CANCER IMMUNOTHERAPY USING γδT CELLS

Dealing with diversity

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Cancer immunotherapy using γδT cells
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CHAPTER 1

General introduction

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IMMUNOTHERAPY TO TREAT CANCER: THE ERA IS NOW

Current treatment options to fight cancer heavily rely on pharmaceutical and radiological interventions that are accompanied by substantial off-tumour toxicity and lack of clinical efficacy. Cancer immunotherapy aims to capture the specificity and memory of the immune system and holds the promise of truly targeted treatment with durable clinical responses. Recent advances in clinical trials and the approval of more and more immunotherapeutic agents by international regulatory agencies have given the field considerable momentum, a fact that is mirrored by the announcement of cancer immunotherapy as the breakthrough of the year 2013 by Science (1).

So far, the vast majority of efforts aimed at utilizing the immune system to reject cancer have focused on components of adaptive immunity, including monoclonal antibodies and αβT cells. The human immune system can theoretically generate up to 10^{11} unique antibodies and some 10^{15} unique αβT cell receptors (αβTCRs) (2), and controlling this vast diversity in antigen specificity for targeted immune interventions has been a major challenge for clinical implementation. Although immunoglobulins are still used in clinical practice for untargeted protection against viral infections, such as in patients with general B-cell deficiencies, the real breakthrough in clinical immunotherapy came with mastering the genetic profile of defined monoclonal antibodies. Among the first therapeutic antibodies to directly target cancer were anti-CD20 (Rituxan or Rituximab) and anti-Her2 (Herceptin or Trastuzumab) antibodies to treat B cell leukemias and breast cancer, respectively. Treatment with these antibodies, recognizing one particular antigen with a defined affinity, has underscored the therapeutic potential of truly antigen-targeted immunotherapy, as impressive clinical benefit has been reported across studies covering the last decade (3,4). The clinical success of these pioneering agents has in recent years led to the development and regulatory approval of additional antibodies to target various cancers (5), propelling antigen-specific antibody-based immunotherapy into mainstream cancer treatment. Similar to the evolution of clinical antibody treatment, first evidence for the anti-tumour potential of adoptively transferred αβT cells originated from the transfer of a very diverse immune population, the so called donor lymphocyte infusions, in the early 1990s, when allogeneic donor αβT cells that were infused in patients after allogeneic stem cell transplantation demonstrated potent anti-leukemia responses (6). By now, these data have been complemented by remarkable clinical results obtained with strategies that aim to mobilize the tumour-reactivity of autologous T cells in cancer patients, either by the adoptive transfer of ex vivo expanded tumour-infiltrating lymphocytes (TILs) (7,8) or the infusion of monoclonal antibodies that stimulate T cell activity, such as the recently approved anti-CTLA4 antibody Ipilimumab (9,10). Additionally, the genetic engineering of T cells with tumour-reactive αβTCRs (11,12) or antibody-based chimeric antigen receptors (CARs) (13) has gained increasing interest in recent years, and the first clinical trials using adoptive transfer of such gene-modified T cells have demonstrated potent and lasting anti-tumour responses in selected patients (14-18).

Importantly, understanding the diversity of adaptive immune repertoires and utilizing very defined specificities for therapeutic interventions has so far been not only the success but

also the downside of such therapies, resulting in highly personalized cancer care that depends on antibody-based strategies (including CAR-engineered T cells) with limited numbers of targetable tumour antigens and αβT cell products that are only clinically applicable to HLA-matched patient populations. Moreover, clinical anti-tumour efficacy of αβT cell-based approaches is so far mainly restricted to particularly immunogenic tumour types, such as melanoma. Thus, there is a compelling need to call to arms alternative immune components for novel cancer immunotherapeutic concepts.

γδT CELLS: THE PROMISING OUTSIDERS

Unconventional γδT cells, a second lineage of T cells that express a unique somatically recombined γδTCR, possess unique features to confront the limitations of adaptive-based immunotherapeutic strategies. γδT cells are rapidly activated upon encounter of pathogen-derived antigens or self molecules that are upregulated on infected or stressed cells, resembling the activation of innate immune cells that sense molecular stress signatures (19,20). Importantly, γδT cells are set apart from conventional αβT cells by the fact that activation of γδT cells does not depend on antigen presentation in the context of classical MHC molecules. A preferential usage of distinct TCR γ and δ chains, which together have the potential to form a tremendous repertoire of ~10^{20} uniquely recombined γδTCRs (2), has formed the basis for the identification of two major γδT cell subsets. γδT cells that carry Vγ9Vδ2+ TCRs are primarily found in peripheral blood, where they constitute a minor fraction of total T cells and respond to non-peptidic intermediates of the mevalonate pathway called phosphoantigens. Other γδT cells express mainly Vδ1+ or Vδ3+ chains paired with diverse γ chains (also called $Vδ2^{neg}$ γδT cells) and are highly enriched at mucosal sites and epithelial tissues. The effector mechanisms of γδT cells are highly similar to those of αβT cells and involve the secretion of high levels of cytokines and lysis of target cells by the release of granzymes and perforin and the engagement of FAS and TRAIL death receptors. Thus, by combining the potent effector functions of adaptive αβT cells with recognition modes that target unique classes of antigens in an innate-like manner, γδT cells are regarded as valuable sentinels that bridge innate and adaptive immunity.

Underlying the interest in γδT cells for use in cancer immunotherapy is a long-standing body of evidence indicating that γδT cells play important roles in tumour immunosurveillance. Human γδT cells display potent in vitro cytotoxicity towards a surprisingly large array of tumours, including cells derived from both solid and haematological origin (20-22). Importantly, γδT cells are also capable of targeting chemotherapy-resistant leukemic cells (23) and to kill leukemic and colon cancer stem cells ((24) and Sebestyen & Kuball, unpublished observation). In vivo evidence for the nonredundant relevance of γδT cells in tumour immune surveillance stems from studies showing that γδT cell-deficient mice are more susceptible for developing cancer (25-27). Moreover, tumour-infiltrating γδT cells (γδTIL) have been observed in cancer patients with various cancers, and isolated γδTILs were shown to efficiently kill autologous tumours ex vivo, while leaving healthy cells unharmed (28-32). Important roles for γδT cells in

tumour host defence are furthermore suggested by clinical data showing that high numbers of γδTILs in tumours of melanoma patients and elevated levels of circulating γδT cells in leukemia patients correlate with increased cancer-free survival (33,34). Taken together, these studies have established a wealth of evidence for the broad tumour-targeting capabilities of γδT cells and have sparked great interest in their application in cancer immunotherapy.

CLINICAL SUCCESS OF γδT CELLS: STUCK IN DIVERSITY?

Given the broad recognition of unique classes of tumour antigens by γδT cells combined with their potent killing capacity, it is no surprise that γδT cells have been the focus of attempts to design novel cancer immunotherapeutic strategies. Of the two major γδT cell subsets, clinical trials conducted so far have exclusively focused on the stimulation of autologous Vγ9Vδ2+ γδT cells that were either activated in vivo using so-called aminobisphosphonate compounds that specifically activate Vγ9Vδ2+ γδT cells, or expanded ex vivo and reinfused into patients. Protocols for the in vivo mobilization of Vγ9Vδ2+ T cells generally involved repeated cycles of intravenous injection of synthetic phosphoantigen (35) or aminobisphosphonates such as pamidronate (36) or zoledronate (37-40), in combination with multiple IL2 injections per cycle. In trials that explored the adoptive transfer autologous Vγ9Vδ2+ T cells, patient PBMCs were cultured ex vivo for two weeks in the presence of aminobisphosphonates (41-43) or synthetic phosphoantigen (44,45) in combination with IL2. Even though these conditions promoted the expansion of Vγ9Vδ2+ T cells, ex vivo expanded cell products contained rather low (on average 50-60%) and highly variable percentages of Vγ9Vδ2+ T cells, and no additional purification of Vγ9Vδ2+ T cells was performed prior to reinfusion into patients. Patients received repeated infusions of expanded cells, in some trials in combination with IL2. Treatment using γδT cells was generally found to be safe using both in vivo and ex vivo stimulation protocols, but clinical responses varied widely across trials and were generally limited, even in patients with cancers generally sensitive to immune responses such as renal cell carcinoma (reviewed in (46-48)). Important limitations included (a) the need for a preselection of patients due to a wide variability in in vitro cytotoxicity of patient γδT cells against autologous tumour tissue (36,41,44), and (b) limited in vivo or ex vivo expansion potential of patient γδT cells (40,41,44,45,49). Moreover, anti-tumour efficacy of γδT cells showed only marginal improvement over standard treatment options (46). Thus, despite the fact that these trials have established the anti-tumour potential of γδT cells in cancer immunotherapy, current therapeutic strategies using these cells clearly suffer from major shortcomings that have so far prevented γδT cells to live up to their clinical promise.

OUTLINE OF THE THESIS

Recent years have seen important progress in the understanding of γδT cell immunobiology and diversity, and have put these cells forward as true “swiss army knives” of immunity.

Nevertheless, detailed knowledge of the molecular activation modes of γδT cells and their surprisingly diverse functions is still limited, and this is likely to contribute to the suboptimal efficacy of γδT cell-based immunotherapies pursued to date. Therefore, this thesis aims to provide novel insights into the molecular and functional diversity of γδT cells and the requirements for their activation, thereby contributing to the design of improved cancer immunotherapies using γδT cells.

Chapter 2 reviews recent progress in the understanding of target recognition mechanisms of innate-like cells, in particular NK cells and γδT cells, and their value for immunotherapy. The chapter focuses on allogeneic stem cell transplantation (allo-SCT), the preferred treatment for patients with poor-prognosis hematological cancers and one of the most effective forms of immunotherapy to date. Both NK cells and γδT cells will be discussed in this chapter, but the focus of the remainder of the thesis will primarily lie with γδT cells.

Chapter 3 continues in the setting of allo-SCT, and describes a surprising functional diversity of γδT cells that respond to cytomegalovirus (CMV) infection, a common complication in patients after allo-SCT. Multiple valuable functions of CMV-elicited γδT cells are identified in this chapter – including CMV-reactivity, cancer-reactivity and the interaction with dendritic cells – that may explain a paradoxical protective effect of CMV infection on leukemic relapse observed in clinical studies. Moreover, by addressing the molecular requirements of γδT cell activation, a novel costimulatory role of CD8αα on γδT cells is presented in this chapter. In **Chapter 4**, the findings of Chapter 3 are summarized and their implications for improving adoptive cellular therapies are discussed, both in the context of allo-SCT as well as cancer immunotherapy in general.

One major hurdle for the clinical translation of γδT cells or their γδTCRs is represented by a limited understanding of their target recognition mechanisms, and addressing this shortcoming will be critical for the development of more efficacious γδT cell-based immunotherapies. In **Chapter 5**, the role of CD8αα in costimulating defined γδTCRs, as identified in Chapter 3, is further characterized. A surprisingly diverse molecular involvement of CD8αα in costimulating γδTCRs is uncovered in this chapter, which adds to the molecular diversity and complexity of the γδT cell subset. In **Chapter 6**, a novel genetic screening method is introduced that allowed the identification of the small GTPase RhoB as a critical player in Vγ9Vδ2+ T cell-mediated tumour recognition. We show that RhoB is differentially regulated in healthy and transformed cells, and interacts with a previously identified key mediator of Vγ9Vδ2+ T cell activation.

An additional challenge to the clinical application of in particular TCR gene-engineered T cells is a lack of GMP-compatible techniques that allow for the selective isolation of T cells that express high levels of introduced TCRs. In **Chapter 7**, an isolation strategy is introduced – here based on a well-defined tumour-specific αβTCR – that facilitates the efficient depletion of cells that express only low levels of transgenic TCRs. Importantly, this approach is based fully on the use of available clinical grade tools, making translation to the clinic straightforward.

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CHAPTER 2

Hunting for translation with innate-like immune cells and their receptors

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ABSTRACT

Allogeneic stem cell transplantation (allo-SCT) has so far been the most effective immunotherapy for hematological malignancies. However, it is becoming increasingly clear that the immunotherapeutic concepts underlying allo-SCT, as well as the traditional dissection of the immune system into innate and adaptive arms, need substantial refinement. More and more cell types migrate into the interface between innate and adaptive immunity, creating new terms such as innate-like lymphocytes. These innate-like cells, which include natural killer (NK) cells and γδT cells, could provide unique advantages to therapeutic interventions aimed at treating hematological malignancies, including protection against tumor relapse and viral infections without causing harmful graft-versus-host disease (GVHD). Recent molecular and conceptual insights into these subpopulations have opened new avenues to exploit their exciting features for the development of new compounds and to revisit current therapeutic standards in the treatment of hematological cancers. This review therefore aims to discuss the rapid progress in the understanding of molecular mechanisms by which NK cells and γδT cells recognize malignancies and viral infections, and the value of this increasing knowledge to complement the battle against life-threatening complications of current strategies to treat cancer.

INTRODUCTION

After the first allogeneic stem cell transplantation (allo-SCT) was conducted more than half a century ago, allo-SCT is still the preferred treatment option for many patients with poor-prognosis hematological malignancies (1). Even though to date it is the most successful adoptive immunotherapy, it is also potentially the most detrimental to patients, as the outcome of allo-SCT is still substantially hampered by life-threatening complications such as graft-versus-host disease (GVHD), a misdirected immune response of donor T cells against recipient healthy tissue, and relapse of the tumor. In addition, an incomplete immune reconstitution early after transplantation and immunosuppressive therapy to counter GVHD renders allo-SCT patients susceptible to viral infections. In particular reactivations of the common cytomegalovirus (CMV) cause substantial mortality and morbidity after transplantation. Immunotherapeutic concepts to tackle these obstacles have focused primarily on components of the adaptive immune system. For example, the adoptive transfer of cytotoxic αβT cells targeting tumor- or virus-associated antigens have so far yielded provocative and promising data but also demonstrated substantial limitations (2-5). However, the MHC-restricted antigen recognition of αβT cells critically depends on a careful genetic matching between donor and recipient, making suitable donor choices challenging. As a consequence, there is a pressing need for a reconsideration of current immunotherapeutic strategies to treat hematological malignancies. NK cells and γδT cells, innate-like cells that combine characteristics of innate and adaptive immunity (6,7), have increasingly come into focus as tools to cope with these requirements. These cells target alternative classes of

antigens on a broad spectrum of tumors and virus-infected cells, while preserving selective recognition to avoid detrimental reactivity toward healthy tissue. Moreover, the mechanisms of virus and tumor recognition by NK and γδT cells do not depend on antigen presentation via classical MHC molecules, and thus largely obviate the need for genetic matching of stem cell donors and recipients. Substantial breakthroughs have been made recently in the understanding of the molecular mechanisms of target recognition by NK cells and γδT cells (8-12), as well as in the elucidation of novel links between the cross-recognition of virus-infected cells and cancer cells (13,14). Importantly, these advances not only shed new light on puzzling clinical observations such as an improved leukemia control in allo-SCT patients with CMV-reactivation (15,16), but also contribute to the development of novel innate-like immunotherapies to improve clinical outcome of patients with hematological cancers. This review therefore aims to summarize the recent advances in NK and γδT cell immunobiology, discuss their common and distinct features, and highlight the exciting therapeutic potential and challenges for these two major innate-like cell populations.

