD is limited, it has been shown that this pathway is able to elicit cytotoxic alloimmunity *in vitro* and *in vivo* (41), and that the transfer of allogeneic HLA:peptide complexes likely occurs in an *in vitro* system of GVHD (42). These ob-

servations suggest that semi-direct allorecognition may be involved in post-transplantation complications.

Prediction of direct HLA recognition by antibodies

Humoral sensitization to HLA class-I and class-II epitopes and the subsequent production of HLA-specific antibodies can occur upon pre-transplant exposure to allogeneic HLA. The presence of DSA before transplantation is related to antibody-mediated rejection, and significantly impairs graft prognosis (15, 16). Therefore, evaluation of HLA-sensitizing events (*i.e.* pregnancies, blood transfusions, and previous transplants) is generally included in standard pre-transplantation screening. Pregnancy is a major contributor to HLA sensitization, as approximately 30% of the pregnancies results in child-specific sensitization towards HLA-A, -B, -C, and/or -DR loci (43). Moreover, the HLA sensitization frequency increases with the number of full-term pregnancies (43). Blood transfusions can induce HLA sensitization in approximately a third of the solid-organ transplantation recipients (44). However, blood transfusions have a less prominent effect on HLA alloimmunization than pregnancy and solid-organ transplantation (44, 45). In addition to the classical sensitizing events, HLA alloantibodies can also be raised against epitopes in allergens, ingested proteins, and microorganisms that are cross-reacting with HLA (46). Although the presence of these 'natural' DSA in kidney recipients is associated with the induction of mild episodes of antibody-mediated rejection, these patients have favorable graft outcome (47). Therefore, the existence of these 'natural' DSA prior to transplantation is currently not a contraindication for transplantation (47).

Although DSA-detection methods are important tools for risk assessment prior to transplantation, pre-transplant evaluation of preformed DSA remains challenging. For example, antibodies might become undetectable at the moment of transplantation due to the decay of antibody levels over time (48). The clinical relevance of these pre-existing low DSA levels is highly variable; some preformed DSA will elicit HLA alloreactivity *in vivo*, whereas others will not. Currently used detection methods may thus not detect the whole repertoire of clinically relevant DSA. Moreover, risk assessment of high DSA levels is also complicated. The complement-dependent cytotoxicity (CDC) crossmatch assay (reviewed in (49)) is a potent manner to measure the presence of clinically relevant antibodies, whereas other DSA-detection assays, like the HLA-based enzyme-linked immunoabsorbent assay (ELISA) and Luminex-based assays (50, 51), provide valuable but limited information about the clinical relevance of identified DSA. Currently, the CDC assay seems to be a potent indicator for alloreactivity, while *in vitro* DSA-detection methods can further support the matching procedure for solid-organ transplantation. Combining *in vitro* assays with an *in silico* prediction method allows identification of acceptable HLA mismatches towards which a recipient will likely not develop antibody-mediated responses.

In vitro DSA screening assays

Assessment of humoral sensitization to allogeneic HLA was initially performed by the CDC crossmatch assay (49). This assay measures the presence of preformed or *de novo* formed antibodies through their induction of complement-dependent lymphocyte killing. A positive CDC test was associated with a significantly impaired outcome after kidney transplantation (49, 52). Despite its potency to mimic the *in vivo* situation, CDC crossmatch assays lack sensitivity (49) and may show false-positive results (53). To overcome these problems, a

more sensitive assay was developed: the flow cytometry-based crossmatch (FCXM) assay (54). However, both FCXM and the classical CDC crossmatch test correlate equally well to clinical outcome after kidney transplantation (55).

The lack of sensitivity and specificity of cytotoxicity crossmatch assays has led to development of solid-phase assays, such as the HLA-based ELISA and Luminex assays (50, 51). These solid-phase methods, particularly Luminex, are very sensitive and specific; relevant anti-HLA class-I and class-II antibody profiles in solid-organ transplant recipients can be identified and monitored over time. Combining antibody profiles that are present in solid-organ transplant recipients, with HLA typing of the donor, designated as virtual crossmatching, allows identification of DSA, and therefore might be useful in risk stratification prior to solid-organ transplantation (reviewed in (56, 57)). Unfortunately, estimation of the clinical relevance of DSA detected with solid-phase assays remains challenging (56), as tools to discriminate between non-detrimental DSA and deleterious DSA are lacking. Nevertheless, the presence of class-I and class-II DSA detected by Luminex in the absence of positive CDC assay is suggested to be indicative for impaired graft outcome in kidney transplantation (58).

HLAMatchmaker

In vitro CDC-based DSA-detection assays have their limitations; these assays are not suitable to determine HLA-mismatch permissibility for highly sensitized transplantation candidates. Because of the high sensitization levels in those individuals, CDC assays often become almost completely positive, which complicates selection of suitable CDC-negative donors that will not elicit HLA alloreactivity *in vivo*. Therefore, alternative *in silico* methods were sought to predict acceptability of HLA-mismatched combinations.

An established and well-accepted *in silico* method is HLAMatchmaker. The *in silico* algorithm HLAMatchmaker is based on the principle that HLA-specific alloantibodies can bind to distinct amino-acid polymorphisms (immunogenic epitopes) present on HLA antigens (20,21). Multiple polymorphic amino-acid residues on the molecular surface of HLA antigens have been identified. Some of these residues are inaccessible for antibodies, since they are located near the cell membrane or within peptide-binding groove of the HLA molecule, while other residues are fully accessible for antibodies (20, 21). HLAMatchmaker uses this knowledge to predict which HLA mismatches are not able to induce complications in transplantation recipients by defining the acceptable mismatches (20, 21). Initially, HLAMatchmaker defined immunogenic epitopes as antibody-accessible, linear sequences of amino-acid polymorphisms (triplets) (20, 21). Triplets that are present in donor HLA antigens, but not in the recipient HLA antigens, were considered to elicit humoral responses (20, 21). On the other hand, triplets that are present in both donor and recipient HLA antigens, were considered as acceptable (20, 21). Thus, HLAMatchmaker provides a tool for identification of acceptable HLA mismatches.

The clinical applicability of HLAMatchmaker in matching strategies has been extensively evaluated. It has been shown that the triplet version of HLAMatchmaker is a potent indicator for the presence and magnitude of allogeneic HLA-directed antibody responses in renal transplantation and during pregnancy (59, 60). In contrast, the number of triplet mismatches was not indicative for the induction of T-cell alloreactivity (61). This lack of correlation between triplets and T-cell alloreactivity is probably caused by alternative epitope binding by T cells or by the involvement of larger polymorphic sequences in T-cell alloreactivity (61). With regard to HSCT, the number of triplets did neither correlate to acute GVHD,

nor engraftment, nor survival (62).

Despite its applicability in HLA-matching strategies, the triplet version of HLAMatchmaker represents an incomplete repertoire of immunogenic epitopes, as only linear sequence positions are implemented (22). This hiatus has resulted in the development of a redefined version of HLAMatchmaker that identifies eplets (22). Eplets are immunogenic HLA epitopes that are critical for antibody binding and consist of polymorphic amino-acid patches located at the molecular surface of HLA molecules (22). These polymorphic patches may consist of polymorphisms in linear sequence positions and three-dimensional polymorphic patches in discontinuous sequence positions. Therefore, implementation of eplets into the algorithm has led to a more accurate definition of structural HLA epitopes.

Evaluation of the eplet version of HLAMatchmaker has shown a similar performance in predicting allogeneic HLA acceptability compared to the triplet version (60). Nevertheless, the eplet version of HLAMatchmaker provides further discrimination of highly divergent HLA specificities (60). Although conflicting results were reported with regard to the prognostic information that is provided by HLAMatchmaker on graft outcome ((63-66), reviewed in (67)), it is generally accepted that HLAMatchmaker is a suitable tool to analyze serum antibodies and to identify acceptable mismatches in solid-organ transplantation (67). However, HLAMatchmaker is inappropriate for HSCT donor selection (62).

In addition to the number of eplets as determined by HLAMatchmaker, additional determinants can be used to define allogeneic HLA acceptability, for instance physiochemical properties of polymorphic amino acids (68). Differences in physiochemical properties between mismatches, including electrostatic potential and hydrophobicity, are useful to predict HLA class I- and class II-specific alloantibody responses prior to solid-organ transplantation (68-70). With higher physiochemical disparity between HLA mismatches, the risk of antibody development increases after kidney transplantation (68-70). These observations suggest that differences in physiochemical properties between polymorphic amino acids may be relevant in defining acceptable HLA mismatches. However, evidence to support clinical relevance is currently lacking.

Prediction of direct T-cell recognition

The presence of T cells directly recognizing intact allogeneic HLA molecules was previously shown in individuals suffering from graft rejection after solid-organ transplantation (71,72) and GVHD after HSCT (73). There is compelling evidence that direct T-cell alloreactivity results from cross-reactive T cells that are initially primed by a foreign peptide, for instance a viral peptide (24-31). For example, the HLA-B*08:01-presented EBV peptide FLRGRAYGL is recognized by an EBV-specific TCR (74), that possesses cross-reactive capacities towards HLA-B*44:02-presented peptide EEYQAFTY (24, 75). Thus, the HLA-B*08:01-presented peptide FLRGRAYGL elicits a public immune response. During a public immune response, the immune response directed against an identical epitope is dominated by T cells expressing similar TCRs in multiple subjects (76). Since virus-specific T cells can be detected in high levels in healthy individuals (77), it is likely that cross-reactive virus-specific T cells may be present in both solid-organ transplantation recipients and HSCT donors prior to transplantation. The presence of virus-specific T cells that are cross-reactive with allogeneic HLA in these individuals, may significantly contribute to complications after transplantation. However, most virus-specific T-cell responses do not have the propensity to induce public TCR responses nor predictable cross-reactivity with allogeneic HLA (78). A single viral

infection can therefore result in the establishment of multiple T cells that are cross-reactive to multiple HLA molecules, whereas other viral infections do not give rise to these cross-reactive T cells. In addition, virus-specific T cells with the same antigen specificity, but different TCRs, elicit different unpredictable patterns of alloreactivity (26, 79).

The molecular mechanism behind T-cell cross-reactivity is complex and currently incompletely understood. T-cell cross-reactivity assumably arises due to structural homology of HLA:peptide complexes (reviewed in (78)) rather than sequence homology of the presented peptides. Despite their sequence dissimilarity, the structure of FLRGRAYGL and EEYQAFTY epitopes in the context of their presenting HLA molecules is quite similar (75). Therefore, molecular mimicry likely attributes to the observed cross-reactivity between these epitopes. On the other hand, cross-reactive TCR in mice can dock to self MHC:peptide complexes in a different orientation than to allogeneic MHC:peptide complexes, suggesting that cross-reactivity can be established without molecular mimicry (80). Thus, direct alloreactivity is a complex immune response that can only partially be explained by molecular mimicry. Since the molecular mechanism behind direct T-cell recognition is poorly understood, prediction of alloreactivity based on viral history is complex. Knowledge about viral history is therefore not sufficient to predict direct T-cell alloreactivity directed towards allogeneic HLA. Since direct T-cell allorecognition was studied intensively over the past decades, several alternative approaches to predict direct T-cell alloreactivity *in vitro* and *in silico* have been developed.

Cytotoxic T-lymphocyte precursor assays

Cytotoxic T-lymphocyte precursor assays (CTLp) determine permissibility of HLA mismatches through *in vitro* evaluation of effector cytotoxic T-cell induction. This Chromium 51 (^{51}Cr) release-based assay, initially described by Brunner *et al*. (81), estimates cytotoxic T-cell activity directed against allogeneic HLA (82). Further development of this assay has resulted in an assay that estimates the extent of alloreactive T-cell responses directed towards allogeneic HLA (82). Since individual allogeneic HLAs can be linked to CTLp frequencies, these assays are a useful approach to distinguish between permissible and non-permissible mismatches *in vitro* (83). More importantly, a high CTLp frequency correlates reasonably well with clinical outcome *in vivo*; high CTLp frequencies were associated with graft rejection after solid-organ and tissue transplantation (73, 84, 85), and with GVHD and impaired survival after allogeneic HSCT (82, 83, 86). Association between graft failure and the presence of primed cytotoxic T lymphocytes in sensitized transplant candidates (*e.g.* women after previous pregnancy) was shown as well (87).

Despite the usefulness of CTLp assay in estimating T-cell alloreactivity, the time-consuming and laborious character of this assay is a major drawback (82). In order to overcome these disadvantages, alternative *in silico* approaches have been sought that mimic the CTLp assay result (88). To this end, amino-acid polymorphisms at TCR-recognition and peptide-binding regions between HLA class-I mismatches were analyzed for their physiochemical and/or position characteristics and were correlated to CTLp outcome (88). These analyses resulted in the establishment of a novel algorithm, which aims to predict HLA class I mismatch-specific CTL alloreactivity (88). Although the algorithm can predict CTLp outcome reasonably well, usage of this model for donor selection seems limited; this tool does not predict GVHD development in patients receiving HSCT (89).

T-cell epitope model

The first clinically relevant model that successfully estimates the effect of direct recognition in HSCT has recently been developed. This HLA-DPB1-restricted model is designated as the T-cell epitope (TCE) model (90). This model has been based on *in vitro* data from two alloreactive T-cell clones isolated from an HSCT patient with graft rejection due to an HLA–DPB1 mismatched graft (90). Membrane-bound intact HLA was essential for recognition of the HLA-DPB1 mismatch by the alloreactive T-cell clones; the clones did not respond to B-lymphoblastoid cell lines transduced with a truncated mismatched-HLA-DPB1 construct that did not lead to cell-surface expression of HLA-DPB1 (90). Thus, it seems likely that these two alloreactive T-cell clones recognized the HLA-DPB1 mismatched antigen in a direct manner.

In order to identify patterns of recognition of other alleles, the T-cell clones were further tested for their recognition of other HLA-DPB1 alleles (90). Alleles were divided into three different immunogenic levels: highly immunogenic (*i.e.* both clones recognized the alleles), intermediate immunogenic (*i.e.* one of the clones recognized the allele but the other did not), or non-immunogenic (*i.e.* both clones did not recognize the allele). Since testing of all HLA-DPB1 alleles *in vitro* is very time-consuming, immunogenicity of other HLA-DPB1 alleles was extrapolated, based on similarities between the peptide-binding grooves of the *in vitro* tested alleles and the not-tested HLA-DPB1 alleles.

Subsequently, HLA-DPB1 mismatches were labeled as permissive or non-permissive based on their immunogenic level and the concept of thymic education. For example, when the HLA-DPB1 allele of the donor belongs to the highly immunogenic group, then donor T cells should be educated not to respond to HLA-DPB1 alleles belonging to the highly immunogenic group, and, in theory, will also not respond to lower immunogenic alleles. Therefore, when the recipient has an HLA-DPB1 allele belonging to the same or a lower immunogenic group, then the HLA-DPB1 mismatch will be permissive in the graft-versus-host (GVH) direction. On the other hand, since the HLA-DPB1 allele of the recipient is not immunogenic, recipient T cells are able to respond to (higher) immunogenic alleles of the donor. Thus, such mismatches are non-permissive in the host-versus-graft (HVG) direction.

Non-permissive mismatches defined by the TCE model, are highly correlated to alloreactivity as reflected by GVHD, graft rejection, and transplant-related mortality after HLA-DPB1-mismatched HSCT (4, 90-92). Counterintuitively, in these situations, the direction of the non-permissiveness appears not to be important: both HVG and GVH non-permissive mismatches lead to alloreactivity in the GVH direction (*i.e.* GVHD) (4, 91). Therefore, both HVG and GVH non-permissive mismatches are considered as overall non-permissive (4, 91). The underlying biology of this bidirectional non-permissiveness is currently poorly understood.

HistoCheck

The *in silico* model HistoCheck has been developed to estimate T-cell alloreactivity between HLA class-I and class-II mismatches (93). HistoCheck calculates a matching score for any donor-recipient combination based on their HLA typing, the so-called sequence-similarity matching score (93). The sequence-similarity matching score is determined by comparing differences in amino acids between HLA alleles with regard to their functional similarity and their location in the HLA molecule; amino-acid positions involved in TCR recognition and HLA-peptide binding are implemented in the sequence-similarity matching score (93). As

a high sequence-similarity matching score represents a high level of dissimilarity between donor and recipient (93), correlation of the sequence-similarity matching scores with clinical outcome was expected. However, HistoCheck is not indicative for transplant outcome *in vivo*, as sequence-similarity matching scores showed no correlation with GVHD after HSCT (94-96). The inability of HistoCheck to be indicative for T-cell alloreactivity may be explained by several limitations of this model: HistoCheck does not integrate the presence of alloreactive donor T cells nor viral history in its algorithm. Additionally, the concepts of aforementioned molecular mimicry between HLA:peptide complexes and unconventional docking of TCR, are not included in HistoCheck. Since these aspects of direct T-cell recognition are complex and not fully understood, establishment of reliable, clinically relevant tools to predict direct T-cell recognition remains challenging.

Prediction based on specific amino-acid changes

An alternative approach to predict direct T-cell alloreactivity, is to analyze the impact of amino acids at certain locations within HLA molecules. Several amino-acid substitutions in the peptide-binding domain of HLA class-I molecules are related to an increased risk of GVHD (6), whereas other amino-acid substitutions are related to a diminished relapse risk (7). The effect of specific amino-acid changes on alloreactivity was recently investigated in a large cohort (97). In this study, the impact of changes on HLA class-I positions 9, 99, 116 and 156 for peptide binding alteration and position 77 for killer cell immunoglobulin-like receptor binding, was investigated in recipients of an allogeneic HSCT with a single allelic mismatch at either the HLA-A, -B, or –C locus (97). Particularly amino-acid changes at position 116 in HLA-C were associated with an increased acute GVHD risk (97, 98), but also changes at position 99 for HLA-C and position 9 for HLA-B were associated with clinical T-cell alloreactivity (97). By determining the effect of specific amino acids within the HLA molecule, multiple amino-acid positions have been identified that influence transplantation outcome; this knowledge may be used for donor selection.

Prediction of indirect T-cell recognition

Indirect recognition of allogeneic HLA acts via presentation of peptides derived from allogeneic HLA molecules. Over 350 of these indirectly recognizable HLA-derived peptides have been eluted from HLA (99). T cells recognizing these peptides likely play a role in alloreactivity; the erection of indirectly recognizing T cells after solid-organ transplantation was strongly correlated to both acute (100-102) and chronic graft failure (102, 103). Furthermore, the presence of circulating T cells recognizing allogeneic HLA epitopes in an indirect manner was predictive of rejection (101). As mentioned previously, indirect T-cell recognition is considered to be a slower alloreactive response than direct T-cell recognition (33, 34). The proposed slower rate of indirect T-cell recognition may be related to the idea that indirectly recognizing T cells arise from the naive pool, whereas directly recognizing T cells likely evolve from the memory pool, as the latter T cells are supposedly cross-reactive (104). Since direct recognition has received most attention historically, not many methods are available to predict indirect recognition of HLA disparities; there is no *in vitro* system available, and only one *in silico* model.

PIRCHES model

We have recently developed a model for *in silico* prediction of indirectly recognizable

HLA-derived peptides, the so-called PIRCHES model (Predicted Indirectly ReCognizable HLA Epitopes) (105). Indirect T-cell recognition that targets allogeneic HLA, depends on HLA-derived peptides that differ between host and graft. These HLA-derived peptides are likely presented on shared HLA. HLA-derived peptides that are identical between donor and recipient should be ignored by the alloimmune system, as T cells recognizing these peptides should have been deleted from the repertoire due to thymic selection. Thus, the HVG reaction of graft rejection after solid-organ transplantation should be evoked by donor-specific peptides, whereas GVHD after HSCT should be evoked by recipient-specific peptides. We have designated the donor-specific peptides that can be recognized by the recipient as HVG-PIRCHES, and the recipient-specific peptides that can be recognized by the donor as GVH-PIRCHES.

In order to elicit indirect T-cell recognition, allogeneic HLA proteins need to be processed into peptides and these peptides need to be presented on shared HLA. Since both steps are determined by certain motifs in the protein sequences, both antigen processing and antigen presentation pathways can be predicted via several (computational) tools (106-120). Our PIRCHES model uses these predictions to define permissibility of HLA mismatches. For HLA class-I peptide presentation (designated as PIRCHE-I), the PIRCHES model first determines proteasomal cleavage of all HLA molecules of the donor and recipient into peptides and transport of those peptides via the transporter associated proteins (TAP) into the endoplasmatic reticulum (ER). Subsequently, the binding affinities of the predicted cleavage products to HLA class-I alleles are predicted, as a derivative of peptide presentation by the HLA class-I molecules that are shared between donor and recipient. Prediction of HLA class II-presented epitopes (PIRCHE-II) is restricted to HLA-binding affinity predictions of peptides, since (enzymatic) cleavage patterns have not been clearly defined yet.

On the basis of their performance, we implemented NetChop, NetMHCPan, and NetMHC-II or NetMHCIIPan (reviewed in (106-109)) in our PIRCHES model to predict the number of PIRCHE-I and PIRCHE-II. NetChop is a potent predictor of proteasomal cleavage and TAP transport, whereas NetMHCPan predicts binding affinity to HLA class I. NetMHC-II can predict peptide binding to HLA class-II alleles for which binding data exist, whereas for NetMHCIIPan these data were extrapolated to other alleles. Both HLA class I- and HLA class II-binding predictors have good predictive capacities (106), and are frequently used in to identify viral epitopes (121).

The first construction of the PIRCHES model was based on predicting HLA class I-derived peptide presentation on shared HLA-DR, and used the binding affinity predictions of NetMHC-II (105). After kidney transplantation with HLA class-I mismatches, mismatches that led to allogeneic HLA-specific antibody production correlated to higher numbers of HVG-PIRCHE-II compared to mismatches that did not led to antibody production (105), suggesting that indirect recognition of HLA-derived epitopes was required for HLA-specific IgG antibody production. For HSCT, the situation is more difficult, as alloreactivity post HSCT not only involves CD4+ T-cell recognition and stimulation of B cells, but clearly involves CD8+ T-cell recognition of alloantigens as well (122). The PIRCHES model was therefore extended to PIRCHE-I predictions. Moreover, usage of NetMHCIIPan was incorporated, as NetMHC-II can only predict binding to a limited number of HLA-DR alleles. Indeed, after HLA-mismatched HSCT, high numbers of both GVH-PIRCHE-I and -II are correlated to clinical alloreactivity (Thus *et al*., manuscripts in preparation).

In the current PIRCHES model, we regarded any difference in presentable peptides

derived from donor-versus-recipient alleles as a PIRCHE (*i.e.* only one amino acid difference is regarded a difference). The model can likely be improved when the T-cell recognition is more specifically elucidated. It is well known that some positions of peptides are more important in TCR binding than others (123, 124), as amino acids that are lying deep inside the peptide binding groove of the presenting HLA molecule, are likely not seen by the TCR. Furthermore, polymorphisms leading to different peptide properties (*e.g.* polar versus nonpolar, hydrophobic versus hydrophilic) may lead to more pronounced T-cell recognition. These refinements are currently being studied for their effect on the predictive potential of the model.

Conclusion

HLA mismatches can cause severe post-transplantation complications such as graft rejection (1) and GVHD (5). In these complications, the induction of both antibody production and T-cell recognition may play a role. Interestingly, permissibility of HLA-mismatched combinations is highly variable; some mismatches are poorly tolerated, whereas others are highly permissible. Although the degree of HLA amino-acid sequence disparity varies largely amongst different HLA mismatches depending on the allelic versus antigenic nature of the mismatch and the HLA locus, the number of polymorphic amino-acid residues in itself is not predictive for the permissibility of HLA-mismatched combinations, as multiple additional factors are involved. Both the nature and the position of the amino-acid polymorphisms within the mismatched HLA, as well as their effect on neighboring amino acids, determines the permissibility of HLA-mismatched combinations. Several approaches have been developed to predict the permissibility of HLA mismatches, thereby aiming to improve donor selection procedures. The objective of all these approaches is to predict the development of abovementioned antibody and T-cell recognition of allogeneic HLA.

Several well-established *in vitro* assays can be used to detect DSA that are related to impaired graft survival. In addition to these assays, HLAMatchmaker is a well-validated tool to identify which HLA mismatches do not induce alloreactive humoral responses in transplantation recipients (20). Although HLAMatchmaker is a powerful predictor for acceptable HLA mismatches in solid-organ transplantation, this tool is not suitable for predicting HLA permissibility in the setting of HSCT (62).

With regard to direct T-cell recognition, the risk for clinical alloreactivity can be estimated with the *in vitro* CTLp assay (82). In addition to this *in vitro* assay, several *in silico* approaches aim at predicting direct recognition-based T-cell alloreactivity. For example, the TCE model can assess non-permissive HLA-DPB1 mismatches for HSCT (90). The relevance of the TCE model has not yet been investigated in solid-organ transplantation. Practically, one should note that HLA-DPB1 is rarely typed prospectively in the setting of solid-organ transplantation, as donor availability is more restricted than for HSCT. Although HistoCheck has been developed to estimate direct recognition *in silico* for all HLA loci, this model does not correlate to alloreactivity *in vitro* nor *in vivo* (95). Alternatively, several studies have identified amino-acid positions that are influencing transplantation outcome (97); this information can be implemented in donor selection procedures.

Indirect T-cell recognition can be predicted with the *in silico* PIRCHES model (105). This model predicts HLA-derived epitopes that can be presented on shared HLA class I and II. Both PIRCHE-I and -II are well correlated to alloreactivity after HSCT. With regard to PIRCHE-II, increasing numbers of PIRCHE-II are correlated to antibody production after solid-organ

transplantation (105).

Alloreactivity after transplantation can unlikely be attributed to one single pathway of HLA recognition. To determine the relative contribution of direct and indirect recognition, combining the different methods of predicting alloreactivity would be of interest. Direct and indirect recognition may act synergistically, and therefore the combination of a positive CTLp assay and a high number of PIRCHES may lead to a more pronounced alloreactive response. Furthermore, combining the PIRCHES and the TCE model for HLA-DPB1 mismatches might allow identification of HLA-DPB1 mismatches recognized in both a direct and indirect manner. Moreover, a combination of low PIRCHE-II and low number of eplets as determined by HLAMatchmaker may be favorable in solid-organ transplantation.

In conclusion, over the past decades, many approaches have been developed to predict alloreactivity after transplantation *in vivo*, some attempts leading to more successful predictors than others. The failure of multiple tools to predict alloreactivity is not surprising, as knowledge about alloreactivity is still limited. However, multiple approaches seem to be clinically relevant and some are currently implemented in clinical practice. Further improvement of the definition of HLA-mismatch permissibility, and implementation of these definitions into the donor-selection procedure, will eventually lead to reduced alloreactivity, thereby improving clinical outcome after solid-organ transplantation and HSCT.

Disclosure

The authors have no personal conflict of interest to declare. The UMCU has filed a patent application on the prediction of an alloimmune response against mismatched HLA.

References

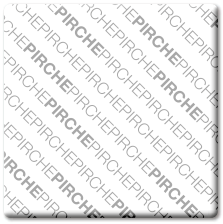
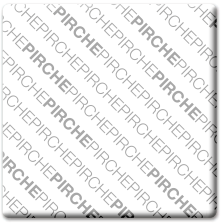
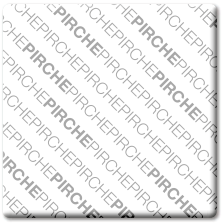
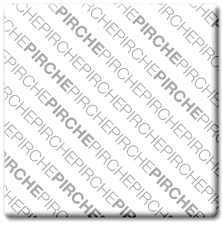
1. Dunn TB, Noreen H, Gillingham K, et al. Revisiting traditional risk factors for rejection and graft loss after kidney transplantation. American.Journal of Transplantation 2013; 11(10): 2132-2143.
2. Rizzari MD, Suszynski TM, Gillingham KJ, et al. Consideration of donor age and human leukocyte antigen matching in the setting of multiple potential living kidney donors. Transplantation 2011; 92(1): 70-75.
3. Vu LT, Baxter-Lowe A, Garcia J, et al. HLA-DR matching in organ allocation: balance between waiting time and rejection in pediatric kidney transplantation. Archives.of Surgery 2011; 146(7): 824-829.
4. Crocchiolo R, Zino E, Vago L, et al. Nonpermissive HLA-DPB1 disparity is a significant independent risk factor for mortality after unrelated hematopoietic stem cell transplantation. Blood 2009; 114(7): 1437-1444.
5. Flomenberg N, Baxter-Lowe LA, Confer D, et al. Impact of HLA class I and class II high-resolution matching on outcomes of unrelated donor bone marrow transplantation: HLA-C mismatching is associated with a strong adverse effect on transplantation outcome. Blood 2004; 104(7): 1923-1930.
6. Kawase T, Morishima T, Matsuo K, et al. High-risk HLA allele mismatch combinations responsible for severe acute graft-versus-host disease and implication for its molecular mechanism. Blood 2007; 110(7): 2235-2241.
7. Kawase T, Matsuo K, Kashiwase K, et al. HLA mismatch combinations associated with decreased risk of relapse: implications for the molecular mechanism. Blood 2009; 113(12): 2851-2858.
8. Morishima Y, Sasazuki T, Inoko H, et al. The clinical significance of human leukocyte antigen (HLA) allele compatibility in patients receiving a marrow transplant from serologically HLA-A, HLA-B, and HLA-DR matched unrelated donors. Blood 2002; 99(11): 4200-4206.
9. Mosaad YM and Kamel H. HLA-DPB1 mismatch and acute graft-versus host disease in HLA-identical sibling donors. The.Egyptian.Journal of Immunology 2005; 12(2): 21-28.
10. Doxiadis II, Smits JM, Schreuder GM, et al. Association between specific HLA combinations and probability of kidney allograft loss: the taboo concept. The. Lancet 1996; 348(9031): 850-853.
11. Mifsud NA, Purcell AW, Chen W, et al. Immunodominance hierarchies and gender bias in direct T(CD8)-cell alloreactivity. American.Journal of Transplantation 2008; 8(1): 121-132.
12. Fleischhauer K, Kernan NA, O'Reilly RJ, et al. Bone marrow-allograft rejection by T lymphocytes recognizing a single amino acid difference in HLA-B44. New England.Journal of Medicine 1990; 323(26): 1818-1822.
13. Macdonald WA, Purcell AW, Mifsud NA, et al. A naturally selected dimorphism within the HLA-B44 supertype alters class I structure, peptide repertoire, and T cell recognition. Journal of Experimental.Medicine 2003; 198(5): 679-691.
14. Heemskerk MB, Cornelissen JJ, Roelen DL, et al. Highly diverged MHC class I mismatches are acceptable for haematopoietic stem cell transplantation. Bone Marrow Transplantation 2007; 40(3): 193-200.
15. Lafaucheur C, Suberbielle-Boissel C, Hill GS, et al. Clinical relevance of preformed HLA donor-specific antibodies in kidney transplantation. American.Journal of Transplantation 2008; 8(2): 324-331.
16. Brand A, Doxiadis IN, and Roelen DL. On the role of HLA antibodies in hematopoietic stem cell transplantation. Tissue Antigens 2013; 81(1): 1-11.
17. Terasaki PI and Cai J. Human leukocyte antigen antibodies and chronic rejection: from association to causation. Transplantation 2008; 86(3): 377-383.
18. Spellman S, Bray R, Rosen-Bronson S, et al. The detection of donor-directed, HLA-specific alloantibodies in recipients of unrelated hematopoietic cell transplantation is predictive of graft failure. Blood 2010; 115(13): 2704-2708.
19. Steele DJ, Laufer TM, Smiley ST, et al. Two levels of help for B cell alloantibody production. Journal of Experimental.Medicine 1996; 183(2): 699-703.
20. Duquesnoy RJ. HLAMMATCHMAKER: a molecularly based donor selection algorithm for highly alloimmunized patients. Transplantation Proceedings. 2001; 33(1-2): 493-497.
21. Duquesnoy RJ. HLAMatchmaker: a molecularly based algorithm for histocompatibility determination. I. Description of the algorithm. Human Immunology 2002; 67(11): 847-862.
22. Duquesnoy RJ. A structurally based approach to determine HLA compatibility at the humoral immune level. Human Immunology 2006; 67(11): 847-862.
23. Warrens AN, Lombardi G, and Lechler RI. Presentation and recognition of major and minor histocompatibility antigens. Transplant.Immunology 1994; 2(2): 103-107.
24. Burrows SR, Khanna R, Burrows JM, et al. An alloresponse in humans is dominated by cytotoxic T lymphocytes (CTL) cross-reactive with a single Epstein-Barr virus CTL epitope: implications for graft-versus-host disease. Journal of Experimental.Medicine 1994; 179(4): 1155-1161.
25. Burrows SR, Silins SL, Moss DJ, et al. T cell receptor repertoire for a viral epitope in humans is diversified by tolerance to a background major histocompatibility complex antigen. Journal of Experimental.Medicine 1995; 182(6): 1703-1715.
26. Burrows SR, Silins SL, Khanna R, et al. Cross-reactive memory T cells for Epstein-Barr virus augment the alloresponse to common human leukocyte antigens: degenerate recognition of major histocompatibility complex-bound peptide by T cells and its role in alloreactivity. European.Journal of Immunology 1997; 27(7): 1726-1736.

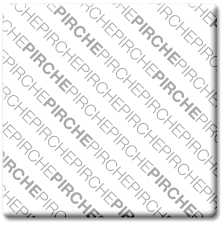
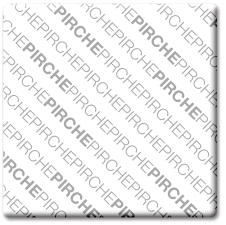
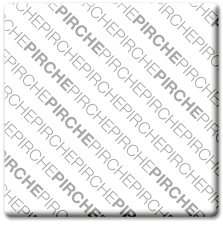
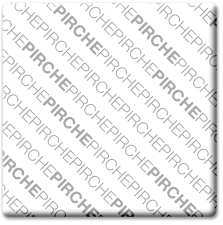
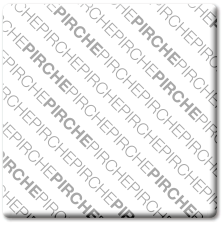
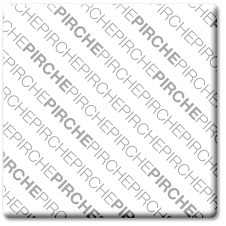
27. Morice A, Charreau B, Neveu B, et al. Cross-reactivity of herpesvirus-specific CD8 T cell lines toward allogeneic class I MHC molecules. PLoS.One. 2010; 5(8): e12120.
28. Landais E, Morice A, Long HM, et al. EBV-specific CD4+ T cell clones exhibit vigorous allogeneic responses. Journal of Immunology 2006; 177(3): 1427-1433.
29. Elkington R and Khanna R. Cross-recognition of human alloantigen by cytomegalovirus glycoprotein-specific CD4+ cytotoxic T lymphocytes: implications for graft-versus-host disease. Blood 2005; 105(3): 1362-1364.
30. Amir AL, van der Steen DM, Hagedoorn RS, et al. Allo-HLA-reactive T cells inducing graft-versus-host disease are single peptide specific. Blood 2011; 118(26): 6733-6742.
31. D'Orsogna LJ, van Besouw MM, van der Meer-Prins EM, et al. Vaccine-induced allo-HLA-reactive memory T cells in a kidney transplantation candidate. Transplantation 2011; 91(6): 645-651.
32. Ali JM, Bolton EM, Bradley JA, et al. Allorecognition pathways in transplant rejection and tolerance. Transplantation 2013; 96(8): 681-688.
33. Game DS and Lechler RI. Pathways of allorecognition: implications for transplantation tolerance. Transplant.Immunology 2002; 10(2-3): 101-108.
34. Bolton EM, Bradley JA, and Pettigrew GJ. Indirect allorecognition: not simple but effective. Transplantation 2008; 85(5): 667-669.
35. Song ES, Linsk R, Olson CA, et al. Allospecific cytotoxic T lymphocytes recognize an H-2 peptide in the context of a murine major histocompatibility complex class I molecule. Proceedings.of the.National.Academy.of Sciences.of the.United.States.of America. 1988; 85(6): 1927-1931.
36. Kievits F and Ivanyi P. A subpopulation of mouse cytotoxic T lymphocytes recognizes allogeneic H-2 class I antigens in the context of other H-2 class I molecules. Journal of Experimental.Medicine 1991; 174(1): 15-19.
37. Fangmann J, Dalchau R, and Fabre JW. Rejection of skin allografts by indirect allorecognition of donor class I major histocompatibility complex peptides. Journal of Experimental.Medicine 1992; 175(6): 1521-1529.
38. Herrera OB, Golshayan D, Tibbott R, et al. A novel pathway of alloantigen presentation by dendritic cells. Journal of Immunology 2004; 173(8): 4828-4837.
39. Morelli AE, Larregina AT, Shufesky WJ, et al. Endocytosis, intracellular sorting, and processing of exosomes by dendritic cells. Blood 2004; 104(10): 3257-3266.
40. Game DS, Rogers NJ, and Lechler RI. Acquisition of HLA-DR and costimulatory molecules by T cells from allogeneic antigen presenting cells. American.Journal of Transplantation 2005; 5(7): 1614-1625.
41. Sivaganesh S, Harper SJ, Conlon TM, et al. Copresentation of intact and processed MHC alloantigen by recipient dendritic cells enables delivery of linked help to alloreactive CD8 T cells by indirect-pathway CD4 T cells. Journal of Immunology 2013; 190(11): 5829-5838.
42. Kim YH, Vyth-Dreese FA, Schrama E, et al. Exogenous Addition of Minor H Antigen HA-1+ Dendritic Cells to Skin Tissues Ex Vivo Causes Infiltration and Activation of HA-1-Specific Cytotoxic T Cells. Biology.of Blood and Marrow Transplantation 2011; 17(1): 69-77.
43. Hönger G, Fornaro I, Granado C, et al. Frequency and determinants of pregnancy-induced child-specific sensitization. American.Journal of Transplantation 2013; 13(3): 746-753.
44. Hyun J, Park KD, Yoo Y, et al. Effects of different sensitization events on HLA alloimmunization in solid organ transplantation patients. Transplantation Proceedings. 2012; 44(1): 222-225.
45. Kakaiya RM, Triulzi DJ, Wright DJ, et al. Prevalence of HLA antibodies in remotely transfused or alloexposed volunteer blood donors. Transfusion 2010; 50(6): 1328-1334.
46. Molares-Buenrostro LE, Terasaki PI, Marino-Vázquez LA, et al. "Natural" human leukocyte antigen antibodies found in nonalloimmunized healthy males. Transplantation 2008; 86(8): 1111-1115.
47. Sicard A, Amrouche L, Suberbielle C, et al. Outcome of kidney transplantations performed with preformed donor-specific antibodies of unknown etiology. American.Journal of Transplantation 2014; 14(1): 193-201.
48. van Kampen CA, Versteeg-vd Voort Maarschalk MF, Langerak-Langerak J, et al. Kinetics of the pregnancy-induced humoral and cellular immune response against the paternal HLA class I antigens of the child. Human Immunology 2002; 63(6): 452-458.
49. Gebel HM, Bray RA, and Nickerson P. Pre-transplant assessment of donor-reactive, HLA-specific antibodies in renal transplantation: contraindication vs. risk. American.Journal of Transplantation 2003; 3(12): 1488-1500.
50. Kao KJ, Scornik JC, and Small SJ. Enzyme-linked immunoassay for anti-HLA antibodies--an alternative to panel studies by lymphocytotoxicity. Transplantation 1993; 55(1): 192-196.
51. Pei R, Wang G, Tarsitani C, et al. Simultaneous HLA Class I and Class II antibodies screening with flow cytometry. Human Immunology 1998; 59(5): 313-322.
52. Patel R and Terasaki PI. Significance of the positive crossmatch test in kidney transplantation. New England.Journal of Medicine 1969; 280(14): 735-739.
53. Le Bas-Bernardet S, Hourmant M, Valentin N, et al. Identification of the antibodies involved in B-cell crossmatch positivity in renal transplantation. Transplantation 2003; 75(4): 477-482.
54. Rebibou JM, Chabod J, Bittencourt MC, et al. Flow-PRA evaluation for antibody screening in patients awaiting kidney transplantation. Transplant.Immunology 2000; 8(2): 125-128.
55. Christiaans MH, Overhof R, ten HA, et al. No

advantage of flow cytometry crossmatch over complement-dependent cytotoxicity in immunologically well-documented renal allograft recipients. Transplantation 1996; 62(9): 1341-1347.
56. Amico P, Hönger G, Steiger J, et al. Utility of the virtual crossmatch in solid organ transplantation. Current.Opinion.in Organ Transplantation 2009; 14(6): 656-661.
57. Bielmann D, Hönger G, Lutz D, et al. Pretransplant risk assessment in renal allograft recipients using virtual crossmatching. American.Journal of Transplantation 2007; 7(3): 626-632.
58. Otten HG, Verhaar MC, Borst HP, et al. Pretransplant donor-specific HLA class-I and -II antibodies are associated with an increased risk for kidney graft failure. American.Journal of Transplantation 2012; 12(6): 1618-1623.
59. Dankers MK, Witvliet MD, Roelen DL, et al. The number of amino acid triplet differences between patient and donor is predictive for the antibody reactivity against mismatched human leukocyte antigens. Transplantation 2004; 77(8): 1236-1239.
60. Kosmoliaptsis V, Bradley JA, Sharples LD, et al. Predicting the immunogenicity of human leukocyte antigen class I alloantigens using structural epitope analysis determined by HLAMatchmaker. Transplantation 2008; 85(12): 1817-1825.
61. Dankers MK, Heemskerk MB, Duquesnoy RJ, et al. HLAMatchmaker algorithm is not a suitable tool to predict the alloreactive cytotoxic T-lymphocyte response in vitro. Transplantation 2004; 78(1): 165-167.
62. Duquesnoy RJ, Spellman S, Haagenson M, et al. HLAMatchmaker-defined triplet matching is not associated with better survival rates of patients with class I HLA allele mismatched hematopoietic cell transplants from unrelated donors. Biology.of Blood and Marrow Transplantation 2008; 14(9): 1064-1071.
63. Duquesnoy RJ, Takemoto S, de LP, et al. HLAmatchmaker: a molecularly based algorithm for histocompatibility determination. III. Effect of matching at the HLA-A,B amino acid triplet level on kidney transplant survival. Transplantation 2003; 75(6): 884-889.
64. Laux G, Mytilineos J, and Opelz G. Critical evaluation of the amino acid triplet-epitope matching concept in cadaver kidney transplantation. Transplantation 2004; 77(6): 902-907.
65. Haririan A, Fagoaga O, Daneshvar H, et al. Predictive value of human leucocyte antigen epitope matching using HLAMatchmaker for graft outcomes in a predominantly African-American renal transplant cohort. Clinical.Transplantation 2006; 20(2): 226-233.
66. Silva E, Alba A, Castro A, et al. Evaluation of HLA Matchmaker compatibility as predictor of graft survival and presence of Anti-HLA antibodies. Transplantation Proceedings. 2010; 42(1): 266-269.
67. Duquesnoy RJ. Clinical usefulness of HLAMatchmaker in HLA epitope matching for organ transplantation. Current.Opinion.in Immunology 2008; 20(5): 594-601.
68. Kosmoliaptsis V, Dafforn TR, Chaudhry AN, et al. High-resolution, three-dimensional modeling of human leukocyte antigen class I structure and surface electrostatic potential reveals the molecular basis for alloantibody binding epitopes. Human Immunology 2011; 72(11): 1049-1059.
69. Kosmoliaptsis V, Chaudhry AN, Sharples LD, et al. Predicting HLA class I alloantigen immunogenicity from the number and physiochemical properties of amino acid polymorphisms. Transplantation 2009; 88(6): 791-798.
70. Kosmoliaptsis V, Sharples LD, Chaudhry AN, et al. Predicting HLA class II alloantigen immunogenicity from the number and physiochemical properties of amino acid polymorphisms. Transplantation 2011; 91(2): 183-190.
71. Rutten CE, van Luxemburg-Heijs SA, Halkes CJ, et al. Patient HLA-DP-specific CD4+ T cells from HLA-DPB1-mismatched donor lymphocyte infusion can induce graft-versus-leukemia reactivity in the presence or absence of graft-versus-host disease. Biology.of Blood and Marrow Transplantation 2013; 19(1): 40-48.
72. Pietra B, Wiseman A, Bolwerk A, et al. CD4 T cell-mediated cardiac allograft rejection requires donor but not host MHC class II. Journal of Clinical.Investigation. 2000; 106(8): 1003-1010.
73. van Emmerik NE, Vaessen LM, Balk AH, et al. Progressive accumulation of CTL with high avidity for donor antigens during the development of acute cardiac rejection. Transplantation 1996; 62(4): 529-536.
74. Kjer-Nielsen L, Clements CS, Purcell AW, et al. A structural basis for the selection of dominant alphabeta T cell receptors in antiviral immunity. Immunity. 2003; 18(1): 53-64.
75. Macdonald WA, Chen Z, Gras S, et al. T cell allorecognition via molecular mimicry. Immunity. 2009; 31(6): 897-908.
76. Venturi V, Price DA, Douek DC, et al. The molecular basis for public T-cell responses? Nature Reviews. Immunology 2008; 8(3): 231-238.
77. Hislop AD, Annels NE, Gudgeon NH, et al. Epitope-specific evolution of human CD8(+) T cell responses from primary to persistent phases of Epstein-Barr virus infection. Journal of Experimental.Medicine 2002; 195(7): 893-905.
78. D'Orsogna LJ, Roelen DL, Doxiadis II, et al. TCR cross-reactivity and allorecognition: new insights into the immunogenetics of allorecognition. Immunogenetics 2012; 64(2): 77-85.
79. Amir AL, D'Orsogna LJ, Roelen DL, et al. Allo-HLA reactivity of virus-specific memory T cells is common. Blood 2010; 115(15): 3146-3157.
80. Colf LA, Bankovich AJ, Hanick NA, et al. How a single T cell receptor recognizes both self and foreign MHC. Cell 2007; 129(1): 135-146.

81. Brunner KT, Mauel J, Cerottini JC, et al. Quantitative assay of the lytic action of immune lymphoid cells on 51-Cr-labelled allogeneic target cells in vitro; inhibition by isoantibody and by drugs. Immunology 1968; 14(2): 181-196.
82. Jeras M. The role of in vitro alloreactive T-cell functional tests in the selection of HLA matched and mismatched haematopoietic stem cell donors. Transplant.Immunology 2002; 10(2-3): 205-214.
83. Roosnek E, Hogendijk S, Zawadynski S, et al. The frequency of pretransplant donor cytotoxic T cell precursors with anti-host specificity predicts survival of patients transplanted with bone marrow from donors other than HLA-identical siblings. Transplantation 1993; 56(3): 691-696.
84. Herzog WR, Zanker B, Irschick E, et al. Selective reduction of donor-specific cytotoxic T lymphocyte precursors in patients with a well-functioning kidney allograft. Transplantation 1987; 43(3): 384-389.
85. Roelen DL, van BE, van Bree SP, et al. The presence of activated donor HLA class I-reactive T lymphocytes is associated with rejection of corneal grafts. Transplantation 1995; 59(7): 1039-1042.
86. Oudshoorn M, Doxiadis II, van den Berg-Loonen PM, et al. Functional versus structural matching: can the CTLp test be replaced by HLA allele typing? Human Immunology 2002; 63(3): 176-184.
87. van Kampen CA, Roelen DL, Versteeg-vd Voort Maarschalk MF, et al. Activated HLA class I-reactive cytotoxic T lymphocytes associated with a positive historical crossmatch predict early graft failure. Transplantation 2002; 74(8): 1114-1119.
88. Jöris MM, van Rood JJ, Roelen DL, et al. A proposed algorithm predictive for cytotoxic T cell alloreactivity. Journal of Immunology 2012; 188(4): 1868-1873.
89. Jöris MM, Lankester AC, von dem Borne PA, et al. Translating in vitro prediction of cytotoxic T cell alloreactivity to hematopoietic stem cell transplantation outcome. Transplant.Immunology 2014; 30(2-3): 59-64.
90. Zino E, Frumento G, Marktel S, et al. A T-cell epitope encoded by a subset of HLA-DPB1 alleles determines nonpermissive mismatches for hematologic stem cell transplantation. Blood 2004; 103(4): 1417-1424.
91. Fleischhauer K, Shaw BE, Gooley T, et al. Effect of T-cell-epitope matching at HLA-DPB1 in recipients of unrelated-donor haemopoietic-cell transplantation: a retrospective study. The.Lancet Oncology 2012; 13(4): 366-374.
92. Fleischhauer K, Locatelli F, Zecca M, et al. Graft rejection after unrelated donor hematopoietic stem cell transplantation for thalassemia is associated with nonpermissive HLA-DPB1 disparity in host-versus-graft direction. Blood 2006; 107(7): 2984-2992.
93. Elsner HA, DeLuca D, Strub J, et al. HistoCheck: rating of HLA class I and II mismatches by an internet-based software tool. Bone Marrow Transplantation 2004; 33(2): 165-169.
94. Shaw BE, Barber LD, Madrigal JA, et al. Scoring for HLA matching? A clinical test of HistoCheck. Bone Marrow Transplant. 2004; 34(4): 367-368.
95. Askar M, Sobecks R, Morishima Y, et al. Predictions in the face of clinical reality: HistoCheck versus high-risk HLA allele mismatch combinations responsible for severe acute graft-versus-host disease. Biology.of Blood and Marrow Transplantation 2011; 17(9): 1409-1415.
96. Spellman S, Klein J, Haagenson M, et al. Scoring HLA class I mismatches by HistoCheck does not predict clinical outcome in unrelated hematopoietic stem cell transplantation. Biology.of Blood and Marrow Transplantation 2012; 18(5): 739-746.
97. Pidala J, Wang T, Haagenson M, et al. Amino acid substitution at peptide-binding pockets of HLA class I molecules increases risk of severe acute GVHD and mortality. Blood 2013; 122(22): 3651-3658.
98. Ferrara GB, Bacigalupo A, Lamparelli T, et al. Bone marrow transplantation from unrelated donors: the impact of mismatches with substitutions at position 116 of the human leukocyte antigen class I heavy chain. Blood 2001; 98(10): 3150-3155.
99. Peptide database. SYFPEITHI 2013. Available from: www.syfpeithi.de
100. Suciu-Foca N, Ciubotariu R, Itescu S, et al. Indirect allorecognition of donor HLA-DR peptides in chronic rejection of heart allografts. Transplantation Proceedings. 1998; 30(8): 3999-4000.
101. Liu Z, Colovai AI, Tugulea S, et al. Indirect recognition of donor HLA-DR peptides in organ allograft rejection. Journal of Clinical.Investigation. 1996; 98(5): 1150-1157.
102. Ciubotariu R, Liu Z, Colovai AI, et al. Persistent allopeptide reactivity and epitope spreading in chronic rejection of organ allografts. Journal of Clinical.Investigation. 1998; 101(2): 398-405.
103. Hornick PI, Mason PD, Baker RJ, et al. Significant frequencies of T cells with indirect anti-donor specificity in heart graft recipients with chronic rejection. Circulation 2000; 101(20): 2405-2410.
104. Afzali B, Lechler RI, and Hernandez-Fuentes MP. Allorecognition and the alloresponse: clinical implications. Tissue Antigens 2007; 69(6): 545-556.
105. Otten HG, Calis JJ, Kesmir C, et al. Predicted indirectly recognizable HLA epitopes presented by HLA-DR correlate with the de novo development of donor-specific HLA IgG antibodies after kidney transplantation. Human Immunology 2013; 74(3): 290-296.
106. Zhang L, Udaka K, Mamitsuka H, et al. Toward more accurate pan-specific MHC-peptide binding prediction: a review of current methods and tools. Briefings.in Bioinformatics. 2012; 13(3): 350-364.
107. Nielsen M, Lundegaard C, Lund O, et al. The role of the proteasome in generating cytotoxic T-cell epitopes: insights obtained from improved predictions of proteasomal cleavage. Immunogenetics 2005; 57(1-2): 33-41.