TARGET RECOGNITION BY NK CELLS AND γδT CELLS

NK cells: activating NK receptors and their ligands

NK cells were initially identified as lymphocytes with non-MHC-restricted cytotoxicity against tumor cells without the need for prior sensitization (7). Since then, accumulating evidence has suggested important functions for NK cells in immunity against tumor and viruses, both by exerting direct cytotoxicity to transformed and infected cells as well as by the secretion of cytokines. In humans, two major NK cell subsets have been identified based on expression of the adhesion molecule CD56 and the Fc receptor CD16. Potent cytotoxicity against transformed and infected cells is provided by the majority of circulating NK cells which are characterized by a CD56dimCD16+ phenotype, while CD56brightCD16- NK cells are enriched in secondary lymphoid tissues and are suggested to play important roles in regulating adaptive immune responses by secreting high levels of cytokines (17). The activation state of NK cells depends on signaling of both activating and inhibitory receptors that concertedly discriminate healthy from diseased cells by sensing stress-induced cellular changes (Fig. 1). Thus, the intricate balance between triggering of a variety of antagonistic stress-surveillance mechanisms by target cells either prompts NK cell activation or induces tolerance.

Major activating receptors on NK cells, including NKG2D and the natural cytotoxicity receptors (NCRs) NKp30, NKp44 and NKp46, recognize pathogen-derived or self 'stress' molecules on diseased target cells, including tumor cells. NKG2D, by far the best-studied of these receptors, recognizes the stress-induced self-proteins MHC class I related protein A and B (MICA and MICB) (18) and unique long 16 (UL16)-binding proteins (ULBPs) (19), all of which share homology with MHC class I molecules. Surface expression of these NKG2D ligands is selectively increased on transformed cells from hematological and solid origin (18,20), allowing NK cells to distinguish healthy from aberrant cells through NKG2D. In patients treated for hematological malignancies, genetic polymorphisms in ULBPs correlate with

relapse-free survival (21), demonstrating the relevance of NKG2D ligands to clinical outcome. Alongside NKG2D, the NCRs play major roles in tumor cell lysis by NK cells (22-24), although so far only few NCR ligands have been identified. B7H6, a homolog of CD80 (B7-1) and CD86 (B7-2), which is expressed on hematological tumor cells but not healthy cells, has recently been identified as a ligand for NKp30 (25). In addition, NKp30 recognizes HLA-B-associated transcript 3 (BAT3), a nuclear factor that is secreted and translocated to the cell surface in stressed and transformed cells (26). Importantly, expression of alternatively spliced isoforms of NKp30 such as the immunosuppressive NKp30c associates with unfavourable prognosis of cancer patients (9), emphasizing the relevance of NKp30-mediated tumor recognition in vivo. Most recently, a variant isoform of mixed-lineage leukemia-5 (MLL5) has been identified as a ligand for NKp44 (8). Although MLL5 is expressed on all healthy tissues, the variant isoform is selectively expressed on tumor cells, where it is able to induce NKp44-mediated NK cell activation in vitro (8). Evidence suggests that also non-protein stress signatures may be recognized by NCRs, as recent studies demonstrated that NCRs may bind heparan sulfate proteoglycans (HSPG) (27,28). These sugar moieties are ubiquitously expressed on normal tissue cells, but may be overexpressed or presented as unique tumor-associated variants on tumor cells (29), providing NK cells with additional tumor sensing capabilities through NCRs. Although the identification of these NCR ligands has helped the understanding of NCR-mediated NK cell cytotoxicity, it is most likely that other ligands are yet to be identified. Moreover, detailed insights into NCR-mediated NK cell cytotoxicity are just emerging, as

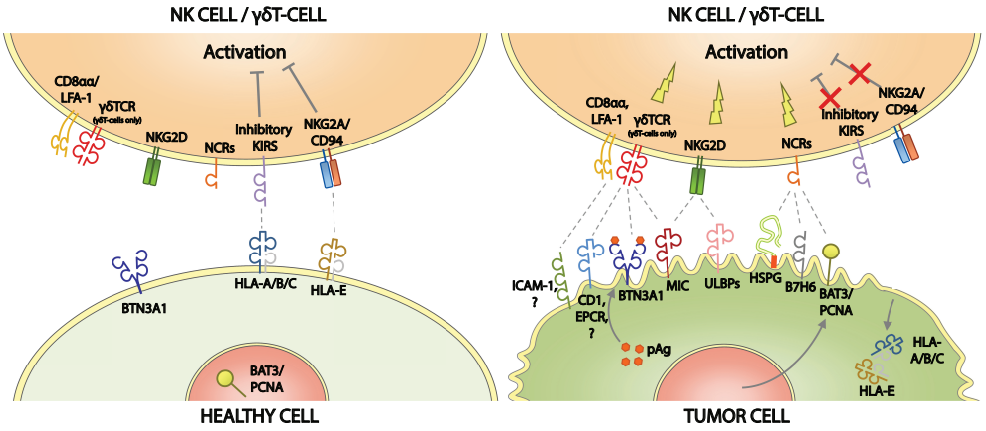

Figure 1. Target recognition by innate-like receptors on NK cells and γδT cells. Selective recognition of malignant and virus-infected cells by NK cells and γδT cells is mediated by both activating and inhibitory receptors. NK and γδT cells do not respond to healthy tissue cells due to ubiquitous expression of MHC class I molecules (HLA-A/B/C/E) that bind inhibitory receptors (KIRs and NKG2A/CD94), as well as the lack of molecular stress signals to activate NKG2D, NCRs or (in the case of γδT cells) the γδTCR (left panel). Upon malignant transformation or viral infection, loss of MHC class I molecules together with the upregulation of stress molecules causes signalling of activating receptors that overrule inhibitory signals, resulting in activation of NK cells and γδT cells. (pAg: phosphoantigen).

exemplified by the identification of proliferating cell nuclear antigen (PCNA) as a ligand for the activating NCR NKp44, an interaction that paradoxically leads to inhibition rather than activation of NK cells through an immunoreceptor tyrosine-based inhibitory motif (ITIM) in NKp44 (30). Thus, substantial gaps still remain in the understanding of NCR-mediated tumor recognition, and this represents a major obstacle for current clinical efforts aimed at using NK cells for the treatment of hematological cancers.

NK cells: inhibitory KIRs and the concept of allogeneic NK cells

In addition to activating receptors, NK cells express inhibitory receptors that continuously sense the presence of MHC class I molecules constitutively expressed on virtually all healthy cells (31). Expression of class I MHC molecules may be down-regulated upon viral infection or malignant transformation to escape detection by conventional T cells, and NK cells are capable of sensing this 'loss of self' via reduced signaling through their inhibitory receptors. In humans, two main types of inhibitory receptors are responsible for the continuous surveillance against missing self (Fig. 1). Inhibitory killer cell immunoglobulin-like receptors (KIRs) detect classical MHC class I molecules, also termed human leukocyte antigens (HLA) -A, -B, and -C, while the NKG2A-CD94 complex binds the non-classical MHC molecule HLA-E (31). The classical HLA molecules comprise a highly polymorphic family of HLA-A, -B and -C alleles, and each of the seven identified inhibitory KIRs preferentially recognizes a distinct subset of HLA alleles. Importantly, each individual inherits a KIR repertoire, or haplotype, with a subset of available KIR alleles, resulting in a wide variability between KIR haplotypes among individuals. Consequently, a hallmark report by Ciccone and colleagues demonstrated that NK cells that express inhibitory KIRs matched to self HLA alleles kill allogeneic cells when their inhibitory KIRs are not engaged due to a mismatch in HLA alleles (32). Implications of such 'alloreactivity' by NK cells have been demonstrated in animal organ transplant models, where KIR/HLA-mismatch leads to attack of the transplant by alloreactive NK cells and subsequent graft rejection (33). In the setting of hematopoietic stem cell transplantation, the impact of NK cell alloreactivity on transplantation outcome has been demonstrated by the introduction of haploidentical transplantation protocols (i.e. donor and recipient share one HLA haplotype but are fully mismatched for the other). In haploidentical transplantation, which can be applied in the absence of a suitable HLA-matched donor, T cells are frequently depleted from the graft to prevent GVHD caused by the substantial lack of HLA matching (1). This results in a prominence of innate-like cells after allo-SCT which is usually not observed after non-T cell depleted transplantations (34-36), and thus allows to study the impact of a juvenile innate-like immune system on the control of leukemia and infections. Remarkably, patients with a KIR/HLA-mismatched stem cell donor generally develop NK cells alloreactive against host cells, including leukemic cells, while having a reduced risk of developing GVHD (37,38). Nevertheless, translating these findings into the clinic has so far been challenging due to the complexity and inter- and intra-individual plasticity of the KIR system (39). This includes, but is not limited to, different avidities of defined KIRs with their HLA counterpart (40). KIR allelic variations (41,42). and most importantly a rapid induction of NK cell tolerance due to a regulatory immune environment which can render active NK cells into useless bystanders

(43,44).

NK cells: can virus-reactive NK cells cross-recognize tumor cells?

Although NK cells were first described for their anti-tumor-reactivity, they play important roles in the immune response against pathogens as well (45). As discussed above, NK cells sense the viral immune-evasive down-regulation of MHC class I molecules, for example by the CMV-encoded proteins UL16 and UL142, through inhibitory KIRs. However, such immune evasion mechanisms may also inhibit surface expression of the MHC class I-related proteins MICA/B and ULBPs, rendering target cells undetectable through NKG2D on NK cells (46,47). Similarly, the CMV tegument protein pp65 serves as a ligand for the activating receptor NKp30, but instead of leading to activation of NK cells this interaction results in the inhibition of NK cytotoxicity by disrupting the NKp30-CD3ζ activating complex (48). Although these mechanisms could in principal impede NK cell-mediated antiviral immunity, for example after an allo-SCT, important roles for NK cells in controlling CMV infection have been implied by the observation that CMV reactivation associates with marked increases in circulating NK cells in allo-SCT patients but also healthy immunocompetent individuals (49,50). These CMV-induced NK cells displayed a cytotoxic CD56dim phenotype and could be characterized by expression of the HLA-E-specific activating NK receptor NKG2C. More evidence for a protective role for NK cells in CMV infection comes from reports showing that the expression of certain activating KIRs (KIRs that lack the inhibitory ITIM domains of inhibitory KIRs but instead associate with activating signalling molecules (31)) associates with decreased CMV reactivation after stem cell transplantation (51,52). Nevertheless, the mechanisms leading to NK-mediated protection from CMV, including the viral or self-ligands recognized by the activating KIRs, so far remain unclear. It is noteworthy that the expansion of cytotoxic NK cells in response to viral infection could have important consequences for tumor control in patients after allo-SCT, as expression of activating KIRs has also been associated with protection from leukemic relapse (53-56). Moreover, the expression of additional tumor-sensing receptors such as NCRs and NKG2D by virus-driven NK cells could allow a broad cross-reactivity not only against viral infection but importantly also against leukemic cells (57,58). However, these hypotheses and indirect assumptions have never been formally investigated in patients receiving an allo-SCT. Thus, to date it is difficult to judge whether NK cells that are expanded upon CMV reactivation truly have a beneficial impact on controlling leukemia, as previously suggested (15).

γδT cells: a surprising clonal diversity in anti-tumor function makes translation challenging

The biological importance of γδT cells is emphasized by a recent report demonstrating that γδT cell signatures occurred in vertebrate ancestors already roughly 500 million years ago (59), thus having survived extremely long evolutionary selection pressures to date. Similar to NK cells and in agreement with their innate-like character, γδT cells are implicated in the rapid response to a variety of disease conditions, including malignant transformation, by lysing target cells and secreting high amounts of cytokines such as IFNγ (6). Underlying this functional resemblance is a surprising overlap in transcriptional profiles of NK cells and γδT cells (60). γδT cells express, like NK cells, activating and inhibitory NK receptors that modulate

their activation (61) (Fig. 1), although the exact involvement of these receptors in γδT cell activation remains puzzling. This is in part due to the expression of an additional unique activating immune receptor by γδT cells, namely the somatically rearranged T cell receptor (TCR). γδT cells exclusively express TCRs composed of a γ and a δ chain, and these γδTCRs can strongly contribute to γδT cell activation alongside NK receptors. For example, engagement of NKG2D serves to augment TCR-mediated activation of γδT cells in some settings (62), while in others NKG2D triggering may be sufficient for γδT cell activation without involvement of the γδTCR (63). Furthermore, as reported for NK cells, NKp30, NKp44 and NKp46 have been shown to be sufficient for inducing γδT cell cytotoxicity against tumor cells, although strikingly the expression of these NK receptors on γδT cells depended on prior activation via the γδTCR (64). Together, these observations have been used to suggest differential and complementary functions of the TCR and NK receptors on γδT cells, where binding of the γδTCR to its cognate antigen is frequently required for cellular activation while NK receptors mediate a further fine-tuning through an additional discrimination between healthy and diseased cells (61).

Definition of cognate antigens for γδTCRs has proven extremely challenging and this lack of knowledge has substantially hampered the progress in preclinical and clinical investigation of this cell population. The general assumption has so far been that the γδTCR recognizes a variety of molecular stress signals on infected or transformed cells. Thus, in contrast to conventional αβTCRs, γδTCRs do not rely on antigen presentation by classical MHC molecules. Two distinct subsets of γδT cells have been identified based on tissue localization and associated expression of defined TCRγ and TCRδ chains. In human peripheral blood the predominant γδT cell subset carries Vγ9Vδ2+ TCRs and comprises 1-5% of circulating T cells, while γδT cells located in epithelial tissues express diverse Vγ chains paired with mainly Vδ1 or Vδ3 chains and represent approximately 50% of all local T cells (65). Although ligands of γδTCRs have only scarcely been identified (Fig. 1), it seems that this apparent distinction based on localization and TCR gene usage is also reflected in the antigens that are recognized by both subsets. Vγ9Vδ2 T cells are activated by self or pathogen-derived non-peptidic prenyl pyrophosphates (also termed phosphoantigens), of which intracellular levels are elevated in tumor cells due to a dysregulation of the mevalonate pathway of isoprenoid synthesis (66). Importantly, recent efforts have implicated a key role for the butyrophilin BTN3A1 in mediating phosphoantigen-dependent activation of Vγ9Vδ2 T cells (10,12). BTN3A1 is a member of the B7 family, like the NKp30-ligand B7H6, and is ubiquitously expressed in healthy tissues. Thus, Vγ9Vδ2 T cells respond to tumor-associated elevations in phosphoantigen levels via BTN3A1, although mechanistically it remains unclear how phosphoantigens and BTN3A1 together form a cellular stress signature that is recognized by Vγ9Vδ2 TCRs. Importantly, work from our group has clearly demonstrated that individual Vγ9Vδ2 TCRs associate with distinct affinities towards their target (67). The functional avidity of individual Vγ9Vδ2 γδT cells towards tumors varies therefore widely between Vγ9Vδ2 γδT cell clones and is mediated by at least three components: (a) clonal diversity in the complementarity-determining-region 3 (CDR3) of γδTCRs of different clones (67), as well as the heterogeneity in the expression of (b) activating NCRs and (c) inhibitory KIRs. This suggests that so far pursued strategies based on the application of unselected γδT cells e.g. from donor peripheral blood might also transfer

largely ineffective or even unwanted cell populations. These new insights have important implications for therapeutic concepts: even though phosphoantigen-reactivity appears to be a universal feature of Vγ9Vδ2 T cells, distinct clones within the polyclonal Vγ9Vδ2 T cell population contribute differentially to tumor-recognition, suggesting that selection of optimal Vγ9Vδ2 T cell subpopulations will be crucial for successful clinical application.
Rather than responding to small phosphoantigens, ligands of γδTCRs on epithelial tissue-localized γδT cells (also called $Vδ2^{neg}$ γδT cells) include MHC class I-related molecules such as MICA/B and CD1 (11,68-71) (Fig. 1). Stress-related upregulation of MICA/B expression is observed on tumors of both epithelial (18) and hematological (20) origin. Interestingly, on γδT cells that express γδTCRs specific for MICA/B, NKG2D competes with the γδTCR for binding to MICA/B (72), suggesting a complex interplay between at least these two receptors in the process of γδT cell activation. Other subsets of $Vδ2^{neg}$ T cells may recognize CD1, a family of MHC class I-like molecules that is involved in the presentation of lipid antigens. In humans, five CD1 molecules are expressed (CD1a to CD1e) and so far, CD1a, c and d have been identified as ligands of $Vδ2^{neg}$ γδTCRs (11,68,70,71). Since few tumors express CD1, recognition of CD1 by γδT cells more likely serves to modulate other immune functions, including adaptive immune responses. For example, $Vδ2^{neg}$ γδT cells that recognize stress-induced self-lipids presented by CD1c on dendritic cells (DCs) produce high levels of TNFα that reciprocally induce the maturation of these DCs (73). These matured DCs were in turn able to efficiently present peptide antigen and to activate naïve αβT cells, demonstrating how γδT cell-mediated stress surveillance could contribute to the initiation of adaptive immune responses (71,73). Immunomodulatory roles of γδT cells are further emphasized by observations that γδT cells are themselves capable of functioning as antigen-presenting cells (APCs) with efficacies that rival DCs (74,75), and of directly interacting with B cells and T cells (65). Thus, γδT cells might not only shape adaptive immune responses under physiological conditions but could also serve as vehicle for alternative therapeutic vaccines, being easily accessible and at least as potent as DCs.

γδT cells: a valuable cross-reactivity to viral infection and cancer

Important roles for γδT cells have been described in immunity against viruses, including CMV (76). In both healthy individuals as well as immunocompromised patients such as after organ transplantation, CMV infection associates with marked expansions of γδT cells of the $Vδ2^{neg}$ subset (77,78). We and other have recently expanded these observations to patients with hematological malignancies, where CMV reactivation after allo-SCT correlated with increased numbers of circulating $Vδ2^{neg}$ γδT cells (13,79). CMV-reactive γδT cells also expanded upon CMV reactivation in patients receiving umbilical cord blood grafts, suggesting that CMV-reactive γδT cells can also be obtained from a naïve innate-like immune repertoire (13), and emphasizing the value of such third-party stem cell sources for use in allo-SCT. Importantly, subsets of CMV-reactive γδT cells are capable of cross-recognizing solid (80) as well as hematological (13) cancers, underscoring the potential clinical value of such γδT cells in e.g. the setting of allo-SCT, where these cells could provide protection against both CMV disease and leukemic relapse. Indeed, recent studies in large allo-SCT cohorts show a

strong favourable association between CMV reactivation after allo-SCT and a reduced risk of leukemic relapse (15,16), and CMV- and leukemia-crossreactive γδT cells provide a likely explanation for these puzzling observations (81). However, little is known about the antigenic signature that is recognized by γδT cells on CMV-infected cells. The γδTCR plays an important role in surveillance against CMV infection, as demonstrated recently by the identification of the novel γδTCR ligand endothelial protein C receptor (EPCR) which is upregulated on the cell surface of infected cells (14). Strikingly, EPCR expression is also increased on epithelial tumor cells and facilitated the cross-recognition of solid tumor cells by $V\delta2^{neg}$ γδT cells. Expression of EPCR on target cells alone was not sufficient for activation of $V\delta2^{neg}$ γδT cells however, which also required costimulation via LFA-1/ICAM-1 (14). In this line, we have recently identified CD8αα, which is commonly expressed on γδT cells, as a costimulatory molecule for activation of defined tumor-reactive Vδ1 TCRs by hematological malignancies (13), although the ligand of CD8αα on tumor cells remains unclear. In addition to the γδTCR, other receptors such as KIRs are likely to be involved in the sensing of CMV infection, as we reported that recognition of leukemic tumor cells, but not CMV-infected cells, was mediated by the γδTCR of cross-reactive γδT cell clones (13).

In summary, the innate-like NK cell and γδT cell immune populations are capable of responding rapidly to a wide variety of infections and solid and hematological malignancies, and they have been attributed direct and indirect roles in tumor immunosurveillance and disease. This is mediated by shared but also unique receptors that in part recognize related antigens (such as the B7 family members BTN3A1 and B7H6, and the MHC-like molecules MICA/B and CD1), reflecting unifying tumor-sensing mechanisms of these innate-like immune cells. The emerging insights into cross-reactivity of innate-like cells to malignancies and viral infection combined with the lack of classical MHC-restriction in the process of antigen recognition, put NK cells, γδT cells and their individual receptors in a new spotlight as attractive tools to overcome the obstacles associated with hematological cancers and allo-SCT. Consequently, efforts to apply these innate-like immune cells in the clinic are growing exponentially.

CLINICAL RESULTS USING INNATE-LIKE CELLS AGAINST HEMATOLOGICAL CANCERS

NK cells: heterogeneity in clinical outcome

The first NK cell-based clinical trials to address relapse after hematopoietic stem cell transplantation depended on the administration of low dose interleukin-2 (IL-2) to activate patient NK cells in vivo (82,83) (Table 1). Overall however, before the emerging insights into KIR/HLA-mismatching and NK cell alloreactivity a rather limited efficacy of such NK cell-based strategies has been reported across trials (82,84). Consequently, the concept of HLA/KIR-mismatching was implemented in haploidentical transplantation trials by taking advantage of the genetic disparity between leukemia patients and the stem cell donor. Transplantation of haploidentical stem cell grafts containing alloreactive NK cells to leukemia

Table 1. Key clinical results using innate-like cells to treat hematological cancers

Cell type	Strategy	Type of cancer	Clinical outcome	Remarks	Reference
NK cells	Repeated administration of low dose IL2 after autologous stem cell transplantation	NHL and breast cancer	No clinical evaluation, but a ~10-fold increase in circulating CD56hi NK cells with increased ex vivo lytic capacity was observed	Phase I trial to determine feasibility of IL2 administration for in vivo NK cell activation	(83)
	Haploidentical allo-SCT with and without KIR ligand incompatibility between donors and recipients	ALL and AML	HLA/KIR mismatch: 0% relapse in AML patients HLA/KIR match: 75% relapse in AML patients	The first trial to avoid NK cell self-tolerance by exploiting HLA/KIR mismatch between donor and recipient	(38)
	Adoptive transfer of haploidentical NK cells in a non-transplantation setting	HL, AML, RCC and melanoma	CR in 26% of AML patients	Demonstrated the potential value of allogeneic NK cell infusion irrespective of the use of allo-SCT	(90)
	Retrospective anal ysis of KIR/HLA-mismatches in HLA-matched allo-SCT	AML	HLA/KIR mismatch: 27% relapse in AML patients HLA/KIR match: 33% relapse in AML patients	Showed that also in HLA-matched allo-SCT a mismatch between activating KIRs and HLA-C	(91)
γδT cells	Administration of aminobisphosphonate and low-dose IL2 to activate autologous γδT cells	NHL and MM	33% PR, but only in patients selected for positive in vitro γδT cell proliferation	The first trial demonstrating therapeutic potential of in vivo activation of γδT cells	(94)
	Repeated administration of autologous ex vivo expanded γδT cells and IL2	MM	65% SD	The only trial focused on hematological malignancies to infuse ex vivo expanded γδT cells so far	(95)
	Administration of aminobisphosphonate and low-dose IL2 to activate autologous γδT cells	AML, RCC and melanoma	25% PR in AML patients	Treatment resulted in objective clinical responses in AML patients, but not in RCC and melanoma patients	(93)

CR: complete response; PR: partial response; SD: stable disease; RCC: renal cell carcinoma; Allo-SCT: allogeneic stem cell transplantation; ALL: acute lymphoid leukemia; AML: acute myeloid leukemia; NHL: non-Hodgkin lymphoma; HL: Hodgkin lymphoma; MM: multiple myeloma

patients associated with reduced relapse risk and increased survival in the absence of GVHD (38,85), although transplantation parameters appear to be of critical importance, as studies using different protocols failed to replicate these findings (86,87). Of note, the beneficial effect of KIR/HLA-mismatch is most prominent in grafts depleted of T cells rather than non-depleted grafts (86,88), presumably because the beneficial effect of alloreactive NK cells is overruled by alloreactivity of T cells in the graft (89). The activation of NK cells seems to be crucial for anti-tumor efficacy as well, as suggested by the observation that infusion of IL-2-preactivated allogeneic NK cells, obtained from haploidentical donors by lymphapheresis, combined with a repeated IL-2 injection regime induced in vivo NK cell expansions and complete remission

in ~30% of poor-prognosis leukemia patients (90). More recently also in HLA-matched (i.e. non-haploidentical) transplantations the mismatch between activating KIRs on NK cells transplanted as part of a stem cell graft and defined HLA-C alleles has been suggested as a possible mechanism to increase the graft-versus-tumor effect by increasing allo-reactivity of NK cells (91). However, the usage of such strategies critically depends on the genetic background of donors and recipients, making suitable donor choices challenging.

γδT cells: first steps into the clinic

To date, all trials using γδT cells focused on autologous Vγ9Vδ2 γδT cells that were either activated in vivo by injecting cancer patients with aminobisphosphonates (compounds that specifically activate Vγ9Vδ2 γδT cells) combined with IL-2, or activated and amplified ex vivo and adoptively transferred into the patient (92). Most clinical trials have been conducted on solid tumors, with only three trials reported so far to apply γδT cells in the hematological setting (Table 1). Two trials relied on the administration of low dose IL-2 together with aminobisphoshonate to activate autologous γδT cells in patients with diverse hematological malignancies in a non-transplantation setting (93,94). Treatment was generally well-tolerated, and clinical responses, including partial remission and disease stabilization, were observed in both trials. In one additional small-scale pilot study, autologous γδT cells which had been activated ex vivo with aminobisphosphonate and IL-2 were applied to non-transplanted myeloma patients, and disease stabilization was observed in ~65% of patients (95). Despite these promising results, clinical outcome has generally been limited. Possible causes for a lack of clinical efficacy can be found in trials aimed at applying γδT cells against solid tumors, where γδT cells have been found to be incapable of (a) expanding to sufficient cell numbers in a substantial number of patients in vivo or ex vivo (96), (b) homing properly to tumor sites (97), and (c) displaying sufficient anti-tumor cytotoxicity (98). As for NK-cell based therapies, factors limiting clinical outcome may also include a missing genetic disparity between γδT cell donor and recipient and the application of heterogeneous cell populations.

In the context of allo-SCT to treat hematological malignancies, we and others have employed an 'innate-enriched' approach where allogeneic stem cell grafts were depleted of αβT cells and B cells, but not γδT cells and NK cells (trial-registration NTR2463 and NTR3079, J. Kuball, and ref. (99), Fig. 2). Previous work has shown that increased numbers of donor γδT cells after stem cell transplantation correlates with better overall survival of leukemia patients without increased risk of GVHD (100). Although survival data of currently running trials are yet unavailable, these trials have pointed not only to direct effector functions but more importantly also to intriguing immunomodulatory roles of γδT cells. Firstly, γδT cells can activate NK cells to kill tumors (101), and thus might be able to solve the crucial problem of impaired NK cell activation after adoptive transfer that is usually faced in so far pursued clinical protocols. Secondly, αβTCR/CD19-depleted but usually not CD3/CD19-depleted haploidentical stem cell grafts rapidly reconstitute a broad donor αβT cell repertoire in the absence of αβT cell-mediated GVHD (J. Kuball, unpublished observations, and ref. (99)). Although the mechanisms behind this observation are yet unclear, we reported that repopulating donor γδT cells after allo-SCT are able to partially mature DCs in an antigen-

independent fashion (13). Given that partial maturation of DCs favors induction of αβT cell tolerance rather than alloreactivity (102), αβTCR/CD19-depleted grafts that still contain γδT cells may contribute to a tolerized αβT cell reconstitution after allo-SCT via interaction with DCs. Considering the first clinical data it is therefore tempting to speculate that γδT cells mediate a well-balanced 'innate immune-crosstalk' which is able to kick-off a shaped adaptive immune response that does not associate with extensive GVHD, but allows to at least partially control infections such as CMV. However, whether the reconstituting immune-repertoire has the ability to sufficiently control leukemia remains unclear.

TOWARDS ENGINEERING OF INNATE ANTI-TUMOR IMMUNITY

Without any doubt, the non-MHC-restricted recognition of a wide variety of hematological tumors and viruses by NK cells and γδT cells makes these innate-like cells very attractive candidates for the development of innovative therapies for hematological malignancies, as well as for tackling the current complications of allo-SCT due to relapse, infection or GVHD. However, technical obstacles arise through the largely underappreciated diversity of innate-like cells, as obtaining sufficient numbers of the right NK and/or γδT cells from generally scarce immune populations consequently requires extensive ex vivo expansion, frequently leading to exhaustion of cells before reinfusion into the patient (103). Thus, limited and highly variable clinical responses in trials are most likely a consequence of the fact that these strategies so far relied on poorly defined, heterogeneous cell preparations that express varying levels of activating and inhibitory receptors. As discussed above, receptors such as γδTCRs may appear identical at first sight, but can mediate different affinities to the very same ligands. In addition, recent evidence shows that γδT cells have the potential to differentiate into regulatory cells that suppress adaptive anti-tumor responses (104,105), and that they may be involved in the pathogenesis of inflammatory diseases by producing high amounts of pro-inflammatory cytokines such as interleukin-17 (IL-17) (106,107). Thus, cellular therapeutic products contain so far not only cytotoxic but also regulatory and pro-inflammatory subsets with partially opposite biological impacts resulting in a potential self-neutralization of the product in vivo. Most importantly, many interactions of innate-like immune cells depend on a certain genetic mismatch which is usually not found in an autologous situation. Reactivity is furthermore frequently shut down with a progressing in vivo education of innate-like cells (31), resulting in loss of potency of such strategies over time. To overcome these obstacles, recent preclinical efforts have focused on designing immune cells to express defined tumor-reactive innate-like receptors, thus allowing the selection of non-MHC-restricted receptors with the highest anti-tumor and anti-viral activity without causing detrimental GVHD, as well as the uncoupling of defined receptors from potential education or silencing mechanisms of parental cells.