108. Hoof I, Peters B, Sidney J, et al. NetMHCpan, a method for MHC class I binding prediction beyond humans. Immunogenetics 2009; 61(1): 1-13.
109. Nielsen M, Lundegaard C, Blicher T, et al. NetMHCpan, a method for quantitative predictions of peptide binding to any HLA-A and -B locus protein of known sequence. PLoS.One. 2007; 2(8): e796.
110. Nielsen M and Lund O. NN-align. An artificial neural network-based alignment algorithm for MHC class II peptide binding prediction. BMC.Bioinformatics. 2009; 10: 296.
111. Nielsen M, Lundegaard C, and Lund O. Prediction of MHC class II binding affinity using SMM-align, a novel stabilization matrix alignment method. BMC.Bioinformatics. 2007; 8.
112. Nielsen M, Justesen S, Lund O, et al. NetMHCIIpan-2.0 - Improved pan-specific HLA-DR predictions using a novel concurrent alignment and weight optimization training procedure. Immunome.Research. 2010; 6(1).
113. Brusic V, Rudy G, Honeyman G, et al. Prediction of MHC class II-binding peptides using an evolutionary algorithm and artificial neural network. Bioinformatics. 1998; 14(2): 121-130.
114. Parker KC, Bednarek MA, and Coligan JE. Scheme for ranking potential HLA-A2 binding peptides based on independent binding of individual peptide side-chains. Journal of Immunology 1994; 152(1): 163-175.
115. Rammensee H, Bachmann J, Emmerich NA, et al. SYFPEITHI: database for MHC ligands and peptide motifs. Immunogenetics 1999; 50(3-4): 213-219.
116. Nussbaum AK, Kuttler C, Hadeler KP, et al. PAProC: a prediction algorithm for proteasomal cleavages available on the WWW. Immunogenetics 2001; 53(2): 87-94.
117. Kesmir C, Nussbaum AK, Schild H, et al. Prediction of proteasome cleavage motifs by neural networks. Protein Engineering. 2002; 15(4): 287-296.
118. Peters B, Bulik S, Tampe R, et al. Identifying MHC class I epitopes by predicting the TAP transport efficiency of epitope precursors. Journal of Immunology 2003; 171(4): 1741-1749.
119. Buus S, Lauemøller SL, Worning P, et al. Sensitive quantitative predictions of peptide-MHC binding by a 'Query by Committee' artificial neural network approach. Tissue Antigens 2003; 62(5): 378-384.
120. Zhang GL, Petrovsky N, Kwoh CK, et al. PRED(TAP): a system for prediction of peptide binding to the human transporter associated with antigen processing. Immunome.Research. 2006; 2.
121. Chen P, Rayner S, and Hu KH. Advances of bioinformatics tools applied in virus epitopes prediction. Virologica.Sinica. 2011; 26(1): 1-7.
122. Ferrara JL, Levine JE, Reddy P, et al. Graft-versus-host disease. The.Lancet 2009; 373(9674): 1550-1561.
123. Sant'Angelo DB, Robinson E, Janeway CA, et al. Recognition of core and flanking amino acids of MHC class II-bound peptides by the T cell receptor. European. Journal of Immunology 2002; 32(9): 2510-2520.
124. Rudolph MG, Stanfield RL, and Wilson IA. How TCRs bind MHCs, peptides, and coreceptors. Annual. Review of Immunology 2006; 24: 419-466.




3


Chapter 3

Identifying permissible HLA-mismatches: Predicted Indirectly ReCognizable HLA Epitopes

Kirsten A. Thus,[1] Hanneke W. M. van Deutekom,[2] Jorg J. A. Calis,[2] Eric Borst,[1] Can Keşmir,[2] Machteld Oudshoorn,[3] Bronno van der Holt,[4] Jan Cornelissen,[5] Jürgen Kuball,[1,6] and Eric Spierings[1]

[1]Laboratory of Translational Immunology,
University Medical Center Utrecht, Utrecht, the Netherlands
[2]Department of Theoretical Biology and Bioinformatics,
Utrecht University, Utrecht, the Netherlands
[3]Europdonor Foundation and Department of Immunohematology and Blood Transfusion,
Leiden University Medical Center, Leiden, the Netherlands
[4]HOVON Data Center,
Erasmus MC Cancer Institute-Clinical Trial Center, Rotterdam, the Netherlands
[5]Department of Hematology,
Erasmus MC-Daniel Den Hoed Cancer Center, Rotterdam, the Netherlands
[6]Department of Hematology,
University Medical Center Utrecht, Utrecht, the Netherlands

Abstract

HLA-mismatches in hematopoietic stem-cell transplantation (HSCT) correlate with impaired overall survival (OS). We aimed at identifying permissible HLA-mismatches. To this end, we analyzed a novel computational method that identifies Predicted Indirectly ReCognizable HLA-Epitopes (PIRCHE). PIRCHE are predicted peptides derived from the patient's mismatched-HLA molecules that can be presented by donor-patient shared HLA. We studied the correlation between numbers of PIRCHE and clinical outcome, relative to HLA-matched HSCT. A Dutch multicenter cohort of 685 patients was retrospectively studied. PIRCHE were determined either presented on HLA class I (PIRCHE-I) or class II (PIRCHE-II) antigens for patients receiving a single HLA-mismatched unrelated donor transplant (N=249), using HLA-A, -B, -C, -DRB1 and -DQB1 high-resolution typings. Patients were divided into three equal-sized groups according to their PIRCHE score (low, medium and high). The clinical outcome of these groups was evaluated and compared to a reference group of HLA-matched transplantations (N=436). The primary endpoints studied were OS and non-relapse mortality (NRM).

Patients with low PIRCHE-I and -II had comparable OS (HR 1.33, 95%-CI 0.92-1.94, p=0.13) and NRM (HR 0.90, 95%-CI 0.54-1.51, p=0.69) as patients transplanted with an HLA-matched donor. Patients with medium or high PIRCHE-I or -II had significantly increased risks of mortality (HR 1.44, 95%-CI 1.09-1.86, p=0.009) and NRM (HR 1.47, 95%-CI 1.06-2.02, p=0.02). Moreover, PIRCHE also dissected HLA mismatches into those with a significantly increased or comparable risk of progression-free survival and acute graft-versus-host disease as HLA-matched transplantations, whereas there were no differences in progression risk between the PIRCHE groups. Single HLA-mismatched transplantations with low PIRCHE have similar OS and NRM as HLA-matched transplantations, whereas the risk of mortality is significantly increased for HLA-mismatched transplantations with higher PIRCHE. PIRCHE inclusion in the donor-selection criteria may lead to the identification of the best permissible HLA-mismatched donor.

Introduction

HLA mismatches are the most important risk factor for detrimental outcome following allogeneic hematopoietic stem-cell transplantation (HSCT) (1). The level of HLA typing has evolved greatly over the past decades (2): from a serological level, analyzing HLA expression on the cell surface of lymphocytes, to DNA-based methods. Consequently, donor and patient are currently being matched on allele-level HLA typing. Transplantation with an HLA-A, -B, -C, -DRB1 allele-level matched unrelated donor (8/8 MUD) led to a significantly better survival compared to transplantation with a donor that was mismatched for one of these loci (7/8) (3, 4). Although HLA-DQ has been defined as a classical transplantation antigen as well (5, 6), more recent studies showed that HLA-DQ mismatches only confer a risk when they are an additive mismatch in the 7/8-matched situation (3, 7). As HLA-DQ mismatching may be associated to impaired outcome (8), donor-selection criteria are often still based on allele-level matching for HLA-A, -B, -C, -DRB1, -DQB1 (10/10) (9), and any mismatch on either one of these loci is usually avoided.

HLA-compatible MUDs are not available for up to 84% of the cases, depending on the ethnicity of the patient (10). Therefore, there is a need to identify permissible HLA mismatches. Such permissible HLA mismatches have been identified to some extent in large epidemiological studies: transplantation with these mismatches resulted in similar clinical outcome as HLA-matched transplantations (11, 12). However, with 8654 HLA protein variants identified today (IMGT database, accessible via: www.imgt.org, accession date 12-12-2014), epidemiologically identifying all permissible HLA mismatches is a challenging and hardly possible task.

In silico models that assess the permissibility of HLA-mismatches may facilitate universal selection criteria of HLA-mismatched donors. Such *in silico* models can be constructed based on the concept that HLA mismatches are recognized by the donor T cell, and that this T-cell recognition results in a detrimental outcome (13). Mismatched HLA can be recognized by the donor T cell in a direct or indirect manner (14). During direct recognition, the donor T cell recognizes the intact mismatched HLA molecule on the cell surface of the patient's cells, whereas during indirect recognition, T cells recognize peptides derived from the mismatched HLA that are presented by a shared-HLA molecule. So far, *in silico* models aiming at predicting mismatched HLA-directed alloreactivity, focused on direct recognition of HLA disparities (15, 16). None of the investigated direct recognition models correlated with clinical alloreactivity (17-19).

Indirect recognition may be better predictable than direct recognition. To facilitate the identification of indirectly recognizable HLA epitopes, we have developed a novel computational strategy based on well-validated prediction tools (20-24). The potentially presented HLA-derived epitopes predicted by our method are designated as "Predicted Indirectly ReCognizable HLA Epitopes" (PIRCHE). The PIRCHE method classifies PIRCHE presented either by donor-patient shared-HLA class I (PIRCHE-I) or class II (PIRCHE-II), thus independent from the origin of the HLA mismatch. Theoretically, PIRCHE-I lead to CD8+ T-cell responses and PIRCHE-II to CD4+ T-cell responses. Two previous small cohort studies have shown that PIRCHE-I and -II numbers correlate with acute graft-versus-host disease (GVHD) development (25, 26).

For the patients for whom an HLA-compatible MUD is not available, clinicians face the difficult decision of selecting an HLA-mismatched donor, which can lead to impaired survival. Being able to identify permissible HLA-mismatches leading to comparable clinical

outcomes as HLA-matched HSCT, would provide a major step forward for these patients. Therefore, the aim of this study was to investigate whether in 9/10 transplantations, PIRCHE can provide the possibility of identifying permissible HLA mismatches that lead to similar overall survival (OS) and non-relapse mortality (NRM) as 10/10-matched HSCT.

Methods

Study population

The cohort consisted of 685 patients transplanted for malignant diseases with MUDs at 8 Dutch transplant centers from 1989 to 2011. The mean age was 39 years (standard deviation: 19 years), and median follow-up of the patients still alive was 6 years (range 0-20 years). Patients were transplanted for various diseases: 456 (67%) for acute leukemia, 103 (15%) for lymphoma; 73 (11%) for chronic leukemia; and 53 (8%) for other diseases. The majority of 315 (46%) patients was transplanted in early disease stage; 229 (33%) in intermediate; and 100 (15%) in advanced stages. Of the 685 patients, 249 (36%) were transplanted with a 9/10 match, and 436 (64%) were transplanted with a 10/10 match. Table 1 shows the patient, donor and transplant characteristics according to match status. The indication for HSCT, the usage of anti-thymocyte globulin (ATG) and the sex-match status differed significantly between the 9/10 and 10/10 group. Clinical data were collected according to EBMT guidelines (accessible via: www.ebmt.org). Unambiguous allele-level resolution HLA-A, -B, -C, -DRB1 and -DQB1 typing was available for all patients and donors.

Identification of PIRCHE

PIRCHE were identified for each donor-patient pair, as described previously (25, 26). In short, PIRCHE-I were identified in two steps. First, proteasome-mediated cleavage and transportation via the TAP channel were predicted for all donor and patient HLA molecules using NetChop C-term 3.0 (20, 21). Subsequently, peptides were tested for their binding capacity to the HLA-A, -B, and -C molecules that were shared between the donor and patient, using NetMHCpan 2.4 (22, 23). Only peptides with IC50-binding values ≤500nM were accepted as relevant binders. For PIRCHE-II, the nonameric binding cores of potential 15-meric HLA-DRB1 binders were predicted with NetMHCIIPan 2.0 (27, 28), considering IC50-binding values ≤1000nM as being relevant. Only unique patient-derived peptide-HLA complexes were classified as being a PIRCHE. The PIRCHE prediction algorithm is accessible via: www.pirche.org. The exact HLA mismatches and their PIRCHE scores have been listed in Table S1.

For the determination of PIRCHE, complete sequences of exon 1-8 for HLA class I and exon 1-6 for HLA class II are required. For most HLA alleles in our cohort, these complete sequences were available via the IMGT database (accessible via: www.imgt.org). To improve our PIRCHE determinations, protein sequences of HLA alleles that were not completely documented in the IMGT database, were estimated based on a nearest neighbor principle. In this nearest neighbor extrapolation, incomplete protein sequences were completed based upon the complete sequences from the HLA allele with the highest homology score for the documented partial protein sequences.

Definitions

The primary endpoints were OS, defined as time from HSCT to death due to any cause; and NRM, defined as mortality without previous progression of the primary malignancy, treating

	10/10, N(%)	9/10, N(%)	p
Number of patients	436 (64)	249 (36)	
Age at HSCT, mean (SD)	40 (20)	38 (19)	0.24
Diagnosis			
Acute leukemia	303 (70)	153 (61)	**0.01**
Chronic leukemia	49 (11)	24 (10)	
Lymphoma	60 (14)	43 (17)	
Other	24 (6)	29 (12)	
Patient sex			
Male	260 (60)	151 (61)	0.80
Female	176 (40)	98 (39)	
Sex mismatch			
Yes	59 (14)	55 (22)	**<0.01**
No	370 (86)	192 (78)	
HSCT year			
1989-1996	26 (6)	15 (6)	0.38
1997-2004	121 (28)	57 (23)	
2004-2011	290 (67)	177 (71)	
Source			
BM	143 (33)	76 (31)	0.54
PBSC	293 (67)	173 (70)	
Conditioning			
MA	227 (52)	127 (51)	0.78
RIC	207 (48)	121 (49)	
ATG			
Yes	232 (53)	179 (72)	**<0.01**
No	204 (47)	70 (28)	
Disease status			
Early	212 (51)	103 (45)	0.32
Intermediate	140 (34)	89 (39)	
Late	63 (15)	37 (16)	
CMV mismatch			
Yes	167 (39)	95 (40)	0.91
No	258 (61)	144 (60)	
EBMT risk score			
1	33 (8)	18 (8)	0.05
2	46 (11)	32 (14)	
3	111 (27)	53 (23)	
4	107 (26)	44 (19)	
5	79 (19)	47 (21)	
6 or 7	32 (8)	33 (15)	

Table 1: baseline characteristics of 10/10 and 9/10 groups. Differences between the 10/10 and 9/10 group were tested with chi-square for categorical variables and student's T test for the continuous variable age. Patients receiving a 9/10 match were significantly more often transplanted because of a myeloproliferative disease, and received significantly more often a female-male transplantation and ATG in their conditioning regimen compared to patients receiving a 10/10 match. There was a trend for a higher EBMT risk classification in patients receiving a 9/10 match compared to a 10/10 match. HSCT: hematopoietic stem cell transplantation. Acute leukemia: acute myeloid leukemia (41%); acute lymphoblastic leukemia (26%); myelodysplastic syndrome (24%); other (8%). Chronic leukemia: chronic myeloid leukemia (100%). Lymphoma: non-Hogdkin (64%), chronic lymphocytic leukemia (26%); Hodgkin (10%). Other: multiple myeloma (57%); myeloproliferative neoplasia (43%). Sex mismatch: female donor for male patient. BM: bone marrow. PBSC: peripheral blood stem cells. MA: myeloablative. RIC: reduced intensity conditioning. ATG: anti-thymocyte globulin. CMV mismatch: patient seropositive, donor seronegative or patient seronegative, donor seropositive.

progression as a competing risk. Secondary endpoints were: progression-free survival (PFS), defined as time from HSCT to progression or death, whichever came first; acute GVHD; and progression. Grading of acute GVHD was defined according to international consensus criteria (29), treating progression and NRM as competing risks. For progression of the primary malignancy, NRM was treated as a competing risk.

Statistical analysis

Kaplan-Meier analysis and log-rank tests were performed to univariately analyze the effect of PIRCHE on OS. Multivariate Cox proportional hazard models were carried out to study time-dependent effects on OS and PFS. Models were checked for proportional hazards assumption and no violations were found. A center effect was adjusted using a gamma-frailty term. Competing risk analysis were performed for NRM, acute GVHD and progression. Statistical models evaluated the following clinical predictors for inclusion: patient age (continuous), disease stage (early, intermediate, advanced), time to transplantation (continuous), donor-patient sex-match status, patient KIR ligand status (8), patient and donor CMV status, year of transplantation (in three groups), conditioning regimen intensity (myeloablative versus reduced intensity), stem-cell source (bone marrow versus peripheral blood stem cells), and GVHD prophylaxis with ATG. Stratification was used to account for heterogeneity of diagnosis. A p-value below 0.05 was considered statistically significant. Statistical procedures were performed with SPSS version 20.0 (SPSS Inc, Chicago, IL, USA), apart from the time-dependent analyses that were performed with R version 3.1.2 (R Foundation for Statistical Computing, Vienna, Austria).

Results

Patient and donor characteristics

The 249 patients transplanted with a 9/10-MUD had significantly impaired OS compared to the 436 patients transplanted with a 10/10-MUD (HR 0.71, 95%-CI 0.56-0.91, p<0.001). This poorer OS is mainly due to an increased probability of NRM for the 9/10 compared to the 10/10 group (HR 1.30, 95%-CI 0.96-1.75, p=0.09, Figure 1A). This risk of NRM related to HLA-mismatches is likely the result of alloreactivity. To study whether the numbers of PIRCHE could dissect the 9/10 group into less or more alloreactive mismatches (*i.e.* permissible and non-permissible mismatches), the numbers of PIRCHE-I and -II were predicted for all patient-donor pairs. The numbers of PIRCHE-I and -II ranged from 0 to 17 and 0 to 48, respectively. Theoretically, a higher number of PIRCHE leads to an increased probability of T-cell recognition. The effect of higher numbers of PIRCHE on alloreactivity is likely not linear and, moreover, PIRCHE numbers were not normally distributed. Therefore, to enable studying a potential dose-dependent effect of PIRCHE, patients were divided in three equal-sized groups based on the distribution of PIRCHE: presenting low, medium or high PIRCHE, as listed in Table 2. The NRM rates of these groups were compared using the low PIRCHE group as a reference group.

Primary outcome: effect of PIRCHE on non-relapse mortality

Within the 9/10 group, both the PIRCHE-I as well as PIRCHE-II groups were associated to NRM risk (p=0.06, p=0.02, for PIRCHE-I and -II, respectively; Table 3). Theoretically, low PIRCHE-I or -II result in the lowest risk of alloreactivity. We therefore studied the risk of NRM for medium and high PIRCHE relative to the low PIRCHE group. High PIRCHE-I had

higher risks of NRM compared to low PIRCHE-I (HR 1.80, 95%-CI 0.97-3.34, p=0.06), for medium PIRCHE-I a similar trend was observed (HR 1.59, 95%-CI 0.89-2.85, p=0.12). High PIRCHE-II had a significantly increased risk of NRM compared to low PIRCHE-II (HR 1.94, 95%-CI 1.10-3.43, p=0.02), again followed by a similar trend for medium PIRCHE-II (HR 1.54, 95%-CI 0.85-2.80, p=0.16). Thus, both PIRCHE-I and -II appear correlated to the risk of NRM in 9/10 transplantations.

To study the risk of PIRCHE-I and -II relative to the HLA-compatible group, the NRM rates of the PIRCHE groups were compared to the 10/10 group. Patients with low PIRCHE-I or -II had similar NRM incidences as 10/10 transplantations, whereas there was an increased risk of NRM for patients with medium or high numbers of PIRCHE-I or -II (Figure 1B, C). Moreover, patients with low PIRCHE-I and -II had similar hazards of NRM as 10/10 transplantations, whereas medium and high PIRCHE-I and medium PIRCHE-II had a trend for an increased hazard of NRM, and high PIRCHE-II had significantly increased NRM (HR 1.64, 95%-CI 1.09-2.47, p=0.02; Table 3). Thus, low PIRCHE-I and -II had consistently similar NRM as 10/10 transplantations, whereas there were trends for increased probabilities of NRM in patients in the medium PIRCHE-I and -II groups and significantly increased risks in the high PIRCHE-I and -II groups.

Table 2: PIRCHE distribution of the 9/10 group.

PIRCHE groups		Distribution	
		N	%
PIRCHE-I low	0	88	35
PIRCHE-I medium	1-4	80	32
PIRCHE-I high	5-17	81	33
PIRCHE-II low	0-2	88	35
PIRCHE-II medium	3-13	77	31
PIRCHE-II high	14-48	84	34

PIRCHE groups	Within 9/10 group			Compared to 10/10 HSCT		
	HR	95%-CI	p	HR	95%-CI	p
10/10				1 (ref)		**0.03**
PIRCHE-I low	1 (ref)		0.06	0.97	0.60-1.57	0.90
PIRCHE-I medium	1.59	0.89-2-85	0.12	1.43	0.95-2.17	0.09
PIRCHE-I high	1.80	0.97-3.34	0.06	1.51	0.98-2.33	0.06
10/10				1 (ref)		**0.02**
PIRCHE-II low	1 (ref)		**0.02**	0.92	0.58-1.46	0.73
PIRCHE-II medium	1.54	0.85-2.80	0.16	1.39	0.88-2.20	0.16
PIRCHE-II high	1.94	1.10-3.43	**0.02**	1.64	1.09-2.47	**0.02**

Table 3: Hazard ratios of NRM for PIRCHE tertiles compared to low PIRCHE or a 10/10 match. Patients in the PIRCHE-I and -II high and medium groups had higher risks of non-relapse mortality compared to low PIRCHE-I and -II and 10/10-matched transplantations, whereas patients in the PIRCHE-I and -II low groups have similar non-relapse mortality as 10/10-matched transplantations. NRM: non-relapse mortality. HR: hazard ratio. 95%-CI: 95%-confidence interval of hazard ratio. Multivariate models included for 9/10 group: KIR ligand status (only for PIRCHE-I), CMV serostatus of the patient, year of transplantation, conditioning regimen intensity, patient age; for the overall group: conditioning regimen intensity, patient age. All models included stratification to correct for heterogeneity in diagnoses.

PIRCHE-I and -II determine permissibility of mismatches

Since both PIRCHE-I and -II were associated to NRM, we hypothesized that the combination of PIRCHE-I and -II may be a more accurate marker to assess the permissibility of HLA mismatches, as it potentially reflects the net effect of both, HLA class-I and -II presented epitopes. As we aimed to determine which HLA mismatches result in similar outcome as HLA matches, only HLA mismatches generating both low PIRCHE-I and -II were considered permissible (N=76), whereas mismatches resulting in medium or high (higher) PIRCHE-I or -II were designated as non-permissible (N=173). Patients with a permissible PIRCHE mismatch received less frequent ATG and more often a CMV-matched donor compared to patients with a non-permissible PIRCHE mismatch (Table 4). Both ATG and CMV status of patient and donor were not significantly associated to the tested clinical outcomes, and therefore these uneven distributions have likely not impacted our results.

Indeed, patients with a permissible PIRCHE mismatch demonstrated similar OS and NRM as patients after 10/10-matched HSCT, whereas patients with a non-permissible mismatch had a significantly increased hazard of overall mortality (HR 1.44, 95%-CI 1.09-1.86, p<0.01), and an increased risk of NRM (HR 1.47, 95%-CI 1.06-2.02, p=0.02, Figure 1D, E). To summarize, permissible PIRCHE mismatches, defined as low PIRCHE-I and -II, had a similar survival rate as those transplanted with a 10/10-matched donor; in contrast to non-permissible PIRCHE mismatches, who had a significantly increased probability of overall and non-relapse mortality.

Secondary endpoints

Additionally, the effects of permissible or non-permissible PIRCHE mismatches on the secondary endpoints PFS, acute GVHD and progression were studied (Figure 1E). Patients with a permissible mismatch had a similar probability of PFS as 10/10-matched HSCT, whereas patients with a non-permissible mismatch had impaired PFS (HR 1.38, 95%-CI 1.06-1.80, p=0.02). Moreover, patients with a permissible PIRCHE mismatch did not have an increased probability of acute GVHD compared to 10/10 transplantations, whereas patients with a non-permissible PIRCHE mismatch had (HR 1.71, 95%-CI 1.23-2.39, p<0.01; HR 1.84, 95%-CI 1.04-3.27, p=0.04; for grade II-IV and III-IV, respectively). Progression rates were comparable amongst all three groups (10/10, permissible and non-permissible mismatches). In conclusion, patients with higher PIRCHE-I or -II had a significantly increased probability of alloreactivity compared to 10/10 transplantations, as reflected by increased risks of acute GVHD and NRM, resulting in impaired PFS and OS. Patients with low PIRCHE-I and -II had comparable clinical outcomes as patients transplanted with a 10/10-MUD.

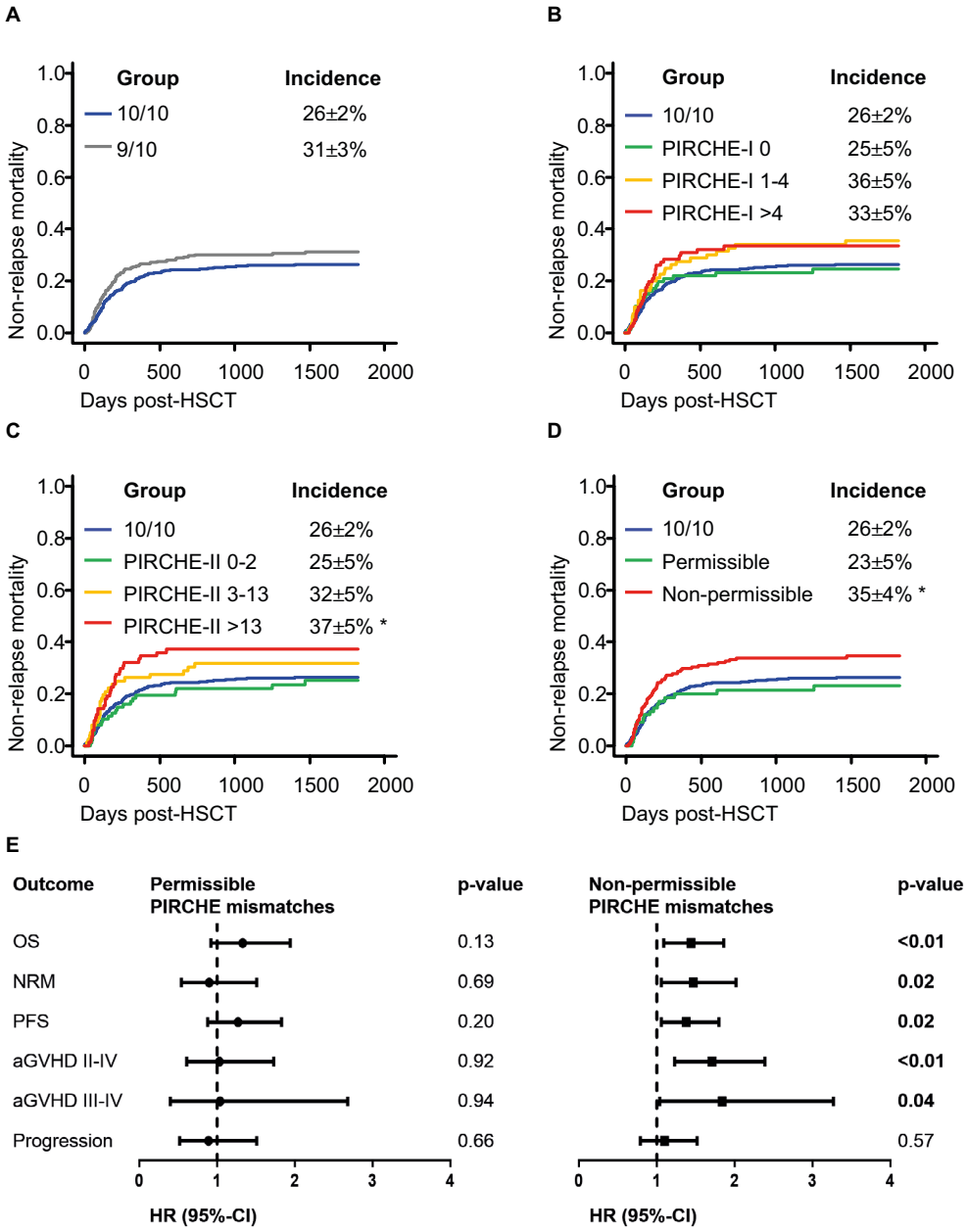


Figure 1: Non-relapse mortality incidence curve for (A) 9/10 versus 10/10 transplantations; (B) PIRCHE-I groups versus 10/10; (C) PIRCHE-II groups versus 10/10; (D) Permissible and non-permissible PIRCHE mismatches versus 10/10; and (E) hazard ratios of permissible and non-permissible PIRCHE mismatches. Patients receiving a 9/10-MUD had higher non-relapse mortality incidences compared to patients receiving a 10/10-MUD. Patients in the PIRCHE-I and -II low groups had comparable non-relapse mortality as patients receiving a 10/10-MUD. Patients in the PIRCHE-I and -II medium and high groups had higher non-relapse mortality incidences than the 10/10 group. Since PIRCHE-I and -II low had similar clinical outcome as the 10/10 group, they were combined into one group of permissible PIRCHE mismatches. Mismatches resulting in higher PIRCHE-I or -II were regarded non-permissible.

Figure 1 (continued): In multivariate analyses, patients with a permissible PIRCHE mismatch had comparable clinical outcomes as those receiving a 10/10-MUD (indicated with a HR of 1, the dotted line), whereas patients with a non-permissible PIRCHE mismatch had significantly increased risks of detrimental outcome, apart from relapse. In the non-relapse mortality curves, cumulative incidences are stated ± standard error. *: Gray-test p<0.05. OS: overall survival. PFS: progression-free survival. NRM: non-relapse mortality. aGVHD: acute graft-versus-host disease. Permissible PIRCHE mismatch: PIRCHE-I and -II low. Non-permissible PIRCHE mismatch: PIRCHE-I or -II higher. HR: hazard ratio. 95%-CI: 95%-confidence interval of hazard ratio. Multivariate models included for OS: year of transplantation, disease status prior to transplantation, patient age, KIR ligand status; for NRM: conditioning regimen intensity, patient age; for PFS: year of transplantation, patient age, disease status prior to transplantation, KIR ligand status; acute GVHD grade II-IV: peripheral blood stem cells as a stem cell source; acute GVHD grade III-IV: peripheral blood stem cells as a stem cell source, conditioning regimen intensity, donor CMV status; relapse: year of transplantation, conditioning regimen intensity, disease status prior to transplantation. Cox proportional hazard models (OS, PFS) included a gamma frailty term to correct for center effects and all models included stratification to correct for heterogeneity in diagnoses.

Discussion

The aim of this study was to investigate whether permissible HLA mismatches can be identified by the PIRCHE model. In this large multi-center cohort, we demonstrate that 9/10-MUD HSCT with low PIRCHE-I and -II is associated to clinical outcomes comparable to those observed after 10/10-MUD HSCT. Conversely, 9/10-MUD HSCT with higher PIRCHE-I or -II is associated with detrimental clinical outcomes when compared to 10/10-matched transplantations. Meanwhile, low PIRCHE-I and -II are not associated with progression. The present findings may provide an important improvement in current donor-selection procedures. To date, there are no clear guidelines available for selection of HLA-mismatched donors. The PIRCHE model potentially allows for universally and easily applicable selection criteria for permissible HLA mismatches that lead to improved OS and reduced NRM. Thus, PIRCHE may facilitate *a priori* selection of HLA-mismatched donors with a permissible mismatch.

PIRCHE did not generate a dose-dependent risk classification in this study. As PIRCHE theoretically predict the probability of donor T-cell recognition, high PIRCHE may lead to a higher chance of T-cell recognition and thus more detrimental outcomes than medium PIRCHE, likewise for medium and low PIRCHE. Although a trend for a dose-dependent effect was observed (Table 3), the size of the present cohort did not allow more detailed studies on multiple risk classifications based on PIRCHE numbers. Larger HLA-mismatched cohorts are required to study the potential dose-dependent effect of PIRCHE, and to further define potential cut-off values of PIRCHE numbers for donor-selection purposes.

The predictive potential of our approach will likely benefit from future improvements of HLA-binding algorithms. These improvements might focus on developing reliable predictions of presentation for HLA-DQ proteins. The present PIRCHE model only analyses binding to HLA-DR, as reliable binding predictions are not available for many HLA-DQ proteins. Moreover, whereas HLA-DR proteins consist of a non-polymorphic α-chain and a polymorphic β-chain, HLA-DQ proteins consist of both a polymorphic α- and β-chain (30). Consequently, to properly assess binding to HLA-DQ proteins, HLA-DQA typing should be included in the typing procedure. Thus, before the PIRCHE predictions can be expanded to HLA-DQ binding, the binding algorithms and the current typing procedures have to be broadened.

HLA-C mismatches lead to a poorly understood disproportional increased risk of alloreactivity (4); whereas the relevance of HLA-DQB1 matching for outcome of HSCT is debatable (3). In our study, both HLA-C and HLA-DQB1 mismatches were included, as they are considered in the donor-selection procedure of all participating centers. Interestingly, HLA-A, -C,

and -DQB1 mismatches led to the highest numbers of both PIRCHE-I and -II in this cohort (Table S1). These high numbers of PIRCHE derived from HLA-C may explain the increased risk of alloreactivity by HLA-C mismatches (25). Research in larger HLA-C mismatched cohorts is required to confirm this hypothesis. In addition, we hypothesize that the discrepant reports on HLA-DQB1 mismatching and HSCT outcome, might be influenced by variable numbers of HLA-DQB1 derived PIRCHE in the individual studies. A meaningful suggestion for future studies would therefore be to compare the number of PIRCHE derived from all HLA mismatches and correlate these to clinical outcome, instead of analyzing locus-specific HLA mismatches that may or may not correlate with alloreactivity.

To the best of our knowledge, our study is the first to present a universal approach for the prediction of permissible HLA mismatches that does correlate with improved OS (reviewed in (14)). Large cohort studies have identified some specific permissible HLA-mismatch combinations (11, 12); however the data obtained from these studies cannot be translated to donor-patient HLA combinations that were not or hardly present in these studies. Interestingly, one of these well characterized permissible HLA mismatches (HLA-C*03:03-*03:04), led to a low number of PIRCHE in the present study (PIRCHE-I: median 0, range 0-2; PIRCHE-II: median 0, range 0-4; Table S1). Thus, the previously identified permissible HLA mismatches, may (partly) be permissible due to a low number of PIRCHE.

In summary, our study demonstrates that PIRCHE correlate with clinical alloreactivity. In particular, patients presenting 0 PIRCHE-I and less than 3 PIRCHE-II have similar clinical outcomes as patients transplanted with an HLA-compatible unrelated donor. Moreover, determining the number of PIRCHE for potential donors prior to HSCT may allow reduction of mortality after HLA-mismatched HSCT by avoiding donors that can recognize higher numbers of PIRCHE. The present results indicate that selection of HLA-mismatched donors with permissible mismatches may be possible, and warrant prospective evaluation of large HLA-mismatched cohorts.

Declaration of interests

The University Medical Center Utrecht has filed a patent application on the prediction of an alloimmune response against mismatched HLA. ES has been listed as inventor on this patent application. The other authors have no personal conflict of interest to declare.

Acknowledgements

Grateful acknowledgement is made to all the clinicians and HLA laboratories that provided clinical data. We also thank dr. Louis Bont and dr. Andries Bloem for critically reading the manuscript. We further acknowledge support from our funding agency: The Netherlands Organization for Scientific Research, Computational Life Sciences Program (grant number 635.100.025) to CK and JJC.

	Permissible mismatch, N(%)	Non-permissible mismatch, N(%)	p
Number of patients	76 (32)	173 (69)	
Age at HSCT, mean (SD)	38 (19)	39 (19)	0.08
Diagnosis			
Acute leukemia	52 (68)	101 (58)	0.16
Chronic leukemia	3 (4)	21 (12)	
Lymphoma	14 (18)	29 (17)	
Other	7 (9)	22 (13)	
Patient sex			
Male	44 (58)	107 (62)	0.56
Female	32 (42)	66 (38)	
Sex mismatch			
Yes	60 (79)	132 (77)	0.76
No	16 (21)	39 (23)	
HSCT year			
1989-1996	4 (5)	11 (6)	0.48
1997-2004	14 (18)	43 (25)	
2004-2011	58 (76)	119 (69)	
Source			
BM	24 (32)	52 (30)	0.81
PBSC	52 (68)	121 (70)	
Conditioning			
MA	42 (56)	85 (49)	0.32
RIC	33 (44)	88 (51)	
ATG			
Yes	47 (62)	132 (76)	**0.02**
No	29 (38)	41 (24)	
Disease status			
Early	34 (48)	69 (43)	0.75
Intermediate	25 (35)	64 (41)	
Advanced	12 (17)	25 (16)	
CMV mismatch			
Yes	21 (29)	74 (45)	**0.02**
No	52 (71)	92 (55)	
EBMT risk score			
1	6 (9)	12 (8)	0.14
2	13 (18)	19 (12)	
3	21 (30)	32 (21)	
4	7 (10)	37 (24)	
5	13 (18)	34 (22)	
6 or 7	11 (16)	22 (14)	

Table 4 (previous page): baseline characteristics of PIRCHE groups. Differences between the permissible and non-permissible mismatch group were tested with chi-square for categorical variables and student's T test for the continuous variable age. Patients with a permissible PIRCHE mismatch received less often ATG and had less often a CMV mismatch with the donor compared to patients with a non-permissible PIRCHE mismatch. Sex mismatch: female donor for male patient. BM: bone marrow. PBSC: peripheral blood stem cells. MA: myeloablative. RIC: reduced intensity conditioning. ATG: anti-thymocyte globulin. CMV mismatch: patient seropositive, donor seronegative or patient seronegative, donor seropositive.

References

1. Bray RA, Hurley CK, Kamani NR, et al. National marrow donor program HLA matching guidelines for unrelated adult donor hematopoietic cell transplants. Biol. Blood Marrow Transplant. 2008; 14(9 Suppl): 45-53.
2. Spellman S, Setterholm M, Maiers M, et al. Advances in the selection of HLA-compatible donors: refinements in HLA typing and matching over the first 20 years of the National Marrow Donor Program Registry. Biol.Blood Marrow Transplant. 2008; 14(9 Suppl): 37-44.
3. Lee SJ, Klein J, Haagenson M, et al. High-resolution donor-recipient HLA matching contributes to the success of unrelated donor marrow transplantation. Blood 2007; 110(13): 4576-4583.
4. Flomenberg N, Baxter-Lowe LA, Confer D, et al. Impact of HLA class I and class II high-resolution matching on outcomes of unrelated donor bone marrow transplantation: HLA-C mismatching is associated with a strong adverse effect on transplantation outcome. Blood 2004; 104(7): 1923-1930.
5. Petersdorf EW, Longton GM, Anasetti C, et al. Definition of HLA-DQ as a transplantation antigen. Proc. Natl.Acad.Sci.U.S.A 1996; 93(26): 15358-15363.
6. Gajewski J, Gjertson D, Cecka M, et al. The impact of T-cell depletion on the effects of HLA DR beta 1 and DQ beta allele matching in HLA serologically identical unrelated donor bone marrow transplantation. Biol. Blood Marrow Transplant. 1997; 3(2): 76-82.
7. Fernandez-Vina MA, Klein JP, Haagenson M, et al. Multiple mismatches at the low expression HLA loci DP, DQ, and DRB3/4/5 associate with adverse outcomes in hematopoietic stem cell transplantation. Blood 2013; 121(22): 4603-4610.
8. Furst D, Muller C, Vucinic V, et al. High-resolution HLA matching in hematopoietic stem cell transplantation: a retrospective collaborative analysis. Blood 2013; 122(18): 3220-3229.
9. Petersdorf EW. Optimal HLA matching in hematopoietic cell transplantation. Curr.Opin.Immunol. 2008; 20(5): 588-593.
10. Gragert L, Eapen M, Williams E, et al. HLA match likelihoods for hematopoietic stem-cell grafts in the U.S. registry. N.Engl.J.Med. 2014; 371(4): 339-348.
11. Kawase T, Morishima Y, Matsuo K, et al. High-risk HLA allele mismatch combinations responsible for severe acute graft-versus-host disease and implication for its molecular mechanism. Blood 2007; 110(7): 2235-2241.
12. Fernandez-Vina MA, Wang T, Lee SJ, et al. Identification of a permissible HLA mismatch in hematopoietic stem cell transplantation. Blood 2014; 123(8): 1270-1278.
13. Ferrara JL, Levine JE, Reddy P, et al. Graft-versus-host disease. Lancet 2009; 373(9674): 1550-1561.
14. Geneugelijk K, Thus KA, and Spierings E. Predicting Alloreactivity in Transplantation. J.Immunol.Res. 2014; 2014: 159479.
15. Elsner HA, DeLuca D, Strub J, et al. HistoCheck: rating of HLA class I and II mismatches by an internet-based software tool. Bone Marrow Transplant. 2004; 33(2): 165-169.
16. Joris MM, van Rood JJ, Roelen DL, et al. A proposed algorithm predictive for cytotoxic T cell alloreactivity. J.Immunol. 2012; 188(4): 1868-1873.
17. Shaw BE, Barber LD, Madrigal JA, et al. Scoring for HLA matching? A clinical test of HistoCheck. Bone Marrow Transplant. 2004; 34(4): 367-368.
18. Spellman S, Klein J, Haagenson M, et al. Scoring HLA Class I Mismatches by HistoCheck Does Not Predict Clinical Outcome in Unrelated Hematopoietic Stem Cell Transplantation. Biol.Blood Marrow Transplant. 2011.
19. Joris MM, Lankester AC, von dem Borne PA, et al. Translating in vitro prediction of cytotoxic T cell alloreactivity to hematopoietic stem cell transplantation outcome. Transpl.Immunol. 2014; 30(2-3): 59-64.
20. Kesmir C, Nussbaum AK, Schild H, et al. Prediction of proteasome cleavage motifs by neural networks. Protein Eng 2002; 15(4): 287-296.
21. Nielsen M, Lundegaard C, Lund O, et al. The role of the proteasome in generating cytotoxic T-cell epitopes: insights obtained from improved predictions of proteasomal cleavage. Immunogenetics 2005; 57(1-2): 33-41.
22. Nielsen M, Lundegaard C, Blicher T, et al. NetMHCpan, a method for quantitative predictions of peptide binding to any HLA-A and -B locus protein of known sequence. PLoS.One. 2007; 2(8): e796.
23. Hoof I, Peters B, Sidney J, et al. NetMHCpan, a method for MHC class I binding prediction beyond humans. Immunogenetics 2009; 61(1): 1-13.
24. Nielsen M, Justesen S, Lund O, et al. NetMHCIIpan-2.0 - Improved pan-specific HLA-DR predictions using a novel concurrent alignment and weight optimization training procedure. Immunome.Res. 2010; 6: 9.
25. Thus KA, te Boome L, Kuball J, et al. Indirectly Recognized HLA-C Mismatches and Their Potential Role in Transplant Outcome. Front Immunol. 2014; 5: 210.
26. Thus KA, Ruizendaal MT, de Hoop TA, et al. Refinement of the Definition of Permissible HLA-DPB1 Mismatches with Predicted Indirectly ReCognizable HLA-DPB1 Epitopes. Biol.Blood Marrow Transplant. 2014; 20(11): 1705-1710.
27. Nielsen M, Lundegaard C, and Lund O. Prediction of MHC class II binding affinity using SMM-align, a novel stabilization matrix alignment method. BMC.Bioinformatics. 2007; 8: 238.
28. Nielsen M and Lund O. NN-align. An artificial neural network-based alignment algorithm for MHC class II peptide binding prediction. BMC.Bioinformatics. 2009; 10: 296.
29. Przepiorka D, Weisdorf D, Martin P, et al. 1994 Consensus Conference on Acute GVHD Grading. Bone

Marrow Transplant. 1995; 15(6): 825-828.
30. Williams TM. Human leukocyte antigen gene polymorphism and the histocompatibility laboratory. J.Mol.Diagn. 2001; 3(3): 98-104.