For example, engineering immune cells using NK receptors has been explored. Chimeric receptors linking NKG2D to the cytoplasmic domain of CD3ζ have been used to redirect αβT cells to various cancers (108,109) (Fig. 2). Importantly, owing to the recognition of multiple

tumor antigens by NKG2D, transduced T cells were capable of suppressing in vivo tumor growth of multiple related tumor types (110). In the context of treating hematological malignancies, autologous or, in an allo-SCT setting, donor αβT cells could therefore be equipped with such chimeric receptors, be expanded and infused into the patient. Nevertheless, such engineered αβT cells still express endogenous αβTCRs with the potential to cause GVHD, a drawback that may be avoided by using NK cells (111) or γδT cells as an alternative vehicle for such

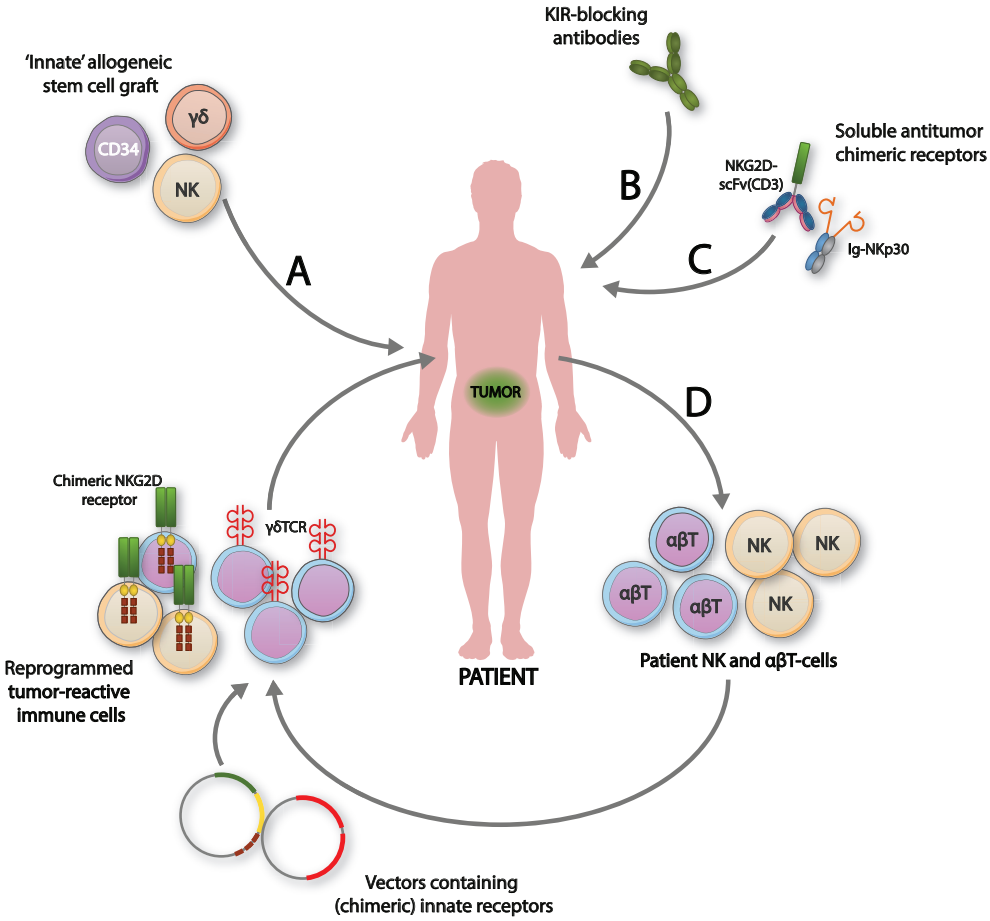

Figure 2. Designing immunotherapies in the context of hematological malignancies using innate-like cells and receptors. In the setting of an allo-SCT, 'innate' stem cell grafts depleted of αβT cells and B cells may be engineered to improve adaptive immune reconstitution and anti-tumor protection in the absence of graft-versus-host disease (A). Alternatively, blocking antibodies against inhibitory KIRs (B) and soluble chimeric receptors that exploit the antigen recognition specificity of receptors such as NKG2D or NCRs (C) may be engineered, produced and administered to patients. Finally, T cells or NK cells isolated from cancer patients may be engineered to express tumor-reactive innate-like receptors such as defined γδTCRs or chimeric NKG2D receptors, and after expansion be reinfused into the patient (D).

receptors. As an alternative to primary NK cells, NK cell lines have been generated that display potent cytotoxicity towards various tumors due to a lack of inhibitory KIR expression (112), and such cell lines have been tested in clinical trials (113). NK cell lines furthermore readily allow genetic engineering to express tumor-reactive (chimeric) receptors (114). In alternative attempts, soluble versions of NK receptors have been generated, such as a bispecific fusion protein that engages tumor cells through an NKG2D domain and recruits and stimulates T cells through an anti-CD3 single-chain variable fragment (115). Chimeric NCRs mimicking antibody-based immunotherapy have also been generated, such as a fusion protein where the tumor-recognizing extracellular domain of NKp30 is fused to the constant region of IgG to allow recruitment of antibody-mediated immune components (116). Importantly, infusion of these chimeric NK receptors into tumor-bearing mice substantially reduced tumor burden. Additionally, soluble anti-KIR antibodies have been developed in order to overcome in vivo silencing of innate immune cells and such very interesting compounds are currently tested in clinical trials (117) (Fig. 2).

As an alternative strategy, work from our laboratory demonstrates that αβT cells can be efficiently reprogrammed against a broad range of solid and hematological tumor cells by introducing defined Vγ9Vδ2 or Vδ1 TCRs (13,20,67) (Fig. 2). Working with γδTCRs overcomes the major drawbacks of current T cell engineering concepts using αβTCRs, namely the classical HLA-restriction of αβTCRs and the formation of potentially auto-reactive TCRs due to mispairing of introduced with endogenous αβTCR chains (118,119). γδTCRs are furthermore, in contrast to chimeric antigen receptors (120), genetically unmodified and therefore non-immunogenic. Expression of γδTCRs in αβT cells moreover induces the down-regulation of surface expression of the endogenous αβTCR, resulting in reduced in vitro alloreactivity against HLA-mismatched healthy cells (20,67). γδTCR-engineered αβT cells obtained from for example allogeneic stem cell donors therefore will presumably have a reduced risk of causing αβTCR- mediated GVHD in allo-SCT patients, although this premise will need to be tested in clinical trials. γδTCRs can also be further engineered by a technique we termed combinatorial-γδTCR-chain exchange (CTE), where TCRγ and TCRδ chains of distinct tumor-reactive γδTCRs are newly combined to generate high-affinity γδTCRs with enhanced functional avidity towards tumors but not healthy cells (67). Finally, this strategy overcomes draw-backs of γδT cells by taking advantage of the proliferative capacity of αβT cells, which unlike that of γδT cells, is still preserved in advanced stage patients (118). In this way, cells engineered with innate receptors are uncoupled from further education which usually occurs in innate immune cells.

CONCLUDING REMARKS

Taken together, NK cells and γδT cells represent innate-like immune populations with highly diverse contributions to the immunosurveillance against cancer and infection, and with unique advantages for the application in the context of hematological malignancies and allo-SCT. Their functions are mediated by a crucial cross-talk not only between these two

subsets but importantly also with components of adaptive immunity. In addition, it becomes increasingly clear that a careful genetic matching is not only vital for strategies taking advantage of adaptive but also of these innate-like immune cells. Focusing on engineering of immune cells with innate-like recognition features could therefore overcome limitations of many current adoptive immunotherapies by: (a) focusing on defined receptors with sufficient affinity and broad reactivity to multiple tumor types (b) that do not require to be completely HLA-matched to the recipient patient; (c) uncoupling engineered immune cells from silencing of conventional innate-like cells by e.g. KIRs; and (d) allowing the generation of high numbers of cytotoxic immune cells or compounds within the time constraints of developing disease. Clinical trials will need to be pursued in order to test efficacy and safety of the application of such strategies.

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CHAPTER 3

γδT cells elicited by CMV reactivation after allo-SCT cross-recognize CMV and leukemia

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ABSTRACT

Human cytomegalovirus (CMV) infections and relapse of disease remain major problems after allogeneic stem cell transplantation (allo-SCT), in particular in combination with CMV-negative donors or cordblood transplantations. Recent data suggest a paradoxical association between CMV-reactivation after allo-SCT and reduced leukemic relapse. Given the potential of Vδ2-negative γδT cells to recognize CMV-infected cells and tumor cells, the molecular biology of distinct γδT cell subsets expanding during CMV-reactivation after allo-SCT was investigated. Vδ2^{neg} γδT cell expansions after CMV-reactivation were observed not only with conventional but also cordblood donors. Expanded γδT cells were capable of recognizing both CMV-infected cells as well as primary leukemic blasts. CMV- and leukemia-reactivity were restricted to the same clonal population, whereas other Vδ2^{neg} T cells interact with dendritic cells (DCs). Cloned Vδ1-TCRs mediated leukemia-reactivity and DC-interactions, but surprisingly not CMV-reactivity. Interestingly, CD8αα expression appeared to be a signature of γδT cells after CMV exposure. However, functionally CD8αα was primarily important in combination with selected leukemia-reactive Vδ1-TCRs, demonstrating for the first time a co-stimulatory role of CD8αα for distinct γδTCRs. Based on these observations, we advocate the exploration of adoptive transfer of unmodified Vδ2^{neg} γδT cells after allo-SCT to tackle CMV-reactivation and residual leukemic blasts, as well as application of leukemia-reactive Vδ1-TCR-engineered T cells as alternative therapeutic tools.

INTRODUCTION

Human cytomegalovirus (CMV) is a widely prevalent herpesvirus that, after primary infection, persists life-long in the human host. Although infections are asymptomatic in most immunocompetent individuals, reactivation of the virus in immunocompromised patients after allogeneic stem cell transplantation (allo-SCT) can lead to life-threatening complications including colitis and pneumonia (1). Moreover, CMV infection is associated with increased risk of acute graft-versus-host disease (GVHD) (2,3) and predisposes to secondary infections due to CMV-induced immunesuppression (4). Paradoxically, recent evidence shows that CMV reactivation after allo-SCT reduces the risk of leukemic relapse (5,6), suggesting an unexpected favorable association between CMV infection and clearance of tumor.

Multiple cell populations have been reported to be involved in clearance of CMV infection. A vast body of information has been gathered for CMV-specific αβT cells and NK-cells (7). For NK-cells it has been hypothesized that they may cross-recognize CMV-infected cells and cancer cells by responding to CMV-infected residual AML blasts (6), which may contain considerable CMV copy numbers. An alternative population that might also contribute to a better control of leukemia after CMV-reactivation is represented by γδT cells. In recent years numerous studies have established the importance of γδT cells, a minor T cell population in peripheral blood but prominently present at sites of CMV replication such as epithelial tissues, in both anti-viral immunity and tumor-surveillance (8). Contrary to αβT cells, activation of γδT cells

does not rely on antigen-presentation by MHC but is instead mediated by pathogen-derived antigens or self molecules that are upregulated on infected, transformed or otherwise stressed cells. In adult peripheral blood the major γδT cell subset expresses T cell receptors (TCRs) composed of Vδ2 and Vγ9 gene segments (therefore also referred to as Vδ2^{pos} γδT cells) and is activated by small, nonpeptidic phosphoantigens of pathogen or self origin (9,10). In contrast, γδT cells that reside in epithelial tissues express TCRs composed of mainly Vδ1 or Vδ3 chains paired with diverse Vγ chains, and a proportion of these γδT cells (collectively called Vδ2^{neg} γδT cells) expresses CD8αα (11,12).

The involvement of γδT cells in the immune response against CMV has been established by studies in transplant patients as well as healthy individuals, showing that CMV infection associates with marked in vivo expansions of specifically Vδ2^{neg} γδT cells that are reactive against CMV-infected cells (13-17). Furthermore, expansion of Vδ2^{neg} T cells upon CMV infection was shown to correlate with clearance of the virus (18). In addition to the anti-CMV response, numerous studies have implicated γδT cells in tumor host defense: γδT cells have been found to infiltrate tumors of diverse origin in vivo (19-21) and both Vδ2^{neg} and Vδ2^{pos} subsets have been abundantly shown to be cytotoxic to cancer cells in vitro (10,22-24).

Taken together these reports have strongly established the importance of Vδ2^{neg} γδT cells in the immune response against CMV and in tumor-surveillance. In the present study we therefore evaluated the potential anti-leukemia capacity of γδT cells that expand upon CMV-reactivation in a population of patients with hematological malignancies receiving allo-SCT from either conventional or cordblood donors. We show that in this cohort CMV-reactivation after allo-SCT associates with in vivo expansions of CMV-reactive Vδ2^{neg} γδT cells. These CMV-induced γδT cells are capable of cross-recognizing hematological cancers, and thus may explain the favorable effect of CMV-reactivation on risk of leukemic relapse. In addition, such cells can serve as tools either from third party donors to tackle CMV infection and leukemia or by taking advantage of here-identified receptors to redirect T cells against leukemia.

METHODS

Cell lines and antibodies (see Supplementary Methods)

Patients, allo-SCT and blood sampling

A cohort of 26 patients with various hematological diseases (Supplementary Table 1), who received an allo-SCT at the UMC Utrecht, from December 2005 until August 2008, was analyzed. Allo-SCT was given as curative or as rescue treatment to patients younger than 70 years with available HLA-matched related or unrelated donors, or with cordblood grafts. Patients were treated according to clinical protocols approved by the local ethics board and gave their informed consent. Outcome of allo-SCT of these patients was retrospectively analyzed in terms of hematopoietic recovery, viral reactivations, acute and chronic GVHD and progression free and overall survival (25,26). After allo-SCT, patients were weekly monitored

for 3 months for CMV-reactivation by real-time automated CMV-DNA PCR using a TaqMan® probe. For patients with conventional stem cell donors, PBMCs of these time points were isolated and stored in liquid nitrogen until phenotypic analysis or expansion. Blood samples of cordblood patients were collected 50-100 days after transplantation. Absolute counts of CD3+, γδTCR+ and Vδ2+ T cells were determined using TRUcount tubes® (BD), according to the manufacturer's protocol. PBMCs were stained for γδTCR, Vδ2, CD3, CD4, CD8α, CD16, CD25, CD27, CD45RO, CD56, CD80, and HLA-DR. The cohort of newborns with CMV infection has been described recently (17).

Expansion and isolation of γδT cell lines
γδT cells were isolated and expanded using a previously described REP-protocol (27) (see Supplementary Methods).

Functional T cell and DC maturation assays
IFNγ-ELISPOT, 51Chromium-release and DC maturation assays were performed as previously described (28,29) (see Supplementary Methods).

Cloning of γδTCRs and retroviral transduction of T cells
γδTCRs were isolated and sequenced as described in Supplementary Methods. Clone TCRs, Vγ9Vδ2-TCR clone G115 (30) and a HLA-A*0201-restricted $WT1_{126-134}$-specific αβTCR (31) were transduced into αβT cells as described (27,29,32) (see Supplementary Methods).

Statistical analyses
Differences were analyzed using indicated statistical tests in GraphPad Prism 5.0 for Windows (GraphPad Software Inc.).

RESULTS

CMV-reactivation after allo-SCT associates with expansion of $V\delta2^{neg}$ γδT cells in both CMV-positive and CMV-negative stem cell donors
In order to test whether an increase in $V\delta2^{neg}$ γδT cells is observed during CMV-reactivation after allo-SCT, blood samples of 26 patients with umbilical cordblood (n=10) and adult stem cell donors (n=16, Supplementary Table 1) were collected after allo-SCT and analyzed by flow cytometry. Nine patients (56%) with adult stem cell donors developed a CMV-reactivation within 3 months after allo-SCT. In agreement with previous work in the context of allo-SCT (14) but also other transplantation settings (13,16), CMV-reactivation but not EBV-reactivation associated with a significant increase in absolute numbers of circulating donor γδT cells in patients with conventional adult stem cell donors (Fig. 1A, left panel; Supplementary Table 2). Expression analysis of CD45RO and CD27 indicated significantly lower percentages of naïve (CD45ROnegCD27pos) γδT cells in CMV-reactivating patients (Supplementary Fig. 1A), suggesting expansion of effector cells in these patients. Also all patients with cordblood

grafts, thus CMV-naïve donors, that developed a CMV-reactivation (n=6) had significantly increased numbers of circulating γδT cells when compared to time-matched non-CMV-reactivating patients (n=4; Fig. 1A, right panel), although due to logistic challenges no time-course evaluation was possible in this cohort. In both patient populations the increase in γδT cells was due to an increase in the Vδ2^{neg} subset (Fig. 1B), while Vδ2^{pos} γδT cells did not significantly differ between CMV-reactivating and non-reactivating patients (adult grafts: median 2.17 versus 2.39 cells/μl, P = 0.37; cordblood grafts: median 4.38 versus 0.81, P =

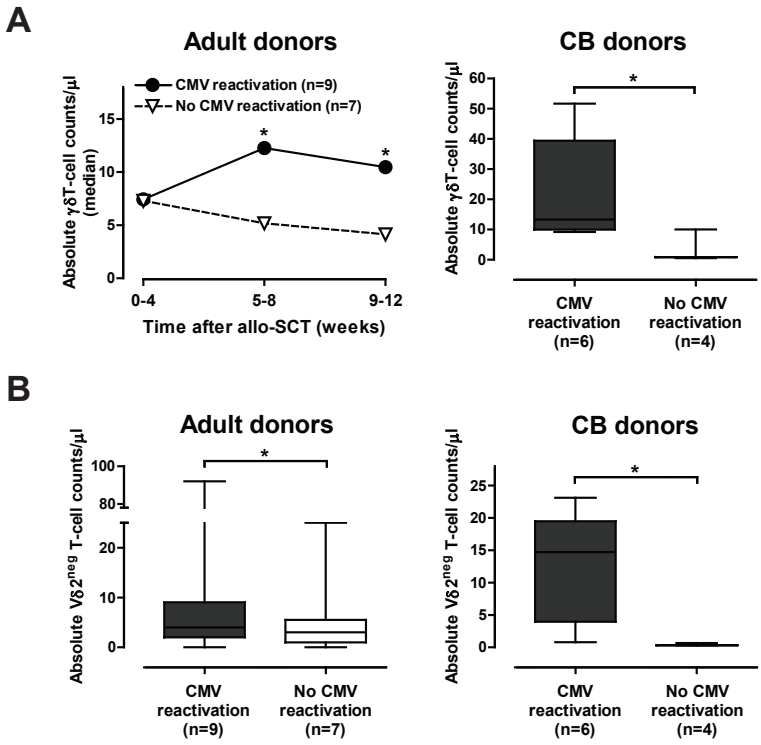

Figure 1. Selective expansion of Vδ2neg γδT cells in patients with CMV-reactivation after allo-SCT. (A) Blood samples of patients with conventional adult stem cell donors were collected weekly after allo-SCT (left panel) or during CMV-reactivation in patients with cordblood-derived grafts (right panel), and presence of γδT cells was analyzed by flow cytometry. In the left panel, median values of all patients are presented. For patients with conventional donors, most CMV-reactivations were observed in the second and third month after transplantation. (B) Presence of Vδ2neg γδT cells was analyzed in patients with conventional and cordblood donors by flow cytometry. In patients with conventional stem cell donors (left panel) Vδ2neg γδT cells were measured in the second and third month after allo-SCT, in patients with cordblood grafts at the same timepoint as in (A). In box plots, the line at the middle is the median, the box extends from the 25th to 75th percentile, and the whiskers extend down to the lowest value and up to the highest. Mann Whitney U test was used to assess differences between CMV-positive and CMV-negative patients, and significant differences are indicated (* P < 0.05).

0.38). No differences were observed in total CD3+ T cell numbers between CMV-reactivating and non- reactivating patients (adult grafts: median 437 versus 355 cells/μl, P = 0.80; cordblood grafts: median 58 versus 97 cells/μl, P = 0.38). In order to assess whether the increase in Vδ2^{neg} γδT cells during CMV- reactivation was mainly driven by γδT cells expressing a public Vγ8Vδ1-TCR, which has been reported to play a substantial role in congenitally infected newborns (17), clonality of increased cell fractions was analyzed by spectratyping. However, when analyzing clonality of Vδ1, Vδ2 and Vδ3 γδT cells no such enrichment was observed in selected patients (Supplementary Fig. 2). Interestingly, the increase in γδT cells in CMV-reactivating patients preceded the increase of αβT cells, as a significant difference in αβT cells between CMV-reactivating and non-reactivating patients was not observed until 3 months after allo-SCT (Supplementary Fig. 1B).

Together, these data confirm in a rather small but apparently representative cohort that CMV-reactivation after transplantation of CMV-seropositive stem cell grafts associates with a significant increase of donor Vδ2^{neg} γδT cells. Notably, Vδ2^{neg} γδT cells were also elicited during CMV-reactivation when CMV-naïve cordblood grafts were used.

Patient-derived Vδ2^{neg} γδT cells specifically recognize CMV-infected and transformed cells ex vivo

To functionally evaluate whether Vδ2^{neg} γδT cells that expanded in vivo upon CMV-reactivation could indeed contribute to an anti-CMV immune response, γδT cells were isolated from CMV-reactivating patients and analyzed ex vivo. Patient-derived bulk, Vδ2^{neg}, and Vδ2^{pos} γδT cell subsets were coincubated with CMV-infected fibroblasts and γδT cell activation was measured by IFNγ-ELISPOT. γδT cells isolated from patients with conventional stem cell grafts secreted significantly higher levels of IFNγ upon contact with CMV-infected cells when compared to uninfected controls (Fig. 2A). In line with previous studies (13,14), CMV-reactivity of patient-derived γδT cells was mediated exclusively by Vδ2^{neg} γδT cells, but not Vδ2^{pos} γδT cells (Fig. 2A). Importantly, γδT cells isolated from cordblood patients produced IFNγ in response to and were able to specifically lyse CMV-infected cells (Fig. 2B).

Previously it has been reported that γδT cells that expand upon CMV-reactivation are able to cross-recognize solid cancer cells (33,34), however cross-reactivity with leukemic cells has not been reported. Therefore, patient-derived Vδ2^{neg} and Vδ2^{pos} γδT cells were coincubated with a variety of hematological cancer cell lines and primary acute myeloid leukemia (AML) blasts. Indeed, CMV-reactive Vδ2^{neg} γδT cells were able to specifically recognize lymphoma (Daudi), leukemia (BV173, K562 and KCL22), and myeloma (U266) cell lines, and most importantly primary AML blasts (Fig. 2C). In contrast, Vδ2^{pos} γδT cells from selected patients responded to hematological cell lines but not to primary AML samples, although reactivity could be induced after treating AML cells with the bisphosphonate pamidronate, a compound that induces the accumulation of Vδ2^{pos} γδT cell-activating phosphoantigens in treated cells (29) (data not shown). In summary, CMV-reactivation after allo-SCT associates with an increase in multipotent Vδ2^{neg} γδT cell populations from both CMV-positive and naïve stem cell donors that are able to recognize both CMV-infected cells and hematological tumor cells.

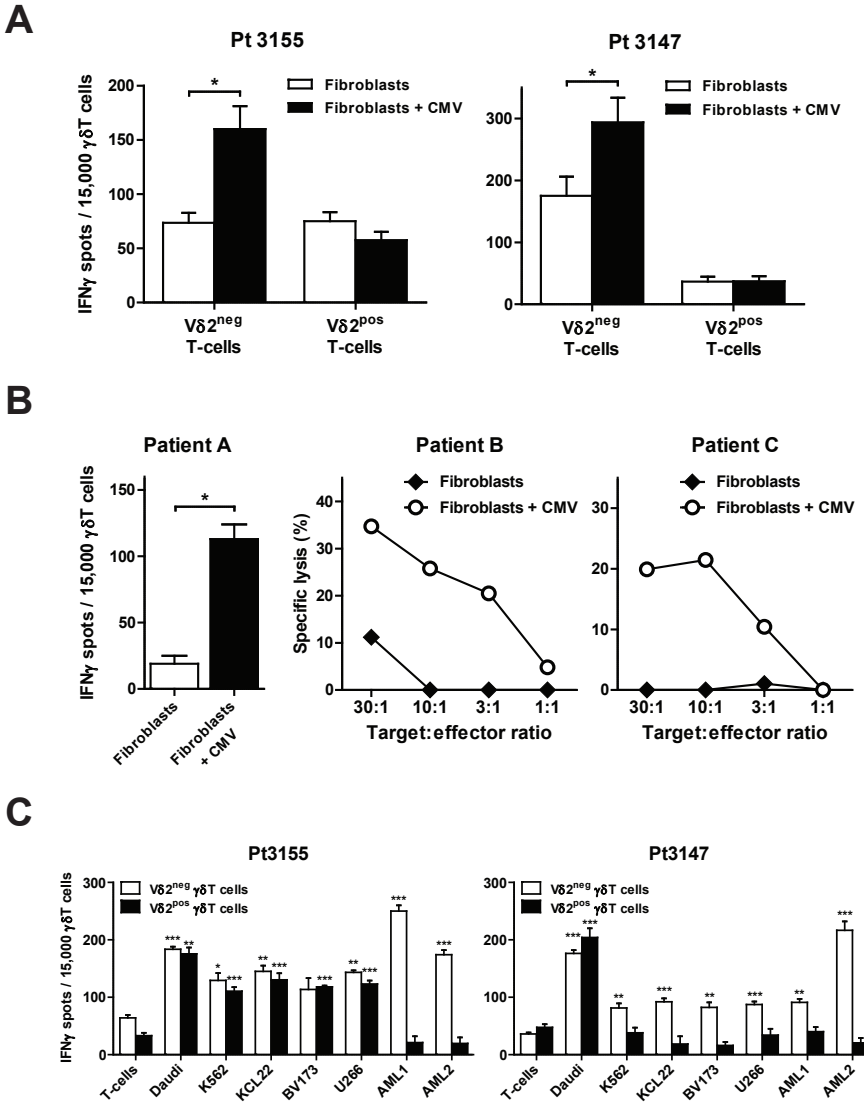

Figure 2. Specific recognition of CMV-infected and leukemic cells by patient Vδ2neg γδT cells. (A) γδT cells isolated from patients with conventional adult stem cell donors were expanded and cultured ex vivo before MACS-sorting and use in functional analysis. Sorted Vδ2pos or Vδ2neg γδT cells were subsequently cocultured for 18 hours with CMV-infected or -uninfected human foreskin fibroblasts and γδT cell activation was measured by IFNγ-ELISPOT. Results from two representative patients are shown. (B) Left panel: γδT cells from patients with cordblood transplantations were tested for CMV-reactivity as in (A). Unsorted γδT cells isolated from these patients predominantly (up to 90%) consisted of Vδ2neg T cells, as determined by flow cytometry. Right panel: Killing capacity of γδT cells from cordblood patients against CMV-infected fibroblasts was determined by coincubating γδT cells and CMV-infected fibroblasts in a 4-6hr 51Chromium-release assay. Uninfected fibroblasts served as control. Data from three different patients

Figure 2. (continued) are shown. (C) MACS-sorted Vδ2pos and Vδ2neg γδT cells from the same patients as in (A) were used to test anti-tumor recognition. Vδ2pos or Vδ2neg γδT cells were cocultured with indicated hematological cancer cell lines or primary leukemic blasts (at 3:1 target:effector ratio) in IFNγ-ELISPOT. For both γδT cell populations healthy unsorted T cells served as negative control target. Error bars represent SEM. Student t test (A, B) or one-way ANOVA (C) was used to assess differences between γδT cell responses (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

CMV-reactive Vδ2^{neg} γδT cell clones cross-recognize leukemic cells, including primary AML blasts

To investigate whether cross-reactivity of Vδ2^{neg} γδT cells to leukemic blasts and CMV-infected fibroblasts is restricted to different clonal populations, Vδ2^{neg} γδT cells were cloned by limiting dilution. All generated clones carried Vδ1^{pos} γδTCRs and expressed the natural killer receptor NKG2D (Fig. 3A and Supplementary Fig. 3). Two clones (B11 and E1) heterogeneously expressed CD8αα. CMV-reactivity of generated clones was subsequently analyzed by coincubation with either CMV-infected or uninfected fibroblasts. Two Vδ1^{pos} γδT cell clones (B11 and E1) responded to CMV-infected fibroblasts by increased IFNγ production, while two other clones (D3 and E2) did not (Fig. 3B). None of the clones produced TNFα in response to CMV-infected cells (data not shown). Next, CMV-reactive Vδ1^{pos} γδT cell clones B11 and E1 were coincubated with the hematological tumor cell lines U266, T2 (T- and B-lymphoblastoid cell line), EBV-LCL (Epstein-Barr virus-transformed lymphoblastoid cell line) or primary AML blasts. Both CMV-reactive clones displayed a potent IFNγ-response against all (clone B11) or most (clone E1) tested tumor cell lines as well as primary AML samples (Fig. 3C) but not healthy fibroblasts. However, leukemia-reactivity was not a feature of all isolated clones, as clones D3 and E2 did not produce IFNγ or TNFα in response to leukemic cell lines or blasts (data not shown). Together, these data suggest that here-isolated CMV-reactive clones are able to cross-recognize hematological tumor cells.