ID	Mismatched locus	Patient HLA-typing	Donor HLA-typing	PIRCHE-I	PIRCHE-II
354	C	03:04	07:02	7	44
548	C	03:04	03:03	0	0
4889	C	03:04	03:03	0	0
5540	C	04:01	15:02	8	25
5924	A	26:01	25:01	0	1
7471	C	06:02	03:04	10	19
7505	DQB1	03:02	03:01	6	20
7609	DRB1	04:14	04:01	4	4
9341	C	15:02	14:02	8	17
9350	DQB1	03:02	03:01	6	5
9878	C	02:02	07:02	3	7
10048	A	02:06	02:01	0	0
11230	C	03:04	05:01	4	11
11327	B	15:07	15:01	1	2
14219	C	16:02	15:02	4	12
14220	C	05:01	07:04	10	30
15014	C	03:03	07:01	4	18
15139	A	02:01	68:01	0	0
16061	C	02:02	15:02	4	9
16468	DRB1	11:04	11:01	0	1
16474	C	01:02	02:02	4	15
17112	DRB1	11:01	11:04	1	0
18172	DQB1	03:02	03:01	3	13
18680	C	03:02	03:03	5	8
18952	C	15:02	14:02	8	27
19667	B	14:02	14:01	0	0
20272	DQB1	03:01	03:02	5	12
20762	DRB1	14:54	14:01	0	0
20822	C	03:04	03:03	0	0
25195	C	07:01	01:02	0	0
27068	C	03:04	01:02	4	2
28920	A	02:01	03:01	0	0
29158	C	03:03	03:04	2	1
30217	C	07:06	16:01	2	2
30906	A	68:01	02:01	3	13
31117	C	07:02	07:01	0	0
31346	C	03:03	01:02	0	23
31466	DQB1	03:02	03:01	5	24

ID	Mismatched locus	Patient HLA-typing	Donor HLA-typing	PIRCHE-I	PIRCHE-II
31976	C	03:04	07:02	7	16
32845	C	01:02	02:02	2	10
33319	DQB1	05:01	05:04	4	9
34094	C	07:01	16:01	8	47
34324	B	44:02	44:35	0	0
34766	B	35:02	35:01	1	4
35139	A	31:01	24:02	11	48
35172	A	68:01	01:01	9	31
35186	DQB1	03:01	03:02	3	20
35474	A	68:02	68:01	3	11
35630	C	07:04	05:01	1	7
35645	C	03:04	03:03	0	0
35988	C	04:01	03:03	5	15
36110	C	07:02	02:02	11	35
36152	C	12:03	04:01	7	14
36381	DRB1	04:07	11:01	15	14
36408	DRB1	11:03	11:01	0	4
36502	C	03:03	04:01	0	1
36663	C	01:02	08:02	8	16
36771	C	14:02	03:04	7	5
36815	A	23:01	24:02	2	6
36881	B	35:02	35:01	0	5
37249	C	15:02	05:01	1	1
37351	A	31:01	24:02	15	18
37352	A	68:02	24:02	2	25
37599	C	04:01	16:01	7	27
37994	DQB1	03:02	03:01	8	14
38744	DRB1	01:02	01:01	1	4
38821	C	14:02	15:02	3	17
38869	DQB1	03:03	03:02	0	0
38899	C	01:02	05:01	5	10
38996	C	03:03	03:04	1	4
39249	B	51:01	56:01	7	14
39520	C	07:01	03:03	0	0
39623	DQB1	03:04	03:01	0	0
39665	DQB1	03:02	03:01	11	14
39744	A	24:02	02:01	0	0
39852	C	16:01	04:01	3	13

ID	Mismatched locus	Patient HLA-typing	Donor HLA-typing	PIRCHE-I	PIRCHE-II
39866	C	16:02	14:02	3	7
40042	C	03:04	03:03	0	1
40115	C	14:02	02:02	1	14
40183	DQB1	05:02	05:03	2	5
40204	C	02:02	15:02	8	3
40241	C	14:02	03:04	0	4
40302	C	03:04	04:01	6	13
40418	DQB1	02:02	03:03	10	16
40618	DRB1	11:04	11:01	0	3
40663	B	15:17	08:01	5	21
40706	A	24:02	03:02	7	7
40893	B	27:02	27:05	2	14
41039	DRB1	04:01	04:04	0	1
41071	A	29:01	29:02	0	2
41133	A	68:01	68:02	0	3
41193	A	24:02	02:05	9	19
41291	A	26:01	02:01	13	16
41293	C	03:04	03:03	0	0
41368	A	68:01	68:02	1	7
41470	A	24:02	02:01	0	0
41564	C	05:01	14:02	12	31
41592	C	03:04	04:01	6	9
41682	C	15:02	16:02	16	11
41737	C	03:04	03:03	0	0
41748	C	15:02	07:02	17	15
42280	A	24:03	24:02	0	0
42310	A	23:01	24:02	2	10
42335	DQB1	06:04	06:02	1	10
42614	C	07:04	05:01	9	27
42945	C	03:04	05:01	3	17
43029	C	02:02	04:01	5	10
43052	B	27:05	27:02	0	3
43053	A	68:01	03:01	11	18
43090	DQB1	02:02	03:03	3	37
43127	C	03:04	07:02	4	13
43134	A	11:01	02:01	7	27
43159	A	32:01	31:01	0	0
43307	DQB1	04:02	02:01	4	24

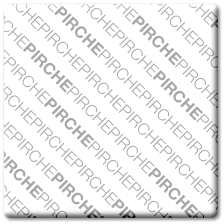
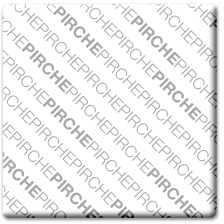
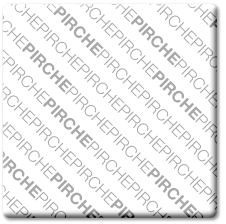
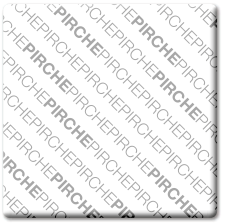
ID	Mismatched locus	Patient HLA-typing	Donor HLA-typing	PIRCHE-I	PIRCHE-II
43383	A	68:01	03:02	4	6
43502	C	03:04	06:02	4	13
44155	A	68:01	03:01	1	31
44501	DRB1	04:04	04:01	5	13
44553	A	03:01	11:01	1	1
44742	A	30:02	32:01	3	2
44787	A	02:01	31:01	0	0
44981	A	01:01	23:01	0	0
45023	A	33:03	68:01	14	24
45042	DQB1	06:02	06:03	2	7
45150	B	44:04	44:03	2	5
45488	C	07:02	03:03	13	43
45718	A	34:02	25:01	0	8
45913	DQB1	03:02	03:01	0	0
46006	C	03:04	03:03	0	0
46183	C	05:01	04:01	3	24
46372	B	15:07	15:01	0	1
46427	DRB1	12:01	11:03	3	4
46433	C	07:02	07:01	0	0
46616	B	35:03	35:01	0	0
46631	A	26:01	02:01	0	15
46666	DQB1	03:02	03:01	2	14
46759	C	02:02	07:02	13	32
46831	A	03:01	24:02	0	0
46939	DQB1	03:03	02:02	0	0
46940	DQB1	03:01	03:02	7	11
46983	B	39:06	39:01	1	1
46984	A	31:01	33:03	0	0
47019	DQB1	03:02	03:01	2	10
47050	B	40:01	44:02	6	21
47051	C	03:03	01:02	4	15
47276	C	01:02	03:04	3	6
47281	DQB1	06:02	06:03	0	0
47473	C	03:04	12:05	0	0
47602	C	15:02	14:02	14	19
47708	DQB1	03:02	03:01	5	5
47723	DQB1	03:01	03:02	1	16
47799	C	12:03	07:01	9	21

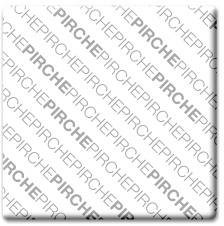
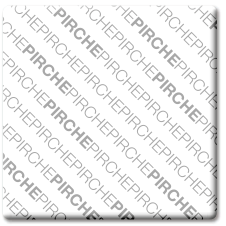
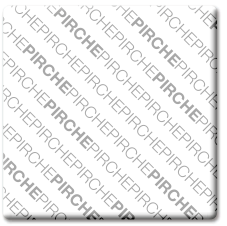
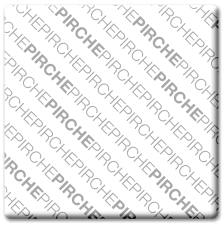
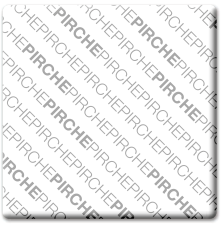
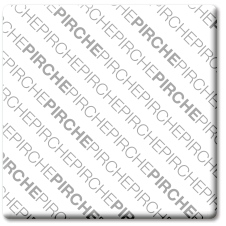
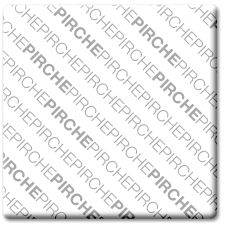
ID	Mismatched locus	Patient HLA-typing	Donor HLA-typing	PIRCHE-I	PIRCHE-II
47816	C	05:03	05:01	0	0
47953	B	37:01	47:01	4	12
47960	DQB1	06:09	06:04	2	4
47983	A	24:02	02:01	2	18
48413	C	03:03	03:04	0	2
48548	B	35:03	35:01	0	0
48682	DQB1	03:01	03:02	6	12
48744	A	02:29	02:06	1	3
48760	A	02:29	02:01	0	0
48784	DQB1	02:02	03:03	0	0
48831	A	34:02	01:01	4	12
48868	A	02:01	24:02	1	6
49120	A	02:17	02:01	0	2
49126	B	35:02	37:01	9	8
49171	B	39:05	39:01	0	0
49172	B	18:03	18:01	0	0
49303	C	01:02	15:02	5	11
49331	C	06:02	07:01	10	23
49337	C	03:03	03:04	0	1
49616	C	03:04	03:03	0	0
49733	A	25:01	01:01	8	30
49809	DRB1	04:01	04:02	0	0
50216	C	14:02	15:02	4	8
50218	A	31:01	36:01	7	37
50519	C	02:02	06:02	6	16
50543	DQB1	03:02	03:01	9	9
50565	B	35:03	35:01	0	0
50763	B	44:29	44:02	3	0
50863	C	04:01	03:04	17	28
51136	C	07:02	03:04	9	17
51137	C	03:04	03:03	0	0
51270	B	35:03	35:01	0	2
51276	C	04:01	06:02	12	18
51504	DQB1	03:02	03:05	0	0
51706	C	05:01	07:02	3	15
52575	A	24:02	02:03	7	48
52712	DQB1	06:02	06:03	4	7
52799	B	18:01	18:03	0	4

ID	Mismatched locus	Patient HLA-typing	Donor HLA-typing	PIRCHE-I	PIRCHE-II
53030	DQB1	02:01	03:02	9	14
53144	DQB1	03:02	03:01	1	20
53836	B	35:03	57:01	0	0
53881	C	03:62	03:03	0	0
54385	C	03:04	03:03	0	0
54595	DRB1	04:03	04:04	0	0
54930	A	24:02	02:01	6	19
55008	C	03:04	03:03	0	0
55695	C	03:03	03:04	1	1
55745	B	27:02	40:02	0	7
55787	B	50:01	07:02	5	22
55859	B	44:27	44:02	0	0
55870	C	03:04	03:03	0	0
55913	C	03:04	07:01	0	0
56026	A	29:02	26:01	12	31
56131	DQB1	03:01	03:02	3	17
56194	DQB1	05:02	05:02	3	22
56195	A	68:01	24:02	2	24
56197	B	27:05	27:13	4	4
56202	A	03:02	02:01	9	13
56239	DQB1	03:01	03:02	0	0
56456	C	12:03	07:02	0	0
56669	DRB1	08:03	08:10	0	0
56675	DQB1	06:02	02:01	0	0
56825	C	06:02	03:04	3	8
56909	DQB1	06:03	06:02	3	4
57084	A	03:01	32:01	0	0
57193	C	03:03	04:01	3	21
57223	DRB1	11:03	11:01	0	0
57249	DQB1	03:01	03:02	7	8
57252	C	05:01	07:01	13	24
57300	A	29:02	24:02	6	14
57330	A	03:01	32:01	4	26
57518	DQB1	03:01	03:02	9	20
57732	A	32:01	01:01	4	8
57745	C	02:02	01:02	6	20
57831	B	51:01	35:01	2	3
58078	DQB1	03:01	05:03	0	0

ID	Mismatched locus	Patient HLA-typing	Donor HLA-typing	PIRCHE-I	PIRCHE-II
58186	B	35:01	35:02	0	0
58217	DRB1	12:01	11:03	6	4
58465	A	30:01	31:01	0	0
58825	B	35:01	35:08	0	0
58890	C	06:02	03:04	4	7
58944	DQB1	03:01	03:02	0	0
59390	C	14:02	15:02	3	19
59935	C	01:02	15:02	12	22
59969	A	31:01	02:01	14	18
59986	A	01:01	02:01	0	0
60100	C	14:02	15:02	6	8
60113	C	04:01	03:04	5	12
60141	B	08:01	40:01	0	0
60144	DQB1	06:02	06:03	3	8
60335	DQB1	02:02	03:03	7	20
60417	A	23:01	24:02	2	1
60554	C	02:02	16:01	6	16
60761	DQB1	06:03	06:04	0	3
61229	C	07:02	07:04	3	3
61441	A	68:01	30:01	8	37
61697	C	04:30	04:01	0	0

Table S1: HLA-mismatches included in this study.

4








Chapter 4

Refinement of the definition of permissible HLA-DPB1 mismatches with Predicted Indirectly ReCognizable HLA-DPB1 Epitopes

Kirsten A. Thus,[1] Mieke T. A. Ruizendaal,[1] Talitha A. de Hoop,[1] Eric Borst,[1] Hanneke W. M. van Deutekom,[2] Liane te Boome,[3] Jürgen Kuball,[1,3] and Eric Spierings[1]

[1]Laboratory of Translational Immunology, University Medical Center Utrecht, Utrecht, the Netherlands
[2]Department of Theoretical Biology & Bioinformatics, Utrecht University, Utrecht, the Netherlands
[3]Department of Hematology, University Medical Center Utrecht, Utrecht, the Netherlands

Biology of Blood and Marrow Transplantation 2014; 20 (11): 1705-1710

Abstract

Hematopoietic stem-cell transplantation with HLA-DPB1 mismatched donors leads to an increased risk of acute graft-versus-host disease (GVHD). Studies have indicated a prognostic value for classifying HLA-DPB1 mismatches based on T-cell-epitope (TCE) groups. The aim of this study was to determine the contribution of indirect recognition of HLA-DP derived epitopes, as determined with the Predicted Indirectly ReCognizable HLA Epitopes (PIRCHE) method. We therefore conducted a retrospective single center analysis on 80 patients transplanted with a 10/10 matched-unrelated donor that was HLA-DPB1 mismatched.

HLA-DPB1 mismatches that were classified as GVH nonpermissive by the TCE algorithm, correlated to higher numbers of HLA class-I as well as HLA class-II presented PIRCHE (PIRCHE-I and -II) compared with permissive or host-versus-graft (HVG) nonpermissive mismatches. Patients with acute GVHD grade II-IV presented significantly higher numbers of PIRCHE-I compared with patients without acute GVHD ($p<0.05$). Patients were divided into two groups; based on presence or absence of PIRCHE. Patients with PIRCHE-I or -II have an increased hazard of acute GVHD when compared with patients without PIRCHE-I or -II (hazard ratio (HR) 3.19, 95%-confidence interval (CI) 1.10-9.19, $p<0.05$, HR 4.07, 95%-CI 0.97-17.19, $p=0.06$, respectively). Patients that were classified as having an HLA-DPB1 permissive mismatch by the TCE model, had an increased risk of acute GVHD when comparing presence of PIRCHE-I with absence of PIRCHE-I (HR 2.96, 95%-CI 0.84-10.39, $p=0.09$). We therefore conclude that the data presented in this study describe an attractive and feasible possibility to better select permissible HLA-DPB1 mismatches, by including both a direct and an indirect recognition model.

Introduction

Hematopoietic stem-cell transplantation (HSCT) with a matched-unrelated donor that is mismatched for HLA-DPB1 leads to an increased risk of acute graft-versus-host disease (GVHD) (1-6). It is well established that certain HLA-DP mismatches more frequently lead to this effect of T-cell related alloreactivity than others (2, 5, 6). HLA mismatch-induced T-cell alloreactivity can be evoked via two routes of mismatched antigen recognition: direct and indirect recognition. Direct recognition occurs when donor T cells recognize an intact mismatched HLA molecule that is present on the cell surface of a recipient's cell. These directly recognizing T cells are likely viral peptide-specific memory T cells showing crossreactivity towards allogeneic HLA, due to molecular mimicry (7).

Direct recognition of HLA-DP mismatches can be predicted with the T-cell-epitope (TCE) model, developed by Fleischhauer *et al*. (2, 5). This TCE model is based upon crossreactivity patterns of alloreactive T cells isolated from a patient that had rejected his graft (2). In this model, HLA-DPB1 alleles of donor and recipient are divided into three (or four) groups, either predicted to have high, intermediate or low immunogenic potential. HLA-DPB1 mismatches are subsequently classified as permissive or nonpermissive based on the concept of thymic education of T cells. Mismatches are defined as permissive if they belong to the same immunogenicity group, and as nonpermissive if they belong to groups with different immunogenicity. The direction of nonpermissiveness is based on the direction of immunogenicity: when the recipient has a higher TCE-assigned immunogenicity than the donor, mismatches are designated as GVH nonpermissive; when the recipient has a lower TCE-assigned immunogenicity than the donor, the mismatch is designated as host-versus-graft (HVG) nonpermissive. Several studies have shown that HLA-DPB1 mismatches classified as nonpermissive by the TCE model are associated with both an increased risk of acute GVHD (2, 5, 6), as well as an increased risk of overall mortality (5, 6).

HLA-molecules can also be recognized indirectly. Indirect recognition has not been included in the TCE model. During indirect recognition, donor T cells recognize polymorphic peptides derived from the mismatched HLA molecule presented by an HLA molecule that is shared between donor and recipient. T cells specific for such polymorphic HLA-derived peptides have been found frequently during graft failure after solid organ transplantation (8-12). Indirect recognition can be predicted *in silico* as well: our group has previously developed a model that predicts the numbers of peptides derived from mismatched HLA-alleles that can be presented by shared HLA. These peptides have been designated as Predicted Indirectly ReCognizable HLA-Epitopes (PIRCHE) (13-15). Increasing numbers of PIRCHE are correlated to an increased risk of alloreactivity, as reflected by increased probabilities of acute GVHD after HSCT (15), and by the development of donor-specific antibodies after kidney transplantation (13).

We hypothesize that nonpermissibility of HLA-DPB1 mismatches can, next to the TCE model, be explained by indirect recognition of the HLA-DPB1 mismatched alleles. To investigate whether PIRCHE can indeed provide an additive explanation for non-permissibility of certain HLA-DPB1 mismatch combinations, the number of HLA-DPB1-derived PIRCHE was determined in patients transplanted with an HLA-A, -B, -C, -DRB1, -DQB1 (10/10)-matched, HLA-DBP1-mismatched unrelated donor. The numbers of PIRCHE were studied for their correlation with permissiveness as determined by the TCE model and to clinical measures of alloreactivity (acute and chronic GVHD, relapse/progression of the original disease and transplant-related mortality (TRM)).

Material and Methods

Patients

All adult patients receiving a peripheral blood stem-cell transplant from a 10/10 (matched for HLA-A, -B, -C, -DRB1, -DQB1 on 4-digit level) matched-unrelated donor after non-myeloablative conditioning, between 2007 and 2012 in the University Medical Center Utrecht, were included in this analysis (Supplementary Tables 1, 2 and 3). Nonmyeloablative conditioning consisted of total body irradiation of 2 Gy on one day, ATG (Genzyme, Cambridge, MA, USA) 2 mg/kg/day for four days, and Fludarabine 30 mg/m^2/day for three days. Patients received an unmanipulated peripheral blood stem cell graft. Immunosuppressive therapy consisted of Cyclosporine A 4.5 mg/kg twice daily until day 120, which was then tapered by a 10% dose reduction per week in the absence of GVHD. Cyclosporine A was combined with Mycophenolate Mofetil 15 mg/kg, three times a day until day 84, and if there was no GVHD, tapered and stopped in two weeks. All patients received antibiotic prophylaxis, including Co-Trimoxazole 480 mg twice a day and Valacyclovir 500 mg twice a day, as previously reported (16). Donor age was mean 36.5 years (range 22-45), and disease stage prior to HSCT was complete remission for 32 patients (40%), partial remission in 32 patients (40%), progressive in 2 (3%) and not available for the remainder of 14 patients (18%).

HLA typing

Sequencing-based high-resolution HLA-typing was performed prior to HSCT for all included patients and donors for the HLA-A, -B, -C, -DRB1, and -DQB1 loci by PCR and sequencing, using the SBTexcellerator® HLA-A, -B, -C, -DRB1 and -DQB1 kits (GenDx, Utrecht, the Netherlands). Purified sequencing products were electrophoresed using a 3730 DNA-Analyzer (Applied Biosystems, Foster City, CA, USA) and sequences were analyzed using the SBTengine® software (GenDx). All protocols were executed according to the manufacturers' guidelines. All allele and genotype ambiguities at the 4-digit level were resolved and all null alleles were excluded.

HLA-DPB1 typing was performed retrospectively using sequencing-based typing (for the used primers see Supplementary Table 4). Homozygotic typings were confirmed with sequence-specific oligonucleotide technologies (One Lambda, Canoga Park, CA, USA). After HLA-DPB1 typing, for 18 individuals (11%), ambiguities could not be resolved. For all these unresolved ambiguities, the locations of the polymorphic residues were analyzed. All of the polymorphisms were located outside positions affecting the immunogenicity as predicted by the TCE model and more than nine amino acids apart. Consequently, all allelic combinations possible with these ambiguities lead to identical TCE and PIRCHE assignment. We therefore included these pairs using their ambiguous HLA-DPB1 typings.

TCE classification

HLA-DPB1 mismatches were classified as either permissive, nonpermissive in the GVH or in the HVG direction, as described previously (2, 5, 6, 17). To this end, HLA-DPB1 typing data were entered in the online tool available for determining TCE groups (http://www.ebi.ac.uk/ipd/imgt/hla/dpb.html [accessed January 2013]), which uses the TCE three-group model. For 2 (3%) donor-recipient combinations, permissiveness could not be determined with this tool, because one of their HLA-DPB1 alleles was not included in the TCE model (HLA-DPB1*35:01 and DPB1*36:01).

PIRCHE determination

PIRCHE were identified for each donor-recipient pair as described previously (14;15). In short, for HLA class-I presented PIRCHE (PIRCHE-I), processing by the proteasome and transportation via the TAP channel of the amino-acid sequences of all donor and recipient HLA molecules (sequences as defined by the international ImMunoGeneTics information system [IMGT]: ftp://ftp.ebi.ac.uk/pub/databases/ipd/imgt/hla [accessed December 2012]), was predicted using NetChop C-term 3.0 (18, 19). Predicted processed nonameric peptides were tested for their binding capacity to the HLA class-I molecules using NetMHCpan 2.4 (20, 21). Peptides with IC_{50}-binding values ≤ 500 nM were accepted as relevant binders (22). For HLA class-II presented PIRCHE (PIRCHE-II), the nonameric binding cores of potential HLA-DRB1 binders (15-mers) were predicted with NetMHCIIPan 2.0 (23-25), considering IC_{50}-binding values ≤ 1000nM as being relevant (26). For each donor-recipient pair, only unique recipient-specific peptide-HLA complexes were counted as PIRCHE.

Statistical analyses

The primary clinical endpoint tested was incidence of acute GVHD grades II to IV (27). Secondary clinical endpoints were: extensive or limited chronic GVHD (16), relapse/progression of the primary malignant disease (for patients transplanted for a malignant disease only, n = 76 (as defined by the HOVON study group (28-32))), TRM, and overall survival.

Differences in distribution of PIRCHE in the TCE-assigned permissive groups were tested with Mann-Whitney U tests comparing the three TCE groups (HVG nonpermissive, GVH nonpermissive and permissive) with each other. These tests were also performed to compare the distribution of PIRCHE amongst clinical outcomes.

Cumulative incidence curves were constructed for primary and secondary clinical endpoints. Relapse and non-relapse mortality were regarded competing risks for acute GVHD, mortality related to relapse was regarded a competing risk for chronic GVHD, TRM was regarded a competing risk for relapse, mortality related to relapse and mortality due to other causes were regarded competing risks for TRM. Differences in cumulative incidences were tested with the Gray test.

The time-dependent association of TCE-assigned nonpermissiveness and numbers of PIRCHE with development of primary and secondary endpoints was tested with Cox regression analyses. Models were adjusted for (when relevant): age of the recipient at transplantation, primary disease, cytomegalovirus status, development of acute GVHD II-IV for chronic GVHD, and development of acute and chronic GVHD for relapse. Backward selection was used to determine which variables should be included in the model; the likelihood ratio test was used to select the relevant variables ($p<0.10$). Variables were also included when they were associated with the variable of interest (either TCE classification or PIRCHE), as tested with ANOVA for the continuous variable age and chi-square for categorical variables: primary disease, gender, cytomegalovirus status, Epstein-Barr virus status (serostatus of both recipient and donor and the match status between them).

Statistical procedures were performed with SPSS statistics software version 20.0 (SPSS Inc, Chicago, IL, USA), and competing risk analyses were performed with R version 3.0.0 (The R Foundation for Statistical Computing, Vienna, Austria). $P<0.05$ was considered to be statistically significant.

Results

Within our cohort of 88 patients transplanted with 10/10 matched-unrelated donors within a uniform reduced intensity regimen (16), 8 (9%) recipients were transplanted with an HLA-DPB1 matched donor. Of the 80 mismatched pairs, 2 (3%) pairs could not be analyzed with the classical direct recognition (TCE) model because for one of the alleles immunogenicity could not be predicted. Of the remaining 78 (97%) pairs with a TCE designation, 54 (69%) were classified as permissive, 12 (15%) as GVH nonpermissive and 12 (15%) as HVG nonpermissive (Supplementary Table 1). In order to assess the impact of indirect recognition, the numbers of PIRCHE were determined as described in Methods. Recipients presented a median of 1 PIRCHE-I (range, 0 to 7) and 3.5 PIRCHE-II (range, 0 to 20).

To correlate the presence of PIRCHE with clinical outcome, donor-recipient pairs were divided into two PIRCHE groups, according to the absence or presence of PIRCHE. Baseline characteristics were evenly distributed amongst the TCE groups and the absence or presence of PIRCHE, apart from age at HSCT (patients with GVH nonpermissive mismatches were younger than those with HVG nonpermissive or permissive mismatches, Supplementary Tables 1, 2, and 3). Models testing TCE-assigned permissiveness were therefore corrected for age at HSCT.

The correlation of the TCE model with acute GVHD

The correlation between direct recognition as predicted with the TCE groups and acute GVHD was investigated with univariate cumulative incidence analyses and in multivariate Cox regression models. TCE permissive mismatches displayed the lowest incidence of acute GVHD, although the cumulative incidences were not significantly different amongst the three TCE groups, and not when comparing permissive with GVH and HVG nonpermissive combined (Figure 1). In multivariate analyses, which were corrected for age of the recipient, TCE groups were not significantly associated with acute GVHD (Table 1).

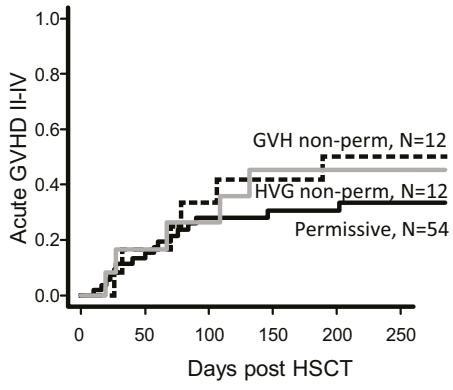


Figure 1: Cumulative acute GVHD incidence curves for the three TCE classifications. Although HVG (gray line) and GVH (dashed line) nonpermissive mismatches have a higher incidence of acute GVHD than permissive (black solid line) mismatches, this was not significantly different. Non-perm indicates nonpermissive.

The correlation of the PIRCHE model with acute GVHD

The influence of indirect recognition on acute GVHD was assessed by correlating numbers of PIRCHE to the incidence of acute GVHD grade II-IV. Recipients with acute GVHD grade II-IV presented significantly higher numbers of PIRCHE-I (p=0.05) but not of PIRCHE-II (Figure 2A,B). When analyzing the presence of PIRCHE, PIRCHE-I and -II were significantly associated with increased incidences of acute GVHD compared with recipients without PIRCHE (p=0.01 and p=0.04, respectively, Figure 2C,D). In Cox regression analyses, the presence of PIRCHE-I was associated with an increased hazard of acute GVHD compared with absence (hazard ratio [HR] 3.19, 95%-confidence interval [CI] 1.10-9.19, p=0.03). For PIRCHE-II there was a similar trend (HR 4.07, 95%-CI 0.97-17.19, p=0.06, Table 1). Thus, the presence of PIRCHE is correlated with increased acute GVHD risks.

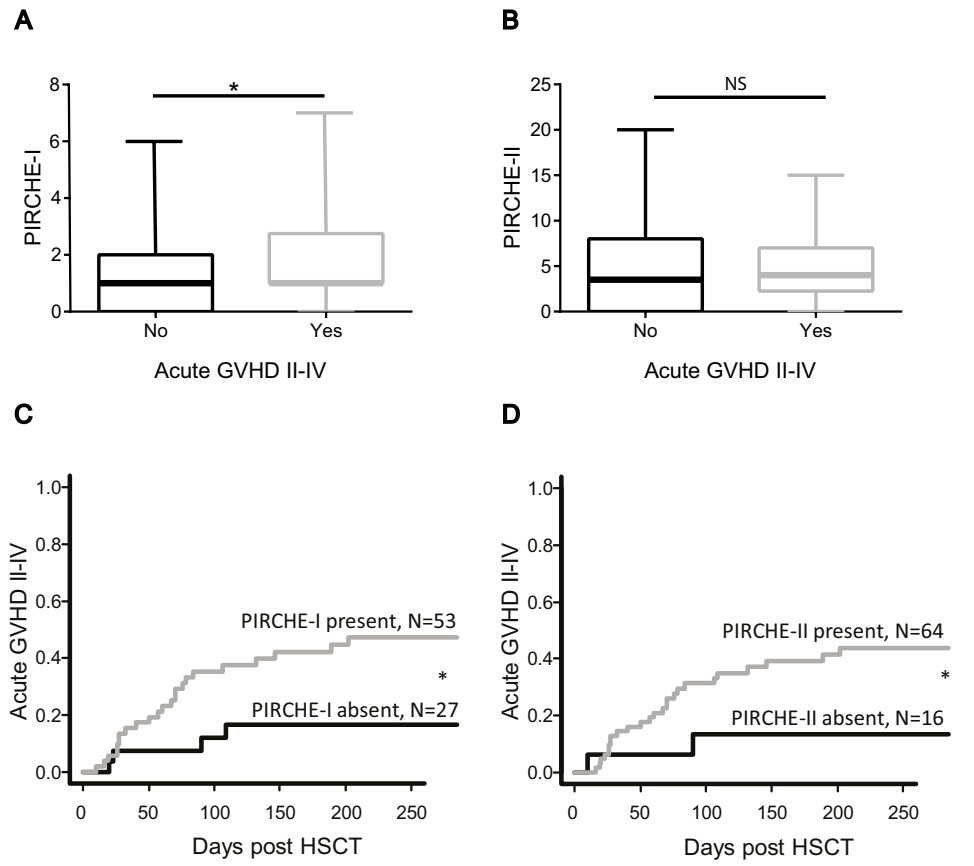


Figure 2: The association of the PIRCHE model with GVHD. Boxplot showing the correlation between acute GVHD and numbers of PIRCHE-I (A) or PIRCHE-II (B). The boxes represent the 25th-75th percentile, the horizontal lines the median and the whiskers all PIRCHE values from minimum to maximum. Recipients developing acute GVHD presented significantly higher numbers of PIRCHE-I, but not of PIRCHE-II. Cumulative acute GVHD incidence curves for the absence and presence of PIRCHE-I (C) or for the absence and presence of PIRCHE-II (D). Cumulative incidence of acute GVHD was significantly increased for recipients presenting PIRCHE-I (gray line), compared with those without PIRCHE-I (black line), similar for PIRCHE-II. * = p<0.05.

Comparing the TCE and PIRCHE model

To test whether the sensitivity of the direct recognition (TCE) and indirect recognition (PIRCHE) models differ, receiver operator characteristic curves were constructed and the areas under the curve (AUC) calculated as a measure of predictive capacity. Only PIRCHE-I significantly predicted acute GVHD (TCE AUC, 0.586, p=0.21; PIRCHE-I AUC, 0.641, p=0.04; PIRCHE-II AUC, 0.580, p=0.25).

To investigate the relationship between the TCE and PIRCHE model, the correlation between the TCE groups and numbers of PIRCHE was analyzed. Pairs with TCE-predicted GVH nonpermissive mismatches had higher numbers of PIRCHE-I when compared with both permissive or HVG nonpermissive mismatches (p<0.01). There was a non-significant increase in numbers of PIRCHE-II when comparing GVH nonpermissive with permissive mismatches, however, PIRCHE-II were significantly higher in the GVH nonpermissive group when compared with HVG nonpermissive (p<0.01), and significantly lower in the HVG nonpermissive compared with the permissive group (p=0.03). When analyzing the presence of PIRCHE, the TCE permissive group contained 22 (41%) pairs without PIRCHE-I, 12 (22%) without PIRCHE-II, and 32 (59%) and 42 (78%) pairs with PIRCHE-I or PIRCHE-II, respectively. The TCE and PIRCHE classification are thus correlated, although the PIRCHE model designates mismatches as nonpermissible when they are permissive according to the TCE model.

To study whether the PIRCHE model can further define HLA-DP mismatches, the correlation of PIRCHE with acute GVHD within the TCE-permissive mismatches was studied. Within the TCE permissive mismatches, recipients without PIRCHE-I had a lower risk of acute GVHD when compared with recipients with PIRCHE-I (HR 2.96, 95%-CI 0.84-10.39, p=0.09, Figure 3, Table 1). This effect was not observed for PIRCHE-II. Of the 16 (30%) recipients that had developed acute GVHD despite the permissive TCE classification, 13 (81%) have PIRCHE-I, and are thus more adequately classified by PIRCHE-I. A similar strategy could not be performed for the TCE nonpermissive group, as all recipients with GVH nonpermissive mismatches have PIRCHE-I and -II, and there were little patients without PIRCHE-I or -II in the HVG nonpermissive group (5 and 4, respectively).

Secondary endpoints

The TCE and PIRCHE model were studied for their correlation with other clinical outcomes. Neither TCE nonpermissiveness nor PIRCHE were associated with an increased hazard of chronic GVHD, in multivariate models corrected for cytomegalovirus, recipient age and

Variable	HR	95%-CI	P
TCE model			
GVH nonpermissive (1)	1.82	0.68-4.87	NS
HVG nonpermissive (1)	1.37	0.50-3.76	NS
PIRCHE-I			
PIRCHE-I present	3.19	1.10-9.19	**<0.05**
PIRCHE-II			
PIRCHE-II present	4.07	0.97-17.19	NS
PIRCHE-I within TCE permissive			
PIRCHE-I present	2.96	0.84-10.39	NS

Table 1: Association of the TCE and PIRCHE model with acute GVHD. Hazard ratios of the different TCE and PIRCHE groups as determined with Cox regression analyses. Presence of PIRCHE-I was significantly associated to an increased hazard of acute GVHD compared with absence of PIRCHE-I. There was a trend for an increased hazard of acute GVHD for presence compared with absence of PIRCHE-II. Within the TCE permissive group, presence of PIRCHE-I was associated to an increased hazard of acute GVHD compared with absence PIRCHE-I. HR: hazard ratio. 95%-CI: 95%-confidence interval of hazard ratio. NS: not significant. 1: reference: permissive mismatches.

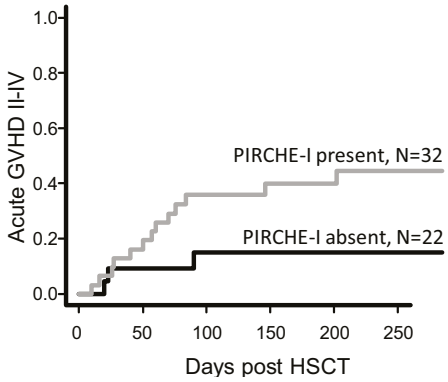


Figure 3: Cumulative acute GVHD incidence curves for the absence and presence of PIRCHE-I within the TCE-permissive group. Within the TCE-permissive group, recipients presenting PIRCHE-I (gray line) have an increased probability (p=0.06) of acute GVHD compared with those without PIRCHE-I (black line).

acute GVHD development (data not shown). Neither the TCE classification nor the presence of PIRCHE were significantly associated to TRM in our cohort, in multivariate models that were corrected for the primary disease group and age of the recipient (data not shown). In multivariate models, corrected for age of the recipient and primary disease, neither TCE nonpermissiveness nor the presence of PIRCHE was associated with overall survival (data not shown).

The TCE model correlates to relapse; HVG nonpermissive mismatches lead to a significantly increased incidence of relapse when compared with GVH nonpermissive mismatches or permissive mismatches (p=0.03 and p<0.001, respectively). In multivariate analyses, which were corrected for the development of chronic GVHD and age of the recipient, HVG nonpermissive mismatches were clearly associated with an increased hazard of relapse when compared with permissive mismatches (HR 3.71, 95%-CI 1.50-9.21, p<0.01). PIRCHE were not correlated to relapse, neither in univariate nor in multivariate analysis. To summarize, TCE-assigned HVG nonpermissive mismatches are correlated with an increased relapse risk, whereas PIRCHE do not correlate to other endpoints than acute GVHD.

Discussion

HLA-DP mismatches are correlated to alloreactivity after HSCT (1-6, 33-36). It is recognized that directly recognizable HLA-DPB1 mismatches, as predicted with the TCE model, correlate with inferior outcomes after HSCT (2, 5, 6, 33). The present study was initiated to investigate whether indirectly recognizable HLA-DPB1 mismatches predict non-permissible mismatches, next to the TCE model. In our local cohort of 10/10 matched-unrelated stem-cell transplants, predicted indirectly recognizable HLA-DPB1 mismatches, as determined via the PIRCHE concept, indeed correlate with acute GVHD. Moreover, our observations indicate that the PIRCHE model is able to further define HLA-DP permissibility within the TCE permissive group.

Despite a clear association between the TCE and PIRCHE predictions, the PIRCHE model appears to more adequately classify HLA-DPB1 mismatches as permissible and non-permissible *in vivo*. These PIRCHE-predicted improvements are likely explained by the lengths of the polymorphic parts of the HLA-DPB1 alleles that are considered in both models. The TCE model is based upon polymorphisms within the peptide-binding groove and T-cell receptor contact regions only; the six hypervariable regions of the alpha-1 domain encoded

by exon 2 (2). However, PIRCHE can also be derived from polymorphic regions outside the peptide-binding groove. Inclusion of these polymorphic regions apparently enhances the predictive capacity of the PIRCHE model when compared with the TCE model. Inclusion of polymorphisms outside exon 2 in an indirect model for antigen recognition is justified by peptide elution data; DP-derived peptides encoded by exon 1 have been frequently eluted from HLA (SYFPEITHI database, [http://www.syfpeithi.de/bin/MHCServer.dll/FindYourMotif.htm], accessed May 2014). Because the PIRCHE model considers a greater part of the HLA-DP alleles, it is a more stringent model, consequently predicting clinically relevant non-permissibility at a higher frequency.

Investigating the interaction between the classical direct recognition (TCE) model and our here newly proposed indirect recognition (PIRCHE) model would be of interest, as the two models theoretically aim to predict two different routes of DP-antigen recognition. One could speculate that these two immune responses are complementary or even enhance each other *in vivo*. Hence, additive or even synergistic effects may be expected. However, due to the strong correlation between the TCE and PIRCHE model in this study, and the small cohort size, interactions between the two models are difficult to test. Observations in the current study may even implicate that the PIRCHE model provides an explanation for the previous findings of the TCE model, as all patients with a GVH nonpermissive TCE mismatch had PIRCHE-I and -II. Future studies conducted on larger patient cohorts allow studying the interactions between nonpermissive mismatches according to the TCE model and presence of PIRCHE, and will elucidate what the predictive capacity of the combination of these two models is, or even if they truly predict independently.

Both the TCE and PIRCHE model have some practical constraints. The TCE model is restricted by the fact that cellular recognition patterns have not been determined for all HLA-DP alleles. In our cohort, we could therefore not predict permissiveness in two of the cases. To resolve this problem, it is necessary to expand the immunogenicity predictions to not yet tested HLA-DPB1 alleles, possibly *in silico*. The PIRCHE model is limited by the requirement of full exon 1-6 sequences, since PIRCHE can also be derived from regions outside exon 2. In the present study, the amino acid sequences of four HLA-DPB1 alleles (HLA-DPB1*20:01, 33:01, 36:01, 138:01) present in 10 (6%) individuals could only partly be included in the PIRCHE model. We therefore may have over- or underestimated the numbers of PIRCHE for these pairs. Our study underlines the need for submission of complete exon 1-6 sequences to the IMGT database, to prevent these gaps in the sequence knowledge. Such data will allow more concise studying of the role of PIRCHE in clinical outcomes.

Furthermore, the current study only predicts nonameric PIRCHE-I. Although HLA class I molecules can bind peptides of other lengths, the current HLA binding predictors are most reliable for nonameric peptides. Future improvements on binding predictions for non-nonameric peptides may facilitate studies on the effect of these non-nonameric PIRCHE-I.

The current study excludes HLA-DPA1 derived PIRCHE. It is highly likely that many of the HLA-DPB1-mismatched pairs have an additional HLA-DPA1 mismatch, which could lead to potential PIRCHE. Future studies should also include analyzing the effect of HLA-DPA1 derived PIRCHE, which may lead to further improvement of the predictive potential.

Mismatching for HLA-DP may lead to increased graft-versus-leukemia effects, since HLA-DP is preferentially expressed on the hematopoietic cell lineage (37). Theoretically, high numbers of DP-derived PIRCHE as well as TCE nonpermissive mismatches may lead to such an increased anti-tumor effect. In the present study, we did not find a reduced relapse risk

in patients with an increased chance of alloreactivity targeting the HLA-DP mismatch (*i.e.* TCE GVH nonpermissive or high PIRCHE). In that perspective, it is noteworthy that HLA-DP is expressed on the vast majority of leukemic cells, but with considerable variety. For example, HLA-DP expression is lower on AML than on B-ALL or B-CLL cells (37). The majority of the acute leukemia patients in our study (82%) suffered from AML, a low HLA-DP expressing leukemia. Research in specific disease subgroups is warranted to analyze the anti-tumor effect of TCE nonpermissiveness and of high numbers of DP-derived PIRCHE, in order to correct for differences in HLA-DP expression levels.

To conclude, our data show a correlation between indirectly recognizable HLA-DP mismatches, as determined with the PIRCHE model, and acute GVHD. These data might be useful to complement currently used models for direct recognition in order to provide a superior donor selection. Extended studies on large cohorts are essential to refine the integration of these two models in donor-selection procedures, both with respect to acute GVHD and graft-versus-leukemia effects, and longer term effects like chronic GVHD and TRM.

Acknowledgements

We would gratefully like to acknowledge dr. Martin Stern for his help with the competing risk analyses, and Anette van Dijk for her help with gathering the clinical data.

References

1. Shaw BE, Potter MN, Mayor NP, et al. The degree of matching at HLA-DPB1 predicts for acute graft-versus-host disease and disease relapse following haematopoietic stem cell transplantation. Bone Marrow Transplant. 2003; 31(11): 1001-1008.
2. Zino E, Frumento G, Marktel S, et al. A T-cell epitope encoded by a subset of HLA-DPB1 alleles determines nonpermissive mismatches for hematologic stem cell transplantation. Blood 2004; 103(4): 1417-1424.
3. Shaw BE, Gooley TA, Malkki M, et al. The importance of HLA-DPB1 in unrelated donor hematopoietic cell transplantation. Blood 2007; 110(13): 4560-4566.
4. Shaw BE, Gooley T, Madrigal JA, et al. Clinical importance of HLA-DPB1 in haematopoietic cell transplantation. Tissue Antigens 2007; 69 Suppl 1: 36-41.
5. Crocchiolo R, Zino E, Vago L, et al. Nonpermissive HLA-DPB1 disparity is a significant independent risk factor for mortality after unrelated hematopoietic stem cell transplantation. Blood 2009; 114(7): 1437-1444.
6. Fleischhauer K, Shaw BE, Gooley T, et al. Effect of T-cell-epitope matching at HLA-DPB1 in recipients of unrelated-donor haemopoietic-cell transplantation: a retrospective study. Lancet Oncol. 2012; 13(4): 366-374.
7. Amir AL, van der Steen DM, Hagedoorn RS, et al. Allo-HLA-reactive T cells inducing graft-versus-host disease are single peptide specific. Blood 2011; 118(26): 6733-6742.
8. Liu Z, Colovai AI, Tugulea S, et al. Indirect recognition of donor HLA-DR peptides in organ allograft rejection. J.Clin.Invest 1996; 98(5): 1150-1157.
9. Vella JP, Vos L, Carpenter CB, et al. Role of indirect allorecognition in experimental late acute rejection. Transplantation 1997; 64(12): 1823-1828.
10. Ciubotariu R, Liu Z, Colovai AI, et al. Persistent allopeptide reactivity and epitope spreading in chronic rejection of organ allografts. J.Clin.Invest 1998; 101(2): 398-405.
11. Suciu-Foca N, Ciubotariu R, Itescu S, et al. Indirect allorecognition of donor HLA-DR peptides in chronic rejection of heart allografts. Transplant.Proc. 1998; 30(8): 3999-4000.
12. Hornick PI, Mason PD, Baker RJ, et al. Significant frequencies of T cells with indirect anti-donor specificity in heart graft recipients with chronic rejection. Circulation 2000; 101(20): 2405-2410.
13. Otten HG, Calis JJ, Kesmir C, et al. Predicted indirectly recognizable HLA epitopes presented by HLA-DR correlate with the de novo development of donor-specific HLA IgG antibodies after kidney transplantation. Hum.Immunol. 2013; 74(3): 290-296.
14. Geneugelijk K, Thus KA, and Spierings E. Predicting Alloreactivity in Transplantation. J.Immunol.Res. 2014; 2014: 159479.
15. Thus KA, Te Boome L, Kuball J, et al. Indirectly Recognized HLA-C Mismatches and Their Potential Role in Transplant Outcome. Front Immunol. 2014; 5: 210.
16. van Dorp S, Pirsma F, Wolfl M, et al. Rituximab treatment before reduced-intensity conditioning transplantation associates with a decreased incidence of extensive chronic GVHD. Biol.Blood Marrow Transplant. 2009; 15(6): 671-678.
17. Zino E, Vago L, Di TS, et al. Frequency and targeted detection of HLA-DPB1 T cell epitope disparities relevant in unrelated hematopoietic stem cell transplantation. Biol.Blood Marrow Transplant. 2007; 13(9): 1031-1040.
18. Kesmir C, Nussbaum AK, Schild H, et al. Prediction of proteasome cleavage motifs by neural networks. Protein Eng 2002; 15(4): 287-296.
19. Nielsen M, Lundegaard C, Lund O, et al. The role of the proteasome in generating cytotoxic T-cell epitopes: insights obtained from improved predictions of proteasomal cleavage. Immunogenetics 2005; 57(1-2): 33-41.
20. Nielsen M, Lundegaard C, Blicher T, et al. NetMHCpan, a method for quantitative predictions of peptide binding to any HLA-A and -B locus protein of known sequence. PLoS.One. 2007; 2(8): e796.
21. Hoof I, Peters B, Sidney J, et al. NetMHCpan, a method for MHC class I binding prediction beyond humans. Immunogenetics 2009; 61(1): 1-13.
22. Buus S, Lauemoller SL, Worning P, et al. Sensitive quantitative predictions of peptide-MHC binding by a 'Query by Committee' artificial neural network approach. Tissue Antigens 2003; 62(5): 378-384.
23. Nielsen M, Lundegaard C, and Lund O. Prediction of MHC class II binding affinity using SMM-align, a novel stabilization matrix alignment method. BMC.Bioinformatics. 2007; 8: 238.
24. Nielsen M and Lund O. NN-align. An artificial neural network-based alignment algorithm for MHC class II peptide binding prediction. BMC.Bioinformatics. 2009; 10: 296.
25. Nielsen M, Justesen S, Lund O, et al. NetMHCIIpan-2.0 - Improved pan-specific HLA-DR predictions using a novel concurrent alignment and weight optimization training procedure. Immunome.Res. 2010; 6: 9.
26. Southwood S, Sidney J, Kondo A, et al. Several common HLA-DR types share largely overlapping peptide binding repertoires. J.Immunol. 1998; 160(7): 3363-3373.
27. Glucksberg H, Storb R, Fefer A, et al. Clinical manifestations of graft-versus-host disease in human recipients of marrow from HL-A-matched sibling donors. Transplantation 1974; 18(4): 295-304.
28. Ossenkoppele GJ, Graveland WJ, Sonneveld P, et al. The value of fludarabine in addition to ARA-C and G-CSF in the treatment of patients with high-risk myelodysplastic syndromes and AML in elderly patients. Blood 2004; 103(8): 2908-2913.
29. Cheson BD, Pfistner B, Juweid ME, et al. Revised

response criteria for malignant lymphoma. J.Clin.Oncol. 2007; 25(5): 579-586.
30. Cornelissen JJ, van der HB, Verhoef GE, et al. Myeloablative allogeneic versus autologous stem cell transplantation in adult patients with acute lymphoblastic leukemia in first remission: a prospective sibling donor versus no-donor comparison. Blood 2009; 113(6): 1375-1382.
31. Lowenberg B, Ossenkoppele GJ, van PW, et al. High-dose daunorubicin in older patients with acute myeloid leukemia. N.Engl.J.Med. 2009; 361(13): 1235-1248.
32. Lokhorst HM, van der HB, Zweegman S, et al. A randomized phase 3 study on the effect of thalidomide combined with adriamycin, dexamethasone, and high-dose melphalan, followed by thalidomide maintenance in patients with multiple myeloma. Blood 2010; 115(6): 1113-1120.
33. Fleischhauer K, Locatelli F, Zecca M, et al. Graft rejection after unrelated donor hematopoietic stem cell transplantation for thalassemia is associated with nonpermissive HLA-DPB1 disparity in host-versus-graft direction. Blood 2006; 107(7): 2984-2992.
34. Shaw BE, Marsh SG, Mayor NP, et al. HLA-DPB1 matching status has significant implications for recipients of unrelated donor stem cell transplants. Blood 2006; 107(3): 1220-1226.
35. Shaw BE, Arguello R, Garcia-Sepulveda CA, et al. The impact of HLA genotyping on survival following unrelated donor haematopoietic stem cell transplantation. Br.J.Haematol. 2010; 150(3): 251-258.
36. Shaw BE, Mayor NP, Russell NH, et al. Diverging effects of HLA-DPB1 matching status on outcome following unrelated donor transplantation depending on disease stage and the degree of matching for other HLA alleles. Leukemia 2010; 24(1): 58-65.
37. Ibisch C, Gallot G, Vivien R, et al. Recognition of leukemic blasts by HLA-DPB1-specific cytotoxic T cell clones: a perspective for adjuvant immunotherapy post-bone marrow transplantation. Bone Marrow Transplant. 1999; 23(11): 1153-1159.
38. Shiina T, Suzuki S, Ozaki Y, et al. Super high resolution for single molecule-sequence-based typing of classical HLA loci at the 8-digit level using next generation sequencers. Tissue Antigens 2012; 80(4): 305-316.

Supplementary data

Variable	Permissive N(%)	GVH non-permissive N(%)	HVG non-permissive N(%)	P
Age, mean in years	55	49	57	**0.01**
Disease				NS
Acute leukemia	15 (28)	1 (8)	1 (8)	
Chronic leukemia	6 (11)	0 (0)	1 (8)	
Lymphoma	10 (19)	3 (25)	3 (25)	
Plasma cell disorder	9 (17)	4 (33)	4 (33)	
MDS/MPN	12 (22)	3 (25)	2 (17)	
Bone marrow failure	2 (4)	1 (8)	1 (8)	
Patient gender, female	16 (30)	4 (33)	5 (42)	NS
Donor gender, female	17 (31)	5 (42)	3 (25)	NS
Gender mismatch	5 (9)	2 (17)	0 (0)	NS
Patient CMV seropositive	36 (67)	8 (75)	8 (75)	NS
Donor CMV seropositive	20 (37)	7 (58)	4 (33)	NS
CMV mismatch	15 (28)	3 (25)	4 (33)	NS
Patient EBV seropositive	53 (98)	12 (100)	11 (92)	NS
Donor EBV seropositive	35 (65)	8 (75)	10 (83)	NS
EBV mismatch	6 (11)	1 (8)	1 (8)	NS

Supplementary Table 1: Distribution of variables amongst TCE groups. Distributions of variables were tested amongst the three TCE groups with ANOVA for age and chi-square for categorical variables. Patients in the GVH nonpermissive group were significantly younger compared with both other groups. Gender mismatch: female donor for male recipient. CMV or EBV mismatch: patient seropositive, donor seronegative. NS: not significant.