The interaction of Vδ2^{neg} γδT cells with DCs is clonally segregated from CMV- and leukemia-reactivity and is mediated by individual γδTCRs

Because isolated clones D3 and E2 did not show a cytokine response against CMV-infected fibroblasts nor leukemic cells (data not shown), we hypothesized that such clones elicited after CMV infection are involved in maturation of dendritic cells (DCs) (35), and thereby may aid in mounting adaptive immune responses. Therefore, monocyte-derived immature DCs were cultured alone or in the presence of Vδ1^{pos} γδT cell clones and expression of the maturation markers CD80 and CD86 on DCs was measured after 48 hours. Selectively in the presence of Vδ1 γδT cell clones D3 and E2, but not CMV- and leukemia-crossreactive clones B11 and E1, a substantial and significant increase in CD80/CD86+ DCs was observed compared to immature DCs alone (Fig. 3D), resembling the phenotype of DCs matured by the classical maturation cocktail (prostaglandin E2, IL-1β, IL-6 and TNFα) (36). However, no detectable production of IL12p70 was induced by Vδ1 γδT cell clones (data not shown). Importantly, induction of this characteristic mature phenotype of DCs by clones D3 and E2 was observed in the absence of

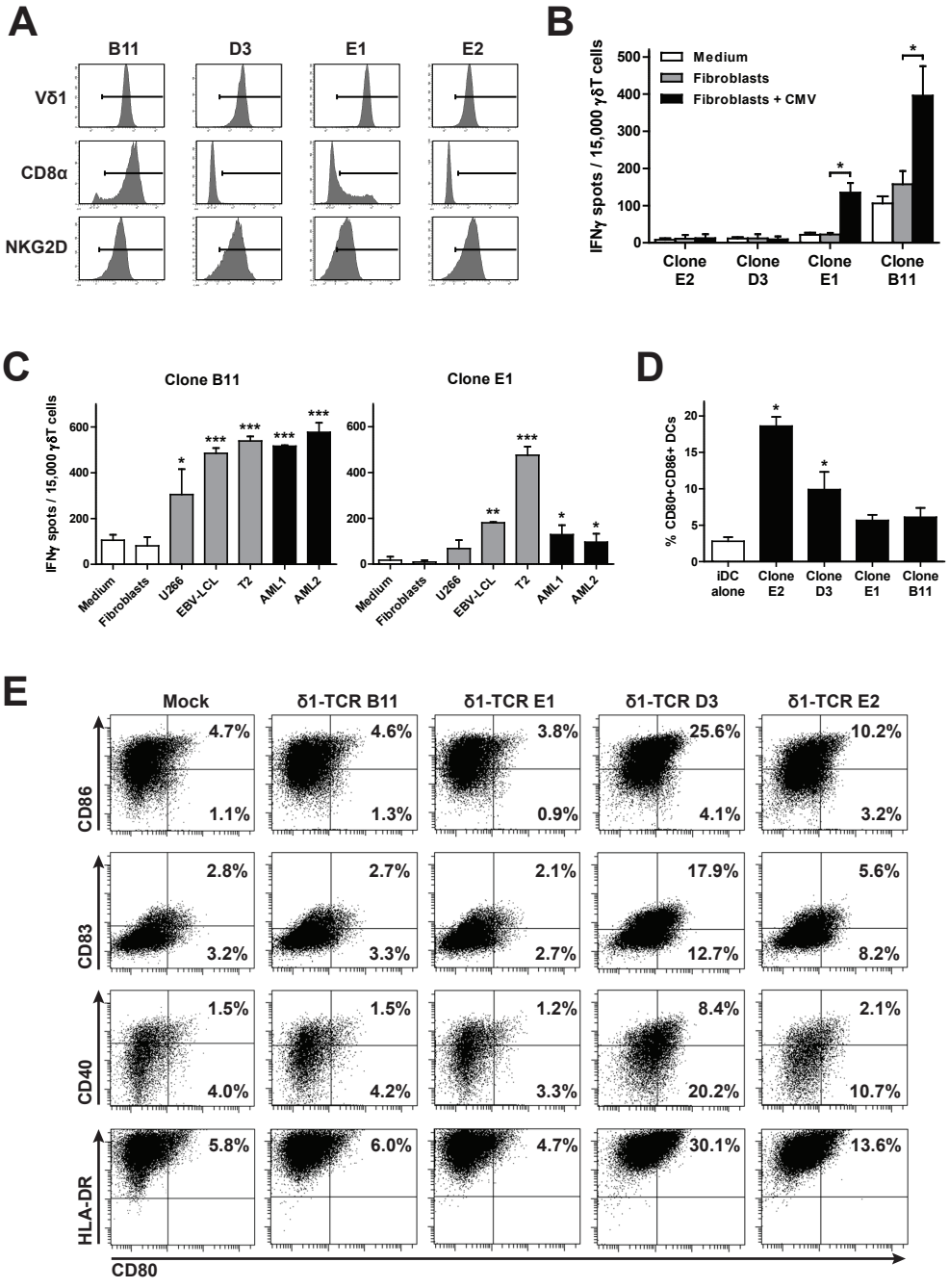

Figure 3. CMV-reactive Vδ2neg γδT cell clones cross-recognize cancer cells, but do not interact with DCs. (A) Vδ2neg γδT cell clones were generated by limiting dilution and phenotyped by flow cytometry. (B) CMV-reactivity of generated clones was determined by incubating clones alone or in

Figure 3. (continued) combination with CMV-infected or uninfected fibroblasts (at 3:1 target:effector ratio) for 18 hours in an IFNγ-ELISPOT assay. (C) CMV-reactive Vδ2neg γδT cell clones E1 and B11 were cultured alone, with hematological cancer cell lines or with primary leukemic blasts for 18 hours and IFNγ-production was determined by ELISPOT. Healthy human fibroblasts served as negative control. (D) Vδ2neg γδT cell clones were incubated with immature DCs for 48 hours and the percentage CD80/CD86 double-positive DCs was measured by flow cytometry. (E) TCR γ- and δ-chains of original Vδ2neg T cell clones were sequenced and retrovirally transduced into αβT cells. Transfer of DC-interacting capacities was tested by culturing mock-transduced T cells or T cells expressing clone-derived δ1-TCRs with immature DCs for 48 hours and measuring expression of maturation markers by flow cytometry. Error bars represent SEM. Student t test (B) or one-way ANOVA (C, D) were applied to assess differences between γδT cell responses (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

CMV-infected cells or CMV virions in cocultures, indicating that maturation of DCs by $V\delta2^{neg}$ γδT cell clones is independent of CMV-antigen.

To test whether induction of maturation markers on DCs by $V\delta1^{pos}$ T cell clones D3 and E2 is mediated by their γδTCRs, Vγ- (Vγ4, Vγ8 and Vγ9) and Vδ1-chains of here-generated clones were sequenced (Supplementary Table 3) and retrovirally transduced into αβT cells. In agreement with our previous data on Vγ9Vδ2 TCRs (29), all clone-derived δ1-TCRs were efficiently expressed in both CD4+ and CD8+ αβT cells and down-regulated endogenous αβTCRs (Supplementary Fig. 4). The involvement of individual δ1-TCRs in the induction of the mature-like phenotype of DCs was subsequently analyzed by incubating transduced T cells with immature monocyte-derived DCs. Selectively δ1-TCRs that were isolated from clones E2 and D3 but neither δ1-TCRs E1 and B11 nor mock-transduced cells induced a marked (~3.5 to 9-fold) upregulation of CD80/CD86 on DCs (Fig. 3E) and increased TNFα secretion in culture supernatants (Supplementary Fig. 5A). In addition, a higher mean expression of CD40, CD83 and HLA-DR was detected on DCs after coincubation with E2-TCR- and D3-TCR-transduced T cells, but not T cells expressing δ1-TCRs E1 and B11. This phenotype depended on both CD1c, a lipid-presenting molecule previously reported to be involved in $V\delta2^{neg}$ γδT cell-mediated DC maturation (35), and TNFα (Supplementary Fig. 5B). As was observed in experiments with original clones, DCs did not produce detectable levels of IL12p70 (data not shown). Taken together, these data show that distinct clonal populations within the expanded $V\delta2^{neg}$ γδT cell subset are responsible for CMV- and leukemia-reactivity and for interacting with DCs, and that the $V\delta2^{neg}$ γδT cell-DC interaction involves defined δ1-TCRs.

Cancer-reactivity, but not CMV-reactivity, is mediated by distinct γδTCRs

To formally confirm that individual γδTCRs of Vδ1 clones B11 and E1 mediate CMV-reactivity, as reported after in primo utero CMV-infection (17), αβT cells transduced with clone-derived δ1-TCRs and the previously reported CMV-reactive Vγ8Vδ1-TCR (17) were incubated with CMV-infected or uninfected fibroblasts. Surprisingly, only T cells expressing the public CMV-reactive Vγ8Vδ1-TCR but neither here-cloned CMV-reactive nor non-reactive δ1-TCRs produced IFNγ after contact with CMV-infected cells (Fig. 4A and data not shown), suggesting that CMV-recognition by original clones B11 and E1 must rely on alternative surface receptors.

In order to test the mechanism involved in tumor recognition, clones B11 and E1 were tested for expression of NKp30, a receptor recently reported to be involved in anti-tumor reactivity by Vδ1^{pos} γδT cells (22). However, here-isolated clones did not express NKp30 (Supplementary Fig. 3). Thus, an alternative mechanism must mediate tumor-reactivity and could include the individual γδTCRs. Therefore, δ1-TCR- and mock-transduced αβT cells were cocultured with hematological cancer cell lines or primary AML blasts and T cell activation was determined by IFNγ-ELISPOT. Selectively T cells transduced with δ1-TCRs of CMV- and cancer-reactive clones B11 and E1 but not mock-transduced T cells were able to recognize both hematological cancer cell lines and primary AML cells, while healthy T cells were not recognized (Fig. 4B). Importantly, cancer-reactivity of both δ1-TCRs could be extended to solid cancers, since pharyngeal (Fadu) and breast cancer (MDA-MB231) cell lines also activated γδTCR- transduced T cells (Fig. 4B). T cells transduced with δ1-TCRs D3 or E2 produced neither IFNγ nor TNFα against tested cancer cells (data not shown). Thus, cancer-reactivity of selected Vδ1^{pos} γδT cell clones is mediated by their respective δ1-TCRs and this reactivity can be transferred to

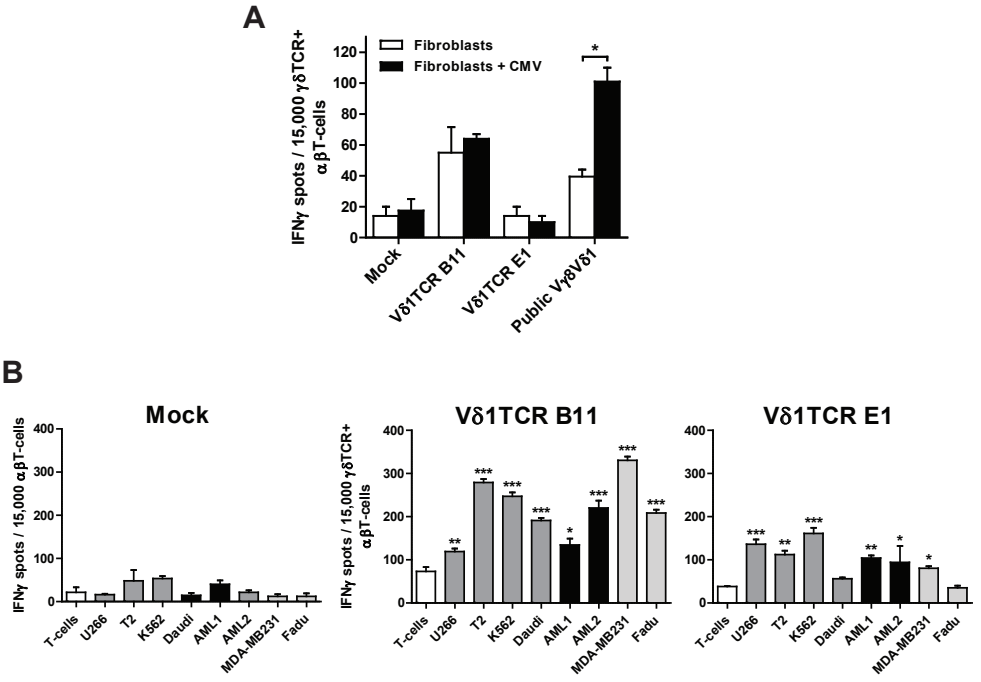

Figure 4. Isolated δ1-TCRs transfer cancer-reactivity, but not CMV-reactivity, to αβT cells. (A) αβT cells transduced with empty vector, with a public CMV-reactive δ1-TCR or with clone-derived δ1-TCRs were incubated for 18 hours with CMV-infected or uninfected foreskin fibroblasts and IFNγ-secretion was measured by ELISPOT. (B) αβT cells transduced with empty vector or with either the B11 or E1 δ1-TCR were cultured with primary AML blasts and hematological and solid cancer cell lines in an IFNγ-ELISPOT. Healthy T cells were used as negative control. Error bars represent SEM. Student t test (A) or one-way ANOVA (B) were used and significant differences are indicated (* P < 0.05; ** P < 0.01; *** P < 0.001).

previously non-reactive αβT cells.

CD8αα functions as critical coreceptor for selected tumor-reactive δ1-TCRs

δ1-TCRs isolated from clones B11 and E1 can be suitable tools to redirect αβT cells against leukemias as reported for Vγ9Vδ2-TCR clone G115, which reprograms both CD4+ helper and CD8+ cytotoxic αβT cells against a broad panel of tumor cells (29). Thus, we questioned whether also here-isolated tumor-reactive Vδ1^{pos} γδTCRs are able to redirect both subsets of αβT cells, CD4+ and CD8+, against cancer cells. CD4+ and CD8+ T cells expressing tumor-reactive δ1-TCRs B11 or E1 were therefore separated and incubated with T2 or Daudi target cell lines. CD4+ and CD8+ αβT cells transduced with δ1-TCR E1 produced similar levels of IFNγ in response to tumor target cells (Fig. 5A). In sharp contrast, δ1-TCR B11 was able to reprogram CD8+ but not CD4+ T cells, even though the introduced B11 TCR was expressed at slightly higher levels in CD4+ than in CD8+ T cells (Supplementary Fig. 4B), as reported previously (29,37). This suggested that for full T cell activation this γδTCR requires a molecule present on CD8+ but not CD4+ αβT cells, such as NKG2D or CD8. To address this, CD8+ T cells transduced with the B11 δ1-TCR were preincubated with blocking antibodies against CD8α, CD8β, or NKG2D and subsequently coincubated with Daudi or T2 target cells. Blocking of NKG2D, which is expressed on most CD8+ but not CD4+ αβT cells and can amplify αβ- and γδT cell responses (38,39), had only an effect on target cell recognition when T cells were transduced with a γ9δ2TCR as reported (29) (data not shown). Strikingly, blocking CD8α but not CD8β resulted in a marked decrease in IFNγ-secretion when compared to T cells pretreated with control antibody (Fig. 5B). Blocking capacity of CD8β antibody was confirmed by inhibiting MHC class I-restricted αβT cells. CD8α-blocking on CD4+ T cells expressing the B11 or E1 δ1-TCRs served as additional negative controls and did not influence T cell responses. Thus, the CD8α but not CD8β domain is important in the ligand interaction of the B11 δ1-TCR. These data indicate that depending on the particular γδTCR, tumor-reactivity is mediated by CD8α-dependent and -independent mechanisms, suggesting e.g. different affinities of here-cloned TCRs to their ligands.