Variable	PIRCHE-I absent N(%)	PIRCHE-I present N(%)	p
Age, mean in years	55	54	NS
Disease			NS
Acute leukemia	5 (18)	12 (23)	
Chronic leukemia	4 (15)	3 (6)	
Lymphoma	7 (26)	10 (19)	
Plasma cell disorder	5 (19)	13 (25)	
MDS/MPN	5 (19)	12 (23)	
Bone marrow failure	1 (4)	3 (6)	
Patient gender, female	10 (37)	15 (28)	NS
Donor gender, female	11 (41)	16 (30)	NS
Gender mismatch	3 (11)	6 (11)	NS
Patient CMV seropositive	22 (78)	33 (62)	NS
Donor CMV seropositive	11 (41)	21 (40)	NS
CMV mismatch	9 (33)	14 (26)	NS
Patient EBV seropositive	26 (96)	51 (96)	NS
Donor EBV seropositive	18 (67)	36 (68)	NS
EBV mismatch	4 (15)	5 (9)	NS

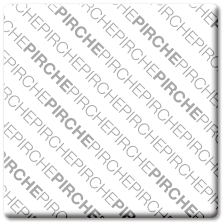
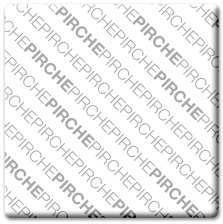
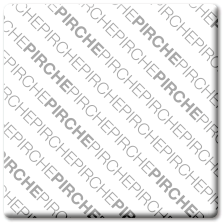
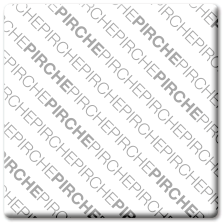
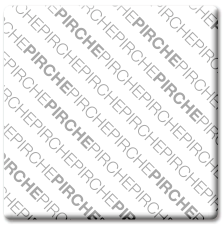
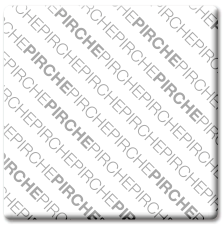
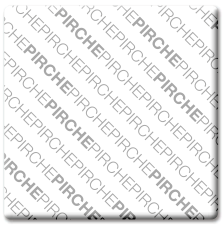
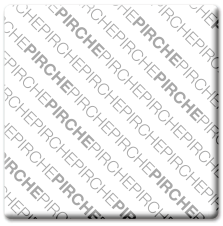
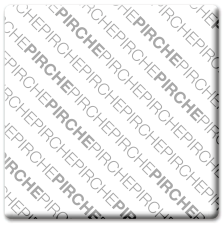
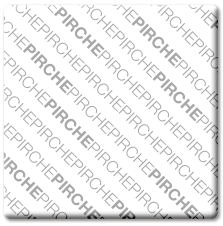
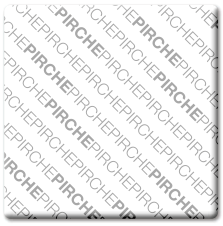
Supplementary Table 2: Distribution of variables amongst PIRCHE-I groups. Distributions of variables were tested amongst the two PIRCHE-I groups with ANOVA for age and chi-square for categorical variables. Gender mismatch: female donor for male recipient. CMV or EBV mismatch: patient seropositive, donor seronegative. NS: not significant.

Variable	PIRCHE-II absent N(%)	PIRCHE-II present N(%)	p
Age, mean in years	557	54	NS
Disease			NS
Acute leukemia	4 (25)	13 (20)	
Chronic leukemia	2 (13)	5 (8)	
Lymphoma	3 (19)	14 (22)	
Plasma cell disorder	3 (19)	15 (23)	
MDS/MPN	4 (25)	13 (20)	
Bone marrow failure	0 (0)	4 (6)	
Patient gender, female	6 (38)	19 (30)	NS
Donor gender, female	7 (44)	20 (31)	NS
Gender mismatch	2 (13)	7 (11)	NS
Patient CMV seropositive	13 (81)	41 (64)	NS
Donor CMV seropositive	8 (50)	24 (38)	NS
CMV mismatch	5 (31)	18 (28)	NS
Patient EBV seropositive	15 (94)	62 (97)	NS
Donor EBV seropositive	11 (69)	43 (67)	NS
EBV mismatch	3 (19)	6 (9)	NS

Supplementary Table 3: Distribution of variables amongst PIRCHE-II groups. Distributions of variables were tested amongst the two PIRCHE-II groups with ANOVA for age and chi-square for categorical variables. Gender mismatch: female donor for male recipient. CMV or EBV mismatch: patient seropositive, donor seronegative. NS: not significant.

Product	Primer sequence
Amplification	
Intron 1-3'UTR (38)	FW: 5' CTCAGTGCTCGCCCCTCCCTAGTGAT 3'
	RV: 5' GCACAGTAGCTTTCGGGAATTGACCA 3'
Exon 2	FW: 5' GAGAGTGGCGCCTCCGCTC 3'
	RV: 5' CCGGCCCAAAGCCCTCACTC 3'
Exon 2-3	GenDx core kit.
Exon 1	FW: 5' CAGAGCAAAGAAAACGCATAAT 3'
	RV: 5'GAGAATAGGCCAGTAGGGTAGC 3'
Sequencing	
Exon 1	FW: 5' TCCCTTTAGCGAGTCC 3'
	RV: 5' GCCAGTAGGGTAGCAAG 3'
Exon 2	FW: 5' TGGCGCCTCCGCTCATG 3'
	RV: 5' CCAAAGCCCTCACTCA 3'
Exon 3	FW: 5' GAAGGACAATCTCAAATTC 3'
	FW: 5' GAAGGACAATCTCAAATGC 3'
	RV: 5' CCTTCAAACTATGCTC 3'
Exon 4	FW: 5' GATATGCTGCATCAGG 3'
	RV: 5' CCTCATGCATCTGGC 3'

Supplementary Table 4: primer sequences as used for PCR and sequencing-based HLA-DPB1 typing. For the amplification of the HLA-DP loci in 86% of the cases primers amplifying from intron 1 to 3'UTR were used. If amplifications were unsuccessful, the GenDx core kit (8%) was used, amplifying exon 2-3, or exon 2 amplification only. To resolve ambiguities in the latter 2 amplification strategies, exon 1 was amplified if required. All sequence reactions were performed with the primers as mentioned. FW: forward primer. RV: reverse primer.






5







Chapter 5

Indirectly recognized HLA-C mismatches and their potential role in transplant outcome

Kirsten A. Thus,[1] Liane te Boome,[2] Jürgen Kuball[1,2] and Eric Spierings[1]

[1]Laboratory of Translational Immunology,
University Medical Center Utrecht, Utrecht, the Netherlands
[2]Department of Hematology,
University Medical Center Utrecht, Utrecht, the Netherlands

Frontiers in Immunology 2014; 5: 210

Abstract

HLA-C mismatches are clearly associated to alloreactivity after hematopoietic stem-cell transplantation; in a number of large cohorts, HLA-C mismatches are correlated to an increased risk of acute graft-versus-host disease (GVHD) or even impaired survival. While for HLA-A and -B both antigenic as well as allelic mismatches are associated with an increased risk of acute GVHD, such an increased risk is only observed for antigenic HLA-C mismatches and not for allelic mismatches. These observations raise the question what sets HLA-C apart from HLA-A and -B. The difference may well be related to the reduced levels of cell-surface expression of HLA-C as compared to HLA-A and -B, possibly due to, amongst other factors, a limited peptide-binding capacity. This limited peptide-binding capacity may retain HLA-C in the ER and enhance degradation of the HLA-C protein. Once degraded, HLA-C-derived peptides can be presented to the immune system via other HLA-alleles and are thus available for indirect recognition. Indeed, such HLA-C derived peptides have previously been eluted from other HLA alleles. We have recently developed an approach to predict indirect recognition of HLA molecules, by establishing the numbers of Predicted Indirectly ReCognizable HLA Epitopes (PIRCHES). The number of PIRCHES presented on HLA class I and II (PIRCHE-I and -II respectively), are highly correlated to clinical measures of alloreactivity, such as acute GVHD. In the present “Hypothesis & Theory”, we reviewed the current knowledge on HLA-C mismatches and alloreactivity. Moreover, we speculate about the role of direct and indirect recognition of HLA-C and the consequences for donor selection in HLA-C mismatched stem-cell transplantation.

Introduction

HLA-C is a classical HLA class-I protein, thus expressed on nucleated cells and is able to present peptides to T cells. Like the other classical HLA class-I proteins (HLA-A and -B), HLA-C consists of a polymorphic heavy chain and the non-polymorphic β2-microglobulin. The coding region for the heavy chain is located on chromosome six, in close vicinity of the HLA-B locus. HLA-C and -B alleles are therefore often inherited in non-random combinations, the so called linkage disequilibrium.

Under normal conditions, HLA-C is expressed at low levels on the cell surface. This low expression level is likely the result of multiple factors: the HLA-C heavy chain messenger RNA is unstable (1); the HLA-C heavy chain does not associate efficiently with the β2-microglobulin (2-4); and HLA-C presents a rather restricted repertoire of peptides due to a very restricted α1 domain (5, 6). Due to the restricted peptide repertoire and the inefficient association with β2-microglobulin, HLA-C is often retained within the endoplasmatic reticulum (ER) and degraded (4, 6, 7). Next to presenting peptides, HLA-C also serves as a ligand for natural killer (NK) cell receptors: Killer Immunoglobulin-like Receptors (KIR). HLA-C binding to KIRs can act as a negative or positive signal for the NK cells. It is often proposed that the negative signal is the main function of HLA-C and that therefore HLA-C cell surface expression levels are low (for a comprehensive review regarding the function of HLA-C in relation to KIR, see (8))(7).

Despite the low expression level of HLA-C, HLA-C mismatches are clearly associated to alloreactivity after hematopoietic stem-cell transplantation (HSCT): in a number of large cohorts HLA-C mismatches are correlated to an increased risk of acute graft-versus-host disease (GVHD) or even impaired survival (Figure 1AB) (9-13).

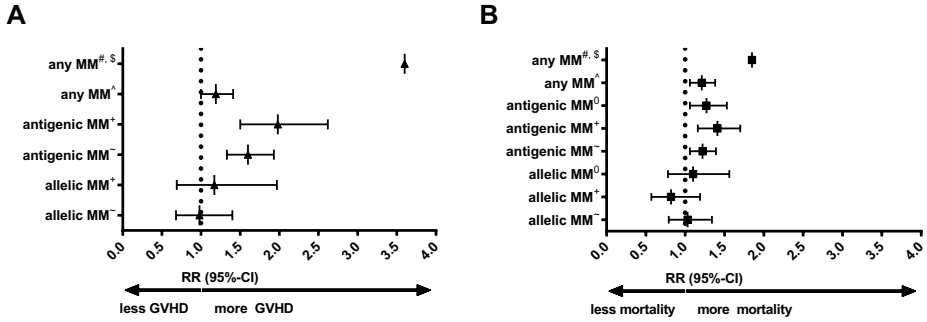


Figure 1 (A) Forest plot for relative risk of acute GVHD grade III-IV of HLA-C mismatches compared to HLA-matched transplants. (B) Forest plot for relative risk of mortality of HLA-C mismatches compared to HLA-matched transplants. #: (10) 33 cases of an HLA-C mismatch (either allelic or antigenic, also in combination with other mismatched loci) were compared to 78 10/10 matches. $: Authors indicated hazard ratios. ^: (11) 749 HLA-C mismatches (either allelic or antigenic) were compared to 108 8/8 matches. +: (9) 189 HLA-C antigenic mismatches and 61 HLA-C allelic mismatches were compared to 1243 8/8 matches. ¯: (12) 382 HLA-C antigenic mismatches and 96 HLA-C allelic mismatches were compared to 1840 8/8 matches. 0: (13) 300 HLA-C antigenic and 57 HLA-C allelic mismatches were compared to 1511 10/10 matches. 10/10 match: donor and recipient matched on high-resolution level for HLA-A, -B, -C, -DRB1, -DQB1. 8/8 match: donor and recipient matched on high-resolution level for HLA-A, -B, -C, -DRB1. MM: mismatch. RR: relative risk, relative to either 8/8 or 10/10 matches, as indicated.

Interestingly, for other HLA class-I mismatches (HLA-A and -B) both low-resolution level (antigenic) as well as high-resolution level (allelic) mismatches are associated with an increased risk of acute GVHD; whereas for HLA-C mismatches this increased risk is only observed for HLA-C antigenic mismatches (9, 12). The effect of HLA-C mismatches on alloreactivity may be explained by NK-cell recognition, however, the exact role of missing KIR ligands in HLA-C mismatched HSCT remains to be elucidated (14). On the other hand, development of acute GVHD clearly involves antigen recognition by T cells (as reviewed in (15)). The aim of this "Hypothesis & Theory" paper is to provide a potential explanation for the high immunogenicity of HLA-C antigenic mismatches, despite the low cell-surface expression levels. This potential explanation is based on how T-cell recognition might be involved in the alloreactivity related to HLA-C mismatches.

T-cell alloreactivity

HLA mismatches can lead to T-cell induced alloreactivity via two routes: direct or indirect recognition. Direct recognition is the process where the donor T cell recognizes the intact mismatched HLA molecule on the cell surface of recipient's cells. Direct recognition is unlikely in the case of HLA-C mismatches because of the low cell-surface expression levels. Indirect recognition occurs when the mismatched HLA protein is processed within the cell and is presented as peptides by HLA molecules. At least 59 peptides derived from HLA-C have been eluted from HLA (16). When the mismatched HLA-C derived peptides differ from self peptides, they can be recognized by T cells.

Our group has recently developed an approach to predict indirect recognition of mismatched HLA, by establishing the numbers of Predicted Indirectly ReCognizable HLA Epitopes (PIRCHES) (17). The number of PIRCHES presented on HLA class I and II, PIRCHE-I and -II respectively, are highly correlated to clinical measures of alloreactivity, such as acute GVHD and transplant-related mortality (Thus *et al.*, manuscripts in preparation).

HLA-C derived PIRCHES

We hypothesize that the thus far unexplained substantial alloreactivity of HLA-C mismatches evolves due to indirect recognition of HLA-C. Indirect recognition may furthermore explain the observation that HLA-C antigenic mismatches specifically lead to alloreactivity, as antigenic mismatches likely lead to a higher number of indirectly recognizable epitopes compared to allelic mismatches. To support these hypotheses, we analysed our local cohort of patients transplanted with an unrelated single HLA-mismatched donor (a 9/10) after non-myeloablative conditioning. All patients and donors were typed for HLA-A, -B, -C, -DRB1, -DQB1, at ultra-high (4-digit) resolution level, resolving all ambiguities. For retrospective high-resolution HLA-C typing of one HSCT pair, no remaining DNA was available. For this single situation, the high-resolution HLA-typing of this donor-recipient pair was deduced based upon the low-resolution HLA-C typing using the HLA-B/-C association probability (18). The majority of the 48 patients included in these analyses, were transplanted with a mismatch for HLA-C (N=20, 42%, Table 1).

PIRCHES were determined in the previously described manner (17) with some adaptations; differences in the current study are the incorporation of NetMHCIIPan 2.0, and NetChop for predicting processing of peptides with a processing probability of >0.5 and NetMHCPan 2.4 to select potential binders with an IC_{50} value <500nM for predicting PIRCHE-I (19-22).

Mismatch locus	N (%)
HLA-A	10 (21)
HLA-B	6 (13)
HLA-C	20 (42)
HLA-DRB1	2 (4)
HLA-DQB1	10 (21)

Table 1: The number and percentage of patients, per mismatched locus. We investigated our complete local cohort of patients transplanted with a single HLA-mismatched unrelated donor (a 9/10 match) after non-myeloablative conditioning, for various underlying diseases. The majority (42%) was transplanted with an HLA-C mismatch.

We first analyzed the numbers of PIRCHE-I and -II separate per mismatched HLA locus (Figure 2AB). HLA-C mismatches yielded the highest numbers of PIRCHE-I (Figure 2A), although the numbers of PIRCHE-I derived from HLA-C were not significantly different when compared to those derived from the other loci, likely due to the low patient numbers. The number of PIRCHE-II derived from HLA-C were significantly higher than those derived from HLA-B and HLA-DQB1 (Figure 2B, $p=0.04$ and $p<0.01$, respectively). The majority of the HLA-C mismatches were antigenic mismatches (N=18, 90%). The abundance of antigenic HLA-C mismatches may explain the high PIRCHE numbers, as the antigenic HLA-C mismatches led to significantly higher numbers of PIRCHES than the allelic mismatches ($p=0.03$ and $p=0.02$ for PIRCHE-I and -II respectively). Allelic HLA-C mismatches always resulted in 0 PIRCHE-I, whereas the number of PIRCHE-II did not exceed 1. Antigenic HLA-C mismatches led to a median of 6 PIRCHE-I (range 0-11), and a median of 18 PIRCHE-II (range 1-32) (Figure 2CD).

To investigate whether indirect recognition of HLA-C predicts alloreactivity, we selected the HLA-C mismatched transplantations only. We subsequently analyzed whether the risk of alloreactivity is related to the number of PIRCHES instead of the allelic versus antigenic definition. To this end, we redefined the HLA-C mismatches into low or higher number of PIRCHES. We defined 0 PIRCHE-I as low PIRCHE-I, as this was the number of PIRCHES derived from the allelic mismatches, and we defined ≤1 PIRCHE-II as low PIRCHE-II, as 1 was the maximum number of PIRCHE-II derived from the allelic mismatches. Interestingly, we have previously shown that these cut-offs were also the cut off values of the lowest tertiles of HLA-DPB1 derived PIRCHES (manuscript in preparation).

For all transplant recipients, the numbers of PIRCHES were correlated to acute GVHD development. We observe a trend for patients in the higher PIRCHE-I or -II group having an increased probability of acute GVHD compared to the low PIRCHES (Figure 3AB). Patients presenting low HLA-C derived PIRCHE-I or -II (N=3) did not develop acute GVHD. This difference is, although striking, not significant, likely due to low patient and event numbers (6 events). The number of PIRCHES is not associated to the severity of acute GVHD in this cohort, although such an association requires a larger study population; we observed only 3 cases of clinically severe acute GVHD (grade III-IV).

Potential implications for donor selection

For HSCT donor-selection procedures, potential donors are at first mainly typed on a low to intermediate resolution level for HLA-A, -B, and -DRB1. Based on donor-recipient matching for these loci, a limited number of donors are selected for further high-resolution typing, which includes typing of the other loci. For patients with rare HLA-B/-C associations, it will be very challenging to find a donor matched for both HLA-B and -C, due to the strong linkage disequilibrium between HLA-B and -C. As HLA-B matching is considered earlier in the donor selection procedure than HLA-C, patients with rare HLA-B/-C associations are

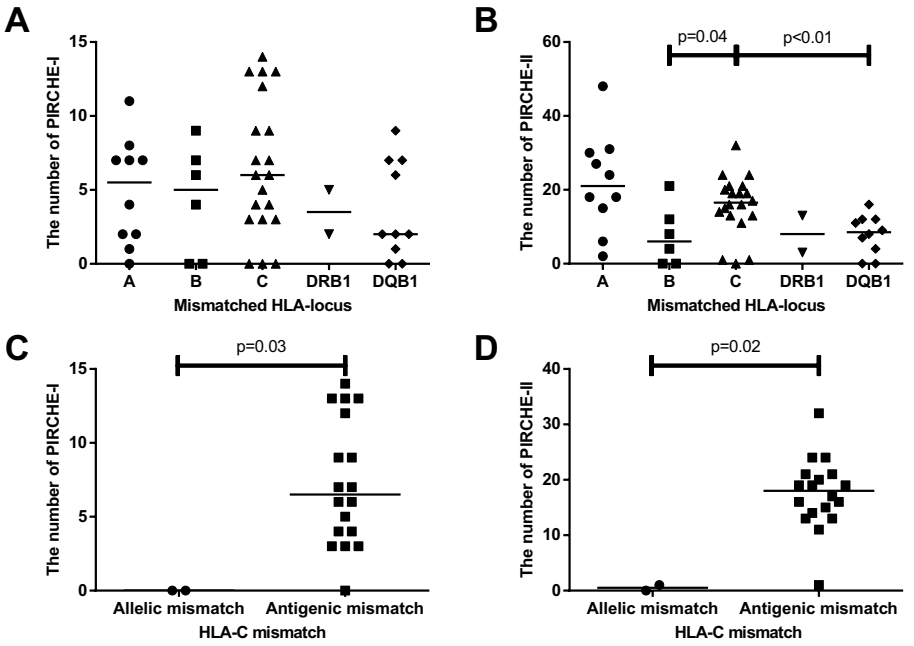


Figure 2 (A) The number of PIRCHE-I displayed by the mismatched locus they are derived from. (B) The number of PIRCHE-II displayed by the mismatched locus they are derived from. (C) The number of PIRCHE-I derived from an HLA-C allelic versus antigenic mismatch. (D) The number of PIRCHE-II derived from an HLA-C allelic versus antigenic mismatch. Horizontal lines indicate the median value, differences between groups were tested with Mann-Whitney U Tests. Patients with an HLA-C mismatch had a significantly higher number of PIRCHE-II compared to patients with an HLA-B or –DQB1 mismatch. Patients with HLA-C antigenic mismatches, had higher number of PIRCHE-I and -II compared to allelic mismatches.

more frequently transplanted with an HLA-C mismatch. These HLA-C mismatches are often antigenic mismatches, as can be expected from the observed HLA-B/-C associations (18). Our data indicate that these antigenic HLA-C mismatches very frequently lead to high numbers of PIRCHES, due to the low level of homology between the two mismatched alleles. We propose that a mismatch for HLA-B may be considered in these cases, as in some situations the HLA-B mismatches can lead to lower numbers of PIRCHES than HLA-C mismatches.

To support the above-mentioned option, we performed a theoretical analysis. To this end, we analyzed the possibility to identify an alternative 9/10 mismatched donor for those HLA-C mismatched cases that had an increased probability of acute GVHD (*i.e.* PIRCHE-I>0 and PIRCHE-II>1, N=17), using haplotype frequency tables (18). For these patients, we aimed at a theoretical HLA-B mismatch instead of an HLA-C mismatch. For 13 (76%) patients we could identify a potential HLA-B mismatched donor (Table 2). In 7 (54%) of these cases, the HLA-B mismatch led to a lower number of PIRCHE-I than the selected HLA-C mismatched donor, and in 9 (69%) of the cases the HLA-B mismatch yielded a lower number of PIRCHE-II than the HLA-C mismatch (Figure 4AB). The numbers of PIRCHE-II related to an HLA-B mismatch instead of an HLA-C mismatch, are significantly reduced for the HLA-B mismatched cases (p=0.03). Thus, HLA-B mismatches can lead to a lower probability of indirect recognition than HLA-C mismatches. We hypothesize that the effect of HLA mismatches does not depend on a locus-specific effect, but is rather related to the resulting PIRCHES.

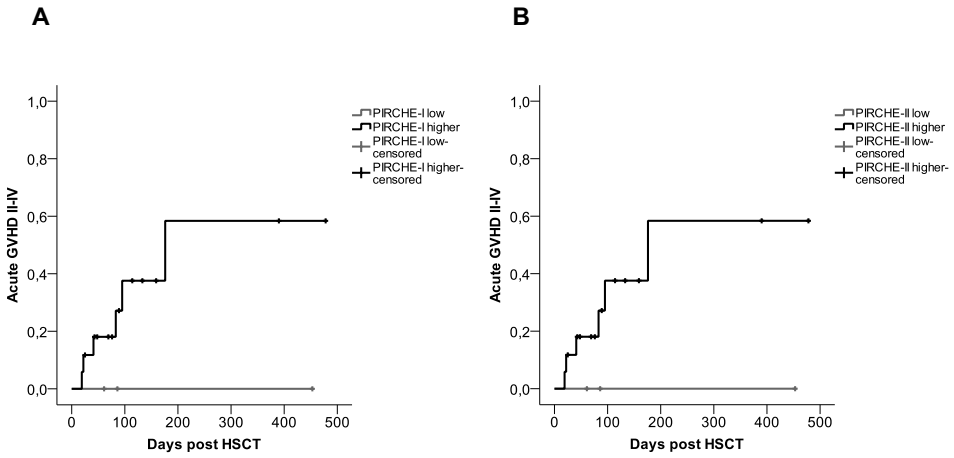


Figure 3 (A) Probability of acute GVHD by HLA-C derived PIRCHE-I low or PIRCHE-I higher. (B) Probability of acute GVHD by HLA-C-derived PIRCHE-II low or PIRCHE-II higher. Kaplan-Meier curves were constructed to analyze the probability of developing acute GVHD II-IV for patients in the low (in grey) and higher (in black) PIRCHE groups. Patients with low PIRCHE-I did not develop acute GVHD. Patients with low PIRCHE-II did not develop acute GVHD. Probabilities of acute GVHD II-IV were not significantly different amongst the low or higher PIRCHE groups as tested with log-rank tests. GVHD: graft-versus-host disease. HSCT: hematopoietic stem-cell transplantation.

Discussion

HLA-C mismatches lead to substantial alloreactivity, despite the low cell-surface expression levels of HLA-C. Particularly, antigenic HLA-C mismatches lead to high risks of complications (Figure 1). In our local cohort of patients transplanted with a single HLA mismatch, we show that HLA-C mismatches lead to higher numbers of indirectly recognizable epitopes (PIRCHES) than when mismatches are located on other loci (Figure 2). Furthermore, patients presenting HLA-C derived PIRCHE-I or more than one HLA-C derived PIRCHE-II are at a higher risk of developing acute GVHD. Indirect recognition of HLA-C mismatches may therefore provide an explanation for the alloreactive complications observed after HLA-C mismatched transplants.

In theory, HLA-C allelic mismatches may lead to direct recognition by donor T cells, as the T-cell receptor (TCR) contact residues likely remain similar amongst allelic mismatches. The polymorphisms in allelic mismatches will mostly reside within the peptide-binding groove, and can thus lead to different peptide presentation repertoires. Because of the self-HLA restriction of the TCR, the T cell may still bind to the allelic mismatch and can then recognize the different peptide repertoire as foreign. With antigenic mismatches, the self and allogeneic HLA will contain large numbers of polymorphic residues, and the TCR may not bind to the mismatched allogeneic HLA anymore. When the TCR cannot bind to the allogeneic HLA, recognition of the mismatch will not occur. Therefore, direct recognition seems more likely in the case of allelic mismatches. In line with this suggestion, previous *in vitro* studies have proposed that one should rather mismatch largely (antigenic) instead of only for a small number of polymorphic residues (allelic)(23). The latter study clearly showed that the chance of the donor developing cytotoxic T-lymphocytic precursors *in vitro* was more likely in less polymorphic mismatches, suggesting a greater probability of direct recognition by donor T cells *in vivo*. However, as HLA-C cell-surface expression is low, devel-

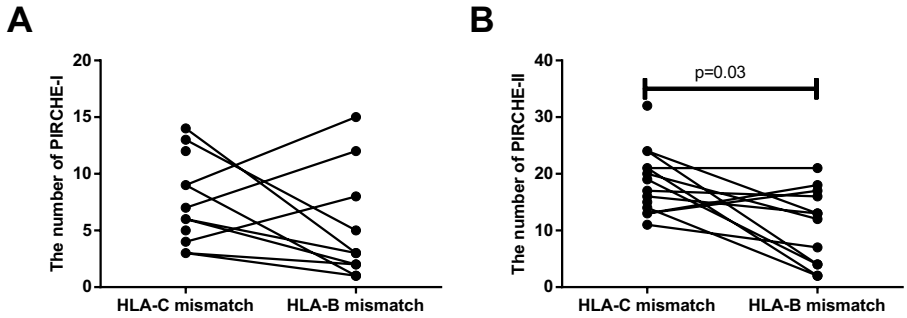


Figure 4 (A) The number of PIRCHE-I for the selected HLA-C mismatch, compared to a potential HLA-B mismatch. (B) The number of PIRCHE-II for the selected HLA-C mismatch, compared to a potential HLA-B mismatch. For 17 patients with high numbers of mismatched HLA-C derived PIRCHE-I and -II, we analyzed whether we could potentially find a mismatched HLA-B alternative donor. We found that in 7 (54%) of these cases we could reduce the number of PIRCHE-I with this strategy and in 9 (69%) of these cases we could reduce the number of PIRCHE-II. The numbers of PIRCHE-II are significantly lower when we would have chosen the potential HLA-B mismatch instead of the selected HLA-C mismatch (Wilcoxon matched-pairs signed ranked test, p=0.03).

opment of direct recognition is less probable. In line with this assumption, the previously reported HistoCheck model for direct recognition of HLA class-I mismatches, did not predict alloreactive complications after HLA-C mismatched HSCT (nor for HLA-A and -B mismatches) (24). Similarly, the scores obtained with this direct recognition model showed no correlation to acute GVHD development in our cohort (p=0.97). These observations further support the hypothesis that indirect recognition may be an important route of HLA-C mismatches evoking alloreactivity.

HLA-C cell-surface expression is low due to, amongst other factors, a limited peptide presentation profile and subsequent unstable association with β2-microglobulin. This instability leads to prolonged HLA-C presence in the ER and finally degradation of the protein. When HLA-C is degraded, it can thereafter be presented on other HLA proteins as peptides. Indeed, HLA-C derived epitopes are frequently diluted from other HLA alleles (16). These HLA-C derived PIRCHES can lead to alloreactivity. As HLA-C allelic mismatches lead to a low number of PIRCHES and antigenic mismatches to a high number of PIRCHES, indirect recognition of mismatched HLA-C may explain the risk related to antigenic HLA-C mismatches and the absence of this relationship for allelic HLA-C mismatches.

Recently, another study explained the absence of immunogenicity of HLA-C allelic mismatches by the predominance of the HLA-C*03:03/03:04 mismatch combination in this group (25). In this study, a negative impact on clinical outcomes was observed for HLA-C antigenic mismatches and any mismatch on HLA-A, -B, or -DRB1, whereas HLA-C*03:03/03:04 mismatches had similar outcomes as HLA-A, -B, -C, -DRB1 matched (8/8) transplantations. In contrast, HLA-C allelic mismatches other than HLA-C*03:03/03:04 did lead to an increased probability of acute GVHD. These data may also be explained by the indirect recognition model, as the HLA-C*03:03/03:04 mismatch leads to a difference in only one amino acid, and therefore likely yields a low number of indirectly recognizable epitopes. Indeed, in our cohort, two patients were transplanted with an HLA-C*03:03/03:04 mismatch, leading to low PIRCHE-I and -II (Table 2).

HLA-C mismatches can not only lead to T-cell recognition; B-cell recognition may alternatively lead to alloreactivity upon HLA-mismatched HSCT. The development of HLA-C

specific antibodies is correlated to complications after HLA-mismatched organ transplantation (26). HLAMatchmaker is a well-validated *in silico* tool for analyzing such HLA-specific antibody responses (27, 28). The number of eplets defined by HLAMatchmaker is correlated to the antibody reactivity against mismatched HLA (29). Although HLA antibodies may play a role in HSCT outcome, the HLAMatchmaker algorithm is not suitable to predict GVH-directed alloreactivity in HLA-mismatched HSCT (30). Particularly for HLA-C, a correlation between GVHD and HLAMatchmaker scores may be unlikely; low cell-surface expression of HLA-C also limits the potential of binding by HLA-C specific antibodies to the mismatched alleles. Indeed, in our small cohort of HLA-C mismatches, HLAMatchmaker scores are also not correlated to acute GVHD development (p=0.77).

HLA-C mismatched donors are more frequently selected than HLA-B mismatched donors, due to the previously mentioned donor selection procedures. We have proposed that HLA-B mismatches may in some situations lead to a lower probability of indirect recognition than HLA-C mismatches, and that therefore an HLA-B mismatch may be preferred. Although some studies indicate a particularly strong effect of HLA-B mismatched transplantations on detrimental outcomes (13); literature remains inconclusive regarding a higher risk of HLA-B mismatches compared to other mismatched loci (9, 11, 12). Before implementing the proposed strategy of selecting donors with the lowest numbers of PIRCHES, regardless of the locus that is mismatched, the effect of PIRCHES per mismatched locus should be studied in a large cohort. Such studies would also allow investigations on the risk of alloreactivity due to direct recognition in cases with zero PIRCHES, reflecting the absence of indirect recognition.

To summarize, in this "Hypothesis & Theory" paper, we investigated whether indirect recognition of HLA-C mismatches may explain the risk of alloreactivity in the context of the relatively low cell-surface expression level of HLA-C. We observed a high number of HLA-C derived PIRCHES in the case of antigenic HLA-C mismatches. These high numbers of PIRCHES seem to be correlated to an increased acute GVHD risk. We next investigated whether selection of an HLA-B mismatched donor might lead to lower numbers of indirectly recognizable epitopes compared to the selected HLA-C mismatch. Indeed, for a number of patients we could identify a potential lower immunogenic alternative. It might thus be preferable to select a mismatch that leads to the lowest number of PIRCHES, instead of avoiding mismatches on a specific locus, although this requires confirmation. This strategy may reduce the risk of alloreactive complications. We further propose that future studies investigating the effect of HLA-C, and other mismatches, on alloreactivity after HSCT with different stem-cell sources, need to be conducted in large cohorts in order to verify the clinical relevance of our hypothesis.

Conflicts of interests

The authors have no personal conflict of interest to declare. The UMCU has filed a patent application on the prediction of an alloimmune response against mismatched HLA.

Acknowledgements

We would gratefully like to acknowledge Can Keşmir, Jorg Calis, Hanneke van Deutekom and Eric Borst for their help in the computational development of the PIRCHES model. We also thank the HLA laboratory of the University Medical Center Utrecht for the retrospective high-resolution HLA typing of our cohort.

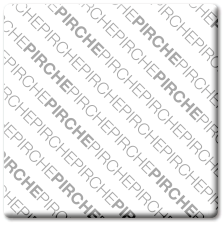
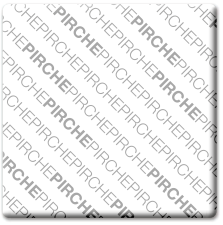
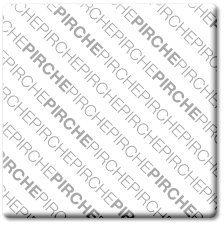
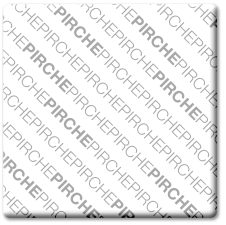
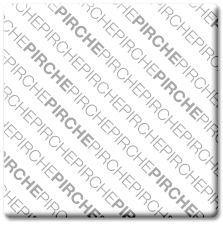
	Patient HLA-typing										Selected HLA-C mismatch			Potential HLA-B mismatch		
Patient ID	**HLA-A**		**HLA-B**		**HLA-C**		**HLA-DRB1**		**HLA-DQB1**		**HLA-C donor**	**PIRCHE-I**	**PIRCHE-II**	**HLA-B donor**	**PIRCHE-I**	**PIRCHE-II**
1	01:01	02:01	15:01	51:01	03:03	**15:02**	13:01	15:01	06:02	06:03	07:02	13	16	NA		
2	02:01	-	40:01	51:01	**03:03**	03:04	09:01	13:02	03:03	06:04	05:01	0	1	NI		
4	01:01	03:01	08:01	*47:01*	07:01	**03:04**	03:01	14:01	02:01	05:03	06:02	4	13	40:01	8	18
10	24:02	24:02	15:01	*44:03*	03:03	**05:01**	11:01	12:01	03:01	03:01	04:01	3	24	44:02	2	4
11	02:01	11:01	07:02	18:03	**02:02**	07:01	11:04	15:01	03:01	06:02	07:02	13	32	NA		
14	03:01	32:01	07:02	15:01	07:02	**03:03**	07:01	13:01	02:02	06:03	01:02	4	15	NA		
16	02:01	29:02	*44:04*	51:01	16:01	**15:02**	10:01	11:01	05:01	03:01	14:02	14	19	44:03	3	4
23	01:01	02:01	08:01	15:01	**03:03**	07:01	03:01	04:01	02:01	03:01	03:04	0	1	NI		
25	03:01	11:01	07:02	*18:01*	07:02	**12:03**	11:01	13:01	03:01	06:03	07:01	9	21	38:01	15	21
29	02:01	-	35:01	*51:01*	**01:02**	04:01	13:02	15:01	06:02	06:04	15:02	5	11	27:05	0	7
30	02:01	03:01	*13:02*	35:01	**02:02**	04:01	04:01	07:01	02:02	03:02	06:02	6	16	27:05	3	13
31	02:01	11:01	15:01	*51:01*	04:01	**12:03**	01:01	04:01	03:02	05:01	03:04	7	13	39:01	12	17
33	02:01	24:02	*40:01*	57:01	06:02	**07:02**	07:01	13:02	03:03	06:04	03:04	9	17	07:02	1	16
34	02:01	-	15:01	44:02	**03:04**	05:01	04:01	04:04	03:01	03:02	03:03	0	0	NI		
36	02:01	03:01	07:02	*07:02*	07:02	**02:02**	04:04	15:01	03:02	06:02	07:02	7	19	27:05	0	0
40	01:01	02:01	*15:01*	38:01	**04:01**	12:03	13:01	13:02	06:03	06:04	12:03	12	14	35:03	0	2
44	11:01	24:02	35:01	*35:03*	04:01	**03:03**	04:07	12:01	03:01	-	04:01	3	21	15:01	1	2
46	11:01	68:01	07:02	*27:05*	**02:02**	07:02	01:01	07:01	03:03	05:01	01:02	6	20	44:02	2	12
47	01:01	02:01	*18:01*	27:05	02:02	**05:01**	11:01	15:01	03:01	06:02	07:01	13	24	44:02	5	13
48	01:01	68:01	44:02	51:01	07:04	**14:02**	01:01	04:04	03:02	05:01	15:02	3	19	NA		

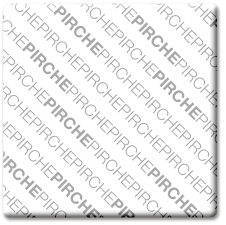
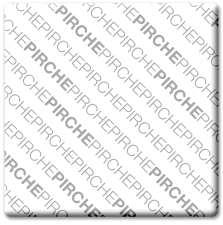
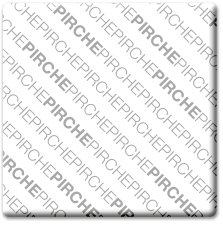
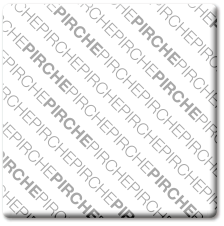
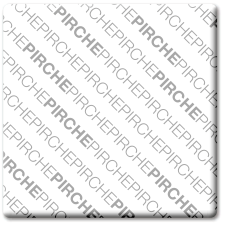
Table 2: HLA typing of the patient, selected HLA-C mismatch and potential HLA-B mismatch alternative. For all our HLA-C mismatched cases, we analyzed the numbers of PIRCHE-I and -II. We next investigated whether we could in theory (based on known haplotypes) identify an HLA-B mismatch instead of an HLA-C mismatch. In this table, HLA-typing of the patient is displayed, in bold the actual HLA-C mismatched allele and in italic the potential HLA-B mismatched allele. The HLA-typing of the (potential) donors is only displayed for the mismatch, as the other alleles have the same typing. NA: potential HLA-B mismatched alternative not available. NI: potential HLA-B mismatch not investigated, as the number of HLA-C derived PIRCHES was low.

References

1. McCutcheon JA, Gumperz J, Smith KD, et al. Low HLA-C expression at cell surfaces correlates with increased turnover of heavy chain mRNA. J.Exp.Med. 1995; 181(6): 2085-2095.
2. Setini A, Beretta A, De SC, et al. Distinctive features of the alpha 1-domain alpha helix of HLA-C heavy chains free of beta 2-microglobulin. Hum.Immunol. 1996; 46(2): 69-81.
3. Shimizu Y and DeMars R. Production of human cells expressing individual transferred HLA-A,-B,-C genes using an HLA-A,-B,-C null human cell line. J.Immunol. 1989; 142(9): 3320-3328.
4. Neefjes JJ and Ploegh HL. Allele and locus-specific differences in cell surface expression and the association of HLA class I heavy chain with beta 2-microglobulin: differential effects of inhibition of glycosylation on class I subunit association. Eur.J.Immunol. 1988; 18(5): 801-810.
5. Zemmour J and Parham P. Distinctive polymorphism at the HLA-C locus: implications for the expression of HLA-C. J.Exp.Med. 1992; 176(4): 937-950.
6. Neisig A, Melief CJ, and Neefjes J. Reduced cell surface expression of HLA-C molecules correlates with restricted peptide binding and stable TAP interaction. J.Immunol. 1998; 160(1): 171-179.
7. Schaefer MR, Williams M, Kulpa DA, et al. A novel trafficking signal within the HLA-C cytoplasmic tail allows regulated expression upon differentiation of macrophages. J.Immunol. 2008; 180(12): 7804-7817.
8. Blais ME, Dong T, and Rowland-Jones S. HLA-C as a mediator of natural killer and T-cell activation: spectator or key player? Immunology 2011; 133(1): 1-7.
9. Woolfrey A, Klein JP, Haagenson M, et al. HLA-C antigen mismatch is associated with worse outcome in unrelated donor peripheral blood stem cell transplantation. Biol.Blood Marrow Transplant. 2011; 17(6): 885-892.
10. Ho VT, Kim HT, Liney D, et al. HLA-C mismatch is associated with inferior survival after unrelated donor non-myeloablative hematopoietic stem cell transplantation. Bone Marrow Transplant. 2006; 37(9): 845-850.
11. Flomenberg N, Baxter-Lowe LA, Confer D, et al. Impact of HLA class I and class II high-resolution matching on outcomes of unrelated donor bone marrow transplantation: HLA-C mismatching is associated with a strong adverse effect on transplantation outcome. Blood 2004; 104(7): 1923-1930.
12. Lee SJ, Klein J, Haagenson M, et al. High-resolution donor-recipient HLA matching contributes to the success of unrelated donor marrow transplantation. Blood 2007; 110(13): 4576-4583.
13. Fürst D, Muller C, Vucinic V, et al. High-resolution HLA matching in hematopoietic stem cell transplantation: a retrospective collaborative analysis. Blood 2013; 122(18): 3220-3229.
14. Velardi A. Role of KIRs and KIR ligands in hematopoietic transplantation. Curr.Opin.Immunol. 2008; 20(5): 581-587.
15. Ferrara JL, Levine JE, Reddy P, et al. Graft-versus-host disease. Lancet 2009; 373(9674): 1550-1561.
16. Peptide database. SYFPEITHI 2013. Available from: http://www.syfpeithi.de
17. Otten HG, Calis JJ, Kesmir C, et al. Predicted indirectly recognizable HLA epitopes presented by HLA-DR correlate with the de novo development of donor-specific HLA IgG antibodies after kidney transplantation. Hum.Immunol. 2013; 74(3): 290-296.
18. Maiers M, Gragert L, and Klitz W. High-resolution HLA alleles and haplotypes in the United States population. Hum.Immunol. 2007; 68(9): 779-788.
19. Nielsen M, Justesen S, Lund O, et al. NetMHCIIpan-2.0 - Improved pan-specific HLA-DR predictions using a novel concurrent alignment and weight optimization training procedure. Immunome.Res. 2010; 6: 9.
20. Kesmir C, Nussbaum AK, Schild H, et al. Prediction of proteasome cleavage motifs by neural networks. Protein Eng 2002; 15(4): 287-296.
21. Hoof I, Peters B, Sidney J, et al. NetMHCpan, a method for MHC class I binding prediction beyond humans. Immunogenetics 2009; 61(1): 1-13.
22. Nielsen M, Lundegaard C, Blicher T, et al. NetMHCpan, a method for quantitative predictions of peptide binding to any HLA-A and -B locus protein of known sequence. PLoS.One. 2007; 2(8): e796.
23. Heemskerk MB, Cornelissen JJ, Roelen DL, et al. Highly diverged MHC class I mismatches are acceptable for haematopoietic stem cell transplantation. Bone Marrow Transplant. 2007; 40(3): 193-200.
24. Spellman S, Klein J, Haagenson M, et al. Scoring HLA class I mismatches by HistoCheck does not predict clinical outcome in unrelated hematopoietic stem cell transplantation. Biol.Blood Marrow Transplant. 2012; 18(5): 739-746.
25. Fernandez-Vina MA, Wang T, Lee SJ, et al. Identification of a permissible HLA mismatch in hematopoietic stem cell transplantation. Blood 2014; 123(8): 1270-1278.
26. Gilbert M, Paul S, Perrat G, et al. Impact of pretransplant human leukocyte antigen-C and -DP antibodies on kidney graft outcome. Transplant.Proc. 2011; 43(9): 3412-3414.
27. Duquesnoy RJ and Marrari M. HLAMatchmaker: a molecularly based algorithm for histocompatibility determination. II. Verification of the algorithm and determination of the relative immunogenicity of amino acid triplet-defined epitopes. Hum.Immunol. 2002; 63(5): 353-363.
28. Duquesnoy RJ, Takemoto S, de Lange P, et al. HLAmatchmaker: a molecularly based algorithm for histocompatibility determination. III. Effect of matching at the HLA-A,B amino acid triplet level on kidney transplant survival. Transplantation 2003; 75(6): 884-889.

29. Dankers MK, Witvliet MD, Roelen DL, et al. The number of amino acid triplet differences between patient and donor is predictive for the antibody reactivity against mismatched human leukocyte antigens. Transplantation 2004; 77(8): 1236-1239.
30. Duquesnoy R, Spellman S, Haagenson M, et al. HLAMatchmaker-defined triplet matching is not associated with better survival rates of patients with class I HLA allele mismatched hematopoietic cell transplants from unrelated donors. Biol.Blood Marrow Transplant. 2008; 14(9): 1064-1071.

6






Chapter 6

PIRCHE-I promote leukemia-free survival after cord blood transplantation: indications for a potential novel donor-selection tool

Kirsten A. Thus,[1] Talitha A. de Hoop,[1] Roel A. de Weger,[2] Marc B. Bierings,[3] Jaap Jan Boelens,[1, 3] and Eric Spierings[1]

[1]Laboratory of Translational Immunology,
University Medical Center Utrecht, Utrecht, the Netherlands
[2]Department of Pathology,
University Medical Center Utrecht, Utrecht, the Netherlands
[3]Pediatric Blood and Marrow Transplantation Program,
University Medical Center Utrecht, the Netherlands

Submitted

Abstract

Unrelated Cord Blood Transplantation (UCBT) provides a curative therapy for patients with hematological malignancies. The effect of HLA mismatches in UCBT is currently subject of debate. HLA-mismatched UCBT may lead to improved leukemia control, but may also lead to graft-versus-host disease (GVHD) resulting in non-relapse mortality (NRM). The aim of this study was to investigate whether indirect recognition of mismatched HLA provides an explanation for the graft-versus-tumor (GVT) effect and risk of GVHD.

The probability of indirect recognition was predicted by the Predicted Indirectly ReCognizable HLA Epitopes (PIRCHE) model. The effect of the numbers of PIRCHE presented on HLA class I and II (PIRCHE-I and -II) was studied in 134 pediatric patients. To study the effects of higher numbers of PIRCHE, patients were divided in two equal-sized groups, using the median number of PIRCHE as cut-off values. Proportional hazard models and competing risk analyses were performed to study the effect of PIRCHE on the clinical outcomes relapse, acute and chronic GVHD, NRM, disease-free and overall survival.

Above median PIRCHE-I were associated to reduced relapse risk (HR 0.26, 95%-CI 0.07-0.94, p=0.04), evaluating the 50 patients transplanted for a malignancy. Both PIRCHE-I and -II were not associated to other clinical outcomes, including GVHD and NRM.

These data suggest that high PIRCHE-I may lead to improved GVT effects after UCBT, without an accompanying GVHD risk. Inclusion of PIRCHE in the UCB-selection criteria may enhance UCBT outcome, which needs to be tested in prospective studies.

Introduction

Unrelated cord blood transplantation (UCBT) provides an attractive alternative for patients in need of allogeneic hematopoietic cell transplantation (HCT), lacking an HLA-matched sibling donor. HLA mismatches are well tolerated in case of UCBT, and an acceptable UCB donor is available for virtually every patient (1).

Whether HLA mismatches in UCBT are favorable or detrimental, is subject of ongoing debate. On the one hand, HLA mismatches may enhance the graft-versus-tumor (GVT) effect, leading to significantly reduced relapse risks after HLA-mismatched UCBT (2-8). On the other hand, HLA-mismatched UCBT has been associated with an increased risk of graft-versus-host disease (GVHD) resulting in non-relapse mortality (NRM) (7, 9). Dissection of the GVT potential of HLA mismatches from the GVHD risk, would greatly enhance UCBT outcome.

Indirect T-cell recognition of HLA mismatches is a potential explanation for both the GVT effect and GVHD. During indirect recognition, donor T cells recognize peptides derived from the mismatched HLA that are presented on shared HLA (10). The Predicted Indirectly ReCognizable HLA Epitopes (PIRCHE) algorithm provides a method to distinguish HLA mismatches into those leading to a high or low probability of indirect T-cell recognition (11-13). PIRCHE can be presented by HLA class I and II (PIRCHE-I and -II, respectively), which theoretically correlate to the probability of CD8+ and CD4+ T-cell recognition, respectively. In adult unrelated donor HCT with peripheral blood-derived stem cells, both PIRCHE-I and -II were correlated to GVHD development (12, 13). Thus, PIRCHE seem to provide the opportunity of dissecting HLA mismatches into those with high and low alloreactive CD8+ and CD4+ T-cell potential.

We hypothesized that the GVT potential and the GVHD risk of HLA mismatches in UCBT are correlated to the numbers of PIRCHE-I and/or -II, and that consequently, PIRCHE may provide a prognostic tool that ultimately improves clinical outcome. To address this issue, all pediatric patients that received an UCBT in the UMC Utrecht were studied for the correlation of PIRCHE-I and -II with clinical outcomes.

Methods

Study population

All consecutive pediatric patients receiving UCBT in the UMC Utrecht from 2004 until 2014 were analyzed for inclusion in this study. Clinical data were collected prospectively. Patients had given written consent in accordance with the Declaration of Helsinki. Allelic HLA-typing was retrospectively performed for all patient and donor pairs for HLA-A, -B, -C, -DRB1, -DQB1 on a two-field unambiguous resolution level. Inclusion criteria were: availability of complete HLA typing and, in case of multiple donor HCT (double unit UCBT, or UCBT combined with haploidentical donor cells), established engraftment of one of the two donors.

Transplantation details

Transplantation procedures of the UMC Utrecht have been described previously (14). In summary, patient-donor pairs were matched with a minimum match grade of 4/6 for HLA-A, -B and -DRB1 (HLA-A and –B serological level and HLA-DRB1 high-resolution level). The majority of patients have received a myeloablative conditioning regimen (either Busulfan-based with Therapeutic Drug Monitoring, N=109, 81%; or Total Body Irradiation-based, N=11, 8%, Table 1), four (8%) of the patients with a malignancy received a non-myeloablative

regimen. All patients were in complete remission prior to HCT. GVHD prophylaxis consisted of Cyclosporin A (target concentration 0.20-0.25 mg/L), one mg/kg prednisolone, and 10 mg/kg anti-thymocyte globulin (ATG, Thymoglobuline) if transplanted prior to 2011. Patients received 10 μg/kg recombinant human granulocyte colony-stimulating factor from seven days post-HCT until neutrophil recovery (defined as three consecutive days neutrophils ≥500/μL). Antimicrobial prophylaxis consisted of ciprofloxacin (seven days prior to HCT until neutrophil recovery), 500 mg/m^2 acyclovir (from seven days prior to HCT until CD4+ T-cell recovery for varicella-zoster virus–positive patients), and two mg/kg twice per week voriconazole (from the start of conditioning until neutrophil recovery), 100 mg/kg cefazolin (from day zero until absence of mucositis), once every three weeks cotrimoxazole from myeloid recovery until at least six months post-HCT.

PIRCHE

PIRCHE were determined as described previously (12, 13). In short, PIRCHE-I were identified in two steps. First, proteasome-mediated cleavage and transportation via the TAP channel were predicted for all donor and patient HLA molecules using NetChop C-term 3.0 (15, 16). Subsequently, peptides were tested for their binding capacity to the HLA-A, -B, and -C molecules that were shared between the donor and patient, using NetMHCpan 2.4 (17, 18). Only peptides with IC_{50}-binding values ≤500nM were accepted as relevant binders (19). For PIRCHE-II, the nonameric binding cores of potential 15-meric HLA-DRB1 binders were predicted with NetMHCIIPan 2.0 (20, 21), considering IC_{50}-binding values ≤1000nM as being relevant (22). Only unique patient-derived peptide-HLA complexes were classified as being a PIRCHE. The PIRCHE prediction algorithm is accessible via www.pirche.org.

For PIRCHE predictions, complete sequences of exon 1-8 for HLA class I and exon 1-6 for HLA class II are required. The model was therefore improved by extending protein sequences of HLA alleles that were not completely documented in the HLA-IMGT database (IMGT database, accessible via: www.imgt.org). Extensions were made based on a nearest neighbor principle, *e.g*. incomplete protein sequences were completed based upon the complete sequences from the HLA allele with the highest homology score for the documented partial protein sequences.

GVH-directed PIRCHE were determined for multiple donor HCT based on the engrafting donor: dominant UCB in case of double UCB (differing dominance in N=2, therefore excluded from the analyses) and UCB in case of haplo-UCBT.

Endpoints and statistical analyses

The data were retrospectively analyzed. Differences in groups of PIRCHE were assessed by χ^2 tests for categorical variables and with a student's T test for continuous variables. The clinical endpoints studied were: relapse, acute and chronic GVHD, NRM, disease-free survival (DFS) and overall survival (OS). For the endpoints related to the GVT effect (relapse and DFS), only patients with a malignancy were studied. The impact of other endpoints was studied in the entire cohort, as all these patients are at risk. The effects of PIRCHE and other donor-, patient- and transplantation-associated variables on the time-dependent clinical outcomes were analyzed using proportional hazard models for chronic GVHD, DFS and OS. Competing risk analyses were performed for relapse, acute GVHD, and NRM. Graft failure and NRM were considered competing risks for relapse; graft failure, relapse and NRM were considered competing risks for acute GVHD; and relapse and graft failure were considered

competing risks for NRM. To study the effect of higher numbers of PIRCHE, patients were divided into two groups, using the median as a cut-off value. Statistical models evaluated the following parameters for inclusion in the multivariate models: patient age, patient sex, conditioning regimen, serotherapy, patient CMV serostatus, primary disease, prior HCT, the number of CD34+ and white blood cells infused/kg, and prior acute GVHD in the model for chronic GVHD. Factors associated with outcome in univariate analyses ($p<0.10$) were included in multivariate models. Kaplan-Meier curves were constructed to study the time-dependent probability of events related to PIRCHE. Statistical procedures were performed with SPSS version 20.0 (SPSS Inc, Chicago, IL, USA), competing risk analyses were performed in R version 3.0.0 (R Foundation for Statistical Computing, Vienna, Austria). P-values <0.05 were considered statistically significant.

Role of the funding source

The funding bodies had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and final responsibility for the decision to submit for publication.

Variable	N (%)
Age at transplantation (year), median (range)	4.93 (0.14-22.74)
WBC infused (10^7/kg), median (range)	6.05 (0.59-33.20)
Patient sex	
Female	54 (40.3)
Male	80 (59.7)
Diagnosis	
Malignancy	50 (37.3)
Inborn error of metabolism	35 (26.1)
Immune deficiency	33 (24.6)
Bone marrow failure	13 (9.7)
Other	3 (2.2)
Conditioning regimen	
TBI/VP16	11 (8.2)
BuCy(MeL)	30 (22.4)
FluBu(Clo)	79 (59.0)
CyFlu	12 (9.0)
Treo-based	2 (1.5)
Match grade	
4/6	18 (13.4)
5/6	64 (47.8)
6/6	51 (38.1)

Table 1: Baseline characteristics. WBC: white blood cells. TBI: total body irradiation. VP16: Etoposide. Bu: Busulfan. Cy: Cyclophosphamide. MeL: Melphalan. Flu: Fludarabine. Clo: Clofarabin. Treo: Treosulfan. BuCy(MeL) was used for patients transplanted before 2009, FluBu(Clo) after 2009. Of the malignancies, the majority was myeloid (N=23, 46%).

Results

Baseline characteristics

In total 156 patients received UCBT in the study period, of which 134 met the inclusion criteria. The median age was five years (range 0-23). Patients were transplanted for various underlying diseases, with malignancy as the main indication. Median follow-up was 1.4 years (range 0-10.6). Detailed clinical characteristics have been listed in Table 1.

The median numbers of PIRCHE were 3.5 for PIRCHE-I (range 0-40) and 13 for PIRCHE-II (range 0-96). Patients with above median PIRCHE-I had significantly more often bone-marrow failure as indication for the HCT (p=0.02), and patients with below median PIRCHE-I were significantly more often male (p=0.01); other baseline characteristics did not significantly differ between the groups of PIRCHE. These differing factors were not associated with the tested clinical endpoints, and therefore the uneven distribution has likely not impacted the analyses.

PIRCHE-I associate with relapse

We hypothesized that a higher number of PIRCHE increases the likelihood of T-cell recognition and thus enhances a GVT effect. To study the effect of PIRCHE on GVT reactivity, the relapse probabilities of two groups of PIRCHE were compared, using the median number of PIRCHE as a cut-off value. Overall, the five year relapse incidence was 22%, which was significantly lower in the group of patients that presented above median PIRCHE-I (11%, p=0.04, Figure 1, hazard ratio (HR) 0.26, 95%-confidence interval (CI) 0.07-0.94, p=0.04, Table 2). PIRCHE-II were not significantly associated to relapse. Thus, above median PIRCHE-I associate with a reduced relapse risk, suggesting that donor CD8+ T cells recognize patient tumor cells, thereby substantiating the well-established crucial role of alloreactive CD8+ T cells in tumor control (23-25).

	PIRCHE-I			PIRCHE-II		
Outcome	**HR**	**95%-CI**	**p**	**HR**	**95%-CI**	**p**
Relapse, N=50	0.26	0.07-0.94	**0.04**	0.37	0.10-1.31	0.12
Acute GVHD II-IV	0.72	0.35-1.46	0.36	0.95	0.47-1.93	0.88
Acute GVHD III-IV (1)	1.11	0.38-3.28	0.84	2.77	0.75-10.26	0.13
Severe chronic GVHD, N=114 (2)	2.20	0.36-13.25	0.39	3.94	0.44-35.36	0.22
DFS, N=50	0.41	0.15-1.13	0.09	0.89	0.33-2.39	0.81
NRM (3)	1.65	0.76-3.57	0.21	1.17	0.52-2.26	0.70
OS (4)	1.09	0.59-2.02	0.77	1.06	0.57-1.97	0.85

Table 2: Effect of above median PIRCHE-I and -II in multivariate models. Number of evaluated patients was 134 unless otherwise indicated. Multivariate models included: 1) CMV status; 2) acute GVHD grade II-IV; 3) prior HCT; 4) prior HCT, number of CD34+ cell infused, number of WBC infused. PIRCHE-I: predicted indirectly recognizable HLA epitopes presented by HLA class I. PIRCHE-II: predicted indirectly recognizable HLA epitopes presented by HLA class II. HR: hazard ratio. CI: confidence interval. GVHD: graft-versus-host disease. DFS: disease-free survival. NRM: non-relapse mortality. OS: overall survival.

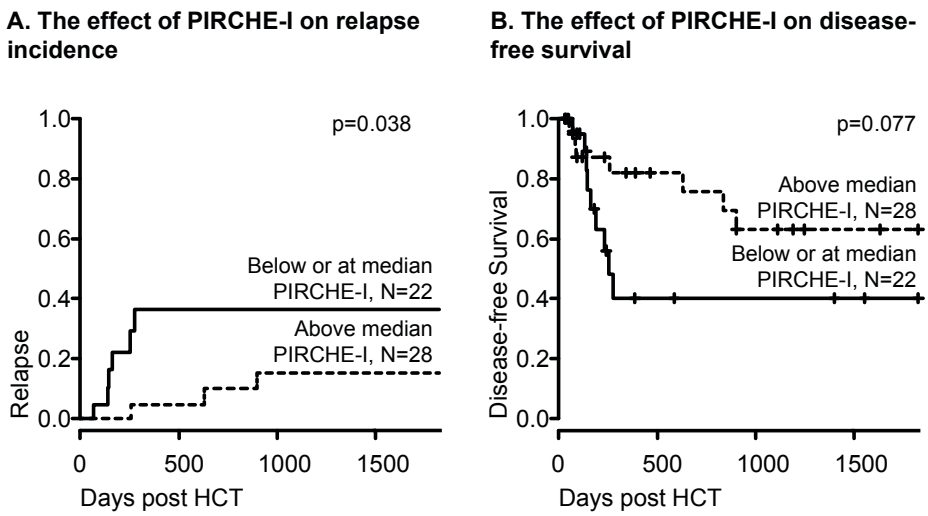


Figure 1: The probability of relapse (A) and disease-free survival (B) by PIRCHE-I groups. Patients with above median PIRCHE-I (dotted line) had a significantly reduced probability of relapse resulting in a trend for improved disease-free survival compared to below or at median PIRCHE-I (solid line). PIRCHE-I: predicted indirectly recognizable HLA epitopes presented by HLA class I. HCT: hematopoietic cell transplantation.

Neither PIRCHE-I nor PIRCHE-II are associated to other endpoints

The positive GVT effect of PIRCHE might clinically be counterbalanced by an increased GVHD risk. We therefore studied the effect of PIRCHE on acute and chronic GVHD in the total study group. Neither PIRCHE-I nor PIRCHE-II were significantly associated to (severe) acute or chronic GVHD development (Table 1). In addition, PIRCHE-I and -II were not significantly associated to NRM and OS (Table 2).

As PIRCHE-I have a positive anti-tumor effect, we studied whether patients with high PIRCHE-I have improved DFS. In patients with a malignancy, there was a trend for patients having an increased probability of DFS when they presented above median PIRCHE-I compared to lower PIRCHE-I (HR 0.41, 95%-CI 0.15-1.13, p=0.09, Table 2, Figure 1).

Discussion

This study was initiated to investigate whether the GVT potential and GVHD risk of HLA mismatches after UCBT is predictable, based on the number of PIRCHE. In this unique, mono-center pediatric patient cohort, PIRCHE-I values above three were significantly associated with a reduced relapse rate. High PIRCHE-I and -II did not result in detrimental (side) effects, as reflected by similar GVHD and NRM. These results therefore suggest that selection of UCB units with high numbers of PIRCHE-I may improve transplant outcome for patients with a malignant disease.

The GVT potential of PIRCHE-I may explain why HLA mismatches in UCBT can be associated to a reduced relapse risk (2-8). The association between PIRCHE-I and relapse suggests that PIRCHE-specific CD8+ T cells may be involved in anti-tumor responses. *In vitro* studies regarding PIRCHE-specific T cells of patients after UCBT may allow elucidating why in some UCBT settings the alloreactivity of HLA mismatches is directed towards the positive anti-tumor response, resulting in improved survival (8), whereas in others it results in GVHD and the detrimental NRM (7, 9). The current data suggest that in our local center especially

tumor-reactive PIRCHE-specific T cells can be identified.

This proof-of-principle study included a limited number of patients with a malignancy. Before PIRCHE can be implemented in the donor-selection procedure, validation studies are warranted, to investigate whether the GVT potential of PIRCHE-I does result in better survival; and whether the effect of PIRCHE-I may be specifically related to, for example, myeloid malignancies, as the GVT effect of donor lymphocyte infusions is mostly restricted to myeloid leukemias (26). These aspects need to be addressed in larger cohorts, with a specific focus on patients transplanted for acute leukemia.

PIRCHE may introduce a new level of matching for UCBT. PIRCHE can be predicted for all patient-donor combinations when high-resolution HLA typing of the donor and patient are available. PIRCHE predictions could be used to assist in the selection procedure when the match grade and cell dose of multiple CB units are considered acceptable, as an extra criterion. Ultimately, PIRCHE may provide an opportunity to select HLA-mismatched CB donors with a high anti-tumor potential.

In conclusion, this study provides data on a potential novel donor-selection method. We have demonstrated that high PIRCHE-I values enhance the GVT potential of HLA-mismatched UCBT without increasing the risk of GVHD and NRM. The results from our local proof-of-principle study require external validation in either retrospective or prospective studies.

Acknowledgements

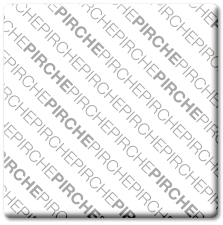
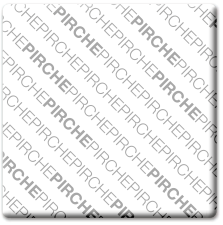
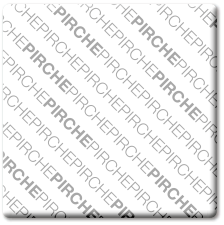
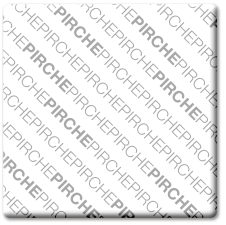
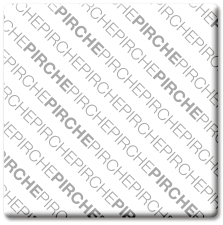
Grateful acknowledgement is made to A. de Wildt, C. Gerhardt and V. Boers-Trilles for their help with acquiring patient data, and to the HLA-laboratory of the UMC Utrecht for their help with performing retrospective high-resolution HLA-typings.

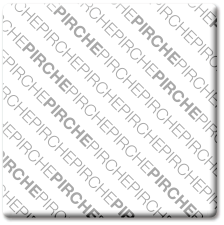
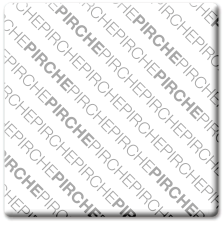
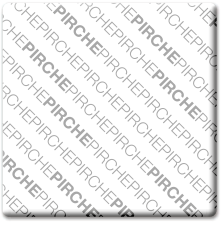
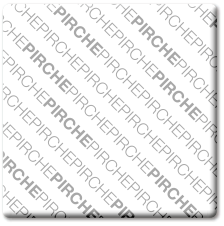
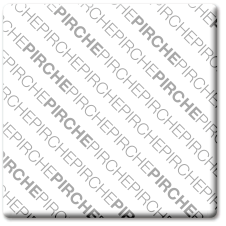
Declaration of interests

The University Medical Center Utrecht has filed a patent application on the prediction of an alloimmune response against mismatched HLA. ES has been listed as inventor on this patent application. The other authors have no conflict of interest to declare.

References

1. Gragert L, Eapen M, Williams E, et al. HLA match likelihoods for hematopoietic stem-cell grafts in the U.S. registry. N.Engl.J.Med. 2014; 371(4): 339-348.
2. Cunha R, Loiseau P, Ruggeri A, et al. Impact of HLA mismatch direction on outcomes after umbilical cord blood transplantation for hematological malignant disorders: a retrospective Eurocord-EBMT analysis. Bone Marrow Transplant. 2014; 49(1): 24-29.
3. Sanz J, Jaramillo FJ, Planelles D, et al. Impact on outcomes of human leukocyte antigen matching by allele-level typing in adults with acute myeloid leukemia undergoing umbilical cord blood transplantation. Biol. Blood Marrow Transplant. 2014; 20(1): 106-110.
4. Atsuta Y, Kanda J, Takanashi M, et al. Different effects of HLA disparity on transplant outcomes after single-unit cord blood transplantation between pediatric and adult patients with leukemia. Haematologica. 2013; 98(5): 814-822.
5. Gluckman E, Rocha V, Arcese W, et al. Factors associated with outcomes of unrelated cord blood transplant: guidelines for donor choice. Exp.Hematol. 2004; 32(4): 397-407.
6. Eapen M, Rubinstein P, Zhang MJ, et al. Outcomes of transplantation of unrelated donor umbilical cord blood and bone marrow in children with acute leukaemia: a comparison study. Lancet 2007; 369(9577): 1947-1954.
7. Eapen M, Klein JP, Sanz GF, et al. Effect of donor-recipient HLA matching at HLA A, B, C, and DRB1 on outcomes after umbilical-cord blood transplantation for leukaemia and myelodysplastic syndrome: a retrospective analysis. Lancet Oncol. 2011; 12(13): 1214-1221.
8. Wagner JE, Jr., Eapen M, Carter S, et al. One-unit versus two-unit cord-blood transplantation for hematologic cancers. N.Engl.J.Med. 2014; 371(18): 1685-1694.
9. Eapen M, Klein JP, Ruggeri A, et al. Impact of allele-level HLA matching on outcomes after myeloablative single unit umbilical cord blood transplantation for hematologic malignancy. Blood 2014; 123(1): 133-140.
10. Hanvesakul R, Maillere B, Briggs D, et al. Indirect recognition of T-cell epitopes derived from the alpha 3 and transmembrane domain of HLA-A2. Am.J.Transplant. 2007; 7(5): 1148-1157.
11. Otten HG, Calis JJ, Kesmir C, et al. Predicted indirectly recognizable HLA epitopes presented by HLA-DR correlate with the de novo development of donor-specific HLA IgG antibodies after kidney transplantation. Hum.Immunol. 2013; 74(3): 290-296.
12. Thus KA, te Boome L, Kuball J, et al. Indirectly Recognized HLA-C Mismatches and Their Potential Role in Transplant Outcome. Front Immunol. 2014; 5: 210.
13. Thus KA, Ruizendaal MT, de Hoop TA, et al. Refinement of the Definition of Permissible HLA-DPB1 Mismatches with Predicted Indirectly ReCognizable HLA-DPB1 Epitopes. Biol.Blood Marrow Transplant. 2014; 20(11): 1705-1710.
14. Lindemans CA, Chiesa R, Amrolia PJ, et al. Impact of thymoglobulin prior to pediatric unrelated umbilical cord blood transplantation on immune reconstitution and clinical outcome. Blood. 2014; 123(1): 126-132.
15. Kesmir C, Nussbaum AK, Schild H, et al. Prediction of proteasome cleavage motifs by neural networks. Protein Eng 2002; 15(4): 287-296.
16. Nielsen M, Lundegaard C, Lund O, et al. The role of the proteasome in generating cytotoxic T-cell epitopes: insights obtained from improved predictions of proteasomal cleavage. Immunogenetics 2005; 57(1-2): 33-41.
17. Nielsen M, Lundegaard C, Blicher T, et al. NetMHCpan, a method for quantitative predictions of peptide binding to any HLA-A and -B locus protein of known sequence. PLoS.One. 2007; 2(8): e796.
18. Hoof I, Peters B, Sidney J, et al. NetMHCpan, a method for MHC class I binding prediction beyond humans. Immunogenetics 2009; 61(1): 1-13.
19. Buus S, Lauemoller SL, Worning P, et al. Sensitive quantitative predictions of peptide-MHC binding by a 'Query by Committee' artificial neural network approach. Tissue Antigens 2003; 62(5): 378-384.
20. Nielsen M, Lundegaard C, and Lund O. Prediction of MHC class II binding affinity using SMM-align, a novel stabilization matrix alignment method. BMC.Bioinformatics. 2007; 8: 238.
21. Nielsen M and Lund O. NN-align. An artificial neural network-based alignment algorithm for MHC class II peptide binding prediction. BMC.Bioinformatics. 2009; 10: 296.
22. Southwood S, Sidney J, Kondo A, et al. Several common HLA-DR types share largely overlapping peptide binding repertoires. J.Immunol. 1998; 160(7): 3363-3373.
23. van der Harst D, Goulmy E, Falkenburg JH, et al. Recognition of minor histocompatibility antigens on lymphocytic and myeloid leukemic cells by cytotoxic T-cell clones. Blood 1994; 83(4): 1060-1066.
24. Falkenburg JH, Goselink HM, van der Harst D, et al. Growth inhibition of clonogenic leukemic precursor cells by minor histocompatibility antigen-specific cytotoxic T lymphocytes. J.Exp.Med. 1991; 174(1): 27-33.
25. Molldrem JJ, Lee PP, Wang C, et al. Evidence that specific T lymphocytes may participate in the elimination of chronic myelogenous leukemia. Nat.Med. 2000; 6(9): 1018-1023.
26. Kolb HJ, Schattenberg A, Goldman JM, et al. Graft-versus-leukemia effect of donor lymphocyte transfusions in marrow grafted patients. Blood. 1995; 86(5): 2041-2050.


7





Chapter 7

Predicted Indirectly ReCognizable HLA Epitopes (PIRCHE) correlate with improved hematological malignancy control after double unit cord blood transplantation

Kirsten A. Thus,[1,*] Todd DeFor,[2,*] Jan Cornelissen,[3,*] Michael Verneris,[2] Jeff Miller,[2] Bronno van der Holt,[4] Hanneke W. M. van Deutekom,[5] Roel A. de Weger,[6] Eric Borst,[1] Judith Somers,[3] John Wagner,[2,#] Jürgen Kuball,[1,7,#] and Eric Spierings[1,#]

[*,#]authors contributed equally
[1]Laboratory of Translational Immunology,
University Medical Center Utrecht, Utrecht, the Netherlands
[2]Division of Pediatric Blood and Marrow Transplant,
University of Minnesota, Minneapolis, USA
[3]Department of Hematology,
Erasmus MC-Daniel Den Hoed Cancer Center, Rotterdam, the Netherlands
[4]HOVON Data Center,
Erasmus MC Cancer Institute-Clinical Trial Center, Rotterdam, the Netherlands
[5]Department of Theoretical Biology and Bioinformatics,
Utrecht University, Utrecht, the Netherlands
[6]Department of Pathology,
University Medical Center Utrecht, Utrecht, the Netherlands
[7]Department of Hematology,
University Medical Center Utrecht, Utrecht, the Netherlands

Abstract

Double unit cord blood transplantation (dUCBT) provides a therapeutic treatment modality for patients with hematological malignancies. Since the vast majority of dUCBT involves HLA mismatches, we determined optimal HLA mismatches for dUCBT with respect to clinical outcome, focusing on indirect recognition of mismatched HLA. To this end, indirect recognition of mismatched HLA was classified by the number of Predicted Indirectly ReCognizable HLA Epitopes presented on HLA class I and II (PIRCHE-I and -II), and correlated to clinical outcomes of two independent dUCBT cohorts. Correlations were first studied in a hypothesis-generating cohort (HC; 66 patients), and confirmed in a validation cohort (VC; 266 patients). Patients were divided in three equally sized PIRCHE groups based on the observed distribution of numbers of PIRCHE in the HC (low, intermediate and high). The risk of disease progression was significantly reduced in the high PIRCHE-I group when compared to the low group (HC: HR 0.33, 95%-CI 0.09-1.19, p=0.09, VC: HR 0.48, 95%-CI 0.28-0.85, p=0.01). This decreased anti-tumor effect of low PIRCHE-I resulted in impaired survival. In the VC, patients with low PIRCHE-I had poorer overall and progression-free survival (OS, PFS) compared to patients with higher PIRCHE-I (OS: HR 0.73, 95%-CI 0.52-1.04 p=0.08; PFS: HR 0.68, 95%-CI 0.49-0.94, p=0.02). To conclude, indirect recognition of HLA impacts progression of hematological malignancies and consequently PFS and OS after dUCBT. These results warrant prospective studies to investigate the effect of PIRCHE-based UCB selection.

Introduction

Transplantation with two units of umbilical cord blood (dUCBT) provides an attractive treatment modality for patients in need of an allogeneic hematopoietic cell transplantation (allo-HCT). A major advantage of dUCBT is the improved tumor control compared to HCT with cells derived from bone-marrow or peripheral blood (1). The enhanced graft-versus-tumor (GVT) effect of dUCBT could be a result of the high level of HLA mismatching (1): it is well known that HLA-mismatches can result in GVT-reactivity after UCBT (2-7). Despite the GVT potential of HLA mismatches, HLA mismatches may lead to an increased probability of graft-versus-host disease (GVHD) (8, 9). To date, dissection of the GVT potential of HLA mismatches from the GVHD risk is not possible. Being able to differentiate HLA mismatches into high and low risk mismatches with respect to the GVT potential and GVHD risk, could enhance UCB unit selection and thus provides a major step forward in improving outcome after dUCBT.

Indirect recognition of HLA mismatches is an important route of alloreactivity. During indirect recognition, donor T cells recognize peptides derived from patient-mismatched HLA presented on shared HLA. T cells recognizing such epitopes have been isolated during GVHD (10), and after solid organ transplantation prior to graft rejection (11-13). Moreover, studies in adult unrelated-donor (URD) HCT indicate that indirect recognition of mismatched HLA plays a role in clinical outcome (14, 15). These latter studies predicted indirectly recognizable HLA epitopes in the context of allo-HCT by a novel computational strategy. Higher numbers of "Predicted Indirectly ReCognizable HLA Epitopes" (PIRCHE) were associated with alloreactivity, as reflected by an increased risk of acute GVHD (aGVHD) development (14, 15). As the PIRCHE model is able to define high and low alloreactive HLA mismatches for URD-HCT, we hypothesized that PIRCHE have the capacity to dissect HLA-mismatches into those with high or low anti-tumor potential for UCBT as well.

The present study addresses the effect of indirect HLA recognition on clinical outcomes after dUCBT. To this end, the relationship between PIRCHE and GVT potential or GVHD risk was studied, in two independent cohorts. Finally, the effect of PIRCHE on progression-free and overall survival (PFS, OS) was investigated in order to generate PIRCHE-based donor selection criteria.

Methods

Study population

The hypothesis-generating cohort (HC) consisted of 66 high-risk Dutch patients that received a dUCBT between 2007 and 2012 (Table 1). Of these 66 patients, 58 (88%) were treated according to the HOVON 106 trial protocol (7, 16). The remaining 8 (12%) were transplanted in the UMC Utrecht outside the trial, with different conditioning regimens. Ultra high-resolution (2-field unambiguous) level HLA-A, -B, -C, -DRB1, -DQB1 typing was available for 37 (56%) patients and donors. In contrast to high-resolution typing, which is restricted to exon 2-3 for class I and exon 2 for class II, 2-field unambiguous level typing includes the entire protein sequence, which is essential for PIRCHE predictions. Therefore, for patient-donor combinations with standard resolution HLA-typing data, unambiguous 2-field level typing was predicted based on known frequencies and associations of HLA-alleles (17). Chimerism analyses were performed on bone-marrow and determined based on quantitative amplification with short tandem repeat polymerase-chain reactions (16).

The validation cohort (VC) consisted of 266 patients after dUCBT in the University of

Minnesota Medical Center for a hematological malignancy between 2003 and 2011 (Table 1). To validate the findings of the HC in a stringent manner, patients were only included when complete 2-field level HLA typing was available, and when single unit dominance was established based on chimerism analyses.

PIRCHE

PIRCHE were determined as described previously (14, 15). For PIRCHE predictions, complete protein sequences of exon 1-8 for HLA class I and exon 1-6 for HLA class II are required. The model was therefore improved as compared to previous reports (14, 15), by extending protein sequences of HLA alleles that were not completely documented in the HLA-IMGT database (IMGT database, accessible via: www.imgt.org). Extensions were made based on a nearest neighbor principle, *i.e.* incomplete protein sequences were completed based upon the complete sequences from the HLA allele with the highest homology score for the documented partial protein sequences. PIRCHE predictions are available via www.pirche.org.

Endpoints and definitions

The primary endpoint was disease progression, in order to study the effect of PIRCHE on anti-tumor potential. Disease progression of the original disease was monitored by morphology in the blood or marrow, and non-relapse mortality (NRM) was regarded a competing event. Secondary evaluated clinical endpoints were: grades II-IV and III-IV aGVHD (18), with progression and NRM as competing events; cGVHD; NRM, defined as mortality without previous progression of disease, with progression as competing event; progression-free survival (PFS), defined as time from dUBCT to progression or death from any cause, whichever came first; and OS, defined as time from dUCBT to mortality. The dominant unit was defined as the unit that was present for >95% in bone-marrow chimerism analyses.

Statistical methods

To investigate the effect of PIRCHE on GVH-directed alloreactivity, only GVH-PIRCHE of the dominant UCB unit were investigated. To study the effects on progression, aGVHD, and NRM, multivariate competing-risks analyses were performed. The effects of PIRCHE on cGVHD, PFS and OS were analyzed in multivariate Cox regression analyses.

Statistical models evaluated the following parameters for inclusion in the multivariate models: patient age, patient sex, sex mismatch (female dominant UCB for male patient), HLA match grade between dominant UCB unit and patient (on 2-field level for 5 loci), conditioning regimen (myeloablative (MA), non-myeloablative with anti-thymocyte globulin (NMA+ATG) and without ATG (NMA-ATG)), patient CMV serostatus, primary disease, origin and accuracy of HLA-predictions (HC only, as in the VC HLA-typing was performed on a high-resolution level for all patients and donors), and disease risk prior to HCT (VC only, as in the HC all patients were considered high risk).

The effect of PIRCHE on alloreactivity was first studied in the HC. For associations in the HC with $p<0.10$, confirmation was sought in the VC, $p<0.05$ was considered statistically significant. Correlations that were observed in both the HC and VC were regarded confirmed. Statistical procedures were performed with SPSS version 20.0 (SPSS Inc, Chicago, IL, USA), and competing risk analyses were performed with R version 3.0.0 (The R Foundation for Statistical Computing, Vienna, Austria).

	HC, N (%)	VC, N (%)	p
N	66	266	
PIRCHE-I, median (range)	8 (0-37)	8 (0-59)	
PIRCHE-II, median (range)	26 (0-138)	25 (0-111)	
Age in years, median (range)	53 (20-65)	44 (0-70)	**<0.01**
Disease			
ALL	10 (15)	62 (23)	**0.01**
AML	35 (53)	108 (41)	
CLL	5 (8)	7 (3)	
CML	4 (6)	7 (3)	
NHL	5 (8)	32 (12)	
HL	-	19 (7)	
MDS	1 (2)	22 (8)	
MM	1 (2)	3 (1)	
MPD	-	5 (2)	
PLL	-	1 (0)	
(V)SAA	5 (8)	-	
Patient sex			
Male	33 (50)	162 (61)	0.11
Female	33 (50)	104 (39)	
Patient CMV serostatus			
Positive	37 (56)	154 (58)	0.97
Negative	27 (41)	111 (42)	
Unknown	2 (3)	1 (0)	
Conditioning regimen			
NMA-ATG	62 (94)	108 (41)	**<0.001**
NMA+ATG	-	51 (19)	
MA	4 (6)	107 (40)	
Matchgrade dominant UCB (out of 10)			
10/10	-	18 (8)	0.05
9/10	2 (3)	18 (8)	
8/10	13 (21)	43 (19)	
7/10	16 (25)	50 (22)	
6/10	20 (32)	43 (19)	
≤5/10	12 (18)	51 (23)	

Table 1: baseline characteristics. HC: Hypothesis-generating cohort. VC: validation cohort. ALL: acute lymphoblastic leukemia, AML: acute myeloid leukemia, CLL: chronic lymphocytic leukemia, CML: chronic myeloid leukemia, NHL: non-Hodgkin lymphoma, HL: Hodgkins lymphoma, MDS: myelodysplastic syndrome, MM: multiple myeloma, MPD: myeloproliferative disorder, PLL: prolymfocytic leukemia, (V)SAA: (very) severe aplastic anemia. NMA: non-myeloablative: consisting of fludarabin, cyclophosphamide, and TBI. MA: myeloablative: consisting of either fludarabin, cyclophosphamide, and TBI; carmustine, etoposide, and cycophosphamide; or busulfan, fludarabin, and melphalan. ATG: anti-thymocyte globulin. Differences between the HC and VC were tested with chi-square for categorical variables and students T-test for the continuous variable age.

Results

PIRCHE-I enhances the GVT effect

Indirect recognition of the patient's mismatched HLA by T cells of the dominant UCB may play a role in the control of hematological malignancies. As PIRCHE predict the likelihood of T-cell recognition, we hypothesized that patients with higher PIRCHE had a reduced probability of progression; this hypothesis was generated in a Dutch HC and validated in a VC from Minnesota (Table 1). Patients were divided in three equal-sized groups (tertiles), based on the observed distribution of numbers of PIRCHE in the HC and thus independent of clinical outcome (low, intermediate, and high, Table 2). These three groups provided the opportunity to study a potential dose-dependent effect of PIRCHE. Baseline characteristics of the three groups of PIRCHE did not significantly differ.

	HC, N(%)	VC, N(%)
PIRCHE-I low (<7)	20 (32)	122 (46)
PIRCHE-I intermediate (7-12)	24 (38)	62 (23)
PIRCHE-I high (>12)	19 (30)	82 (31)
PIRCHE-II low (<20)	21 (33)	102 (38)
PIRCHE-II intermediate (20-35)	21 (33)	83 (31)
PIRCHE-II high (>35)	21 (33)	81 (31)

Table 2: PIRCHE distribution. HC: Hypothesis-generating cohort. VC: validation cohort. PIRCHE: Predicted Indirectly ReCognizable HLA Epitope. Patients were divided in three groups of PIRCHE-I and -II, based on the tertiles of the hypothesis-generating cohort. For the validation cohort, this yielded a distorted distribution, with the majority of patients in the low PIRCHE groups.

In the HC, high PIRCHE-I correlated with a reduced progression risk compared to low (HR 0.33, 95%-CI 0.09-1.19, p=0.09, Table 3). Despite the heterogeneity in the type of hematological malignancies, the VC confirmed a reduced progression rate in the presence of high PIRCHE-I (HR 0.48, 95%-CI 0.28-0.85, p=0.01, Table 3, Figure 1). The size of the VC allowed also to selectively study the impact of PIRCHE-I on progression of acute leukemia; again high PIRCHE-I progressed significantly less than low (HR 0.40, 95%-CI 0.19-0.84, p=0.02). Thus, these two independent cohorts suggest a control of hematological malignancies by CD8+ T cells through the recognition of PIRCHE-I.

PIRCHE-II relates to cGVHD

The positive anti-tumor effect of PIRCHE may be counterbalanced by an increase in the GVHD risk. We therefore next studied the effects of PIRCHE on acute and chronic GVHD. In the HC, high PIRCHE-II correlated with an increased hazard of cGVHD when compared to low (HR 3.81, 95%-CI 1.19-12.17, p=0.02, Table 3), while PIRCHE-I did not. Despite a substantial lower probability of cGVHD in the VC compared to the HC (24% vs. 47%), a similar trend was still observed for both intermediate and high PIRCHE-II being correlated to an increased cGVHD risk (HR 1.70, 95%-CI 0.89-3.25, p=0.11; and HR 1.76, 95%-CI 0.93-3.32, p=0.08, Table 3). As in the VC intermediate and high PIRCHE-II have similar cGVHD incidences (data not shown), these groups were combined into one group of higher PIRCHE-II. Higher PIRCHE-II had an increased hazard of cGVHD compared to low (HR 1.72, 95%-CI 0.98-3.05, p=0.06). In conclusion, PIRCHE-II may associate with cGVHD rates.

Outcome	PIRCHE group	HC					VC				
		N per group	N events (%)	HR	95%-CI	P	N per group	N events (%)	HR	95%-CI	P
Acute GVHD II-IV (1)	Low PIRCHE-I	20		1 (ref)		0.60	122	67 (55)	1 (ref)		0.21
	Intermediate PIRCHE-I	24	12 (50)	1.25	0.56-2.79	0.59	62	37 (60)	1.16	0.77-1.76	0.48
	High PIRCHE-I	19	11 (58)	0.90	0.34-2.36	0.83	82	47 (57)	1.29	0.85-1.97	0.23
	Low PIRCHE-II	21		1 (ref)		0.69	102	61 (60)	1 (ref)		0.11
	Intermediate PIRCHE-II	21	14 (67)	2.86	1.04-7.86	**0.04**	83	46 (55)	0.82	0.53-1.27	0.38
	High PIRCHE-II	21	10 (48)	1.33	0.46-3.90	0.60	81	44 (54)	0.69	0.44-1.09	0.11
Acute GVHD III-IV (2)	Low PIRCHE-I	20		1 (ref)		0.67	122	26 (21)	1 (ref)		0.19
	Intermediate PIRCHE-I	24	4 (17)	1.07	0.24-4.90	0.93	62	12 (19)	0.97	0.48-1.96	0.94
	High PIRCHE-I	19	3 (16)	0.55	0.05-6.69	0.64	82	23 (28)	1.56	0.86-2.86	0.15
	Low PIRCHE-II	21		1 (ref)		0.93	102	21 (21)	1 (ref)		0.76
	Intermediate PIRCHE-II	21	2 (10)	0.85	0.14-5.14	0.86	83	19 (23)	0.96	0.50-1.87	0.91
	High PIRCHE-II	21	4 (19)	1.08	0.13-8.77	0.94	81	21 (26)	1.09	0.57-2.06	0.80
Chronic GVHD (3)	Low PIRCHE-I	20		1 (ref)		0.59	97	26 (27)	1 (ref)		0.99
	Intermediate PIRCHE-I	21	11 (52)	1.12	0.39-3.19	0.84	57	18 (32)	1.04	0.55-1.96	0.90
	High PIRCHE-I	16	9 (56)	0.67	0.21-2.08	0.49	69	23 (33)	1.03	0.56-1.88	0.93
	Low PIRCHE-II	20		1 (ref)		**0.08**	89	20 (23)	1 (ref)		0.17
	Intermediate PIRCHE-II	16	11 (55)	1.89	0.71-5.00	0.20	65	24 (37)	1.70	0.89-3.25	0.11
	High PIRCHE-II	21	11 (61)	3.81	1.19-12.17	**0.02**	69	23 (33)	1.76	0.93-3.32	0.08
Progression (4)	Low PIRCHE-I	18		1 (ref)		**0.08**	122	41 (34)	1 (ref)		**<0.01**
	Intermediate PIRCHE-I	21	8 (38)	0.48	0.16-1.43	0.19	62	19 (31)	0.66	0.37-1.16	0.15
	High PIRCHE-I	19	4 (21)	0.33	0.09-1.19	**0.09**	82	17 (21)	0.48	0.28-0.85	**0.01**
	Low PIRCHE-II	18		1 (ref)		0.65	102	29 (28)	1 (ref)		**<0.01**
	Intermediate PIRCHE-II	19	7 (37)	0.63	0.23-1.73	0.37	83	19 (23)	1.39	0.72-2.67	0.32
	High PIRCHE-II	21	6 (29)	0.81	0.26-2.57	0.72	81	28 (35)	2.35	1.32-4.18	**<0.01**

Table 3 (previous page): Association of PIRCHE with clinical outcome of HCT. HC: Hypothesis-generating cohort. VC: validation cohort. PIRCHE: Predicted Indirectly ReCognizable HLA Epitope. 1: models were corrected for: primary disease, conditioning, CMV status in hypothesis-generating cohort; conditioning, disease risk, matchgrade dominant UCB in validation cohort. 2: models were corrected for: matchgrade dominant UCB in hypothesis-generating cohort; conditioning, patient sex, matchgrade dominant UCB in validation cohort. 3: models were corrected for: conditioning in validation cohort. 4: models were corrected for: patient sex, sex mismatch, CMV status, HLA-typing predicted in hypothesis-generating cohort; acute GVHD, conditioning, patient age in validation cohort. High PIRCHE-I were associated with a desirable reduced relapse rate, whereas high PIRCHE-II was associated with the pathological GVHD.

PIRCHE-based graft-versus-tumor effect may enhance survival

Low PIRCHE-I lead to an increased progression risk, whereas higher PIRCHE-II may associate with an increased cGVHD risk, suggesting that the most detrimental combination possible might be low PIRCHE-I and higher PIRCHE-II. The potential effect of such a detrimental combination on OS and PFS was studied in comparison to other potential combinations: low PIRCHE-I and -II; higher PIRCHE-I, low PIRCHE-II; and higher PIRCHE-I and -II. The HC did not allow studying the effect of these groups on survival due to the limited patient numbers, therefore, this analysis was selectively performed on the VC. The two combinations with higher PIRCHE-I clearly associated with a significantly better 3-year OS and PFS than the most detrimental combination, *i.e.* low PIRCHE-I, higher PIRCHE-II (Table 4). As both groups with higher PIRCHE-I have improved OS and PFS, the PIRCHE-I anti-tumor potential seems to impact survival mostly. Indeed, patients with higher PIRCHE-I have improved survival rates compared to patients with low PIRCHE-I (OS: HR 0.73, 95%-CI 0.52-1.04, p=0.08; PFS: 0.68, 95%-CI 0.49-0.94, p=0.02, Figure 1). The PIRCHE-I higher group does not have an increased risk of NRM (HR 0.71, 95%-CI 0.44-1.15, p=0.16, Figure 1). To conclude, the augmented GVT effect of PIRCHE-I impacts survival after dUCBT, without resulting in higher detrimental GVHD and NRM risks.

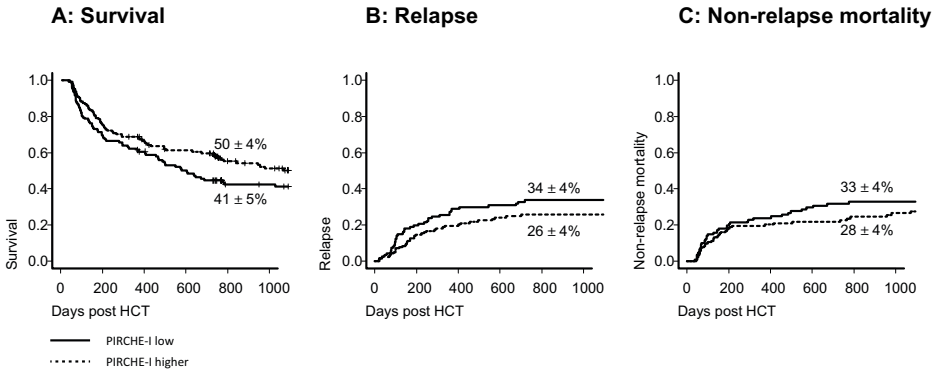


Figure 1: Effect of PIRCHE-I groups on overall survival (A), relapse (B), and non-relapse mortality (C), in the validation cohort. Black lines indicate PIRCHE-I low, dashed lines indicate PIRCHE-I higher. Three year incidence estimates are displayed, ± standard error. Patients in the PIRCHE-I higher group have higher overall survival rates compared to PIRCHE-I low, due to reduced relapse without increased non-relapse mortality. HCT: hematopoietic cell transplantation.

		N per group	N events (%)	HR	95%-CI	P
OS (1)	Low PIRCHE-I, higher PIRCHE-II	54	32 (59)	1 (ref)		0.14
	Low PIRCHE-I and -II	68	36 (53)	0.67	0.39-1.17	0.16
	Higher PIRCHE-I, low PIRCHE-II	34	16 (47)	0.53	0.28-0.99	**0.05**
	Higher PIRCHE-I and -II	110	51 (46)	0.62	0.40-0.99	**0.04**
PFS (2)	Low PIRCHE-I, higher PIRCHE-II	54	36 (67)	1 (ref)		**0.02**
	Low PIRCHE-I and -II	68	41 (60)	0.62	0.37-1.06	0.08
	Higher PIRCHE-I, low PIRCHE-II	34	16 (47)	0.43	0.23-0.79	**<0.01**
	Higher PIRCHE-I and -II	110	56 (51)	0.57	0.37-0.88	**0.01**

Table 4: Association of PIRCHE selection-criteria with survival, in the validation cohort. 1: model was corrected for: CMV status, primary disease, disease risk, matchgrade dominant UCB. 2: model was corrected for: age, CMV status, matchgrade dominant UCB. The reference group is low PIRCHE-I and higher PIRCHE-II. Patients with higher PIRCHE-I, irrespective of the PIRCHE-II status, had significantly improved 3-year PFS and OS compared to patients with low PIRCHE-I and higher PIRCHE-II. OS: overall survival. PFS: progression-free survival. UCB: umbilical cord blood.

Discussion

Alloreactivity after HLA-mismatched HCT has been mainly considered to be the result of direct recognition of an HLA-molecule by a given TCR (19, 20). Our data suggest that indirect recognition of mismatched HLA plays a significant additional role in mismatched transplantation. Indirectly recognized HLA-mismatches, as predicted with the novel computational PIRCHE strategy, associated with anti-tumor potential: a higher number of HLA class-I presented PIRCHE correlated with control of the hematological malignancy, resulting in improved PFS and OS, without leading to an increased risk of GVHD and NRM. Collectively, these data suggest that a priori consideration of the numbers of PIRCHE-I and -II in UCB selection might allow separation of the GVT potential and GVHD risk of HLA mismatches. Such an approach may improve survival of patients after UCBT.

High PIRCHE-I were associated with a reduced progression risk in both cohorts. This observation implies an important role for CD8+ T cells in the GVT effect, which is in line with cumulative evidence regarding the therapeutic effect of anti-tumor specific CD8+ T cells after HCT (21-23). With respect to detrimental GVH alloreactivity, our data suggest an association between PIRCHE-II and cGVHD, supporting a role for donor CD4+ T cells recognizing host tissue, in accordance with the established crucial role of CD4+ T cells in the development of cGVHD (24, 25). Moreover, these data add to previous suggestions that HLA class II is abundantly expressed on host tissue (26). The initial observation in the HC that PIRCHE-II correlated to cGVHD, led to only a trend in the VC, which is likely the result of the two-fold reduced incidence of cGVHD in the VC as compared to the HC. Although the PIRCHE results confirm previous results with regard to the T-cell subsets that are involved in either GVT or GVHD responses, the presence of indirectly recognizing T cells has thus far not been thoroughly investigated in the context of HCT. Nevertheless, such T cells have been detected in one patient after donor lymphocyte infusion (10), and prior to acute and chronic solid organ graft rejection (11-13), hence they can be activated upon HLA-mismatched transplantation. Our data advocate *in vitro* studies of alloreactive T cells recognizing HLA-derived peptides in the context of GVT and GVHD after allo-HCT.

Previous reports investigating PIRCHE in the setting of adult URD-HCT, showed a correlation between aGVHD and PIRCHE-I and -II (14, 15). In contrast, our dUCBT data spe-

cifically indicate that patients with higher PIRCHE-I had significantly improved OS and PFS compared to patients transplanted with low PIRCHE-I, due to a reduced progression risk. These opposing observations between the two stem-cell sources might partially reflect the different cellular composition of and immunological reconstitution after UCBT as compared to URD transplantation (27); as for example UCB is more naïve, and regulatory T cells, potential dissectors of GVT and GVHD effects, are preferentially induced upon UCBT (28). In addition, the data add to previous observations that HLA-mismatches have increased anti-tumor potential in UCBT (2-5), whereas HLA-mismatches are mostly associated with GVHD risk in adult URD-HCT (29, 30). Thus, PIRCHE-segregating of GVT potential from GVHD risk in the context of UCBT in contrast to URD-HCT, is in line with the disparities between the two cell sources with respect to clinical outcome, and warrants further investigation of the different immunological mechanisms responsible.

PIRCHE may provide a part of the rationale as to why HLA mismatches are associated with GVT effects after UCBT. In multiple studies, HLA mismatches were correlated to reduced progression rates after UCBT (2-7), resulting in improved PFS (6). In the present study, high PIRCHE-I correlated to reduced progression risk, suggesting that HLA mismatches providing high numbers of potential CD8+ T-cell epitopes through PIRCHE-I, may lead to GVT effects. Thereby, our results would compare well to previous observations of anti-tumor potential of HLA mismatches, possibly as a consequence of indirect recognition by CD8+ T cells.

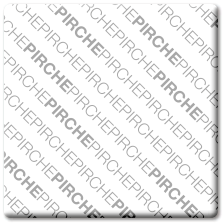
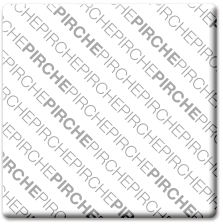
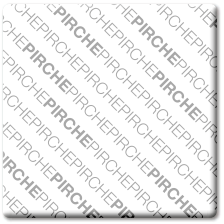
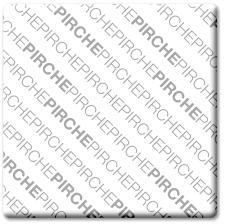
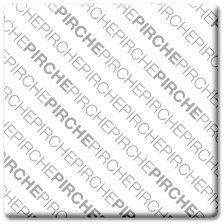
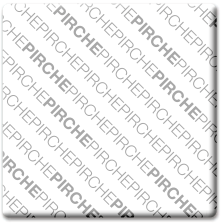
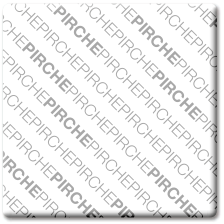
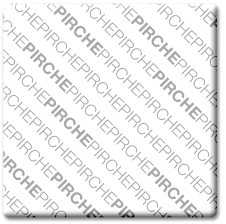
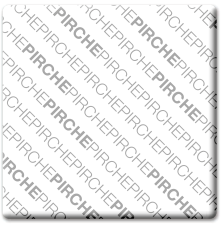
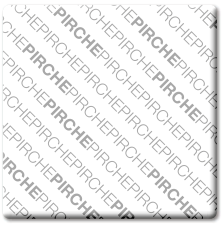
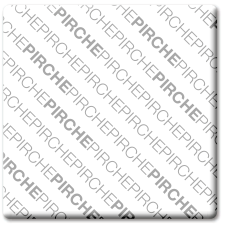
The level of HLA class-I mismatching as such may independently be correlated to GVT potential as well, as this level may predict the probability of direct recognition by CD8+ T cells. However, when we studied the level of mismatching for HLA class I and II separately, only HLA class-II mismatches were significantly associated to reduced progression rates and improved PFS, whereas HLA class-I mismatches were not (data not shown). These results suggest that HLA class-II mismatches can produce PIRCHE-I, thereby activating indirectly recognizing tumor-reactive CD8+ T cells, whereas direct recognition of HLA class-I mismatches may not be involved in this process. Moreover, these results may provide an explanation for the anti-tumor potential of PIRCHE-I, as the expression levels of PIRCHE-I derived from HLA class-II mismatches, are likely higher on hematological tumor cells compared to host tissue, thereby directing the alloreactivity of CD8+ T cells to the tumor cells.