The original clone B11 expressed the CD8αα homodimer but not the CD8αβ heterodimer (Supplementary Fig. 3). To test whether CD8αα was also involved in activation of the original B11 γδT cell clone, clone B11 (CD8+) and clone E1 (CD8low) were cocultured with T2 target cells in the presence of CD8α- or CD8β-blocking antibodies. Similar to the effect on T cells transduced with the B11 δ1-TCR, blocking of CD8α significantly inhibited IFNγ-production by the original clone (Fig. 5C). However, the effect of CD8α-blocking was less pronounced on the original clone compared to B11-transduced αβT cells, most likely due to lower expression of CD8αα on the parental clone when compared to CD8αβ expression on transduced T cells (data not shown). Again, blocking of CD8β did not affect IFNγ-secretion, as expected based on the CD8αα-positive phenotype of clone B11. As was observed in E1-transduced T cells, CD8α/β-blocking did not affect activation of clone E1 (Fig. 5C). To corroborate these observations, additional CD8αα-positive Vδ1 T cell clones were generated from a different donor and the effect of CD8α-blocking on activation of clones was analyzed. Of nine CD8αα+ clones tested, blocking CD8α but not CD8β inhibited activation of one clone that reacted to

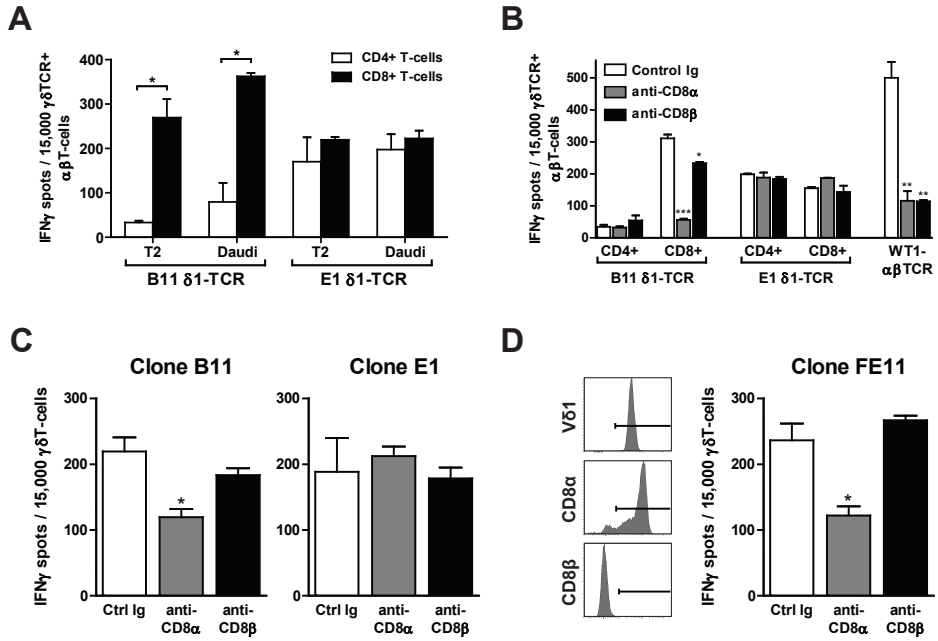

Figure 5. CD8αα acts as a coreceptor for selected δ1-TCRs. (A) CD4+ and CD8+ αβT cells transduced with B11 or E1 δ1-TCRs were sorted and subsequently cocultured with T2 or Daudi cell lines in an IFNγ-ELISPOT. (B) CD4+ and CD8+ transduced αβT cells were coincubated with T2 target cells as in (A), but now in the presence of a control antibody or blocking antibodies against CD8α or CD8β. αβT cells expressing a WT1126-134-specific αβTCR (31) that were coincubated with T2 cells pulsed with 10-6 M WT1126-134 peptide served as positive control for CD8α- and CD8β-blocking. (C) Original clones B11 and E1 were incubated with T2 target cells as in (B). (D) Clone FE11 was generated by limiting dilution, phenotyped by flow cytometry (left panel), and coincubated with SW480 target cells as in (B). Error bars represent SEM. Student t test (A) or one-way ANOVA (B, C, D) were used and significant differences are indicated (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

the colorectal cancer cell line SW480 (clone FE11), as measured by reduced IFNγ secretion (Fig. 5D). CD8α-blocking had no effect on activation of the parental polyclonal Vδ2^{neg}CD8+ γδT cell line of this donor nor of two other donors (data not shown), suggesting that CD8-dependence of defined γδT cell clones is not a general phenomenon yet observed in a substantial fraction (2 out of 10) of isolated clones.

CD8αα was in the majority of isolated clones not functionally involved in tumor-reactivity, questioning whether CD8αα rather plays a general role in CMV-reactivity. In order to assess whether an increase in CD8αα expression on γδT cells might be linked to CMV-infection in vivo, the cohort of conventional stem cell donors was analyzed for CD8 expression by flow cytometry. Strikingly, CMV-reactivating patients had significantly more circulating CD8+ γδT cells compared to non-reactivating patients (Fig. 6A). This observation was confirmed in a complementary cohort of congenitally CMV-infected newborns (Fig. 6B). In this cohort, CD8α

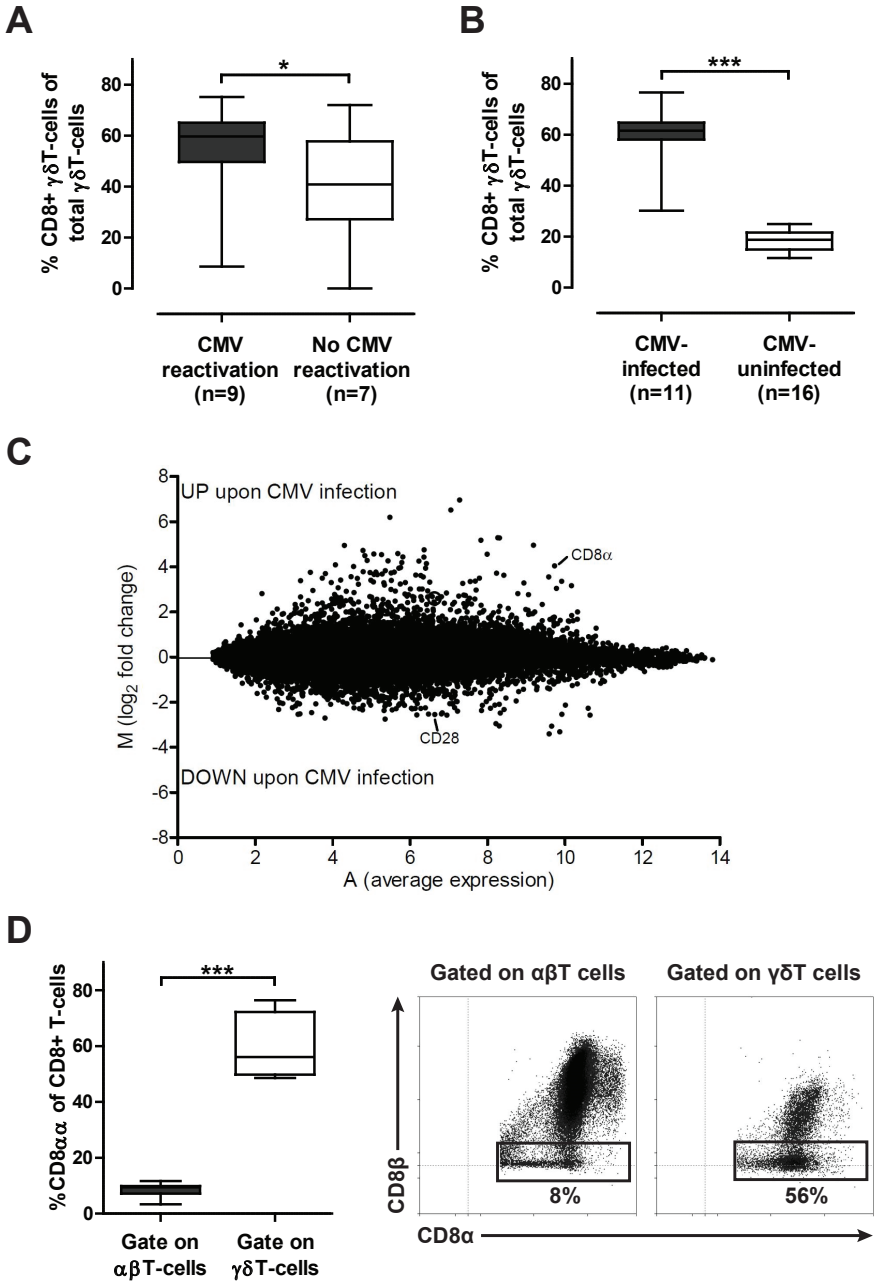

Figure 6. CMV reactivation after allo-SCT and congenital CMV infection associate with increased expression of CD8 on γδT cells. (A) The percentage of CD8+ γδT cells of patients with conventional stem cell donors was measured in the second and third month after allo-SCT by flow cytometry. (B) Cord blood from fetuses congenitally infected (n=11) or not infected (n=16)

Figure 6. (continued) was collected at term delivery and the percentage of γδT cells expressing CD8 was analyzed by flow cytometry. (C) Gene expression analysis of γδT cells derived from three CMV-infected newborns versus γδT cells derived from three CMV-uninfected newborns. MA plot of differentially expressed genes in γδT cells upon CMV infection. M (log2 of fold change) reflects the differential expression of a gene. Positive and negative values indicate genes which are up- and down-regulated, respectively, upon CMV infection. A (mean expression) reflects the overall expression level of a gene. Note that a similar figure, with indication of other genes, has been published before (17). The highly up-regulated expression of CD8α RNA is indicated. The down-regulation of CD28 RNA is indicated as well for comparison. (D) The majority of CD8 on γδT cells of congenitally infected newborns is composed of the CD8αα homodimer. Percentages of CD8+ γδT cells and CD8+ αβT cells expressing the CD8α+CD8β- phenotype were determined by flow cytometry in cordblood samples from eight congenitally infected newborns (left panel). Representative flow cytometry plots (right panel) illustrate the staining patterns of CD8α and CD8β on αβT cells and γδT cells. Mann Whitney U test (A, B) and Student t test (D) were used and significant differences are indicated (* $P < 0.05$; *** $P < 0.001$).

expression on γδT cells associated with a differentiated effector (CD27neg/lowCD28neg) (17) phenotype (Supplementary Fig. 6). Microarray gene expression profiling revealed highly upregulated expression of CD8α but not CD8β upon CMV-infection (Fig. 6C), and flow cytometry on blood samples of infected individuals indeed showed that CMV-associated expression of CD8 on γδT cells is preferentially of the αα homodimer (Fig. 6D). Of note, CD8+ αβT cells sorted from the same CMV-infected newborns did not show increased expression of CD8α (Fig. 6D). To test whether CD8αα plays a functional role in CMV-reactivity by Vδ2^{neg} γδT cells, clones B11 and E1 were coincubated with CMV-infected or uninfected fibroblasts in the presence of CD8α-blocking antibody. However, blocking CD8αα inhibited not only the specific recognition of CMV-infected cells but also the occasionally observed background reactivity of clone B11 but not clone E1 against freshly plated fibroblasts (data not shown), suggesting that CD8αα may interact rather with a general stress-antigen than an antigen specific for CMV infection. In summary, these data show that CD8αα expressed on human Vδ2^{neg} γδT cells associates with CMV-infection in vivo and is able to function as a critical costimulator on selected clones as well as on γδTCR-reprogrammed αβT cells when coincubated with tumor cells.

DISCUSSION

The contribution of Vδ2^{neg} γδT cells to controlling CMV-infection has received considerable attention in recent years, and it is now well-established that these unconventional T cells play important roles in the immune response to CMV-infection (13-15,17,34). Combined with their widely reported reactivity towards a variety of (mainly solid) tumors (19,21,23), this has made Vδ2^{neg} γδT cells a promising cell population for immunotherapeutic application. In the present study we demonstrate that Vδ2^{neg} γδT cells that expand upon CMV-reactivation after allo-SCT are capable of responding to both CMV-infected and leukemic cells. Additionally, by demonstrating that tumor-reactivity of Vδ2^{neg} γδT cells can be transferred by γδTCR

gene-transfer, and by identifying a novel role for CD8αα in the antigen-restriction of γδTCRs, we provide a solid basis for the therapeutic exploration of Vδ2^{neg} γδT cells and their γδTCRs.
Our observation that the occurrence of a single event (i.e. CMV-infection) is able to induce expansion of γδT cell subsets with anti-CMV- and anti-leukemia-reactivity, including reactivity against primary leukemic blasts, provides an alternative explanation for recent unexpected findings of a reduced relapse rate in patients with CMV-reactivation after allo-SCT (5,6). Furthermore, it is in line with a report in kidney transplant patients demonstrating that expansion of Vδ2^{neg} γδT cells following CMV-infection associated with a reduced risk of developing solid cancer post-transplantation (33). γδT cells isolated from these patients reacted against both CMV-infected cells and epithelial tumor cells in vitro. Thus, although CMV-reactivation after allo-SCT is still associated with substantial non-relapse-related mortality (e.g. GVHD, colitis, and secondary infections), reactivation of the virus reduces the risk of mortality due to relapse of leukemia, and we show that one possible link is a CMV-induced expansion of leukemia-reactive γδT cells. This hypothesis is further substantiated by clinical data demonstrating that increased numbers of γδT cells after allo-SCT are associated with improved disease-free survival, without higher incidence of GVHD (40).
Mechanistically, little is known about the requirements for γδT cell activation, and the identity of the molecules on CMV-infected and leukemic cells that are recognized by here-generated γδT cell clones so far remain elusive. Dual-reactivity of Vδ2^{neg} γδT cell clones to CMV and solid cancer cells has been reported and has led to the hypothesis that γδTCRs of dual-reactive cells recognize shared antigens on CMV-infected and transformed cells (34,41). However, our γδTCR-gene transfer experiments show that cancer-reactivity, but not CMV-reactivity is mediated by δ1-TCRs isolated in this study, indicating that alternative immune receptors may be responsible for CMV-reactivity of the original clones or that the γδTCR is involved but depends on additional molecules not expressed on αβT cells. In line with this, it was recently reported that the γδTCR isolated from a CMV-reactive Vγ4Vδ5 clone requires costimulation by CD11a-CD18 (LFA-1) (41). However, here-isolated CMV-reactive clones E1 and B11 as well as αβT cells transduced with their respective γδTCRs expressed high levels of CD11a (see Supplementary Fig. 7), suggesting that other mechanisms must be involved. Alternatively, it was recently shown that Vδ2^{neg} γδT cells could be stimulated by IgG-opsonized CMV virions via the IgG receptor CD16 (FcγRIIIa), independent of γδTCR-engagement (42). However, here-isolated CMV-reactive clones did not express CD16 (see Supplementary Fig. 3).
We report here for the first time that in human γδT cells CD8αα functions as restriction element for target recognition by distinct δ1-TCRs. Although long described to be expressed on γδT cells (11,12), the function of CD8αα on these cells has so far remained unknown. In our experiments, blocking CD8α resulted in a marked and significant inhibition of tumor recognition by different clones and distinct δ1-TCRs, which was not observed when tested on the bulk population. These data put CD8αα into the field of coreceptors for δ1-TCRs for a defined subset of tumor-reactive γδT cells. Moreover, we report that CMV-infection associates with an increase in CD8αα-expressing γδT cells in both allo-SCT patients as well as congenitally infected newborns, suggesting a link between CD8αα and the immune response against CMV in vivo. However, the functional involvement of CD8αα in γδT cell-mediated CMV-reactivity

remains to be further defined. Within the αβT cell compartment, CD8αα-positive T cells are enriched in mucosal tissues such as intestine and these cells are described to display a characteristic innate-like phenotype (43). However, on these cells CD8αα does not function as a classical MHC class I-binding αβTCR coreceptor as CD8αβ does, but more likely serves as suppressor of αβTCR-mediated T cell activation (44). A subset of NK cells also expresses CD8αα, and these cells possess greater killing capacity than CD8αα-negative NK cells (45). This effect was attributed to enhanced resistance to apoptosis that was specifically mediated through CD8αα-signaling (46). Superior cytotoxicity of CD8αα-expressing NK cells has been associated with clinical remission of leukemia patients (47,48), indicating that CD8αα on innate immune cells may be relevant to clinical outcome after allo-SCT. Finally, CD8αα on murine innate-like intestinal αβT cells was shown to enhance αβTCR-mediated T cell activation by binding the non-classical MHC-I molecule thymus leukemia (TL) (49). Thus, the expression of CD8αα on innate(-like) immune cells may indicate a universal role for CD8αα as regulatory receptor in innate immune responses.

To tackle CMV-infections in immuno-compromised patients, several clinical trials have focused on the adoptive transfer of CMV-reactive αβT cells (50,51). However, major obstacles are presented by the MHC-restricted antigen-recognition of αβT cells and the challenge to generate sufficient numbers of CMV-reactive αβT cells within the time constraints of severe infection (52). Our data suggest that Vδ2^{neg} γδT cells are an interesting alternative source of CMV-reactive T cells for such patients as we observe that in vivo generated Vδ2^{neg} γδT cells react against not only CMV-infected cells but also leukemic cells in vitro. Moreover, we demonstrate that CMV-reactive γδT cells can also be obtained from the naïve umbilical cordblood repertoire, underscoring the value of this third-party stem cell source for application in allo-SCT, in particular also for patients with CMV-negative donors. In summary, we advocate the exploration of adoptive transfer of unmodified Vδ2^{neg} γδT cells in CMV- and tumor-immunotherapies and the application of leukemia-reactive Vδ1-TCR-engineered T cells. Clinical trials will need to be pursued in order to test efficacy and safety of the application of such strategies.