To conclude, our study is the first to show that the recognition of HLA-derived peptides by donor T cells may play a role in the GVT effect after dUCBT. In two independent cohorts, PIRCHE-I are clearly associated with GVT effects, while they did not contribute to increased GVHD risks. Thus, PIRCHE might be a novel and unique tool to dissect the GVT potential and GVHD risk and therefore need to be prospectively studied for the possibility of substantially improving donor selection.

References

1. Brunstein CG, Gutman JA, Weisdorf DJ, et al. Allogeneic hematopoietic cell transplantation for hematologic malignancy: relative risks and benefits of double umbilical cord blood. Blood 2010; 116(22): 4693-4699.
2. Gluckman E, Rocha V, Arcese W, et al. Factors associated with outcomes of unrelated cord blood transplant: guidelines for donor choice. Exp.Hematol. 2004; 32(4): 397-407.
3. Cunha R, Loiseau P, Ruggeri A, et al. Impact of HLA mismatch direction on outcomes after umbilical cord blood transplantation for hematological malignant disorders: a retrospective Eurocord-EBMT analysis. Bone Marrow Transplant. 2014; 49(1): 24-29.
4. Sanz J, Jaramillo FJ, Planelles D, et al. Impact on outcomes of human leukocyte antigen matching by allele-level typing in adults with acute myeloid leukemia undergoing umbilical cord blood transplantation. Biol. Blood Marrow Transplant. 2014; 20(1): 106-110.
5. Atsuta Y, Kanda J, Takanashi M, et al. Different effects of HLA disparity on transplant outcomes after single-unit cord blood transplantation between pediatric and adult patients with leukemia. Haematologica. 2013; 98(5): 814-822.
6. Wagner JE, Jr., Eapen M, Carter S, et al. One-unit versus two-unit cord-blood transplantation for hematologic cancers. N.Engl.J.Med. 2014; 371(18): 1685-1694.
7. Somers JA, Braakman E, van der Holt B, et al. Rapid induction of single donor chimerism after double umbilical cord blood transplantation preceded by reduced intensity conditioning: results of the HOVON 106 phase II study. Haematologica 2014; 99(11): 1753-1761.
8. Eapen M, Klein JP, Sanz GF, et al. Effect of donor-recipient HLA matching at HLA A, B, C, and DRB1 on outcomes after umbilical-cord blood transplantation for leukaemia and myelodysplastic syndrome: a retrospective analysis. Lancet Oncol. 2011; 12(13): 1214-1221.
9. Eapen M, Klein JP, Ruggeri A, et al. Impact of allele-level HLA matching on outcomes after myeloablative single unit umbilical cord blood transplantation for hematologic malignancy. Blood 2014; 123(1): 133-140.
10. Amir AL, Hagedoorn RS, van Luxemburg-Heijs SA, et al. Identification of a coordinated CD8 and CD4 T cell response directed against mismatched HLA Class I causing severe acute graft-versus-host disease. Biol.Blood Marrow Transplant. 2012; 18(2): 210-219.
11. Ciubotariu R, Liu Z, Colovai AI, et al. Persistent allopeptide reactivity and epitope spreading in chronic rejection of organ allografts. J.Clin.Invest 1998; 101(2): 398-405.
12. Liu Z, Colovai AI, Tugulea S, et al. Indirect recognition of donor HLA-DR peptides in organ allograft rejection. J.Clin.Invest 1996; 98(5): 1150-1157.
13. Suciu-Foca N, Ciubotariu R, Itescu S, et al. Indirect allorecognition of donor HLA-DR peptides in chronic rejection of heart allografts. Transplant.Proc. 1998; 30(8): 3999-4000.
14. Thus KA, te Boome L, Kuball J, et al. Indirectly Recognized HLA-C Mismatches and Their Potential Role in Transplant Outcome. Front Immunol. 2014; 5: 210.
15. Thus KA, Ruizendaal MT, de Hoop TA, et al. Refinement of the Definition of Permissible HLA-DPB1 Mismatches with Predicted Indirectly ReCognizable HLA-DPB1 Epitopes. Biol.Blood Marrow Transplant. 2014; 11: 1705-1710.
16. Somers JA, Brand A, van Hensbergen Y, et al. Double umbilical cord blood transplantation: a study of early engraftment kinetics in leukocyte subsets using HLA-specific monoclonal antibodies. Biol.Blood Marrow Transplant. 2013; 19(2): 266-273.
17. Maiers M, Gragert L, and Klitz W. High-resolution HLA alleles and haplotypes in the United States population. Hum.Immunol. 2007; 68(9): 779-788.
18. Przepiorka D, Weisdorf D, Martin P, et al. 1994 Consensus Conference on Acute GVHD Grading. Bone Marrow Transplant. 1995; 15(6): 825-828.
19. Amir AL, D'Orsogna LJ, Roelen DL, et al. Allo-HLA reactivity of virus-specific memory T cells is common. Blood 2010; 115(15): 3146-3157.
20. Distler E, Bloetz A, Albrecht J, et al. Alloreactive and leukemia-reactive T cells are preferentially derived from naive precursors in healthy donors: implications for immunotherapy with memory T cells. Haematologica 2011; 96(7): 1024-1032.
21. van der Harst D, Goulmy E, Falkenburg JH, et al. Recognition of minor histocompatibility antigens on lymphocytic and myeloid leukemic cells by cytotoxic T-cell clones. Blood 1994; 83(4): 1060-1066.
22. Falkenburg JH, Goselink HM, van der Harst D, et al. Growth inhibition of clonogenic leukemic precursor cells by minor histocompatibility antigen-specific cytotoxic T lymphocytes. J.Exp.Med. 1991; 174(1): 27-33.
23. Molldrem JJ, Lee PP, Wang C, et al. Evidence that specific T lymphocytes may participate in the elimination of chronic myelogenous leukemia. Nat.Med. 2000; 6(9): 1018-1023.
24. Horwitz ME and Sullivan KM. Chronic graft-versus-host disease. Blood Rev. 2006; 20(1): 15-27.
25. Flynn R, Du J, Veenstra RG, et al. Increased T follicular helper cells and germinal center B cells are required for cGVHD and bronchiolitis obliterans. Blood. 2014; 19;123(25): 3988-3998.
26. Collins T, Korman AJ, Wake CT, et al. Immune interferon activates multiple class II major histocompatibility complex genes and the associated invariant chain gene in human endothelial cells and dermal fibroblasts. Proc.Natl.Acad.Sci.U.S.A 1984; 81(15): 4917-4921.
27. van Heijst JW, Ceberio I, Lipuma LB, et al. Quantitative assessment of T cell repertoire recovery after hematopoietic stem cell transplantation. Nat.Med. 2013; 19(3): 372-377.
28. de Roock S, Hoeks SB, Meurs L, et al. Critical role for programmed death 1 signaling and protein kinase B

in augmented regulatory T-cell induction in cord blood. J.Allergy Clin.Immunol. 2011; 128(6): 1369-1371.
29. Furst D, Muller C, Vucinic V, et al. High-resolution HLA matching in hematopoietic stem cell transplantation: a retrospective collaborative analysis. Blood 2013; 122(18): 3220-3229.
30. Flomenberg N, Baxter-Lowe LA, Confer D, et al. Impact of HLA class I and class II high-resolution matching on outcomes of unrelated donor bone marrow transplantation: HLA-C mismatching is associated with a strong adverse effect on transplantation outcome. Blood 2004; 104(7): 1923-1930.









8




Chapter 8

Complete donor chimerism is a prerequisite for the effect of Predicted Indirectly ReCognizable HLA Epitopes (PIRCHE) on acute graft-versus-host disease

Kirsten A. Thus,[1] Talitha A. de Hoop,[1] Valeria E. Boers Trilles,[1] Jürgen Kuball,[1,2] and Eric Spierings[1]

[1]Laboratory of Translational Immunology,
University Medical Center Utrecht, Utrecht, the Netherlands
[2]Department of Hematology,
University Medical Center Utrecht, Utrecht, the Netherlands

Submitted

Abstract

Predicted indirectly recognizable HLA epitopes (PIRCHE) theoretically predict donor T-cell recognition of mismatched-HLA derived peptides following allogeneic hematopoietic stem-cell transplantation (allo-HSCT), as is evidenced by the correlation between presence of HLA-DPB1 derived PIRCHE and acute graft-versus-host disease (GVHD). Complete donor T-cell chimerism (CC) is associated with an increased GVHD risk compared to mixed patient and donor chimerism (MC). The effect of potential donor T-cell epitopes on GVHD development, may be best distinguishable in CC. This study was initiated to investigate whether the effect of PIRCHE is different in patients with CC compared to those with MC. Indeed, the correlation between PIRCHE and GVHD is present in patients with CC, whereas it is absent in those with MC. The data presented here suggest that chimerism status is important for the detection of potential GVHD epitopes, and add to previous evidence that PIRCHE recognition by donor T cells induces GVHD.

To the editor

Human leukocyte antigen (HLA) mismatches are an important risk factor for the development of graft-versus-host disease (GVHD) following allogeneic hematopoietic stem-cell transplantation (allo-HSCT) (1). GVHD is induced by alloreactive donor T cells that recognize genetically disparate patient tissue (2). HLA mismatches are the single most important genetic difference leading to GVHD (3). HLA mismatches can be recognized by the donor T cells via, amongst other routes, so-called indirect recognition. During indirect recognition, the donor T cells recognize mismatched-HLA derived peptides that are different from self peptides. The probability of indirect T-cell recognition of HLA mismatches can be predicted by calculating the numbers of Predicted Indirectly ReCognizable HLA Epitopes (PIRCHE) (4-6). The number of PIRCHE equals the number of HLA-derived T-cell epitopes, and higher numbers of PIRCHE presented by HLA class-I or -II (PIRCHE-I or -II) are clearly correlated to acute GVHD development (5, 6).

The presence of a vast majority of donor T cells in the patient's hematopoietic system is a significant predictor for acute GVHD, thereby underlining the essential role of alloantigen-specific donor T cells in the induction of GVHD. The descent (donor or patient) of the reconstituting hematopoietic system after allo-HSCT can be quantified via chimerism analyses. In chimerism analyses, complete donor chimerism (CC) is defined as 100% of hematopoietic cells derived from the donor, whereas mixed chimerism (MC) indicates that both patient and donor signals are detectable (7). The degree of donor T-cell chimerism rapidly increases during acute GVHD (8), and CC in the T-cell compartment significantly predicts acute GVHD (9, 10). Thus, although acute GVHD can develop in a state of MC (8), the risk of GVHD is increased in patients with CC.

As complete donor T-cell chimerism associates with GVHD, the effect of potential donor T-cell epitopes on GVHD development, is likely most profound during CC. In that respect, the correlation between PIRCHE, being potential donor T-cell epitopes, and GVHD may be most prominent in patients with CC after allo-HSCT. To investigate this hypothesis, we retrospectively studied a cohort in which previously a correlation was shown between the presence of HLA-DPB1-derived PIRCHE and acute GVHD (6).

Detailed clinical characteristics of the study cohort and of the PIRCHE method were described elsewhere (6). From the previously studied cohort, chimerism data could be analyzed for 73 patients, with a median age of 56 years (range 31-70), of which the majority was male (N=50, 68.5%). Diagnostic chimerism analyses were based on short-tandem repeat polymerase chain reactions on T cells separated from peripheral blood by automated magnetic cell sorting (Auto-MACS, Miltenyi Biotec, Bergisch Gladbach, Germany) (11). CC was defined as 100% donor signal on all time-points measured, MC was defined as >1% of patient and donor signal (11). Chimerism was measured a median of 2 times (range 1-12 measurements), starting at a median of 29 days post-HSCT (range 12-91 days). The majority of first chimerism measurements was performed prior to onset of GVHD (N=62, 85%). Of the total of 73 patients analyzed, 36 (49%) displayed CC and 37 (51%) MC.

To study the correlation between chimerism status and PIRCHE effects, patients were stratified according to chimerism status. Patients with CC that developed grade II-IV acute GVHD, had significantly higher numbers of PIRCHE-I and -II when compared to those with grade 0-I acute GVHD (Mann-Whitney U test, p=0.008, and p=0.036, for PIRCHE-I and -II, respectively, Figure 1). In patients with MC, PIRCHE numbers did not differ between patients with grade II-IV acute GVHD versus those with grade 0-I acute GVHD (p=0.774, and p=0.639

for PIRCHE-I and -II, respectively, Figure 1).

We previously showed a clear correlation between the presence of PIRCHE and acute GVHD development (6). In the present study, we stratified according to chimerism and then studied the effect of PIRCHE presence on GVHD development. Of the patients with CC, 17 (47%) had PIRCHE-I, and 24 (36%) had PIRCHE-II. In patients with MC, 27 (73%) had PIRCHE-I and 30 (81%) PIRCHE-II. In patients with CC, the presence of PIRCHE-I was associated to a higher GVHD incidence (59% versus 5%, for presence or absence of PIRCHE-I, respectively, log-rank test p=0.024), whereas this association was absent in patients with MC (48% versus 30%, p=0.359). A similar, although less striking, difference was found for PIRCHE-II (33% versus 0%, p=0.029; and 50% versus 24%, p=0.092; for presence or absence of PIRCHE-II in CC and MC). Thus, when reanalyzing the previous findings that patients with acute GVHD have higher numbers of PIRCHE and that the presence of PIRCHE is associated with acute GVHD development (6), these correlations were only present in those with CC and absent in those with MC, suggesting that stable engraftment of donor cells is required for PIRCHE-induced GVHD.

We observed a clear concordance of PIRCHE with acute GVHD in patients with CC, but not in patients with MC. This observation suggests that PIRCHE recognition mainly plays a role in situations with 100% donor T cells in the hematopoietic system. The definition of complete donor chimerism as 100% donor cells on all measured time points, is a guideline definition (7), although literature also describes >90% or >95% donor cells as CC (11-13). The percentage of 100% donor signal and the fact that this should be detectable on all measured time points, to establish a PIRCHE effect, can be debated; during 90% or 95% donor chimerism, donor cells also clearly dominate the hematopoietic system. The PIRCHE effect may well be a dose- and time-dependent phenomenon. Larger prospective studies with chimerism percentages as a continuous variable and measurements on predefined time points should be executed to further establish the required percentage of donor cells for the PIRCHE effect.

Graft-versus-host (GVH) directed alloreactivity not only leads to GVHD, but may also substantiate donor engraftment by the elimination of patient lymphoid cells (14). Since the presence of PIRCHE correlates with GVH-directed alloreactivity, PIRCHE-recognition might also contribute to the elimination of patient cells. The success of this elimination can be studied by chimerism analyses, as in CC the elimination is more successful than in MC. Since GVH-PIRCHE indicate a higher probability of donor T-cell responses, we hypothesized that a higher number of GVH-directed PIRCHE associates with a greater probability of CC.

Higher GVH-PIRCHE-I and -II did not significantly correlate to CC. However, a trend was observed that in patients with CC the numbers of GVH-PIRCHE-I were lower compared to patients with MC (p=0.088). In line with this, patients with GVH-PIRCHE-I had a significantly higher odds of MC compared to those without GVH-PIRCHE-I (OR 1.70, 95%-CI 1.07-2.68, p=0.025), an effect which was not present for GVH-PIRCHE-II (OR 1.42, 95%-CI 0.90-2.24, p=0.161).

In contrast to GVH-directed alloreactivity, HVG-directed alloreactivity would enable the patients residual T cells to prevent donor engraftment. We therefore next studied whether a higher number of HVG-directed PIRCHE is correlated to MC. There are some indications that HVG-PIRCHE-I correlate with MC, as in patients with MC the numbers of HVG-PIRCHE-I were higher than in those with CC (p=0.006). Moreover, patients with HVG-PIRCHE-I had a significantly higher odds of MC compared to patients without HVG-PIRCHE-I (OR 1.80, 95%-

CI 1.14-2.83, p=0.012), an effect which was not present for HVG-PIRCHE-II (OR 1.32, 95%-CI 0.83-2.11, p=0.262). To summarize, higher GVH-directed PIRCHE-I inversely associate with CC, whereas higher HVG-directed PIRCHE-I potentially associate with MC.

The interpretation of the independent effects of GVH- and HVG-PIRCHE-I on chimerism status is difficult, as the majority of patients with GVH-PIRCHE-I also have HVG-PIRCHE-I (N=30, 68%). The counterintuitive results of GVH-PIRCHE-I being correlated to MC, may hence be explained by the correlation between GVH- and HVG-PIRCHE-I presence. Nonetheless, the significant correlation of HVG-PIRCHE-I with MC suggests that residual patient T cells recognize the donor cells, thereby preventing complete donor engraftment.

To conclude, this study was initiated to investigate whether the effect of PIRCHE on GVHD is dependent on donor T cells as reflected by the chimerism status. Indeed, PIRCHE appeared to be highly correlated to acute GVHD in patients with CC, but not in those with MC. Thus, our data support a role for the donor hematopoietic system in PIRCHE-reactivity, substantially endorsing the hypothesis that donor cells, most likely T cells, can recognize PIRCHE. Our data provide a step forward in understanding the effect of PIRCHE on GVHD after HLA-mismatched HSCT.

Acknowledgements

Grateful acknowledgement is made to Liane te Boome, Anette van Dijk and Martin Weiland for the clinical data, Mieke Ruizendaal and the HLA laboratory of the UMCU for the HLA typings and Joyce van Loon and Roel de Weger, for the chimerism data.

Conflict of interest

The University Medical Center Utrecht has filed a patent application titled “Method for prediction of an immune response against mismatched human leukocyte antigens”; number: WO 2014072467 A1. Dr. Eric Spierings is mentioned as an inventor on this patent application. The other authors have no personal conflict of interest to declare.

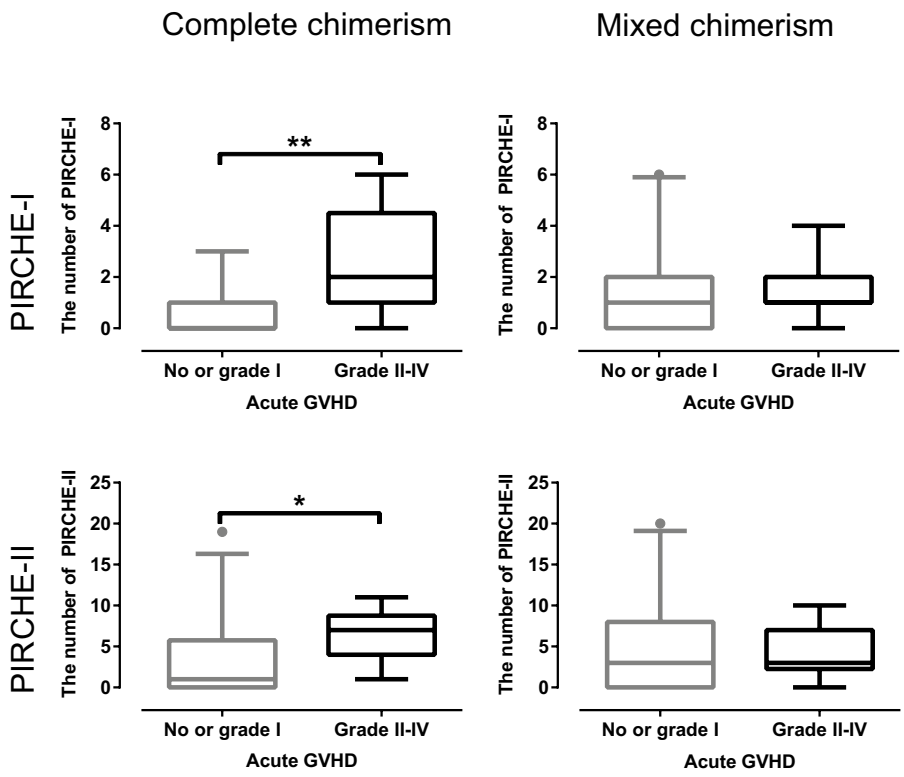
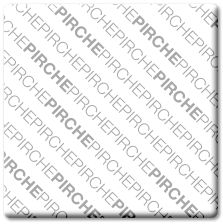
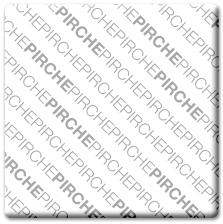
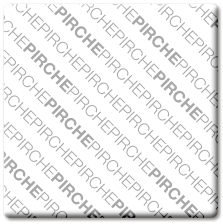
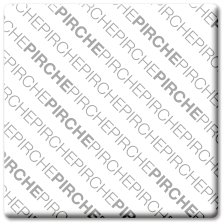
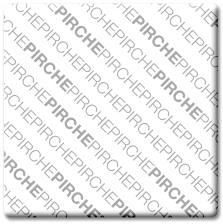
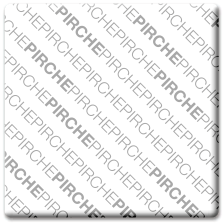
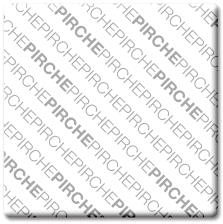
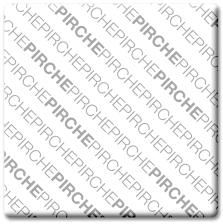
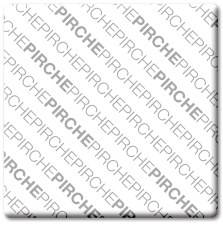
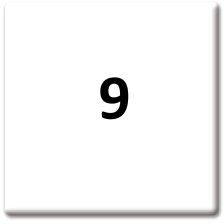
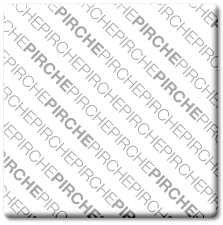
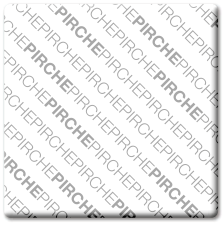


Figure 1: PIRCHE numbers by acute GVHD development for patients with complete donor chimerism (left panels) and with mixed chimerism (right panels). Box and whisker plots: boxes represent the 25-75th percentiles, horizontal lines the median, whiskers the 5-95th percentiles and dots the outliers. Patients with complete chimerism (left panels) had significantly more PIRCHE-I and -II when they developed grade II-IV acute GVHD (PIRCHE-I: median 2, range 0-6, PIRCHE-II: median 7, range 1-11) compared to patients with no or grade I acute GVHD (PIRCHE-I: median 0, range 0-3, PIRCHE-II: median 1, range 0-19). Patients with mixed chimerism (right panels) had similar numbers of PIRCHE-I and -II when they developed grade II-IV acute GVHD (PIRCHE-I: median 1, range 0-4, PIRCHE-II: median 3, range 0-10) compared to patients with no or grade I acute GVHD (PIRCHE-I: median 1, range 0-6, PIRCHE-II: median 3, range 0-20). **: Mann-Whitney $p<0.01$, *: Mann-Whitney $p<0.05$. PIRCHE: predicted indirectly recognizable HLA epitope. GVHD: graft-versus-host disease.

References

1. Lee SJ, Klein J, Haagenson M, et al. High-resolution donor-recipient HLA matching contributes to the success of unrelated donor marrow transplantation. Blood 2007; 110(13): 4576-4583.
2. Ferrara JL, Levine JE, Reddy P, et al. Graft-versus-host disease. Lancet 2009; 373(9674): 1550-1561.
3. Bray RA, Hurley CK, Kamani NR, et al. National marrow donor program HLA matching guidelines for unrelated adult donor hematopoietic cell transplants. Biol. Blood Marrow Transplant. 2008; 14(9 Suppl): 45-53.
4. Otten HG, Calis JJ, Kesmir C, et al. Predicted indirectly recognizable HLA epitopes presented by HLA-DR correlate with the de novo development of donor-specific HLA IgG antibodies after kidney transplantation. Hum.Immunol. 2013; 74(3): 290-296.
5. Thus KA, te Boome L, Kuball J, et al. Indirectly Recognized HLA-C Mismatches and Their Potential Role in Transplant Outcome. Front Immunol. 2014; 5: 210.
6. Thus KA, Ruizendaal MT, de Hoop TA, et al. Refinement of the Definition of Permissible HLA-DPB1 Mismatches with Predicted Indirectly ReCognizable HLA-DPB1 Epitopes. Biol.Blood Marrow Transplant. 2014; 20(11): 1705-1710.
7. Antin JH, Childs R, Filipovich AH, et al. Establishment of complete and mixed donor chimerism after allogeneic lymphohematopoietic transplantation: recommendations from a workshop at the 2001 Tandem Meetings of the International Bone Marrow Transplant Registry and the American Society of Blood and Marrow Transplantation. Biol.Blood Marrow Transplant. 2001; 7(9): 473-485.
8. Petersen SL, Madsen HO, Ryder LP, et al. Chimerism studies in HLA-identical nonmyeloablative hematopoietic stem cell transplantation point to the donor CD8(+) T-cell count on day + 14 as a predictor of acute graft-versus-host disease. Biol.Blood Marrow Transplant. 2004; 10(5): 337-346.
9. Mattsson J, Uzunel M, Remberger M, et al. T cell mixed chimerism is significantly correlated to a decreased risk of acute graft-versus-host disease after allogeneic stem cell transplantation. Transplantation 2001; 71(3): 433-439.
10. Minculescu L, Madsen HO, and Sengelov H. T-Cell Chimerism Is Valuable in Predicting Early Mortality in Steroid-Resistant Acute Graft-versus-Host Disease after Myeloablative Allogeneic Cell Transplantation. Acta Haematol. 2014; 132(2): 187-192.
11. Huisman C, de Weger RA, de VL, et al. Chimerism analysis within 6 months of allogeneic stem cell transplantation predicts relapse in acute myeloid leukemia. Bone Marrow Transplant. 2007; 39(5): 285-291.
12. El-Cheikh J, Vazquez A, Crocchiolo R, et al. Acute GVHD is a strong predictor of full donor CD3+ T cell chimerism after reduced intensity conditioning allogeneic stem cell transplantation. Am.J.Hematol. 2012; 87(12): 1074-1078.
13. Mickelson DM, Sproat L, Dean R, et al. Comparison of donor chimerism following myeloablative and nonmyeloablative allogeneic hematopoietic SCT. Bone Marrow Transplant. 2011; 46(1): 84-89.
14. Storb R, Yu C, Barnett T, et al. Stable mixed hematopoietic chimerism in dog leukocyte antigen-identical littermate dogs given lymph node irradiation before and pharmacologic immunosuppression after marrow transplantation. Blood 1999; 94(3): 1131-1136.











9



Chapter 9

The number of T-cell allo-epitopes associates with CD4+ and CD8+ T-cell infiltration in pediatric cutaneous GVHD

Kirsten A. Thus,[1] Astrid G. S. van Halteren,[2] Titus A. P. de Hond,[1] Marijke R. van Dijk,[3] Robin Q. H. Kloos,[1] Arjan C. Lankester,[2] Marc B. Bierings,[4] and Eric Spierings[1]

[1]Laboratory of Translational Immunology,
University Medical Center Utrecht, Utrecht, the Netherlands
[2]Immunology Laboratory/Pediatric Stem Cell Transplantation Unit,
Willem Alexander Children's Hospital, Leiden University Medical Center,
Leiden, the Netherlands
[3]Department of Pathology,
University Medical Center Utrecht, Utrecht, the Netherlands
[4]Department of Hematology and Stem Cell Transplantation,
Wilhelmina Children's Hospital, Utrecht, the Netherlands

Accepted for publication in a revised version
Cellular Immunology 2015

Abstract

Graft-versus-host disease (GVHD) remains an important complication after hematopoietic stem-cell transplantation (HSCT). Risk factors for GVHD include, but are not limited to, HLA mismatches, a female donor for a male recipient, and stem-cell source. We retrospectively analyzed if HLA- and sex-mismatching quantitatively affects the composition of GVHD-induced cutaneous T-cell infiltrates. We quantified absolute numbers of CD4+ and CD8+ T cells present in tissue sections from skin biopsies derived from 23 pediatric HSCT-recipients with histologically and clinically confirmed acute GVHD. Recipients receiving a sex-mismatched graft displayed an increased number of CD4+ T cells when compared to recipients of a graft from a sex-matched unrelated donor (either adult unrelated donor or cord blood) (p=0.01). The absolute numbers of skin-infiltrating T cells were increased in patients expressing T-cell epitopes derived from the recipient's mismatched HLA molecules, so called predicted indirectly recognizable HLA epitopes (PIRCHE). The combined expression of HLA class I- or II-presented PIRCHE with a sex mismatch resulted in the highest number of skin-infiltrating T cells. Our results provide the first indication that an increased number of major (*i.e.* HLA) and minor (*i.e.* HY) histocompatibility antigen-derived T-cell epitopes expressed by the recipient but not by the donor, is associated with accumulation of CD4+ and CD8+ T cells in the skin. These observations may explain the pronounced effect of sex-mismatching in HLA-mismatched transplants as evidenced by registry-based epidemiological studies.

Introduction

Graft-versus-host disease (GVHD) remains a major limiting factor for successful allogeneic hematopoietic stem-cell transplantation (HSCT) (1). Risk factors for GVHD include, amongst others, stem-cell source, a sex-mismatch (female donor for male recipient), and HLA mismatches (2). The pathophysiological mechanisms via which these risk factors lead to GVHD, are poorly understood.

GVHD evidently involves T-cell recognition of non-self antigens expressed by the cells of the recipient, as depletion of T cells from the graft reduces the development of GVHD (3). In general, HLA mismatches are the best established inducers of GVHD (4). HLA mismatches can be recognized by donor T cells in a direct or indirect manner. During direct recognition, the donor T cell recognizes the mismatched HLA protein expressed on the cell surface. During indirect recognition, the donor T cell recognizes peptides derived from the mismatched HLA protein presented on shared HLA molecules (5). In contrast to the poorly understood process of direct recognition, our group has recently proposed a method that can predict indirect recognition *in silico*, the PIRCHE method. The PIRCHE method predicts so called Predicted Indirectly ReCognizable HLA Epitopes (PIRCHE) (6). We have shown that the number of both HLA class-I (PIRCHE-I) as well as HLA class-II (PIRCHE-II) presented PIRCHE may be associated with the development of GVHD (7, 8).

In addition to HLA mismatches, minor histocompatibility (H) antigens are a second class of allo-antigens which can lead to T-cell recognition in transplant settings (9). Mismatches for, in particular ubiquitously expressed, minor H antigens are well established inducers of GVHD (10). Minor H mismatching occurs for example in sex-mismatched HSCT, when peptides derived from the Y chromosome (HY) presented by HLA of male recipient cells can be recognized by female donor T cells (11, 12).

The relative contribution of donor CD4+ and CD8+ T cells in the development or severity of skin GVHD in relation to graft source is poorly understood, although the presence of both CD4+ and CD8+ T cells in cutaneous GVHD is well established (11, 13). We addressed this issue retrospectively by quantifying the absolute numbers of CD3+CD4+ and CD3+CD8+ T cells in GVHD skin sections in relation to graft source, *i.e.* HLA identical related donor (IRD) or adult matched unrelated donor (MUD) or unrelated cord blood (CB), and sex-mismatching.

Material and Methods

Biopsy selection

A total of 34 archival formalin-fixed paraffin embedded (FFPE) skin biopsies were selected for this study. Biopsies were obtained from pediatric recipients that underwent HSCT in the Willem-Alexander Children's Hospital and in the Wilhelmina Children's Hospital. All biopsies were collected for routine histopathological assessment of acute cutaneous GVHD and before the start of prednisone treatment. Histological assessment was performed on hematoxylin and eosin stained sections. Clinical GVHD was graded according Glucksberg criteria (14). For analysis of GVHD sections, only recipients with both histologically as well as clinically confirmed GVHD were included.

Immunohistochemistry

FFPE sections (4 μm) were subjected to standard deparaffinization in xylol, rehydration, antigen retrieval in EDTA pH 8,0 buffer and overnight staining at room temperature

with primary CD3, CD4 and CD8 antibodies all appropriately diluted in PBS containing 0.5% donkey serum. Antibodies used were CD3 rabbit IgG (clone A0452, Dako, Glostrup, Denmark), CD4 goat IgG (clone AF-379-NA, R&D Systems, Minneapolis, MN, USA) and CD8 mouse IgG2b (clone NCL-CD8-4B11, Novocastra via Leica Biosystems, Nussloch, Germany). The subsequent day, sections where washed in PBS where after the following secondary antibodies were added: donkey-anti-rabbit (Alexa Fluor 546, A10040), donkey-anti-goat (Alexa Fluor 488, A-11055), and donkey-anti-mouse (Alexa Fluor 647, A-31571, all from Life Technologies Thermo Fischer Scientific, Waltham, MA, USA).

Confocal laser microscopy

All sections were mounted with vectashield (Vector Laboratories) and analyzed on a LEICA TCS SP confocal laser scanning system (Leica Microsystems) using a 40x numerical aperture. Single color photographs were taken and combined to generate electronic overlays. For each recipient 7.5-29 photographs (mean 17) were scored for the presence of CD3+CD8+ and CD3+CD4+ T cells. Photographs were scored in a blinded fashion (*i.e.* researchers were unaware of the diagnosis or graft information at the time of counting) by two independent observers (KT and TdH). To account for inter-observer deviations, the average of the two scores was used for analyses. The scores of the two observers were compared and when the percentage of CD4+ or CD8+ T cells between the two scores deviated > 5%, sections were reanalyzed together until consensus was reached (three in total, of which two without GVHD, and one with histological confirmed GVHD). We restricted our analyses of GVHD sections to those with ten or more available photographs (N=23), to correct for differences in size of the sections and numbers of evaluable photographs.

HLA typing

HLA typing was performed on high resolution (2-field) level for unrelated donor transplants (MUD or CB) for HLA-A, -B, -C, -DRB1, -DQB1, and -DPB1 in 13 (81%) of the cases. IRD transplants were high-resolution typed for HLA-A, -B, -C, -DRB1, -DQB1 in 3 (43%) of cases. PIRCHE were predicted based on the available HLA typing as described previously (7, 8). The algorithm used to determine PIRCHE values is available at www.pirche.org. HLA-A1, -A2, -A33, -B7, -B8, -B27, -B52, -B60, -DR15, -DRB3*03:01 (scored positive in case of DRB1*13:02), and -DQ5 were considered HY-presenting alleles (10).

Statistical analysis

Statistical analyses were performed in GraphPad Prism 5.00 (GaphPad Software, La Jolla, CA). Differences in number of CD4+ and CD8+ T cells in infiltrates were tested with Mann-Whitney U Tests. P-values < 0.05 were considered significant.

Results

We first determined whether the numbers of skin-infiltrating T cells, as exemplified in Figure 1, differed between biopsies of recipients with or without histologically confirmed GVHD. As expected, both the number of skin-infiltrating CD4+ as well as the number of CD8+ T cells were higher in the histologically-confirmed GVHD infiltrates, as compared to tissue biopsies from recipients with skin inflammation due to other causes, for example herpes virus infection or a reaction to administered medicines ($p<0.01$ and $p=0.07$, for CD4+ and CD8+ T cells, respectively). The number of CD4+ and CD8+ T cells correlated to each other, meaning

that samples containing high numbers of CD4+ T cells also contained high numbers of CD8+ T cells (for patients without GVHD, the correlation between absolute numbers of CD4+ and CD8+ T cells was R^2=0.83, p=0.06; and for patients with confirmed GVHD this correlation was R^2=0.53, p=0.004). The observation that the number of T cells is higher in histologically-confirmed GVHD, validates, in retrospect, the objectivity of the blinded scoring system.

The numbers of both CD4+ and CD8+ T cells varied considerably in histologically-confirmed GVHD biopsies (range of the mean of two independent observers CD4+: 9.5-495, CD8+: 2-1043). Given the substantial variation in absolute numbers of T cells present in the three different types of grafts used for HSCT (15, 16), we investigated whether donor type or graft source affected the number of skin-infiltrating T cells. Although skin tissue obtained after CB transplantation contained significantly higher numbers of CD4+ T cells compared to MUD transplantations (p=0.02), the numbers of CD8+ T cells in infiltrates of skin biopsies obtained after IRD, MUD or CB HSCT did not significantly differ (data not shown).

As HLA-mismatches are clear inducers of GVHD, we hypothesized that a higher probability of indirect recognition of the HLA mismatch as predicted with the PIRCHE method, leads to a higher number of infiltrating T cells in HLA mismatched HSCT settings. Theoretically, a higher number of PIRCHE-I leads to CD8+ T cell induction whereas a higher number of PIRCHE-II leads to CD4+ T cell induction. The numbers of CD4+ and CD8+ T cells were retrospectively analyzed in infiltrates of recipients receiving unrelated donor HSCT (MUD or CB, N=16) in correlation to the presence of either PIRCHE-I or PIRCHE-II. A trend was observed for an increased number of CD4+ T cells in the recipients with PIRCHE-II and an increased number of CD8+ T cells in the recipients with PIRCHE-I (Figure 2B, C).

We next investigated whether the number of CD4+ and CD8+ T cells differs when we compare skin biopsies obtained after sex-mismatched HSCT with biopsies obtained after sex-matched HSCT. When combining all donor sources, we did not observe a difference in CD4+ and CD8+ T-cell numbers in sex-mismatched HSCT with either an HLA class-I or -II HY-presenting allele versus sex-matched GVHD infiltrates (data not shown).

Multiple studies have indicated that the negative impact of sex-mismatching may be more pronounced or even only present in recipients of (HLA-mismatched) unrelated grafts (10, 11). To investigate the effect of donor-recipient relationship alongside the sex-matching effect, analyses were stratified according to the relationship between the donor and recipient (IRD compared to an unrelated donor), and again the sex-match effects were analyzed in recipients with HY-presenting HLA alleles. Indeed, an increased number of CD4+ T cells is present in the infiltrate of recipients receiving stem cells from an unrelated donor when comparing sex-mismatched versus sex-matched transplantations (Figure 2E). This effect is not present for the IRD transplants (data not shown).

As the risk of GVHD due to a sex-mismatch is most pronounced in HLA-mismatched transplantations, we hypothesized that the combination of a potential HY-epitope being presented next to a PIRCHE leads to a high number of T cells in the infiltrates. To study this hypothesis, the presence of the combination of PIRCHE and HY was compared with absence of PIRCHE and a sex-match in patients that had an HY-presenting allele. A clear increase in the number of CD4+ T cells in the PIRCHE-II and HY-presenting infiltrate, and CD8+ T cells in the PIRCHE-I and HY-presenting infiltrate was observed, compared to absence of PIRCHE and a sex-match (representative images in Figure 1). Note that the number of the sex-match PIRCHE-absent cases is rather low in this retrospective study set up (Figure 2G,H).

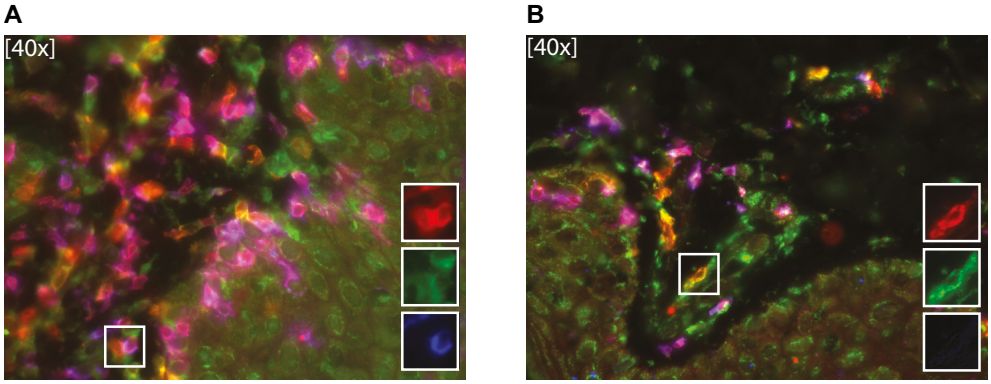


Figure 1 (A) GVHD T-cellular skin infiltrate of a recipient transplanted with a sex-mismatched HLA-mismatched unrelated donor. (B) GVHD T-cellular skin infiltrate of a recipient after HLA mismatched but PIRCHE-II matched, sex-matched unrelated donor. Blue: CD8; Green: CD4; Red: CD3. In yellow CD4+ T cells, in pink CD8+ T cells. Representative images of two sections displaying the significant differences in cellular infiltrates of the skin. The PIRCHE and HY-presenting skin section is dominated by high numbers of T cells whereas the PIRCHE and HY-matched skin section contains significantly less T cells. GVHD: graft-versus-host disease.

Discussion

This study was initiated to improve our knowledge of the pathophysiological mechanisms leading to GVHD and to understand the frequently observed differential numbers of T cells in skin infiltrates of pediatric recipients after HSCT (11, 13). Our results generate the hypothesis that an increasing number of major (*i.e.* HLA) and minor (*i.e.* HY) histocompatibility antigen-derived T-cell epitopes expressed by recipient skin cells, may lead to increased accumulation of CD4+ and CD8+ T cells in the skin. These observations may explain the more pronounced effect of sex-mismatching in HLA-mismatched transplants. Noteworthy, due to the retrospective nature of this study, the status of matching of other minor H antigen mismatches was not established on forehand.

The influx of CD4+ (13) and CD8+ (11) T cells in pediatric skin during GVHD has been identified before. To the best of our knowledge, our study is the first that underlines the essential role of multiple epitopes attracting CD4+ and CD8+ T cells to the site of inflammation. Interestingly, CD4+ T cells seem to preferentially home to the dermis, whereas CD8+ T cells were also observed in the epidermal layer (Figure 1). We speculate that the CD4+ T cells first home to the skin, subsequently attracting CD8+ T cells, thereby inducing profound immune responses in the epidermis. Our results are in line with a previous observation that multiple epitopes derived from different antigens induce the cooperative response of CD4+ and CD8+ T cells resulting in GVHD (17).

Our observations need to be considered cautiously given the diversity in this recipient population considering underlying diseases, conditioning regimens and GVHD prophylaxis, and, potentially consequently, the large variation in T-cell numbers observed (Table 1). Furthermore, the small sample size of the cohort did not allow multivariate analyses. On the opposite, this study is restricted to pediatric recipients, and can therefore be regarded as informative for this specific subgroup of recipients which is rarely studied separately. To substantiate the results presented in this hypothesis-generating study, studies should be conducted in larger and more homogenous study cohorts, allowing prospective stratification for HY and including testing the role of other ubiquitously expressed minor H antigen mismatches.

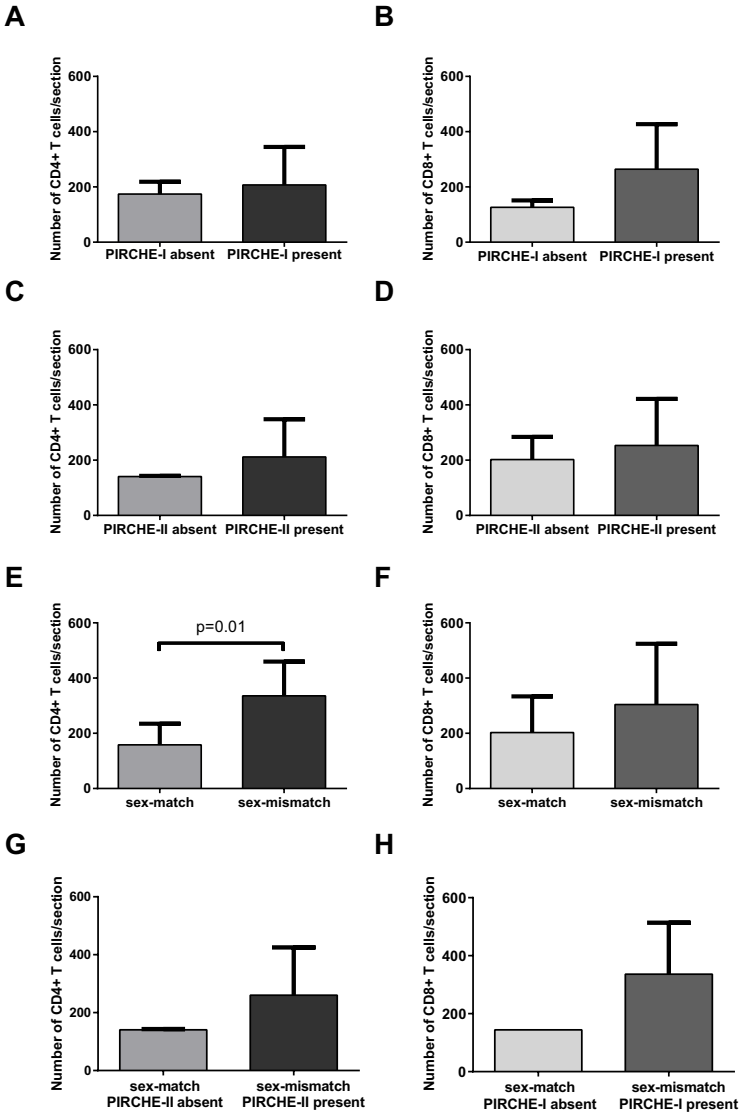


Figure 2 (A) The number of CD4+ T cells according to PIRCHE-I presence. (B) The number of CD8+ T cells according to PIRCHE-I presence. (C) The number of CD4+ T cells according to PIRCHE-II presence. (D) The number of CD8+ T cells according to PIRCHE-II presence. (E) The number of CD4+ T cells according to sex-match status. (F) The number of CD8+ T cells according to sex-match status. (G) The number of CD4+ T cells according to sex-match status and PIRCHE-II presence. (H) The number of CD8+ T cells according to sex-match status and PIRCHE-I presence. There were 2 recipients without PIRCHE-I or -II, and 14 with PIRCHE-I or -II. From the unrelated donor recipients with an HY-presenting allele, 9 were a sex-match and 5 a sex-mismatch. The presence of a PIRCHE-II mismatch associates with an increased number of CD4+ T cells whereas the presence of a PIRCHE-I mismatch associates with an increased number of CD8+ T cells. Recipients after sex-mismatched transplantation have a significantly increased number of CD4+ T cells in their cutaneous graft-versus-host disease infiltrate. The number of T cells appears to be highest in case of both a sex-mismatch and the presence of PIRCHE.

ID	Center	Primary Disease	Graft source	Matchgrade	Sexmismatch	GVHD grade	PIRCHE-I	PIRCHE-II	CD4+ T cells	CD8+ T cells
567	L	aCML Ph+	MUD	8/12	Yes	3	3	17	249	626
581	L	AML rec	IRD	12/12	Yes	2	0	0	352	543
586	L	AML rec CR2	MUD	10/12	Yes	2	5	7	100	234
603	L	ALL pro B	IRD	12/12	No	3	0	0	495	444
658	L	ALL-t PPR	MUD	12/12	No	3	0	0	143	144
659A	L	AML rec	MUD	9/12	No	4	4	14	204	421
659B	L	AML rec	MUD	9/12	No	4	4	14	75	172
676	L	ALL HR Ph+	MUD	9/12	No	3	12	25	104	354
696	L	JMML	MUD	9/12	No	3	6	27	85	25
716	L	AML rec	IRD	12/12	No	3	0	0	200	526
729	L	ALL rec	IRD	12/12	Yes	2	0	0	123	216
732	L	B Thallassemia homozygous	MUD	11/12	Yes	2	4	1	44	370
744	L	ALL rec	IRD	12/12	Yes	3	0	0	28	7
752	L	ALL-T rec	IRD	12/12	Yes	2	0	0	67	242
755	L	X-CGD	MUD	10/12	No	2	4	4	206	146
783	L	LAD type 1	MUD	10/12	Yes	3	3	2	291	327
800	L	JMML	MUD	11/12	No	4	4	0	139	260
1	U	Di George	IRD	8/8	No	2	0	0	139	84
31	U	Chediak Higashi	CB	7/10	No	1	1	15	320	249
57	U	CID	CB	9/12	No	2	1	4	152	56
43	U	CID	CB	8/10	Yes	2	13	8	460	372
51	U	AML CR 2	CB	7/10	Yes	1	7	16	475	89
2	U	MDS	MUD	11/12	Yes	1	0	4	206	109

Table 1 (previous page): Baseline characteristics of recipients with clinical and histopathological acute GVHD that were included in the analyses. L: Willem-Alexander Childrens Hospital, Leiden, the Netherlands. U: Wilhelmina Childrens Hospital, Utrecht, the Netherlands. CML: chronic myeloid leukemia. AML: acute myoloid leukemia. ALL: acute lymphoblastic leukemia. JMML: juvenile myelomonocytic leukemia. X-CGD: X-linked chronic granulomatous disease. LAD: leukocyte adhesion deficiency. CID: combined immunodeficiency. MDS: myelodysplastic syndrome. IRD: identical related donor. MUD: matched unrelated donor. CB: cord blood. Sexmismatch: female donor for male recipient. GVHD grade: maximum acute overall clinical GVHD grade. The number of PIRCHE-I and -II epitopes were predicted based on available HLA-typing (including HLA-DPB1 when available; 8/8 IRD was regarded a complete match). CD4+ and CD8+ T cell numbers are the mean numbers of two observers as independently counted in the skin section.

Acknowledgements

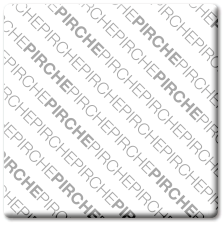
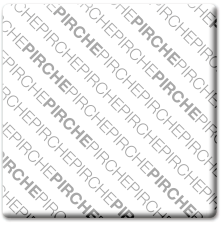
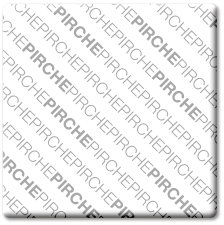
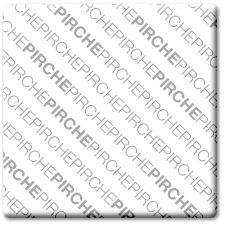
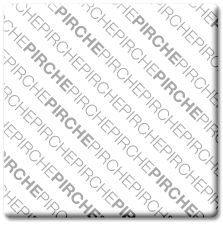
Astrid van Halteren is a scolar of the National Blood Foundation/AABB.

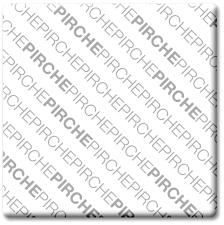
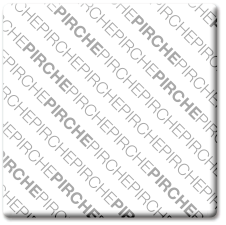
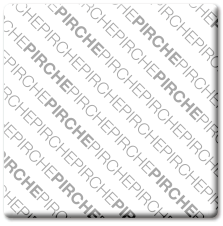
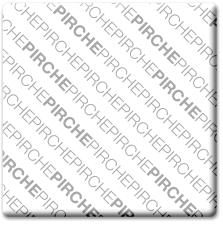
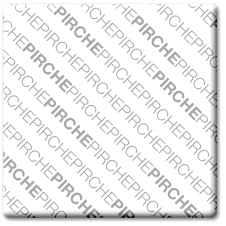
Financial disclosure statement

The University Medical Center Utrecht has filed a patent application titled “Method for prediction of an immune response against mismatched human leukocyte antigens”; number: WO 2014072467 A1. Dr. Eric Spierings is mentioned as an inventor on this patent application. The other authors have no conflict of interest to declare.

References

1. Ferrara JL, Levine JE, Reddy P, et al. Graft-versus-host disease. Lancet 2009; 373(9674): 1550-1561.
2. Jagasia M, Arora M, Flowers ME, et al. Risk factors for acute GVHD and survival after hematopoietic cell transplantation. Blood 2012; 119(1): 296-307.
3. Bayraktar UD, de LM, Saliba RM, et al. Ex vivo T cell-depleted versus unmodified allografts in patients with acute myeloid leukemia in first complete remission. Biol.Blood Marrow Transplant. 2013; 19(6): 898-903.
4. Petersdorf EW. Optimal HLA matching in hematopoietic cell transplantation. Curr.Opin.Immunol. 2008; 20(5): 588-593.
5. Peptide database. SYFPEITHI 2013, Available from: www.syfpeithi.de
6. Geneugelijk K, Thus KA, and Spierings E. Predicting Alloreactivity in Transplantation. J.Immunol.Res. 2014; 2014: 159479.
7. Thus KA, te Boome L, Kuball J, et al. Indirectly Recognized HLA-C Mismatches and Their Potential Role in Transplant Outcome. Front Immunol. 2014; 5: 210.
8. Thus KA, Ruizendaal MT, de Hoop TA, et al. Refinement of the Definition of Permissible HLA-DPB1 Mismatches with Predicted Indirectly ReCognizable HLA-DPB1 Epitopes. Biol.Blood Marrow Transplant. 2014; 20 (11): 1705-1710.
9. Goulmy E, Schipper R, Pool J, et al. Mismatches of minor histocompatibility antigens between HLA-identical donors and recipients and the development of graft-versus-host disease after bone marrow transplantation. N.Engl.J.Med. 1996; 334(5): 281-285.
10. Spierings E, Kim YH, Hendriks M, et al. Multicenter analyses demonstrate significant clinical effects of minor histocompatibility antigens on GvHD and GvL after HLA-matched related and unrelated hematopoietic stem cell transplantation. Biol.Blood Marrow Transplant. 2013; 19(8): 1244-1253.
11. Kim YH, Faaij CM, van Halteren AG, et al. In situ detection of HY-specific T cells in acute graft-versus-host disease-affected male skin after sex-mismatched stem cell transplantation. Biol.Blood Marrow Transplant. 2012; 18(3): 381-387.
12. Mutis T, Gillespie G, Schrama E, et al. Tetrameric HLA class I-minor histocompatibility antigen peptide complexes demonstrate minor histocompatibility antigen-specific cytotoxic T lymphocytes in patients with graft-versus-host disease. Nat.Med. 1999; 5(7): 839-842.
13. Faaij CM, Lankester AC, Spierings E, et al. A possible role for CCL27/CTACK-CCR10 interaction in recruiting CD4 T cells to skin in human graft-versus-host disease. Br.J.Haematol. 2006; 133(5): 538-549.
14. Glucksberg H, Storb R, Fefer A, et al. Clinical manifestations of graft-versus-host disease in human recipients of marrow from HL-A-matched sibling donors. Transplantation 1974; 18(4): 295-304.
15. Chevallier P, Robillard N, Illiaquer M, et al. Characterization of various blood and graft sources: a prospective series. Transfusion 2013; 53(9): 2020-2026.
16. Korbling M and Anderlini P. Peripheral blood stem cell versus bone marrow allotransplantation: does the source of hematopoietic stem cells matter? Blood 2001; 98(10): 2900-2908.





10


Summary
&
General Discussion

Summary

Allogeneic hematopoietic cell transplantation (allo-HCT) with an HLA-mismatched donor leads to an increased risk of alloreactivity and reduced survival compared to HCT with HLA-matched donors. However, for many patients, a completely HLA-matched donor is hard to find due to the enormous polymorphic repertoire of HLA, and therefore HLA-mismatched donors are frequently selected. In these situations, criteria to select the “best” mismatched donor were poorly defined. The aim of this thesis was to develop a predictive method for permissible HLA mismatches. The constructed Predicted Indirectly ReCognizable HLA Epitope (PIRCHE) model is a computational method that predicts the probability of indirect T-cell recognition for all potential HLA-mismatch combinations.

To determine whether PIRCHE can aid in defining permissible HLA mismatches, a large cohort of patients transplanted with a single HLA-mismatched (9/10) adult unrelated donor (URD) was studied (**Chapter 3**). The patients were divided in different PIRCHE groups and the clinical outcome of these groups was compared to HLA-matched (10/10) HCT. Patients in the lowest tertiles of PIRCHE-I and -II had comparable clinical outcome as patients transplanted with an HLA-matched URD. Patients with higher PIRCHE-I or -II numbers had significantly impaired overall survival (OS) compared to 10/10-matched transplantations. These higher PIRCHE-I or -II HLA-mismatches were designated as non-permissible mismatches, and the mismatches resulting in low PIRCHE-I and -II were designated permissible. Non-permissible PIRCHE mismatches were associated with an increased probability of acute graft-versus-host disease (GVHD) and non-relapse mortality (NRM), whereas permissible PIRCHE mismatches were not associated to GVHD and NRM. The reduced toxicity rate of permissible PIRCHE mismatches was not counterbalanced by an increased probability of relapse. This chapter indicates that HLA-mismatched URD selection can be improved by selecting HLA mismatches resulting in low PIRCHE-I and -II.

The effect of HLA-DPB1 derived PIRCHE was compared to HLA-DPB1 mismatches designated as (non-)permissive by a classical direct recognition model, the T-cell epitope model (**Chapter 4**). In 10/10-matched, HLA-DPB1-mismatched URD-HCT, the presence of PIRCHE-I and -II led to an increased acute GVHD risk. The presence of HLA-DPB1 derived PIRCHE-I differentiates high and low risk individuals within the group of mismatches that was identified as permissive by the direct recognition model. PIRCHE were not significantly associated with other clinical outcomes in this cohort. Based on the reduced GVHD risk, these data again advocate selection of URDs with low PIRCHE-I and -II.

PIRCHE may explain the unresolved issue of immunogenic antigenic HLA-C mismatches (**Chapter 5**). HLA-C is often the mismatched locus in 9/10 URD-HCT, due to current donor typing procedures. A number of studies have shown that HLA-C antigenic mismatches are correlated to increased GVHD risks, whereas HLA-C allelic mismatches are not. In a small study group, the numbers of PIRCHE that could be presented from an HLA-C antigenic mismatch were significantly higher than those from an allelic mismatch. A theoretical analysis showed that for some patients transplanted with an HLA-C mismatch, an HLA-B mismatched donor may be a very valid alternative, as the latter mismatches can lead to a lower number of PIRCHE compared to HLA-C mismatches. These data suggest that the PIRCHE model may aid in the identification of the locus to mismatch for during selection of HLA-mismatched donors.

Cord blood (CB) derived hematopoietic cells serve as an alternative cell source for allo-HCT. CB appears to be more tolerant for HLA mismatches, resulting in reduced GVHD risks compared to URD-HCT, and CB may have an improved graft-versus-tumor (GVT) effect. To

study the effect of PIRCHE in CB-HCT, a cohort of pediatric patients transplanted with unrelated CB for various diseases was analyzed (**Chapter 6**). In this group, PIRCHE-I had a specific anti-leukemic effect: patients receiving CB-HCT had a highly reduced relapse risk when the numbers of PIRCHE-I were high. PIRCHE numbers were not associated to other clinical outcomes, indicating that for CB-HCT in case of a malignancy, selecting donors based on high numbers of PIRCHE-I may improve the anti-tumor potential.

One unit of CB does not contain enough cells for heavier patients, and older children and adults are therefore frequently transplanted with two units of CB. The effect of PIRCHE on such double unit CB-HCT was studied in two independent cohorts (**Chapter 7**). This study showed not only a confirmation of the GVT effect of high PIRCHE-I, but also a trend for high PIRCHE-II being associated to an increased chronic GVHD risk. This indicates that GVT and GVHD effects in these patients can potentially be dissected based upon PIRCHE; it seems preferable to select donors with high PIRCHE-I and low PIRCHE-II for patients with a malignancy. Indeed, in a large cohort of patients transplanted for a hematological malignancy with two units of CB, patients with higher PIRCHE-I and low PIRCHE-II had better OS compared to patients with low PIRCHE-I and higher PIRCHE-II.

The PIRCHE model is based upon the concept of donor T-cell recognition of patient mismatched HLA. To study the dependence of the PIRCHE effect on the presence of these donor T cells, the relationship between chimeric status and PIRCHE-induced effects was analyzed (**Chapter 8**). The correlation between HLA-DPB1 derived PIRCHE and acute GVHD after adult URD-HCT, was only present in patients that had reached complete donor T-cell chimerism in their peripheral blood, whereas the correlation between PIRCHE and GVHD was not found in those with mixed donor-patient chimerism. The results from this study indicate that complete donor engraftment is essential for PIRCHE-induced T-cell activation.

The presentation of T-cell epitopes such as PIRCHE may lead to T-cell infiltration in the skin during GVHD. In line with this, pediatric patients with PIRCHE-I or -II had higher numbers of T cells in their cutaneous acute GVHD infiltrates than patients without PIRCHE (**Chapter 9**). The numbers of CD4+ and CD8+ T cells in skin tissue sections were furthermore correlated to other donor characteristics: after CB-HCT, patients had more CD4+ T cells in their infiltrates; after a sex-mismatched HCT, CD4+ T cells dominated the infiltrate; and lastly, the combination of a sex- and PIRCHE-mismatch resulted in the highest number of T cells in infiltrates. This chapter therefore suggests that a higher number of epitopes, *i.e.* PIRCHE and minor histocompatibility antigens, attracts more T cells to the skin during acute GVHD.

In summary, the PIRCHE model was investigated in a number of different HCT strategies. For patients transplanted with hematopoietic cells harvested from the bone marrow or peripheral blood of adult URDs, low numbers of PIRCHE-I and -II consistently led to reduced alloreactivity risks compared to higher numbers of PIRCHE (Chapter 3-5). Patients transplanted with CB, had significantly improved GVT responses when the number of PIRCHE-I was high (Chapter 6-7). Finally, the presence of donor cells in the peripheral blood of patients after allo-HCT was required for the PIRCHE effect (Chapter 8) and patients with PIRCHE had higher numbers of T cells in their cutaneous acute GVHD infiltrates (Chapter 9). In conclusion, PIRCHE were clearly correlated to clinical alloreactivity after different types of HLA-mismatched HCT.

General Discussion

This thesis describes a potential novel method to identify permissible HLA mismatches, that can be used in donor-selection procedures for allogeneic hematopoietic cell transplantation (allo-HCT). The method described in this thesis is based upon the concept of indirect T-cell recognition of mismatched HLA: the process whereby the donor T cell recognizes polymorphic peptides derived from the mismatched HLA. The developed Predicted Indirectly ReCognizable HLA Epitope (PIRCHE) model was investigated in both adult unrelated donor (URD) HCT as well as cord blood (CB) HCT. PIRCHE had distinct effects in different HCT settings: in URD-HCT, low numbers of PIRCHE-I and -II were correlated to reduced graft-versus-host disease (GVHD) rates and subsequently to reduced (transplant-related) mortality risks, without an accompanying increased relapse risk (Chapter 3-5). After CB-HCT, however, high numbers of PIRCHE-I led to increased anti-tumor responses, whereas low numbers of PIRCHE-II were potentially associated to reduced chronic GVHD risks (Chapter 6, 7). Potential explanations for these distinct PIRCHE effects related to the cell source will be discussed below. In addition, the implications of these distinct effects on donor-selection will be discussed and last, the future directions of PIRCHE will be addressed.

Differential effect of PIRCHE in different HCT settings

The different PIRCHE effects in the two HCT settings may be explained by the distinctive compositions of either adult or CB HC. First of all, CB is, from an immunological point of view, very naïve, as the immune system has not yet been exposed to antigens. Despite this naivety, CB cells have excellent antiviral capacity (1), indicating that there is potential to induce specific immune responses. Next to the naivety of the CB graft, CBs are also well known for their capability of regulatory-T cell (Treg) induction upon immune activation (2), which is likely the result of the required tolerant state during pregnancy (3). Furthermore, T cells of adults and fetuses exhibit distinct gene-expression profiles (4), indicating that adult and CB T cells have different developmental potential. Last, T-cell reconstitution differs after adult URD- versus CB-HCT. After adult URD-HCT the early reconstitution phase is dominated by CD8+ T cells, whereas after CB-HCT there is a marked early increase in CD4+ T cells, despite the fact that in general CB-derived CD4+ and CD8+ T cells regenerate faster (1). Thus, the naivety of the graft, the specific induction of Tregs, the distinct gene-expression profiles and the difference in reconstituting potential may all partly explain the differences in PIRCHE effects observed after adult URD- and CB-HCT. For all these factors, we have found some indications of their correlation with cell source-related PIRCHE effects.

First, the naivety of CB as compared to the antigen-experienced adult URDs, may lead to a different probability of direct recognition of mismatched HLA. Direct recognition refers to the process whereby the donor T cell recognizes the intact mismatched-HLA protein on the cell surface presenting a certain peptide, as foreign. Directly recognizing memory-T cells have been studied extensively, resulting in some established knowledge (Chapter 2, and (5-7)): many virus-specific T cells show cross-reactivity towards allogeneic HLA; some viruses induce public T-cell responses (*i.e.* similar T-cell repertoires dominating the immune responses of multiple individuals); and some of these public responses show predictable HLA cross-reactivity. Allo-HCT with cells from antigen-experienced adult donors, will presumably result in rapid T-cell activation through direct recognition by virus-specific memory-T cells. This activation of directly recognizing T cells may cause a pro-inflammatory condition, that potentiates indirectly recognizing T-cell responses. Thus, as the chance of direct recognition in URD-HCT is higher than after the naïve setting of CB-HCT, the directly recognizing T cells

may be involved in the development of PIRCHE-specific responses after URD-HCT, whereas this co-stimulatory activation of directly recognizing T cells may not be present in CB-HCT, resulting in a lower probability of PIRCHE-induced acute GVHD development in the latter setting.

Secondly, the absence of PIRCHE-induced acute GVHD development after CB-HCT might be the result of dampening of the T-cell responses by (antigen-specific) Tregs. Indeed, we have observed that patients with high PIRCHE-II had more Tregs (CD4+FoxP3+) early after CB-HCT compared to patients with lower PIRCHE-II (unpublished observations). This potential relation between Treg and PIRCHE numbers may suggest that transplantation with CB recognizing many PIRCHE, leads to an induction of Tregs, potentially suppressing the activation of T cells that could otherwise have driven acute GVHD development. As after CB-HCT, PIRCHE-I clearly have anti-tumor potential, this Treg suppression likely takes place locally. Identifying PIRCHE-specific T cells after different types of HCT could help elucidate the phenotypes and local effects of PIRCHE-activated T cells.

Last, the fact that the early immune reconstituting phase after adult URD-HCT is dominated by CD8+ T cells, is in agreement with the clear effects of PIRCHE-I on acute GVHD in adult URD-HCT; whereas the domination of CD4+ T cells after CB-HCT, may explain the effect of PIRCHE-II on chronic GVHD after CB-HCT. Moreover, we have identified more CD4+ T cells in cutaneous GVHD infiltrates of patients after CB-HCT compared to URD-HCT (Chapter 9), in line with the CD4+ dominated T-cell reconstitution after CB-HCT as well. The cause of this differential reconstitution is not established, but it is possibly related to the different ontological qualities of URD and CB T cells (1). Thus, although the reasons for the differential T-cell subset reconstitution after different types of HCT are not known, they do relate to our findings of the effects of PIRCHE-I and -II on outcomes after different types of allo-HCT.

PIRCHE and tumor-specificity

The distinctive PIRCHE anti-tumor effect in relation to the HC source, *i.e.* PIRCHE-I association with a GVT effect after CB-HCT only, is in accordance with results regarding the effects of HLA mismatches on HCT outcomes. In adult URD-HCT, HLA mismatches are often not associated to relapse risk (8, 9), whereas it is well established that after CB-HCT, HLA mismatches induce anti-tumor responses (10-13). In addition, it has been suggested that the general anti-tumor effect of allo-HCT is less pronounced after adult URD-HCT than after CB-HCT (14), potentially because CB-HCT considers a higher level of HLA mismatching than URD-HCT. The variation between anti-tumor potential of HLA mismatches in different cell sources is poorly understood, but PIRCHE may provide a first step forward in understanding this aspect.

The GVT potential of PIRCHE in CB-HCT was restricted to PIRCHE-I (Chapter 6, 7), indicating that CD8+ T cells recognize epitopes presented on HLA class I by the tumor cells. Although HLA class I is expressed on virtually all nucleated cells (15), in CB-HCT, PIRCHE-I were not associated to GVHD. On the other hand, HLA class II is mainly expressed on hematological (tumor) cells (15), as these cells function as professional antigen presenting cells. One may therefore expect that HLA class II would be specifically involved in the anti-leukemia responses during HLA-mismatched HCT: this involvement could be demonstrated through anti-tumor responses via either direct or indirect recognition of HLA class-II mismatches. Indeed, after double CB-HCT, the number of HLA class-II mismatches was associated to a reduced relapse risk, whereas the number of HLA class-I mismatches was not (Chapter 7).

In addition to HLA class-II match grade, PIRCHE-I were independently associated to reduced relapse risk as well. These data suggest that PIRCHE-I derived from HLA class-II mismatches result in tumor-specific reactivity of CD8+ T cells. In this context, HLA class-II derived PIRCHE-I potentially serve as semi-hematopoietic restricted minor histocompatibility (H) antigens.

The indication of a correlation between HLA class-II derived PIRCHE-I and relapse, may partly explain the absence of tumor-specificity of PIRCHE-I in URD-HCT: in the investigated single HLA-mismatched transplants, the majority (76%) of cases was matched for HLA class II (Chapter 3). When stratifying according to HLA class-I or -II mismatches in this cohort, again no correlation between high PIRCHE-I and less relapse was found (unpublished observation), indicating that HLA class-II derived PIRCHE-I were not associated to an anti-tumor effect. Since patient numbers in this group were rather low, no permanent conclusions can be drawn yet. Similarly, the presence of PIRCHE-I was not significantly associated with relapse in HLA-DPB1 mismatched HCT (Chapter 4). The latter study did, however, show a trend for a reduced relapse-related mortality rate in case of above 2 HLA-DPB1-derived PIRCHE-I (unpublished observation), indicating that the potential anti-tumor effect of HLA class-II derived PIRCHE-I requires a larger quantity of epitopes. Such a dose-response threshold remains to be confirmed. If indeed high numbers of HLA class-II derived PIRCHE-I are required to induce an anti-tumor effect, this may explain why the anti-tumor potential was only observed in the setting of many mismatches such as CB-HCT, which resulted in much higher PIRCHE numbers when compared to URD-HCT (in double CB-HCT, median PIRCHE-I: 8, range 0 to 48, Chapter 7; in URD-HCT, median PIRCHE-I: 3, range 0 to 17, Chapter 3). In summary, the absence of a significant correlation between PIRCHE-I and relapse in URD-HCT may be due to low numbers of HLA class-II mismatched transplantations, a reduced number of epitopes in case of little HLA mismatches and/or, as stated previously, intrinsic differences between URD and CB grafts.

Myeloid and lymphoblastic leukemias are not equally sensitive to immunotherapy. For example, the graft-versus-leukemia effect of donor lymphocyte infusions (DLI) does lead to durable remission in patients with acute myeloid leukemia (AML) (16, 17), whereas treating relapse after acute lymphoblastic leukemia (ALL) with DLI has never been more successful than chemotherapeutics (17-20). Potentially there is a difference in HLA-expression levels between myeloid and lymphoid blasts, that explains this difference in susceptibility to immunotherapy. AML is clearly sensitive of HLA recognition, as the AML blasts of patients with a relapse after HLA-mismatched allo-HCT often display HLA class-I down regulation (21). In line with this, we have observed that in patients with AML the effect of PIRCHE-I on relapse after CB-HCT was more profound than in patients with ALL (unpublished observation, ongoing study). The differential effect of PIRCHE-I on AML as compared to ALL may indicate that CB selection with high PIRCHE-I does not lead to GVT effects in ALL, potentially due to the different HLA-expression levels of the different leukemias. These results definitely warrant further investigation and may lead to individualized PIRCHE-based donor selection, as the level of HLA expression of the leukemia may determine the susceptibility to immunotherapy.

PIRCHE and donor selection

The interpretation and application of PIRCHE predictions in donor-selection depends on donor source, indication for the allo-HCT and conditioning regimen. As mentioned

previously, the developed PIRCHE scoring system predicts permissible mismatches leading to reduced GVHD risks for adult URD-HCT, but predicts increased GVT effects after CB-HCT. These observations advocate the selection of donors with low PIRCHE-I and -II in case of URD-HCT, and high PIRCHE-I in case of CB-HCT for a malignancy. In any instance, for patients with non-malignant diseases, one should theoretically select the donor with the lowest alloreactive risk and thus low numbers of PIRCHE, although cohort studies verifying the advantage of low PIRCHE in non-malignant diseases have yet to be performed.

Next to donor source and underlying disease, conditioning regimens play an important role in complications after HCT, and should potentially be accounted for when including PIRCHE in the selection criteria. For example, MA conditioning regimens are associated to reduced relapse rates compared to NMA conditioning regimens, whereas the latter regimens are associated with reduced GVHD rates (22). In the first setting one may preferentially want to select donors that reduce the GVHD risk, and thus donors with low PIRCHE. In patients with a high-risk malignancy due to a poor clinical status conditioned with a NMA regimen, one may want to aim for improved anti-tumor reactivity and thus use CB with high numbers of PIRCHE-I. The current data did not allow concise studies on the role of PIRCHE in different conditioning regimens, as the numbers of patients in different subgroups of conditioning regimens were rather low. Elucidating the exact role of conditioning regimens on PIRCHE effects therefore requires larger cohort studies.

Theoretically, PIRCHE-induced alloreactivity is the result of activated donor T cells. As donor T cells should be a prerequisite for the PIRCHE effect, one may assume an absent or reduced PIRCHE effect in case of T-cell depletion or delayed T-cell reconstitution, for example in conditioning regimens containing anti-thymocyte globulin (ATG). Indeed, the effects of PIRCHE seemed most profound in conditioning regimens without ATG (Chapter 7). In addition, the effect of PIRCHE after URD-HCT was only present in case of complete donor T-cell chimerism (Chapter 8). Although these chapters both suggest a relationship between donor T-cell presence and PIRCHE effects, data on the exact timing of donor T-cell reconstitution was not available. Current studies are ongoing to determine more precisely the link between donor T-cell reconstitution and PIRCHE responses. Despite the lack of such detailed studies, the current studies include patients that have received ATG and display an effect of PIRCHE, therefore the data advocate to incorporate PIRCHE-selection criteria in case of ATG administration as well.

For patients for whom an HLA-matched URD is not available, clinicians face the difficult choice between a single HLA-mismatched URD or (double) CB-HCT. Ideally, in these cases, PIRCHE criteria would facilitate the selection of either URD cells or CB units. However, current data are not sufficient to study whether a 9/10-URD with low PIRCHE, to reduce the toxicity, is preferred over a CB with high PIRCHE-I, that increases the anti-tumor potential. When roughly comparing the analyzed low and high PIRCHE 9/10-URD- and double CB-HCT with 10/10-URD-HCT, only the 9/10 high PIRCHE group seems to have impaired overall survival (data not shown). However, these groups are poorly comparable with each other, as due to the retrospective nature of the studies, the 9/10-URD transplantations differ largely from the CB transplantations, a consequence of local HCT procedures and previous study inclusion criteria. Thus, very large cohorts are required to properly study the difference in clinical outcomes of all these potential donors. This question can potentially only be answered by randomized controlled trials that determine which patient is transplanted with a 9/10-URD and which with a (double) CB. Whether such randomized controlled trials are

considered feasible and ethical, remains to be debated.

PIRCHE read out

The precise cut-off value of PIRCHE above or below which the risk of complications will be reduced, was generally not externally validated, apart from Chapter 7, where two separate cohorts were included and the application of an actual number of PIRCHE as a cut-off value was studied. This thesis thus mostly describes whether patients with higher numbers or presence of PIRCHE have increased probabilities of alloreactivity, in a proof-of-principle setting. To study a correlation between higher numbers of PIRCHE and clinical outcomes, arbitrary cut-offs were defined by dividing the patients into two or three groups; either based on equal group sizes (Chapter 3, 6, 7) or on potential biologically relevant cut-offs (Chapter 4, 5). Alternatively, PIRCHE could have been studied as a continuous number, although analyzing PIRCHE as a continuous variable is questionable, as PIRCHE are not normally distributed. Moreover, the correlation between PIRCHE and clinical outcome is likely non-linear. PIRCHE numbers represent a likelihood of T-cell recognition, as in theory each PIRCHE can be recognized by a different T-cell clone, leading to the assumption that more PIRCHE increase the chance of T-cell recognition (Figure 1A). However, one PIRCHE can be sufficient for T-cell activation, parallel to minor H antigen mismatches (23). Once a threshold of a certain number of PIRCHE is reached, increasing the number of PIRCHE above this threshold may not lead to increased risks of alloreactivity, as there is probably a plateau for the probability of T-cell recognition upon a large number of presented epitopes. The effect of higher numbers of PIRCHE on T-cell activation should therefore be carefully modeled, ultimately resulting in precise cut-off values applicable in donor selection.

Ideally the number of PIRCHE is modeled against clinical outcome to study the optimal relevant cut-off values. The probability of T-cell recognition in itself may not be the optimal read out for clinical alloreactivity, as the effect of T-cell activation due to PIRCHE may be very different in the various transplant settings, as was described above. Thus, this thesis gives rise to the idea of an increasing number of PIRCHE being associated to clinical outcome, but the actual number of PIRCHE that leads to alloreactivity needs to be further studied.

Future perspectives

PIRCHE can identify permissible HLA mismatches, as exemplified by the observation that after URD-HCT, low PIRCHE have similar clinical outcome as HLA-compatible transplantations. For CB-HCT, low numbers of PIRCHE-II potentially result in reduced chronic GVHD risks and thus PIRCHE seem to predict permissible mismatches in these transplantations as well. To successfully integrate PIRCHE as a donor-selection criterion, permissible PIRCHE mismatches should be available for every patient. We therefore studied whether we could find a CB unit with low PIRCHE-II for patients that had previously been transplanted with an HLA-mismatched donor in our center. For 86% of these patients, we could identify a permissible PIRCHE mismatch within the top ten of CB units that were considered acceptable based on other criteria (cell dose, HLA match grade; data not shown). A similar study is not yet performed for URDs. Strategies should be developed to generate acceptable PIRCHE mismatches for every patient. Increasing the number of potential donors is one strategy that can lead to this goal. Another strategy would be to improve the model by further classifying the non-permissible mismatches, as not all mismatches resulting in PIRCHE will be equally alloreactive. There are a number of options to achieve improvement of the

PIRCHE model. First of all, the immunogenicity of the various PIRCHE will differ. The current model does not discriminate between high and low immunogenic PIRCHE. Secondly, the actual number of PIRCHE may be better predicted. Furthermore, *in vitro* proof-of-principle by identifying PIRCHE-specific T cells and their phenotypic characteristics would potentially enable understanding the selective GVHD and GVT effects and hence allow further refinement of the predictions. All these aspects are discussed below and may be studied for their possibility of expanding the PIRCHE-acceptable donor pool.

Identifying highly immunogenic PIRCHE

Determination of the immunogenic level of PIRCHE may aid in PIRCHE-based donor selection, as it might provide a possibility to classify HLA-mismatches with identical PIRCHE numbers into low and high alloreactive mismatches. Immunogenicity is the ability of an antigen to induce an immune response (24). A highly immunogenic PIRCHE has a higher probability of T-cell recognition, whereas of less immunogenic PIRCHE many more are needed to induce T-cell activation (Figure 1B). Knowledge on the immunogenicity level of PIRCHE may increase our understanding of the effect of the absolute number of PIRCHE on clinical outcome.

In general, the immunogenicity of an antigen is influenced by multiple factors: the ability to be processed and presented; the presence of a recognizing T cell; the abundance of the epitope; and the interaction between the T-cell receptor (TCR) and the peptide:HLA (pHLA) complex (25). Of these factors, the binding of the peptide to HLA is the most selective event (25), and has therefore received most attention in our current model. The TCR repertoire of the donor is not known prior to HCT, and can therefore not be used to predict immunogenicity in this context. How the other factors could result in prediction of the immunogenicity of PIRCHE is discussed below.

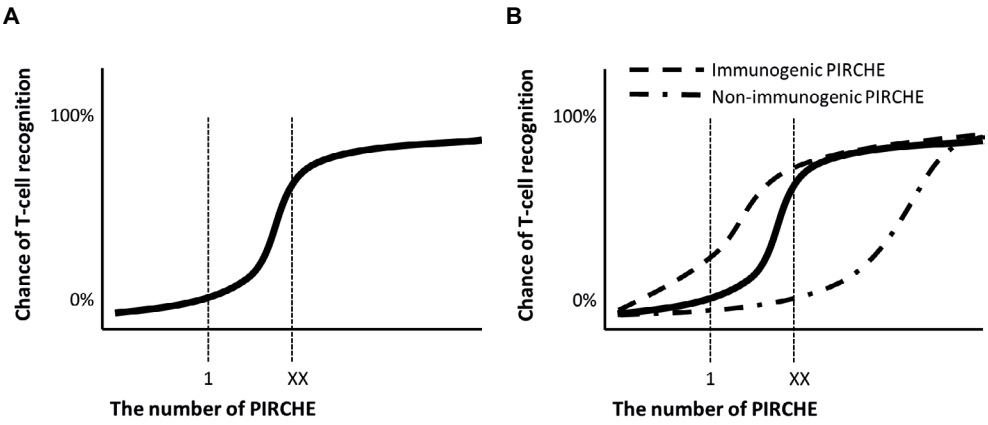


Figure 1 (A) The hypothetical effect of the number of PIRCHE on T-cell recognition. PIRCHE are a method to predict the probability of T-cell recognition. With 1 PIRCHE, there is a (small) possibility that the donor T-cell repertoire will contain a T-cell receptor (TCR) that can recognize that PIRCHE. With increasing numbers of PIRCHE, the chance of such a TCR will increase. At a certain number (XX) of PIRCHE, a plateau will be reached whereby one PIRCHE extra does not really add to an increase in the chance of T-cell recognition. The probability of T-cell recognition may be a surrogate marker of clinical outcome, although the effect of T-cell activation on clinical alloreactivity may be different depending in other transplant and patient characteristics, as was described in the text. **(B) The effect of the number of PIRCHE differs depending on the immunogenicity of each PIRCHE.** Immunogenic PIRCHE may lead to a lower threshold for T-cell recognition, in contrast to non-immunogenic PIRCHE which may need very high numbers before the T cells will be activated.

Peptide processing
The proteasome is responsible for cleavage of proteins into peptides. There are two types of proteasomes: the constitutive proteasome, expressed in most cells, and the immunoproteasome, that can be induced upon inflammation via IFNγ and is the constitutive form in dendritic cells (26). The immunoproteasome has altered cleavage activity than the conventional proteasome (reviewed in (27)). Upon the inflammatory situation after HCT, the immune activation may lead to preferential induction of the immunoproteasome, generating specific cleavage products, thereby resulting in the presentation of specific PIRCHE. Incorporation of immunoproteasomal cleavage predictions may hence offer an opportunity of predicting highly immunogenic PIRCHE. Immunoproteasomeal cleavage products can be predicted (26), although the correlation with actual T-cell epitopes was not better when comparing immunoproteasomal cleavage with the overall (constitutive and immuno-) proteasomal cleavage predictions that are currently applied in our model (26). Thus, the incorporation of these immunoproteasomal predictions specifically, does likely not contribute to the prediction of highly immunogenic PIRCHE.

Abundance of the epitope: HLA expression
The expression level of a given PIRCHE-HLA complex potentially contributes to the immunogenicity, as the chance of T-cell interaction is increased upon higher expression levels. HLA-C, -DRB3, 4, 5, -DQ, and -DP proteins are lower expressed than HLA-A, -B, and -DRB1 proteins (28). This may on the one hand lead to less T-cell exposure for PIRCHE presented by these lower expressed alleles, but on the other hand also to a lower probability of processing into peptides derived from these low expressed proteins. Nonetheless, this thesis describes two cohorts (Chapters 4 and 5) whereby PIRCHE derived from HLA-C and -DPB1 were correlated to acute GVHD development. Thus, PIRCHE derived from low expressed alleles do correlate to alloreactivity. Potentially, the expression levels of the presenting HLA proteins are more important. We have performed some preliminary analyses differentiating the predictive capacity of PIRCHE-I in- and excluding HLA-C presentation, where we did not find clear differences (data not shown). Ideally, studies analyzing the effect of HLA-C presentation, as well as presentation by other loci, should incorporate actual HLA-C expression levels. The expression levels of HLA-C alleles differ, and it was recently shown that the expression level of HLA-C mismatches correlates to alloreactivity (29). Thus, theoretically the expression levels of PIRCHE-HLA complexes may contribute to the immunogenicity, however, this contribution is likely little as we did not find an indication for a correlation between expression levels and outcome yet, in small cohort studies.

Abundance of the epitope: pHLA stability
Currently, a PIRCHE is regarded immunogenic when the peptide can be processed and presented by shared HLA. Binders are selected based upon the predicted IC_{50} value being below a general value as a measure of affinity (500 nM for HLA class-I presentation and 1000 nM for HLA class-II presentation), irrespective of the presenting allele. The downside of using IC_{50} values is that the measurement of these values is based upon competition assays: these assays measure the 50% maximal inhibitory concentration (IC) of the peptide of interest in competition with a standard reference peptide for the presenting HLA allele. The height of the IC_{50} value is thus dependent on the stability of the reference peptide

in the peptide-binding groove, and therefore IC_{50} values from different alleles are not easily comparable with each other. Furthermore, IC_{50} values have allele-specific thresholds (30), which have been established for a limited number of alleles (31). Ideally, one would determine the relevant IC_{50} threshold for every allele (30). Determining these cut-offs to select relevant binders requires extensive investigation, but may improve the specificity of the PIRCHE model.

The stability of a pHLA complex may better predict the probability of T-cell recognition than binding affinity as measured via IC_{50} values. The well-known HA-1 minor H antigen regards two allelic variants, both leading to peptides that bind to HLA-A*02:01 with an IC_{50} value well below 500 nM: the immunogenic HA-1^{H} has an IC_{50} of 30 nM whereas it's non-immunogenic HA-1^{R} counterpart has an IC_{50} of 365 nM (32). Both would be considered binders in the current PIRCHE strategy, however, the non-immunogenic HA-1^{R} was not eluted during peptide elution studies (32). The proposed mechanism for HA-1 immunogenicity is the difference in on/off rate between the two allelic counterparts: the HA-1^{H}-HLA-A2 complex is more stable than the HA-1^{R}-HLA-A2 complex (33). Hence, the stability of pHLA complexes seems an important predictor of immunogenicity. This pHLA stability could be incorporated in the model by using the HLA peptide-binding predictions of the Bioinformatics and Molecular Analysis Section (BIMAS) (34). The BIMAS approach is, however, hampered by the limited number of predictions for HLA class-I alleles and the absence of HLA class-II alleles. Therefore, currently, IC_{50} values offer the best opportunity of predicting the abundance of PIRCHE for every presenting HLA allele.

TCR interaction

The PIRCHE designation of being a potential T-cell epitope can be refined. The current strategy to identify PIRCHE is to compare linear sequences of presented peptides and to designate a peptide as a PIRCHE when there is one amino acid difference between mismatched-HLA derived peptides and self peptides. Although the exact mechanisms of how a TCR recognizes a foreign peptide are unknown, activation of T cells is (partly) dependent on the stability of the binding of the TCR to the pHLA complex (35). This stability is, from the peptide's perspective, mainly determined by a limited number of amino acid positions of the peptide and the physicochemical properties of these specific residues, as described below.

The amino acid residues that are most exposed to the TCR differ for the various HLA class-I (37, 38), as well as class-II (39-42) alleles. In HLA class-I presentation, the center of the peptide is generally more exposed to TCR interaction than the flanking residues (36). However, it has been shown that in a given TCR interaction with an HLA-A*02:01-presented peptide, position 8 was most important (37); whereas for another TCR interaction with an HLA-B*51:01-presented peptide, positions 3-8 were critical (38). For MHC class-II presentation it is commonly accepted that position 5 is most important for TCR recognition, not allowing changes in this residue (39-41). However, the more peripheral positions 2 and 8 of two common diabetes epitopes presented by HLA-DR are very important for TCR recognition (42). Thus, as the critical TCR residues differ, it may be worthwhile to exactly elucidate which residues are T-cell exposed for every presenting HLA allele. Polymorphisms on these critical TCR-exposed residues may lead to a higher probability of activating T cells than less exposed residues, and thus to more immunogenic PIRCHE. A straightforward strategy to investigate the effect of polymorphisms in T-cell exposed residues, is to only analyze amino

acid polymorphisms for positions outside the HLA-anchor positions; this strategy is currently studied by our group. Nevertheless, multiple studies have shown that substitutions in predicted HLA-anchor positions that are buried from the TCR, can lead to lack of TCR binding as well (41, 43). Properly improving the predictions by weighting the most important positions is thus challenging.

Next, not only the position but also the properties of the polymorphic amino acids in the TCR-exposed residues may impact T-cell recognition, as binding of the TCR to the pHLA complex is partly determined by the characteristics of the peptide. For example, the above described TCR that interacted with a peptide presented by HLA-A*02:01, does not allow charged amino-acids in the center of the peptide (37). Moreover, in general, T cells are proposed to have a preference for large and aromatic residues (44). One may therefore assume that amino acid substitutions resulting in changes of charge or size, have a greater impact on T-cell interaction than amino acid polymorphisms that have similar properties as the amino acid they substitute. PIRCHE could therefore be scored based on the properties of the polymorphisms instead of on any polymorphism. Incorporating all these different potential properties of amino acid substitutions and weighting them accordingly, also with respect to their position in the peptide, requires extensive modelling. Recently, a "simple" immunogenicity score has been developed for HLA class-I presented peptides, based on the presence of aromatic and larger residues on positions 4 to 6 of the peptide (44). A first step in testing the relevance of scoring PIRCHE not based on any polymorphic residue but based on the characteristics of these polymorphisms, could be incorporating this immunogenicity score. However, analyzing the effects of different immunogenic levels of PIRCHE next to the effect of PIRCHE numbers, requires larger study cohorts than those described in this thesis.

In summary, some PIRCHE will likely be more immunogenic than others. Being able to classify immunogenicity of PIRCHE may enhance the applicability of the model. We have described a number of possibilities that could allow for the prediction of highly immunogenic PIRCHE. Immunogenicity may well be predicted by scoring pHLA stability and identifying the (properties of) TCR-exposed residues, whereas incorporating immunoproteasomal cleavage predictions and HLA expression levels probably contributes less. When pHLA stability and the immunogenicity score of TCR-exposed residues will become available for every presenting HLA allele, predicting the immunogenicity level of PIRCHE may be achievable.

Refinement of the PIRCHE pool

The accuracy of the predicted PIRCHE pool can possibly be augmented. Currently, some HLA mismatches may have been wrongly defined as resulting in high, low or no PIRCHE. Improving the accuracy of the number of PIRCHE, could increase the model's predictive capacity of clinical outcome even further. Multiple potential refinements of the PIRCHE predictions will be discussed below.

HLA class-II presentation

Current PIRCHE-II predictions only include HLA-DRB1 presentation, while HLA-DQ and -DP might play a role as presenting molecules in indirect recognition of HLA mismatches as well. When the PIRCHE model was constructed, binding predictions were not available for the majority of HLA-DQ and -DP proteins (45), and could therefore not be incorporated.

Only recently HLA class-II binding predictors for all possible HLA-DQ and -DP proteins have become available (46). Further, predicting the complete HLA class II-presented peptide repertoire is challenging in the daily practice of donor selection, due to incomplete HLA typing: despite the fact that the HLA class II peptide-binding groove is formed by both an α- and β-chain, in general, only typing for HLA-DRB1 and -DQB1 is performed prior to HCT. For HLA-DRB1, binding algorithms are nevertheless applicable, since the few different HLA-DRα chains all lead to a similar peptide-binding groove. The HLA-DQ and -DP proteins, however, consist of both a polymorphic α- and β-chain, leading to the potential expression of four different heterodimers of each protein on the cell surface. Consequently, HLA typing of both chains is required to properly predict peptide presentation by HLA-DQ and -DP. As binding to HLA-DQ and -DP is now predictable, typing the HLA-DQ and -DP α-chains can expand the PIRCHE-II pool. Nonetheless, HLA-DQ and -DP are expressed on a much lower level than HLA-DRB1 (as described above), and therefore, expanding the PIRCHE-II predictions to HLA-DQ and -DP presentation does likely not impact the results a lot.

Peptide lengths
Including different peptide lengths may lead to successful expansion of the PIRCHE library, especially in the case of HLA class-I presentation. HLA class-I proteins are not restricted to presenting 9-mers, although only these lengths have currently been studied in the PIRCHE model. The PIRCHE-I immunogenicity predictions become harder when implementing different peptide lengths, as longer peptides have a different conformation in the HLA class-I binding groove than shorter peptides (38, 47, 48). This different conformation leads to a change in the positions that are most TCR exposed. Thus, when incorporating different peptide lengths, the effect of these different lengths on peptide conformation and subsequently TCR interaction should be accounted for. Although the currently incorporated peptide length of 9 amino acids for PIRCHE-I is the most abundant length, other peptide lengths do play a significant role in immunity as well (49). Thus, the ability of predicting all potential peptide lengths, likely leads to more precise PIRCHE predictions.

HLA restriction
The HLA-restriction of TCRs is probably not as strict as assumed in the current PIRCHE model. Currently, PIRCHE are only counted when they are presented by shared HLA alleles, as the donor T cells should be positively selected in the thymus based on interaction with these HLA alleles. However, TCRs can interact with different presenting HLA alleles that have identical critical TCR-binding residues, flanking the peptide-binding groove (48, 50). The effect of binding of PIRCHE to mismatched HLA proteins with identical TCR-binding positions, should be analyzed. Including presentation of PIRCHE by HLA alleles with identical TCR-binding residues, probably only broadens the repertoire in case of allelic mismatches, as in antigenic mismatches the sites flanking the peptide-binding groove are generally also polymorphic.

Input for the PIRCHE model
For PIRCHE predictions, inclusion of the entire amino acid sequence of an HLA protein (*i.e.* from exon 1 to 6 or 8, depending on the locus) is important. However, a large number of HLA alleles in the IMGT database contain only the exon 2-3 sequence, as that is the minimal requirement for inclusion in this database. Ignoring the remainder of the sequence in our

method would generate much more PIRCHE for completely available patient alleles than in our current strategy, as all peptides derived from outside exon 2 and 3 will be regarded PIRCHE. In our studies, we have therefore either sequenced the unknown exons or extrapolated the incomplete sequences, based on a nearest neighbor principle, apart from HLA-DPB1, where extrapolation was not possible with the available sequences. Moreover, locally, we are sequencing every allele completely, including all exons, for every patient and donor. The currently sequenced exons were identical to our predicted sequences, indicating that the extrapolation was successful thus far (data not shown). Meanwhile, we are in the process of uploading the newly sequenced exons and alleles to the IMGT database.

Non-inherited maternal antigens

Implementing non-inherited maternal antigens (NIMA) into PIRCHE predictions may enhance the model with respect to CB-HCT. NIMA play an important role in tolerance after CB-HCT: when CB cells are used for an HLA-mismatched but NIMA-matched patient, patients have an increased probability of survival when compared to transplantation with an HLA and NIMA mismatch (51). This NIMA tolerance is the consequence of the pregnancy, where a fetus is exposed to a non-inherited maternal HLA haplotype. The NIMA tolerance is required to prevent immune responses of the child towards the mother. As the CB is tolerant against the maternal HLA, we hypothesize that the CB is also tolerant against maternal-PIRCHE, and these NIMA-PIRCHE should be regarded self peptides in the PIRCHE strategy. Very preliminary results from a small study showed that the number of PIRCHE substantially decreases when taking into account NIMA-PIRCHE (data not shown, ongoing study), potentially expanding the PIRCHE-acceptable donor pool. Moreover, it seemed that the correlation between PIRCHE numbers and clinical outcome was improved when regarding NIMA-PIRCHE as self peptides (data not shown). Currently, the sample size of this study is being expanded, allowing for more concise studies regarding the effect of NIMA-PIRCHE.

Expanding the self-peptide pool

The PIRCHE method can theoretically be extended to whole genome sequences, both with respect to determining self peptides as well as potential epitopes. PIRCHE are basically minor H antigens, as theoretically any polymorphism in presented peptides derived from a patient and donor can be recognized by a T cell. Some of the currently predicted PIRCHE are possibly actually similar to self-peptides derived from non-HLA proteins and will subsequently not induce T-cell responses. Inclusion of whole genome sequences for PIRCHE analyses may prevent this potential overestimation of PIRCHE. In the near future, whole exomes will likely be available during donor selection, as the costs of whole genome sequencing are diminishing. With the increase of available genes to match donor and patient for, the definition of a complete match will become more and more stringent; there will exist an ever increasing need of methods to predict permissible mismatches. PIRCHE-predictions expanded to any peptide may assist in such permissible mismatching strategies.

To sum up, the validity of the predicted PIRCHE may be improved, by broadening the PIRCHE-II predictions to HLA-DQ and -DP presentation; analyzing different peptide lengths; expanding the definition of HLA presentation; and by broadening the input for the model with respect to HLA sequences, NIMA typing and whole exome sequences. These additions

may improve the sensitivity and the specificity of the model by in some instances decreasing or in other instances increasing the number of predicted PIRCHE. The refinement of the number of PIRCHE should ultimately lead to expansion of the acceptable donor pool.

***In vitro* proof-of-principle**

Identifying T cells that recognize PIRCHE in patients with acute GVHD or substantial anti-tumor responses would help improve the model, as it would enable studying *in vitro* which properties of the PIRCHE lead to (more profound) T-cell activation. Thus far, CD4+ T cells indirectly recognizing HLA class-I and -II mismatches have been shown in recipients of organ allografts (52-55), and in a patient with GVHD that had received multiple DLIs (56). Indirectly recognizing CD8+ T cells have not been identified yet in patients after allo-HCT. Patient material from patients after HCT is rather scarce. We therefore started to study the presence of PIRCHE-specific CD8+ T cells in peripheral blood of healthy donors.

We aimed at showing that indirectly recognizing T cells can be induced in healthy individuals, and therefore also in potential donors of allo-HCT. To this end, T cells from a healthy donor were primed with autologous monocyte-derived dendritic cells loaded with potential PIRCHE. These primed T cells were challenged with T2 cells loaded with the PIRCHE from the priming phase or an HLA-A2-derived self peptide. Significantly more T cells produced IFNγ upon restimulation with three PIRCHE compared to the self-peptide (Figure 2). Thus, PIRCHE-specific T cells can be induced upon priming of a healthy donor.

Alternatively, PIRCHE-specific CD8+ T cells were identified with fluorochome-labeled PIRCHE-tetramers in a multiparous woman; these T cells were specific for PIRCHE derived from the mismatched HLA of her two children (Figure 3A). Furthermore, HLA-specific antibodies were detected in the plasma of this healthy woman. These antibodies were directed against the HLA of the children of which high numbers of PIRCHE-II were presented, whereas such antibodies were neither present against the mismatched HLA resulting in 0 or 1 PIRCHE-II nor matched HLA. This single case observation suggests that PIRCHE-specific CD4+ T cells have provided help to the B cells, that led to the production of HLA-specific IgG antibodies (Figure 3B). This latter finding is in accordance with results from a study regarding kidney transplantation (57). In the latter study, the number of PIRCHE-II derived from HLA mismatches against which antibodies were observed (immunogenic mismatches), was

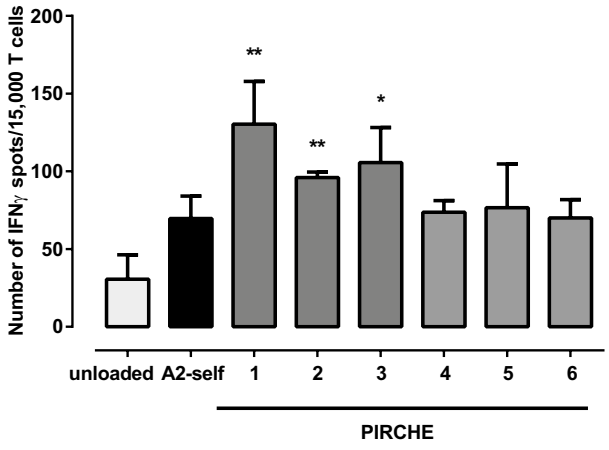


Figure 2: IFNγ ELISpot testing T cells from a healthy donor primed with potential PIRCHE. The number of IFNγ spots per 15,000 T cells, upon challenging them with T2 cells loaded with various peptides. Primed T cells produce significantly more IFNγ upon stimulation with three PIRCHE (1-3) compared to the A2-derived self-peptide, three other PIRCHE (4-6) did not induce significant responses. A2-self: LLLSGALAL (HLA-A2 derived). 1: LMLAMLSSL (HLA-DQB1 derived). 2: YIYNREEFV (HLA-DPB1 derived). 3: AVLGAVVAV (HLA-C derived). 4: YIYNREELV (HLA-DPB1 derived). 5: YIYNRQEYA (HLA-DPB1 derived). 6: AVLGAMVAV (HLA-C derived). **: p<0.05. * p<0.10.

higher than those of mismatches against which antibodies were not formed. Thus, in this healthy multiparous woman, we have found some indications of PIRCHE-specific CD8+ and, indirectly, CD4+ T cells, that were likely induced during pregnancy. Nevertheless, these data regard only one donor and therefore extended investigation of more women is required.

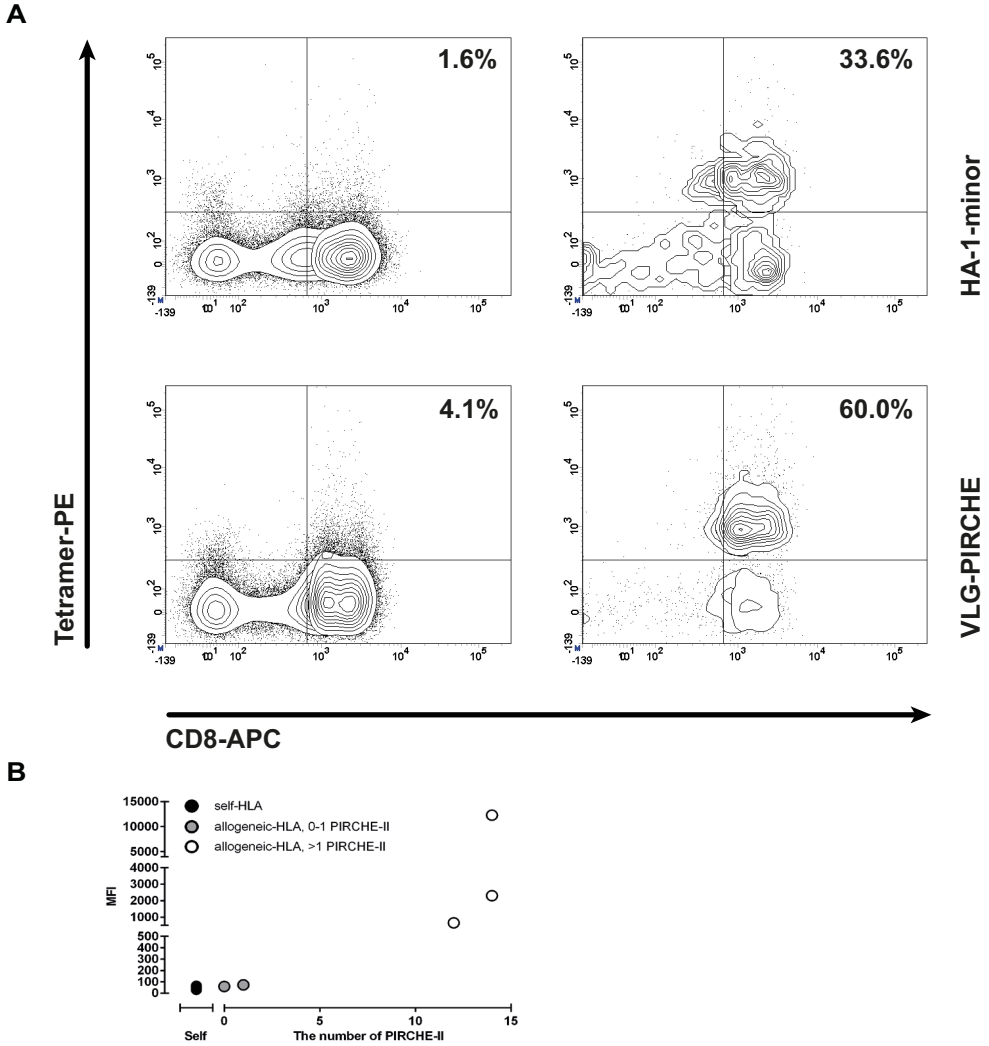


Figure 3 (A) In a healthy multiparous woman, PIRCHE-specific CD8+ T cells were detectable with flourochrome-labeled tetramers. Peripheral blood mononuclear cells were CD8+ T-cell enriched via negative selection with magnetic-activated cell sorting. The CD8+ T-cell enriched fraction was labeled with fluorochrome-labeled tetramers (HA-1 as a control, VLG-PIRCHE as a test) and CD8 and CD45RO antibodies. After enriching the CD8+ tetramer+ fraction with fluorochrome-activated cell sorting (left panels), PIRCHE and minor-specific T cells are clearly detectable (right panels). **(B) In a healthy multiparous woman, HLA-specific antibodies were detectable with Luminex technology, when the numbers of PIRCHE-II were high.** HLA-specific antibodies were detected in plasma, an MFI above 500 was regarded positive. HLA-specific antibodies were detected against three HLA alleles of her children of which high numbers of PIRCHE-II could be presented (in white). Antibodies were not detected against self HLA alleles (in black) or allogeneic HLA with 0 or 1 PIRCHE-II (in grey). MFI: mean fluorescent intensity.

To summarize, PIRCHE-specific CD8+ T cells were induced in a healthy donor upon priming and were present in a healthy multiparous woman, likely induced during previous pregnancies. Although these results are quite preliminary and require extensive confirmation and further testing, they do suggest that PIRCHE-specific CD8+ T cells are inducible in healthy donors. Ultimately, isolating PIRCHE-specific CD4+ and CD8+ T cells from patients with GVHD or without relapse after URD- and CB-HCT, not only aids in improvement of the current model, it also allows studying the specific role of these different T-cell subsets and may result in the development of methods that help prevent PIRCHE-induced GVHD or enhance PIRCHE-I directed anti-tumor responses.

Despite the fact that PIRCHE-specific CD8+ T cells have not yet been identified after allo-HCT, a large number of PIRCHE have been eluted in peptide-elution studies (58). Some of these studies have also been used to establish the computational methods that were incorporated in the PIRCHE predictions. Investigating the reliability of PIRCHE predictions by peptide-elution studies on cells from peripheral blood or EBV-LCL, similar as performed in these studies underlying the PIRCHE model, does therefore not validate the model. On the contrary, *in vitro* studies regarding the presence of PIRCHE in GVHD tissue or on tumor cells, would help to improve the model. It would enable studying the preferential expression of PIRCHE on certain tissues, thereby improving the dissection of the GVHD and GVT effect and help identifying highly immunogenic PIRCHE.

Implementation of PIRCHE

The ultimate goal of this thesis was to improve clinical outcomes of patients that have to be transplanted with HLA-mismatched donors, by enabling selection of permissible HLA mismatches. This thesis described consistent correlations of PIRCHE with clinical outcome, despite the potential changes to the model and the unexplained differences in clinical outcomes after URD- and CB-HCT. Therefore, based on the available data, we propose the following flow of PIRCHE-based donor selection (summarized in Figure 4). The URD search is started in absence of an HLA-identical sibling donor. Depending on the current local protocols, the next option is an HLA-matched URD or CB. In absence of an HLA-match, PIRCHE can provide guidelines. When selecting an HLA-mismatched URD, selection of a donor with the lowest number of PIRCHE-I and -II is preferred; the sample size of our studies was not large enough to compare low PIRCHE-I, high PIRCHE-II with the opposite option. Nonetheless, the associations between PIRCHE and clinical outcome in the URD setting were most substantial for PIRCHE-I, suggesting that low PIRCHE-I may be preferred over low PIRCHE-II. When selecting a CB donor, the underlying disease is of utmost importance for the PIRCHE-based donor-selection advice. For malignant diseases, AML specifically, we advise to select a donor with high PIRCHE-I, to enhance the anti-tumor effects, and low PIRCHE-II, to avoid GVHD. For non-malignant diseases, the best option may be low PIRCHE-I and -II, although the advantage of selecting a CB donor with low PIRCHE-I has not been confirmed yet in this setting.

In our local center, PIRCHE numbers have been monitored in the prospective donor-selection procedure. One of these monitored cases (personal communication by dr. E. Spierings) serves as an example of PIRCHE-based donor selection in daily practice. A patient with HLA-A*02:01, -A*03:01, -B*50:01, -B*35:01, -C*04:01, -C*06:02, -DRB1*01:01, -DRB1*04:08, -DQB1*05:01, -DQB1*03:01 was in need of an allo-HCT. The chance of finding a 10/10 URD was considered low, as HLA-DRB1*04:08 is infrequent in combination with

A*02:01. There were more than 100 potential single allele-mismatched donors, with mismatches on HLA-A, -B or -DRB1. A potential strategy could be to select a donor with an HLA-DRB1 mismatch, as this is the infrequent allele that is hard to match for. However, when implementing PIRCHE in the donor-selection strategy, HLA-DRB1 mismatches led to high numbers of PIRCHE and were thus not preferred. Alternatively, mismatching for HLA-A*02:01 and matching for DRB1*04:08 yielded less PIRCHE (Table 1). Hence, when regarding PIRCHE next to the potential HLA mismatches, the selection of the mismatched locus can be based on a risk classification rather than on the locus that is attractive to mismatch for in order to enhance the finding of potential donors. Thus, including PIRCHE calculations in donor-selection procedures may greatly change and improve current strategies.

In conclusion

This thesis describes a novel method of predicting permissible HLA mismatches prior to HCT that can be implemented in donor-selection procedures. When integrating PIRCHE in the donor-selection criteria, it should be done cautiously, bearing the donor source, indication for HCT and conditioning regimen in mind. A number of changes may lead to improvement of the current strategy, which ultimately results in even more refined risk classifications of HLA mismatches and expansion of the PIRCHE-acceptable donor pool.

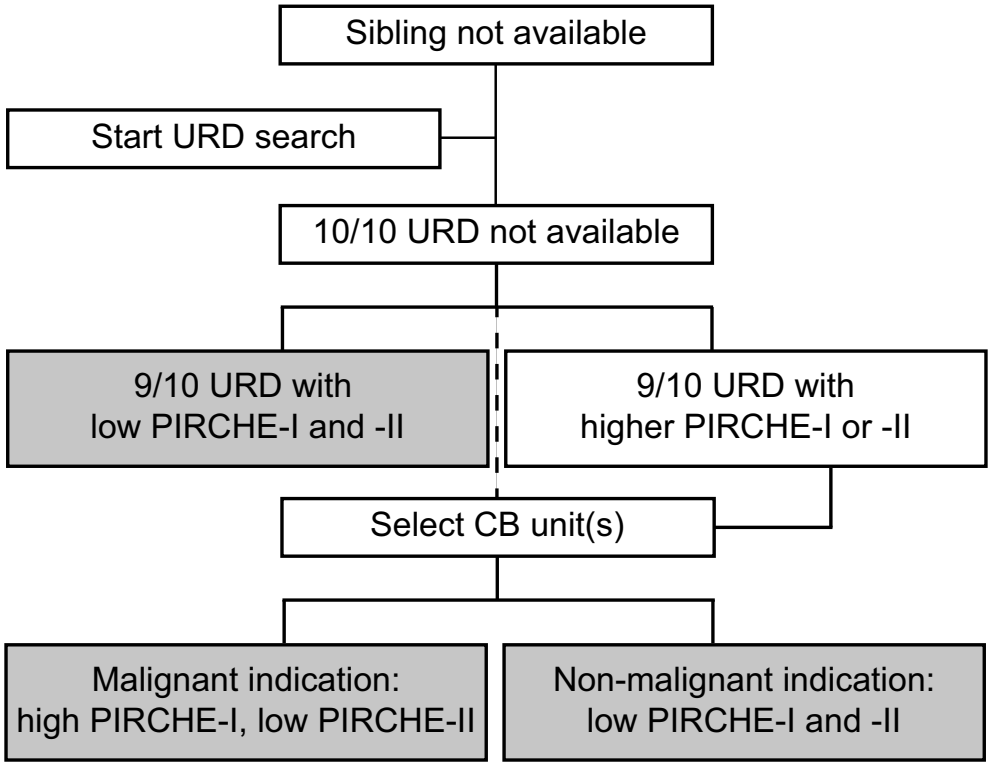


Figure 4: Proposed incorporation of PIRCHE-based donor selection. Flowchart for unrelated donor (URD) and cord blood (CB) selection procedure, grey cells indicate PIRCHE advice.

Potential mismatch combination				
Locus	*Patient HLA typing*	*Potential donor HLA typing*	*PIRCHE-I*	*PIRCHE-II*
A	02:01	02:05	1	3
A	02:01	02:02	1	3
A	02:01	02:08	1	4
A	02:01	25:01	2	18
A	02:01	24:02	3	21
A	02:01	23:01	3	22
A	02:01	11:01	4	30
B	50:01	57:01	6	21
DRB1	04:08	04:01	2	9
DRB1	04:08	13:03	6	14
DRB1	04:08	11:01	6	14
DRB1	04:08	08:03	6	14
DRB1	04:08	11:03	6	14
DRB1	04:08	11:04	6	14
DRB1	04:08	12:01	6	16

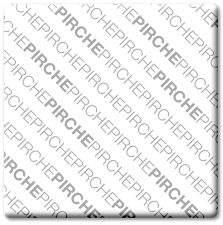
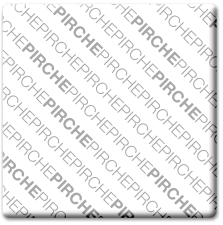
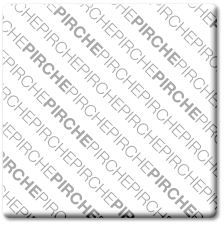
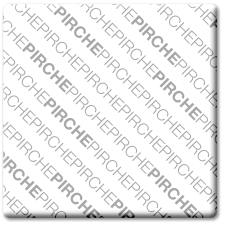
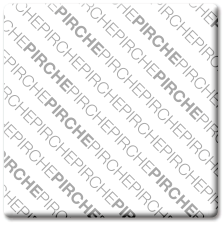
Table 1: Example of PIRCHE implementation in donor-search strategy. Although mismatching for the HLA-DRB1 allele seems logical, as exemplified in the text, when including PIRCHE predictions HLA-A mismatching seems preferred.

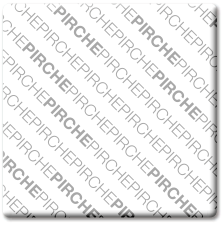
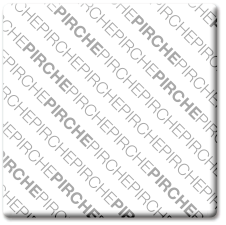
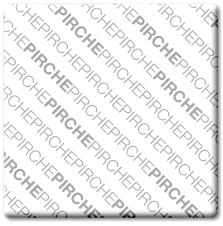
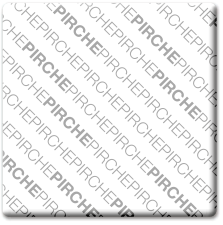
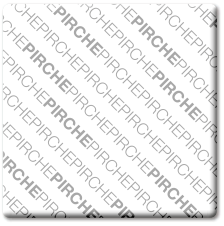
References

1. Chiesa R, Gilmour K, Qasim W, et al. Omission of in vivo T-cell depletion promotes rapid expansion of naive CD4+ cord blood lymphocytes and restores adaptive immunity within 2 months after unrelated cord blood transplant. Br.J.Haematol. 2012; 156(5): 656-666.
2. de Roock S, Hoeks SB, Meurs L, et al. Critical role for programmed death 1 signaling and protein kinase B in augmented regulatory T-cell induction in cord blood. J.Allergy Clin.Immunol. 2011; 128(6): 1369-1371.
3. Mold JE, Michaelsson J, Burt TD, et al. Maternal alloantigens promote the development of tolerogenic fetal regulatory T cells in utero. Science 2008; 322(5907): 1562-1565.
4. Mold JE, Venkatasubrahmanyam S, Burt TD, et al. Fetal and adult hematopoietic stem cells give rise to distinct T cell lineages in humans. Science 2010; 330(6011): 1695-1699.
5. Amir AL, D'Orsogna LJ, Roelen DL, et al. Allo-HLA reactivity of virus-specific memory T cells is common. Blood 2010; 115(15): 3146-3157.
6. D'Orsogna LJ, Roelen DL, Doxiadis II, et al. TCR cross-reactivity and allorecognition: new insights into the immunogenetics of allorecognition. Immunogenetics 2012; 64(2): 77-85.
7. D'Orsogna LJ, Roelen DL, Doxiadis II, et al. Alloreactivity from human viral specific memory T-cells. Transpl. Immunol. 2010; 23(4): 149-155.
8. Lee SJ, Klein J, Haagenson M, et al. High-resolution donor-recipient HLA matching contributes to the success of unrelated donor marrow transplantation. Blood 2007; 110(13): 4576-4583.
9. Furst D, Muller C, Vucinic V, et al. High-resolution HLA matching in hematopoietic stem cell transplantation: a retrospective collaborative analysis. Blood 2013; 122(18): 3220-3229.
10. Cunha R, Loiseau P, Ruggeri A, et al. Impact of HLA mismatch direction on outcomes after umbilical cord blood transplantation for hematological malignant disorders: a retrospective Eurocord-EBMT analysis. Bone Marrow Transplant. 2014; 49(1): 24-29.
11. Gluckman E, Rocha V, Arcese W, et al. Factors associated with outcomes of unrelated cord blood transplant: guidelines for donor choice. Exp.Hematol. 2004; 32(4): 397-407.
12. Somers JA, Braakman E, van der Holt B, et al. Rapid induction of single donor chimerism after double umbilical cord blood transplantation preceded by reduced intensity conditioning: results of the HOVON 106 phase II study. Haematologica 2014; 99(11): 1753-1761.
13. Wagner JE, Jr., Eapen M, Carter S, et al. One-unit versus two-unit cord-blood transplantation for hematologic cancers. N.Engl.J.Med. 2014; 371(18): 1685-1694.
14. Brunstein CG, Gutman JA, Weisdorf DJ, et al. Allogeneic hematopoietic cell transplantation for hematologic malignancy: relative risks and benefits of double umbilical cord blood. Blood 2010; 116(22): 4693-4699.
15. Williams TM. Human leukocyte antigen gene polymorphism and the histocompatibility laboratory. J.Mol.Diagn. 2001; 3(3): 98-104.
16. Steger B, Milosevic S, Doessinger G, et al. CD4(+) and CD8(+)T-cell reactions against leukemia-associated- or minor-histocompatibility-antigens in AML-patients after allogeneic SCT. Immunobiology. 2014; 219(4): 247-260.
17. Kolb HJ, Schattenberg A, Goldman JM, et al. Graft-versus-leukemia effect of donor lymphocyte transfusions in marrow grafted patients. Blood. 1995; 86(5): 2041-2050.
18. Poon LM, Hamdi A, Saliba R, et al. Outcomes of adults with acute lymphoblastic leukemia relapsing after allogeneic hematopoietic stem cell transplantation. Biol.Blood Marrow Transplant. 2013; 19(7): 1059-1064.
19. Collins RH, Jr., Goldstein S, Giralt S, et al. Donor leukocyte infusions in acute lymphocytic leukemia. Bone Marrow Transplant. 2000; 26(5): 511-516.
20. Spyridonidis A, Labopin M, Schmid C, et al. Outcomes and prognostic factors of adults with acute lymphoblastic leukemia who relapse after allogeneic hematopoietic cell transplantation. An analysis on behalf of the Acute Leukemia Working Party of EBMT. Leukemia. 2012; 26(6): 1211-1217.
21. Vago L, Perna SK, Zanussi M, et al. Loss of mismatched HLA in leukemia after stem-cell transplantation. N.Engl.J.Med. 2009; 361(5): 478-488.
22. Schattenberg AV and Levenga TH. Differences between the different conditioning regimens for allogeneic stem cell transplantation. Curr.Opin.Oncol. 2006; 18(6): 667-670.
23. Goulmy E, Schipper R, Pool J, et al. Mismatches of minor histocompatibility antigens between HLA-identical donors and recipients and the development of graft-versus-host disease after bone marrow transplantation. N.Engl.J.Med. 1996; 334(5): 281-285.
24. Weiss DW. Reflections on tumor origin, immunogenicity, and immunotherapy. Cancer Immunol.Immunother. 1984; 18(1): 1-4.
25. Yewdell JW and Bennink JR. Immunodominance in major histocompatibility complex class I-restricted T lymphocyte responses. Annu.Rev.Immunol. 1999; 17: 51-88.
26. Diez-Rivero CM, Lafuente EM, and Reche PA. Computational analysis and modeling of cleavage by the immunoproteasome and the constitutive proteasome. BMC.Bioinformatics. 2010; 11: 479.
27. Rock KL, York IA, Saric T, et al. Protein degradation and the generation of MHC class I-presented peptides. Adv.Immunol. 2002; 80: 1-70.
28. Fernandez-Vina MA, Klein JP, Haagenson M, et al. Multiple mismatches at the low expression HLA loci DP, DQ, and DRB3/4/5 associate with adverse outcomes in hematopoietic stem cell transplantation. Blood. 2013; 121(22): 4603-4610.

29. Petersdorf EW, Gooley TA, Malkki M, et al. HLA-C expression levels define permissible mismatches in hematopoietic cell transplantation. Blood 2014; 124(26): 3996-4003.
30. Paul S, Weiskopf D, Angelo MA, et al. HLA class I alleles are associated with peptide-binding repertoires of different size, affinity, and immunogenicity. J.Immunol. 2013; 191(12): 5831-5839.
31. Buus S, Lauemoller SL, Worning P, et al. Sensitive quantitative predictions of peptide-MHC binding by a 'Query by Committee' artificial neural network approach. Tissue Antigens 2003; 62(5): 378-384.
32. den Haan JM, Meadows LM, Wang W, et al. The minor histocompatibility antigen HA-1: a diallelic gene with a single amino acid polymorphism. Science 1998; 279(5353): 1054-1057.
33. Spierings E, Gras S, Reiser JB, et al. Steric hindrance and fast dissociation explain the lack of immunogenicity of the minor histocompatibility HA-1Arg Null allele. J.Immunol. 2009; 182(8): 4809-4816.
34. Parker KC, Bednarek MA, and Coligan JE. Scheme for ranking potential HLA-A2 binding peptides based on independent binding of individual peptide side-chains. Journal of Immunology 1994; 152(1): 163-175.
35. Krogsgaard M and Davis MM. How T cells 'see' antigen. Nat.Immunol. 2005; 6(3): 239-245.
36. Wucherpfennig KW, Call MJ, Deng L, et al. Structural alterations in peptide-MHC recognition by self-reactive T cell receptors. Curr.Opin.Immunol. 2009; 21(6): 590-595.
37. Piepenbrink KH, Blevins SJ, Scott DR, et al. The basis for limited specificity and MHC restriction in a T cell receptor interface. Nat.Commun. 2013; 4: 1948.
38. Motozono C, Kuse N, Sun X, et al. Molecular basis of a dominant T cell response to an HIV reverse transcriptase 8-mer epitope presented by the protective allele HLA-B*51:01. J.Immunol. 2014; 192(7): 3428-3434.
39. Garboczi DN and Biddison WE. Shapes of MHC restriction. Immunity. 1999; 10(1): 1-7.
40. Reinherz EL, Tan K, Tang L, et al. The crystal structure of a T cell receptor in complex with peptide and MHC class II. Science 1999; 286(5446): 1913-1921.
41. Kersh GJ and Allen PM. Structural basis for T cell recognition of altered peptide ligands: a single T cell receptor can productively recognize a large continuum of related ligands. J.Exp.Med. 1996; 184(4): 1259-1268.
42. De Oliveira DB, Harfouch-Hammoud E, Otto H, et al. Structural analysis of two HLA-DR-presented autoantigenic epitopes: crucial role of peripheral but not central peptide residues for T-cell receptor recognition. Mol.Immunol. 2000; 37(14): 813-825.
43. Reid SW, McAdam S, Smith KJ, et al. Antagonist HIV-1 Gag peptides induce structural changes in HLA B8. J.Exp.Med. 1996; 184(6): 2279-2286.
44. Calis JJ, Maybeno M, Greenbaum JA, et al. Properties of MHC class I presented peptides that enhance immunogenicity. PLoS.Comput.Biol. 2013; 9(10): e1003266.
45. Nielsen M, Justesen S, Lund O, et al. NetMHCIIpan-2.0 - Improved pan-specific HLA-DR predictions using a novel concurrent alignment and weight optimization training procedure. Immunome.Res. 2010; 6: 9.
46. Karosiene E, Rasmussen M, Blicher T, et al. NetMHCIIpan-3.0, a common pan-specific MHC class II prediction method including all three human MHC class II isotypes, HLA-DR, HLA-DP and HLA-DQ. Immunogenetics 2013; 65(10): 711-724.
47. Tynan FE, Burrows SR, Buckle AM, et al. T cell receptor recognition of a 'super-bulged' major histocompatibility complex class I-bound peptide. Nat.Immunol. 2005; 6(11): 1114-1122.
48. Liu YC, Chen Z, Burrows SR, et al. The energetic basis underpinning T-cell receptor recognition of a super-bulged peptide bound to a major histocompatibility complex class I molecule. J.Biol.Chem. 2012; 287(15): 12267-12276.
49. Burrows SR, Rossjohn J, and McCluskey J. Have we cut ourselves too short in mapping CTL epitopes? Trends Immunol. 2006; 27(1): 11-16.
50. IMGT sequence alignment tool. IMGT 2013; Available from: http://www.ebi.ac.uk/ipd/imgt/hla/align.html
51. Rocha V, Spellman S, Zhang MJ, et al. Effect of HLA-matching recipients to donor noninherited maternal antigens on outcomes after mismatched umbilical cord blood transplantation for hematologic malignancy. Biol.Blood Marrow Transplant. 2012; 18(12): 1890-1896.
52. Liu Z, Colovai AI, Tugulea S, et al. Indirect recognition of donor HLA-DR peptides in organ allograft rejection. J.Clin.Invest 1996; 98(5): 1150-1157.
53. Ciubotariu R, Liu Z, Colovai AI, et al. Persistent allopeptide reactivity and epitope spreading in chronic rejection of organ allografts. J.Clin.Invest 1998; 101(2): 398-405.
54. Suciu-Foca N, Ciubotariu R, Itescu S, et al. Indirect allorecognition of donor HLA-DR peptides in chronic rejection of heart allografts. Transplant.Proc. 1998; 30(8): 3999-4000.
55. Hornick PI, Mason PD, Baker RJ, et al. Significant frequencies of T cells with indirect anti-donor specificity in heart graft recipients with chronic rejection. Circulation 2000; 101(20): 2405-2410.
56. Amir AL, Hagedoorn RS, van Luxemburg-Heijs SA, et al. Identification of a coordinated CD8 and CD4 T cell response directed against mismatched HLA Class I causing severe acute graft-versus-host disease. Biol.Blood Marrow Transplant. 2012; 18(2): 210-219.
57. Otten HG, Calis JJ, Kesmir C, et al. Predicted indirectly recognizable HLA epitopes presented by HLA-DR correlate with the de novo development of donor-specific HLA IgG antibodies after kidney transplantation. Hum.Immunol. 2013; 74(3): 290-296.
58. Peptide database. Sypeithi 2013; Available from:

http://www.syfpeithi.de/bin/MHCServer.dll/FindYour-Motif.htm.






&

Nederlandse samenvatting

Dankwoord

List of publications

Curriculum Vitae

Nederlandse samenvatting

Transplantatie met hematopoïetische cellen van een donor (zogenaamde allogene hematopoïetische cel transplantatie, allo-HCT) is een curatieve therapie voor patiënten met maligne en niet-maligne beenmerg aandoeningen en aangeboren afwijkingen van het metabolisme. Een van de belangrijkste complicaties van allo-HCT is transplantatieziekte, deze ziekte ontstaat doordat het immuun systeem van de donor de patiënt als lichaamsvreemd herkent. Transplantatieziekte kan grotendeels voorkomen worden door patiënt en donor HLA te matchen. Echter, door de enorme diversiteit van ons HLA systeem, is het voor een groot deel van de patiënten niet mogelijk om een HLA-gematchte donor te vinden. Voor deze patiënten moeten we op zoek naar de “optimale mismatch”.

HLA mismatches kunnen leiden tot complicaties doordat de donor T cellen het gemismatchte HLA van de patiënt als lichaamsvreemd herkennen, en er een immuunreactie opgewekt wordt. De T cellen kunnen het HLA op twee manier herkennen: zogenaamde directe en indirecte herkenning. Tijdens directe herkenning herkent de T cel het intacte gemismatchte HLA eiwit op het cel oppervlak, waarbij het HLA eiwit een niet-relevant peptide presenteert. Deze vorm van herkenning is waarschijnlijk het gevolg van kruis-reactiviteit: het gemismatchte HLA met een peptide, lijkt op eigen HLA dat een lichaamsvreemd (bijvoorbeeld viraal) peptide presenteert. Tijdens indirecte herkenning wordt een peptide afkomstig van het gemismatchte HLA gepresenteerd op gematcht HLA, waarbij de donor T cel het peptide als lichaamsvreemd herkent. Dit is eigenlijk de gebruikelijke immuunrespons. **Hoofdstuk 2** van dit proefschrift beschrijft deze twee routes van HLA herkenning in detail en focust vooral op hoe ze voorspeld zouden kunnen worden. Kort samengevat is het proces van directe herkenning erg complex en daardoor tot op heden nog onvoorspelbaar. Dit proefschrift beschrijft daarom hoe indirecte herkenning voorspeld kan worden, met het zogenaamde PIRCHE (Predicted Indirectly ReCognizable HLA Epitopes) model.

Het PIRCHE model voorspelt welke peptides afkomstig van gemismatcht HLA gepresenteerd kunnen worden op gematcht HLA, en kijkt daarbij of die peptides verschillen van donor-eigen peptides. In theorie voorspelt een hoger aantal PIRCHE een grotere kans op T cel herkenning. PIRCHE kunnen gepresenteerd worden op HLA klasse I (PIRCHE-I) aan CD8+ T cellen, en op HLA klasse II (PIRCHE-II) aan CD4+ T cellen.

Het PIRCHE model is onderzocht in verschillende vormen van allo-HCT. Voor allo-HCT kunnen de hematopoïetische cellen geoogst worden uit perifeer bloed of beenmerg van een volwassen donor, of uit navelstrengbloed na een bevalling. Hoofdstuk 3-5 van dit proefschrift beschrijven het effect van PIRCHE na volwassen donor HCT, en hoofdstuk 6 en 7 na navelstrengbloed transplantatie. Hoofdstuk 8 en 9, tenslotte, focussen zich op aanwijzingen voor de aanwezigheid van donor T cellen die PIRCHE herkennen bij patiënten met transplantatieziekte.

In **hoofdstuk 3** is het effect onderzocht van PIRCHE na allo-HCT met een enkele HLA mismatch, en zijn de klinische uitkomsten van verschillende PIRCHE groepen (laag, gemiddeld of hoog) vergeleken met die van HLA-gematchte transplantaties. Patiënten met lage PIRCHE-I en -II hadden een kleine kans op transplantatieziekte, leidend tot een overleving die vergelijkbaar was met die van patiënten na HLA-gematchte transplantatie, terwijl patiënten met hoge PIRCHE-I en -II een significant slechtere overleving hadden dan patiënten na HLA-gematchte transplantatie. Dit hoofdstuk suggereert daarom dat we met de keuze voor lage PIRCHE-I en -II HLA-gemismatchte donoren zouden kunnen selecteren die vergelijkbare overlevingskansen bieden als HLA-gematchte donoren.

Klassiek worden voor allo-HCT patiënten en donoren gematcht op beide allelen van HLA-A, -B, -C, -DRB1, en -DQB1, een zogenaamde 10/10 match. Echter, recente studies laten zien dat het matchen voor HLA-DPB1 mogelijk ook tot betere overleving kan leiden. Bovendien is er een model ontwikkeld dat directe herkenning van HLA-DPB1 mismatches kan voorspellen, waarbij de zogenaamde permissive mismatches minder risico op transplantatie-gerelateerde mortaliteit opleveren dan non-permissive mismatches. In **hoofdstuk 4** vergeleken wij de effecten van het PIRCHE model met dit directe herkenningsmodel, het TCE model. PIRCHE-I en -II waren significant geassocieerd met de ontwikkeling van transplantatieziekte, zelfs in het geval van een zogenaamde permissive mismatch volgens het TCE model. Dit hoofdstuk laat zien dat de voorspellingen van het TCE en PIRCHE model aan elkaar gecorreleerd zijn, maar suggereert daarnaast dat PIRCHE óf nauwkeuriger transplantatieziekte voorspellen dan het TCE model, óf dat directe en indirecte herkenning naast elkaar optreden.

De meeste enkele HLA mismatches vinden plaats op het HLA-C locus, vanwege de huidige donor selectie procedures (er wordt eerder in het donor selectie proces naar HLA-A, -B en -DRB1 gekeken, dan naar HLA-C en -DQB1). Interessant is echter dat sommige HLA-C mismatches wel een verhoogd risico op transplantatieziekte geven, ondanks dat bekend is dat HLA-C maar weinig op het cel oppervlak tot expressie komt. Vanwege dit lage expressie niveau lijkt directe herkenning niet zo waarschijnlijk. In **hoofdstuk 5** hebben we onderzocht of indirecte herkenning een mogelijke verklaring voor het HLA-C gerelateerde risico zou kunnen bieden. In een klein cohort leken HLA-C mismatches met een hoger aantal PIRCHE-I en -II tot een groter risico op transplantatieziekte te leiden dan mismatches met weinig PIRCHE-I en -II. Daarnaast hebben we een hypothetische exercitie verricht, om te onderzoeken of voor sommige patiënten een HLA-B mismatch een beter alternatief zou kunnen zijn dan de geselecteerde HLA-C mismatch. Inderdaad zouden sommige HLA-B mismatches tot een lager aantal PIRCHE kunnen leiden dan de geselecteerde HLA-C mismatch. We stellen daarom dat de donor selectie procedure verbeterd zou kunnen worden wanneer er op basis van het aantal PIRCHE gematcht zou worden in plaats van het specifieke locus (HLA-B of -C).

Een alternatieve bron van hematopoïetische cellen is navelstrengbloed. Het voordeel van navelstrengbloed is dat het ligt opgeslagen in vriezers, en daardoor te allen tijde snel beschikbaar is. Daarnaast lijkt navelstrengbloed toleranter voor HLA mismatches, en wordt een navelstrengbloed donor eerder als een match beschouwd dan een volwassen beenmerg of perifeer bloed donor. De belangrijkste determinant voor het slagen van een navelstrengbloed transplantatie is de hoeveelheid cellen die aan de patiënt gegeven wordt, bij onvoldoende cellen lukt het de donor niet om zich te nestelen in het beenmerg van de patiënt. Echter, de hoeveelheid cellen uit één navelstrengbloed oogst is vaak niet voldoende voor zwaardere patiënten. Zwaardere kinderen en volwassen patiënten worden daarom vaak getransplanteerd met twee verschillende donoren. In **hoofdstuk 6** onderzochten wij het effect van PIRCHE na transplantatie met meestal een navelstrengbloed donor bij kinderen, en in **hoofdstuk 7** na transplantatie met twee verschillende navelstrengbloed donoren bij kinderen en volwassenen. Het aantal PIRCHE was na navelstrengbloedtransplantatie niet voorspellend voor het optreden van acute transplantatieziekte, maar transplantatie met hoge PIRCHE-I was geassocieerd met het voorkomen van terugkeer van de maligniteit. De immuunreactie lijkt in dit geval gericht tegen de maligniteit in plaats van de gezonde

weefsels van de patiënt. Deze twee hoofdstukken suggereren dan ook dat het zinvol zou kunnen zijn om bij navelstrengbloed selectie te kiezen voor een donor die veel PIRCHE-I kan herkennen.

In **hoofdstuk 8** onderzochten we of het effect van PIRCHE altijd aanwezig is. Alleen in patiënten waarbij enkel T cellen afkomstig van de donor aantoonbaar zijn, was het aantal PIRCHE geassocieerd met transplantatieziekte, terwijl er geen correlatie tussen PIRCHE en transplantatieziekte was wanneer naast de donor T cellen ook cellen afkomstig van de patiënt waarneembaar waren. Dit suggereert de noodzakelijkheid van voldoende donor T cellen voor het PIRCHE effect.

In **hoofdstuk 9**, tot slot, kwantificeerden wij het aantal CD4+ en CD8+ T cellen in coupes van huidbiopten van kinderen met acute transplantatieziekte. De T cel aantallen waren hoger in patiënten met PIRCHE-I of -II, en ook na een geslachtsgemismatchte transplantatie (vrouwelijke donor voor mannelijke patiënt). Het aantal cellen was het hoogst wanneer er een combinatie van PIRCHE en geslachtsmismatch aanwezig was. Dit hoofdstuk suggereert dat bij meer T cel epitopen (afkomstig van HLA of het Y chromosoom), er tijdens transplantatieziekte meer donor T cellen naar de huid migreren.

Samenvattend laat dit proefschrift een robuuste correlatie zien tussen het aantal PIRCHE en klinische uitkomst na allo-HCT. Wanneer gebruik wordt gemaakt van volwassen ongerelateerde donoren heeft het de voorkeur om met weinig PIRCHE-I en -II te transplanteren om zo transplantatieziekte en daaraan gerelateerde mortaliteit te voorkomen. Echter, in het geval van navelstrengbloed transplantatie geniet het de voorkeur om donoren te selecteren met hoge PIRCHE-I zodat een zo groot mogelijk anti-tumor effect bewerkstelligd wordt.

Dankwoord

"If you want to go fast, go alone. If you want to go far, go together." Dit proefschrift was er niet geweest zonder de bijdragen van een groot aantal mensen. Ik wil graag iedereen bedanken die betrokken is geweest bij de totstandkoming van mijn proefschrift. Eerst en vooral natuurlijk mijn begeleiders. Eric Spierings, bedankt dat je mij de kans hebt gegeven om onderzoek te doen naar een fantastisch onderwerp, heel toepasbaar en met een grote impact op de kliniek, helemaal in mijn straatje. Dank voor de zelfstandigheid die je me hebt gegeven, en de mogelijkheid tot samenwerken met veel verschillende mensen. Een hele leerzame ervaring. Erik Hack, ook dank voor de kans om dit onderzoek te doen, zonder dat er een precieze projectaanvraag of beurs was. Dank voor de kritische noten eens in de zoveel maanden, en voor de stimulerende ondersteuning in het valorisatie traject. Lieber Jürgen, danke schön dat je mij hebt "geadopteerd". Ik waardeer het zeer dat je hebt opgemerkt dat ik een beetje extra begeleiding en collega's om mee te sparren nodig had, en dat je altijd bereid was om waar nodig projecten/mensen te ondersteunen of een duwtje in de rug te geven.

De leescommissie, prof. Bruijnzeel, prof. Nieuwenhuis, prof. Prakken en prof. Bontrop, bedankt voor het beoordelen van mijn proefschrift. Dear prof. Wagner, thank you for reading my thesis.

Eric Borst, mijn computerheld in bange dagen, a.k.a. *U-Match IT-er*. Wat fantastisch dat je bijna al mijn hersenspinsels kunt omzetten in een excel macro, en dat je me altijd supersnel vooruit helpt. Ook namens mijn muisarm, veel dank. Kirsten Geneugelijk, wat fijn dat in jou mijn project voortgang vindt. Je gaat vast nog vele verbeteringen aan het PIRCHE model aanbrengen. Can, Jorg en Hanneke, zonder jullie was het model er zeker niet geweest zoals het er nu is. Ik vond het erg prettig om met jullie samen te werken, en heb er heel veel van geleerd. Everybody from Cytolon, good luck with the future of PIRCHE. Thanks for a fruitful collaboration!

Het hele HLA-lab, en in het bijzonder Talitha, bedankt voor de eindeloze hoeveelheid aan (bij)typeringen. Anna en Walter, veel dank voor het back-up zijn voor het verwerken van huidbiopten. Fijn om te weten dat er mensen bereid zijn bij te springen zodat geen belangrijke, zeldzame materialen gemist worden. De studenten die ik heb begeleid, Mieke, Wouter, Valeria en Titus, dank voor jullie inzet. Nathalie en Jorik, het was kort maar krachtig!

Collega's uit de kliniek, hematologie en Giraf, bedankt. Liane, maar ook Anette, dank voor de hulp bij de enorme hoeveelheid klinische data van de hematologie. Jaap Jan, veel dank voor alles wat ik aan CB geanalyseerd heb. Heel fijn dat je altijd bereid bent om supersnel naar mijn analyses te kijken en me door te verwijzen naar Rick voor extra data. Gezellig om samen te werken en fijn dat je altijd tijd hebt/maakt! Rick, dank voor alle antwoorden na JJs doorverwijzingen. :-) Corinne en Arianne, ook jullie dank voor de klinische data. Marc, dank voor de ondersteuning in van alles, maar in het bijzonder bij het opzetten van de huidbiopten studie. Jammer dat we die hebben moeten staken. Fijn dat ik bij jou terecht kon voor allerhande toekomstadvies. De CTF faciliteit, Ineke, Kasper, Lex, dank voor jullie hulp bij het aanvullen van ontbrekende donor data.

Het Ureka Mega Challenge team, Edward en Michel, bedankt voor de begeleiding. Jan Vos,

Linda, en iedereen die er verder bij betrokken is geweest, ik vond het een onwijs leuk en leerzaam en energie-gevend traject. Jullie enthousiasme en investering heeft een grote bijdrage aan dit boekje (en het vervolg) geleverd.

Kamergenootjes uit het WKZ, wat fijn om op de leukste AIO kamer van het WKZ gezeten te hebben. Bij afwezigheid van een groep was het fijn om jullie om me heen te hebben. Bedankt voor het ontbijt, koffies, diners, dansjes en alle andere gezelligheid, maar ook voor jullie bereidheid mij te helpen bij dingen die ik uit moest vinden. Dear roomies, thanks for everything! Kamergenootjes uit het AZU, viel ik zo maar met mijn neus in de boter om nogmaals op de leukste kamer te zitten ;-) Jullie waren zeker een van de positieve dingen van de verhuizing! Ik hoop dat de nodige etentjes om bij te kletsen voortgang blijven vinden!

Alle andere collega's van het LTI, bedankt voor de zinvolle input tijdens meetings, de gezelligheid tijdens borrels en de hulpvaardigheid op de werkvloer! Group Kuball, thanks to you as well for adopting me! Monique, Saskia, Yvonne Schemkes, Marry en Yvonne van der Knaap, bedankt voor alle ondersteuning. Dank zeker ook aan de mensen met wie ik projecten heb ondernomen die het helaas niet tot mijn proefschrift hebben gemaakt. Tuna, Ruud, en de rest van jullie groep, fijn om met jullie samen te werken voor het vinden van *in vitro* proof of principle. Jammer dat het (mij) niet is gelukt, maar ik ben zeer benieuwd wat de muizenproeven nog verder op gaan leveren. Sytze, ik heb veel van je geleerd. Barbara, grazie mille for the help in setting up the skin biopsy culturing. Someday, someone will be very happy with all those samples in the HSCT biobank.

Alle mensen van buiten het LTI met wie ik heb samengewerkt, dank. Roel de Weger, wat fijn dat je mij als student de kans hebt gegeven om aan stamcel transplantatie te werken omdat ik per se een kinder-oncologie project wilde doen. Indirect is dit project daaraan te danken! Joyce, veel dank voor de nodige DNAtjes voor samples die bijgetypeerd moesten worden, uit de oneindige voorraad van de pathologie. Marijke van Dijk, dank voor de GVHD coupes. Astrid van Halteren, ik heb met veel plezier een aantal projecten op jouw lab gedaan. Wat leuk en leerzaam om samen met en naast jou te pipetteren en analyseren. Machteld Oudshoorn, Annemarie van Walraven en Monique Jöris, dank voor het donor-search prognose project, de CTLp analyse en de HLA Europdonor data. Leonie Powley, prof. van Rood, Cristina Navarette, many thanks for the NIMA data. To be continued! Erik Mezenes, Federica Gianotti, Annalisa Ruggeri, Vanderson Rocha, prof. Gluckman and everybody else from Eurocord, thanks for the Eurocord data! To be continued as well. Todd DeFor, Mike Verneris, prof. Wagner, thanks for the double CB project. Todd, thanks for the very meaningful tips on analysis. Daniel Fürst and mr. Mytilineos, thanks for the great data in the 9 out of 10 project. Hope we will continue soon. Judith Somers, Ronnie van der Holt, prof. Cornelissen en iedereen die verder betrokken is geweest bij de HOVON studies, bedankt. Every other clinician or HLA lab I did not mention but that has made it possible to analyze so many clinical data and HLA typings, thanks! Without such collaborations these studies would not have been possible!

Lieve Michiel en Lotte, bedankt voor al jullie hulp. Michiel, dank dat de 2-1-tjes en cappuccino's met jou altijd gepaard gaan met de nodige relativering, delen van ervaringen en allerhande goede adviezen. Ik heb het altijd heel fijn gevonden om met jou te sparren

en het is fijn om iemand in je omgeving te hebben die het allemaal al een keer heeft mee gemaakt. Ik zal het wekelijkse van me af praten missen! Eigenlijk mis ik het nu al… Lot, wat fijn om een (dokters)maatje in het lab te hebben. Leuk om samen te leren pipetteren, maar vooral dank voor je gezelligheid. Jouw briefjes en berichtjes op de flowkast in de avond- of weekenduren maakten me altijd even aan het lachen, ook als ik eigenlijk geen zin meer had. Een nobelprijs komt tenslotte nooit vanzelf ;-) De nieuwe J&J wil je niet meer worden, maar misschien wel de nieuwe L&K?

Idunezen, ook jullie hebben bijgedragen aan dit boekje. Wat heerlijk om altijd met een paar van je beste vrienden in een team te zitten. Ik heb de nodige frustratie met jullie op de baan van me af kunnen slaan, maar ook kunnen dansen in de Tivoli/Derrick, of eten tijdens een fantastisch (kerst)diner. Ik hoop dat we onze tradities nog lang in ere kunnen houden. In het bijzonder, Mark, wat een mooie kaft!! Leuk dat ik voor de tweede keer van jouw ontwerpskills mocht profiteren. “Dokters” ook jullie zijn geweldige vrienden om te hebben. Wat leuk dat we bijna allemaal promoveren en zo onze ervaringen kunnen delen. Ook alle andere vrienden, dank voor alle gezelligheid en het meeleven!

Lieve pap, mam en Silv, dank dat jullie me altijd steunen, en dat ik jullie nog altijd kan bellen (en dat ook vaak doe) als ik advies nodig heb. Henk, Annemarie en Djura, met jullie heb ik er nog een fantastisch gezin bij. Ook op jullie kan ik altijd rekenen. Familie Thus, Liauw, Groen en Baard, wat fijn om zoveel leuke familie te hebben.

Lieve Jorne, wat fijn dat jij er bent. Wat ik in al mijn impulsiviteit ook bedenk, je staat altijd achter me, en geeft me de kans me te ontwikkelen, maar leert me ook van het leven te genieten. Het eerste deel van de bucket list is inmiddels afgewerkt, maar aan het volgende deel moeten we maar snel beginnen!

List of publications

K. Geneugelijk,[#] K. A. Thus,[#] E. Spierings. Predicting alloreactivity in transplantation. *Journal of Immunology Research* 2014; 2014: 159479. [#]authors contributed equally.

K. A. Thus, L. te Boome, J. Kuball, E. Spierings. Indirectly recognized HLA-C mismatches and their potential role in transplant outcome. *Frontiers in Immunology* 2014; 5: 210.

K. A. Thus, M. T. A. Ruizendaal, T. A. de Hoop, E. Borst, H. W. M. van Deutekom, L. te Boome, J. Kuball, E. Spierings. Refinement of the definition of permissible HLA-DPB1 mismatches with Predicted Indirectly ReCognizable HLA-DPB1 Epitopes. *Biology of Blood and Marrow Transplantation* 2014; 20 (11): 1705-1710.

K. A. Thus, A. G. S. van Halteren, T. A. P. de Hond, M. R. van Dijk, R. Q. H. Kloos, A. C. Lankester, M. B. Bierings, E. Spierings. The number of T cell allo-epitopes associates with CD4+ and CD8+ T-cell infiltration in pediatric cutaneous GVHD. *Cellular Immunology* 2015.

L. te Boome, C. Mansilla, L. E. van der Wagen, C. Lindemans, E. Petersen, E. Spierings, K. A. Thus, K. Westinga, M. Plantinga, M. B. Bierings, A. Broers, M. Cuijpers, G. van Imhoff, J. Janssen, C. Huisman, G. Huls, J. J. Boelens, N. Wulffraat, I. Slaper, J. Kuball. Biomarker profiling of steroid resistant acute GVHD in patients after infusion of mesenchymal stromal cells. *Leukemia* 2015.

K. A. Thus, T. A. de Hoop, R. A. de Weger, M. B. Bierings, J. J. Boelens, E. Spierings. PIRCHE-I promote leukemia-free survival after cord blood transplantation: indications for a potential novel donor selection tool. *Submitted for publication*.

K. A. Thus, T. A. de Hoop, V. E. Boers Trilles, J. Kuball, E. Spierings. Complete donor chimerism is a prerequisite for the effect of Predicted Indirectly ReCognizable HLA Epitopes (PIRCHE) on acute graft-versus-host disease. *Submitted for publication*.

K. Geneugelijk,[*] G. Hönger,[*] H. W. M. van Deutekom, K. A. Thus, C. Keşmir, I. Hösli, S. Schaub,[#] E. Spierings.[#] Predicted Indirectly ReCognizable HLA Epitopes presented by HLA-DRB1 (PIRCHE-II) are related to HLA-antibody formation during pregnancy. *Submitted for publication*. [*,#]authors contributed equally.

Curriculum Vitae

Kirsten Anne Thus was born January 11th, 1987, in 's Graveland, the Netherlands. After graduating from the Gemeentelijk Gymnasium Hilversum in 2005, she started her medical studies at the Utrecht University. During her medical study, her growing interest in foreign (tropical) healthcare was enhanced by a clinical rotation in Obstetrics and Gynecology in Queen Elizabeth Central Hospital and Zomba Central Hospital in Malawi, and a clinical rotation in public health at the Bureau of Public Health in Paramaribo, Surinam.

As part of an honors traject, she performed a research project on dominance after double cord blood transplantation at the Department of Pathology in the University Medical Center Utrecht, under supervision of dr. Roel de Weger. This research project was followed up by a final thesis on how to predict the probability of finding an HLA-matched donor, at the HLA laboratory under supervision of dr. Eric Spierings. Her final clinical rotation was performed at the Department of Pediatric Immunology and Oncology at the Wilhelmina Children's Hospital, Utrecht.

After graduating in 2011, she further dove into her interests of (pediatric) immunology and oncology by starting as a PhD student on the topic of how to improve donor selection for hematopoietic cell transplantation, under supervision of dr. Eric Spierings, prof. dr. Erik Hack and prof. dr. Jürgen Kuball, which has resulted in this thesis. In 2013, she won a valorisation contest of the UMC Utrecht, the Ureka Mega Challenge.

In February 2015 she started as a resident in Pediatrics in the Vlietland Hospital, Schiedam. In June 2015 she will continue her carreer in the Wilhelmina Children's Hospital, Utrecht. Ultimately, she wishes to combine a career as pediatrician with research.