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SUPPLEMENTARY METHODS

Antibodies and flow cytometry

Antibodies used for flow cytometry included: γδTCR-APC (clone B1, BD), γδTCR-PE (clone IMMU510, Beckman Coulter), γδTCR-FITC (clone 11F2, BD), Vδ2-PE and –FITC (clone B6, BD), Vδ1-FITC (clone R9.12, Beckman Coulter), αβTCR-PE-Cy5 (IP26A, Beckman Coulter), CD3-eFluor450 (clone OKT3, eBioscience), CD3-pacific blue (clone SP34-2, BD), CD4-PE-Cy7 (clone RPA-T4, BD), CD8α-APC (clone RPA-T8, BD), CD8α-PE-Cy7 (clone SFCI21Thy2D3, Beckman Coulter), CD8β-PE (clone 2ST8.5H7, BD), CD16-PE (clone CB16, eBioscience), CD27-APC-eFluor780 (clone 0323, eBioscience), CD27-APC (clone L128, BD), CD28-ECD (clone CD28.2; Beckman Coulter), CD40-APC (clone HB14, Biolegend), CD45RO-PE-Cy7 (clone UCHL1, BD), CD56-PE (clone B159, BD), CD80-PE (clone L307.4, BD), CD83-FITC (clone HB15e, BD), CD86-PE-Cy5 (clone IT2.2, eBioscience), NKp30-APC (clone P30-15, Biolegend), NKG2D-APC (clone 1D11, BD), CD158a(NKAT1)-FITC (clone HP-3E4, BD), CD158b(NKAT2)-PE (clone DX27, BD), NKB1(NKAT3)-FITC (clone DX9, BD), HLA-DR-APC-Cy7 (clone L243, Biolegend). All allo-SCT samples were processed with FACSCanto-II or LSR-II flow cytometers (BD) and analyzed with FACSDiva software (BD). Whole cord blood samples derived from infected and uninfected newborns were run on the CyAn flow cytometer and data were analyzed using Summit 4.3 (Dako).

Cell lines and primary acute myeloid leukemia cells

Daudi, K562, KCL22, T2, BV173, SW480, MDA-MB231, U266, foreskin fibroblasts and Phoenix-Ampho cell lines were obtained from ATCC. EBV-LCL was kindly provided by Phil Greenberg (Seattle, WA). Fadu was kindly provided by Niels Bovenschen (UMC Utrecht, The Netherlands). Fibroblasts and Phoenix-Ampho cells were cultured in DMEM supplemented with 1% Pen/Strep (Invitrogen) and 10% FCS (Bodinco), all other cell lines in RPMI with 1% Pen/Strep and 10% FCS. Fresh PBMCs were isolated by Ficoll-Paque (GE Healthcare) from buffy coats supplied by Sanquin Blood Bank (Amsterdam, The Netherlands). Where indicated, foreskin fibroblasts were infected with culture supernatants of fibroblasts previously infected with human CMV strain AD169 at a multiplicity of infection (MOI) of 2. After 24 hours, infected and uninfected fibroblasts were washed before being used in functional assays. Frozen primary acute myeloid leukemia (AML) samples were a kind gift from Matthias Theobald (Mainz, Germany) and were collected in compliance with GCP and Helsinki regulations.

Expansion and isolation of γδT cell lines

PBMCs were stimulated for 14 days with 1μg/ml PHA-L (Sigma-Aldrich), 50 U/ml IL-2 (Novartis Pharma), 5 ng/ml IL-15 (R&D Systems), and irradiated allogeneic PBMCs, Daudi and EBV-LCLs. Fresh IL-2 was added twice a week. After first expansion, polyclonal γδT cell lines were obtained by MACS-isolation (TCRγδ+ T cell isolation kit, Miltenyi Biotec) with a purity of >90% and were further expanded using again the REP-protocol. $V\delta2^{pos}$ and $V\delta2^{neg}$ γδT cell fractions were obtained by MACS-depleting $V\delta2^{pos}$ γδT cells from bulk cultures using Vδ2TCR-PE antibody and anti-mouse IgG microbeads (Miltenyi Biotec). γδT cells isolated from patients receiving

cordblood grafts typically contained up to 90% Vδ2^{neg} γδT cells and were therefore not further MACS-sorted. Vδ2^{neg} γδT cell clones were generated from a CMV-seropositive healthy donor by limiting dilution. All γδT cell cultures were stimulated biweekly using the REP-protocol.

Spectratyping and microarray experiments

Spectratyping analysis and microarray experiments were performed as previously described (1). Microarray data and procedures were deposited at Array Express (www.ebi.ac.uk/arrayexpress) under accession no. E-MEXP-2055.

Dendritic cell maturation assay

Monocytes were isolated from PBMCs by plate adhesion and differentiated into immature dendritic cells (iDCs) by culturing for 4 days in AIM-V medium in the presence of 500 U/ml IL-4 (Peprotech) and 800 U/ml GM-CSF (Peprotech). Next, iDCs were cocultured with T cells at a ratio of 1:1 for 48 hours and expression of CD40, CD80, CD83, CD86 and HLA-DR was measured by flow cytometry. Where indicated, CD1c-blocking antibody (clone L161, Biolegend), TNFα-blocking antibody (clone MAb1, eBioscience), or control antibody was added to cultures at a concentration of 20 μg/ml. Secretion of TNFα and IL12p70 was measured by ELISA (eBioscience).

Functional T cell assays

IFNγ-ELISPOT was performed by coculturing 15,000 T cells and 50,000 target cells (ratio 0.3:1) for 24 hours in nitrocellulose-bottomed 96-well plates (Millipore) precoated with anti-IFNγ antibody 1-D1K (Mabtech). Plates were washed and incubated with biotinylated antibody 7-B6-1 (Mabtech) followed by streptavidin-HRP (Mabtech). IFNγ spots were subsequently visualized with TMB substrate (Sanquin) and spots were quantified using ELISPOT Analysis Software (Aelvis). With regard to γδT cell clones, reactivity to CMV-infected cells and cancer cells was generally determined in the same experiment. Where indicated, blocking of CD8α was performed using 10 μg/ml anti-CD8α antibody clone OKT8 (eBioscience), blocking of CD8β with 10 μg/ml anti-CD8β clone 2ST8.5H7 (Abcam), and NKG2D-blocking with 10 μg/ml anti-NKG2D clone 149810 (R&D Systems).

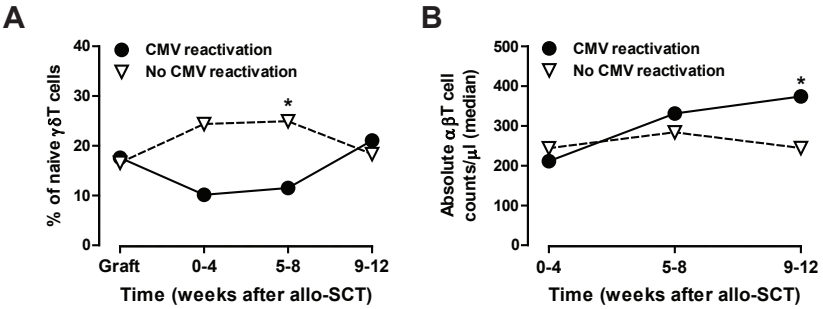
^{51}Chromium-release assays was performed as described (2,3). Target cells were labeled overnight with 150 μCu ^{51}Cr and subsequently incubated with γδT cells in four effector-to-target ratios (E:T) between 30:1 and 1:1. ^{51}Cr-release in supernatant was measured 4-6hr later.

Cloning of γδTCR genes and retroviral transduction of T cells

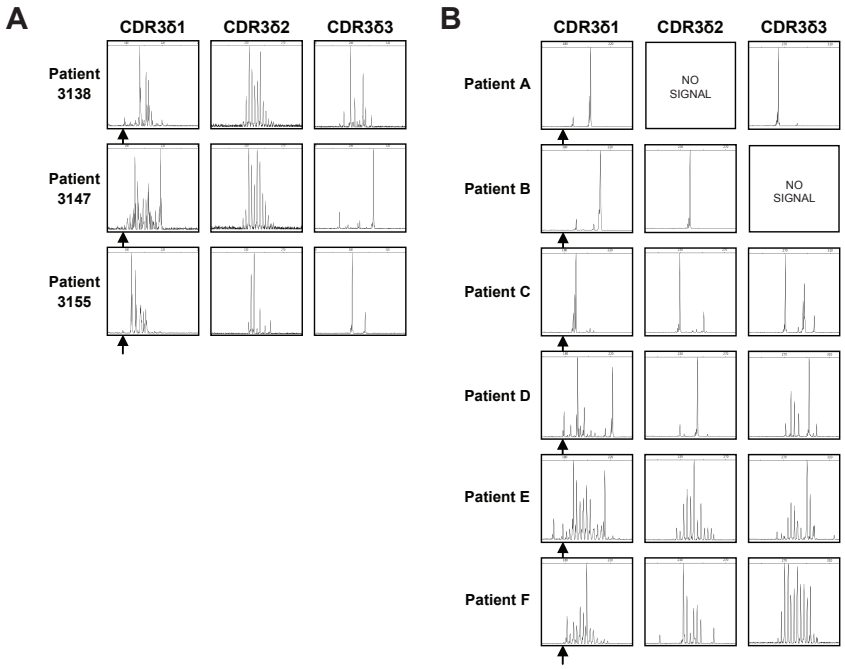
mRNA of γδT cell clones was isolated using the Nucleospin RNA-II kit (Macherey-Nagel) and reverse-transcribed using SuperScript-II reverse transcriptase (Invitrogen). TCRγ- and TCRδ-chains were amplified by PCR using Vδ1 (5'-GATCAAGTGTGGCCCAGAAG-3'), Vγ2-5 (5'-CTGCCAGTCAGAAATCTTCC-3'), Vγ8 (5'-GCTGTTGGCTCTAGCTCTG-3') and Vγ9 (5'-TCCTTGGGGCTCTGTGTGT-3') sense primers, and Cδ (5'-TTCACCAGACAAGCGACA-3') and Cγ (5'-GGGGAAACATCTGCATCA-3') antisense primers. PCR products were sequenced

by Baseclear© (Leiden, the Netherlands). Codon-optimized sequences of clone TCRs were subsequently synthesized by Geneart® (Regensburg, Germany) and subcloned into pBullet. Packaging cells (Phoenix-Ampho) were transfected with gag-pol (pHIT60), env (pCOLT-GALV) (4) and pBullet constructs containing TCRγ-chain-IRES-neomycine or TCRδ-chain-IRES-puromycin, using Fugene6 (Promega). PBMCs preactivated with αCD3 (30 ng/ml) (clone OKT3, Janssen-Cilag) and IL-2 (50 U/ml) were transduced twice with viral supernatant within 48 hours in the presence of 50 U/ml IL-2 and 4 μg/ml polybrene (Sigma-Aldrich). Transduced T cells were expanded by stimulation with αCD3/CD28 Dynabeads ($0.5x10^6$ beads/10^6 cells) (Invitrogen) and IL-2 (50 U/ml) and selected with 800 μg/ml geneticin (Gibco) and 5μg/ml puromycin (Sigma) for one week. Where indicated, CD4+ and CD8+ TCR-transduced T cells were separated by MACS-sorting using CD4- and CD8-microbeads (Miltenyi Biotec). Following selection, TCR-transduced T cells were stimulated biweekly using the REP-protocol (5).

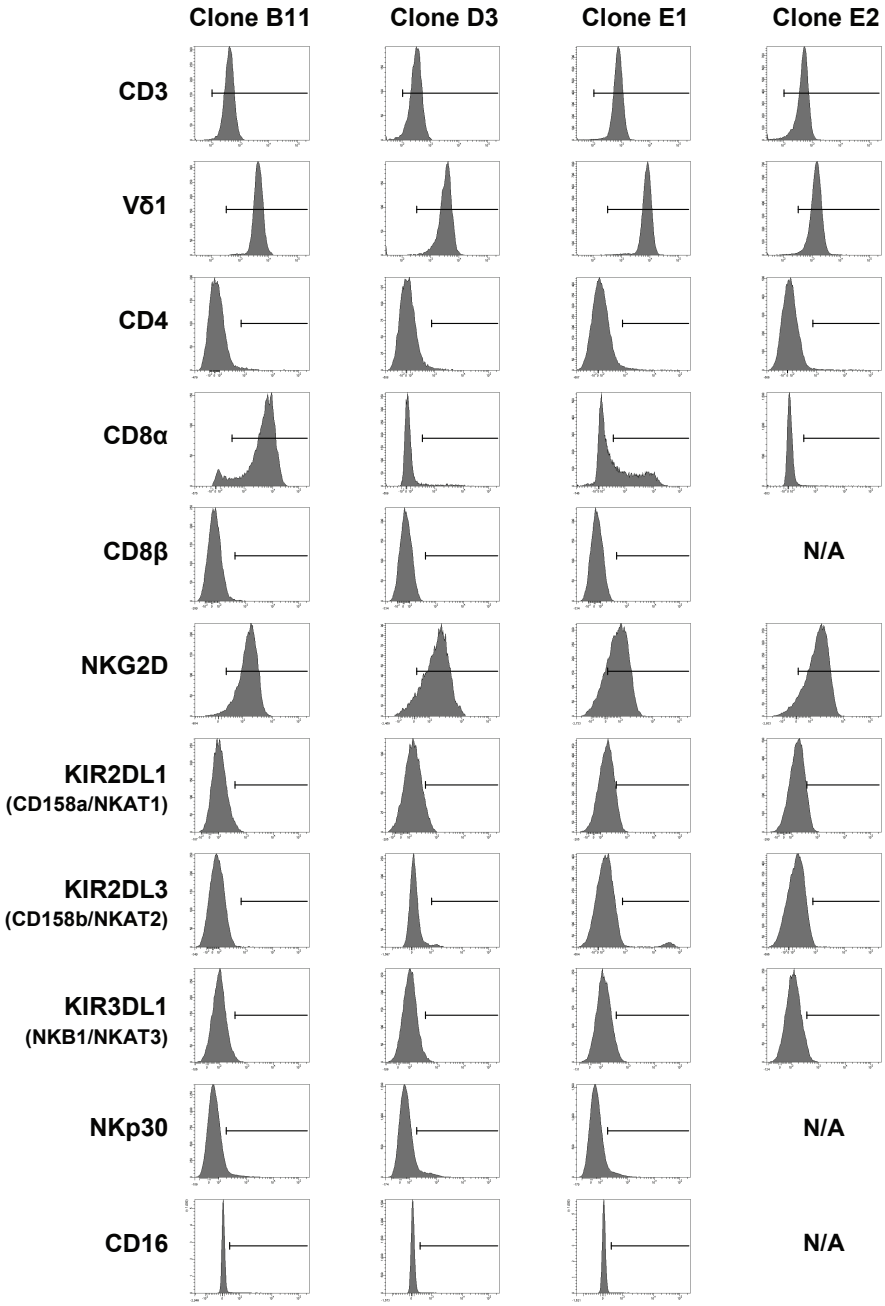
SUPPLEMENTARY DATA



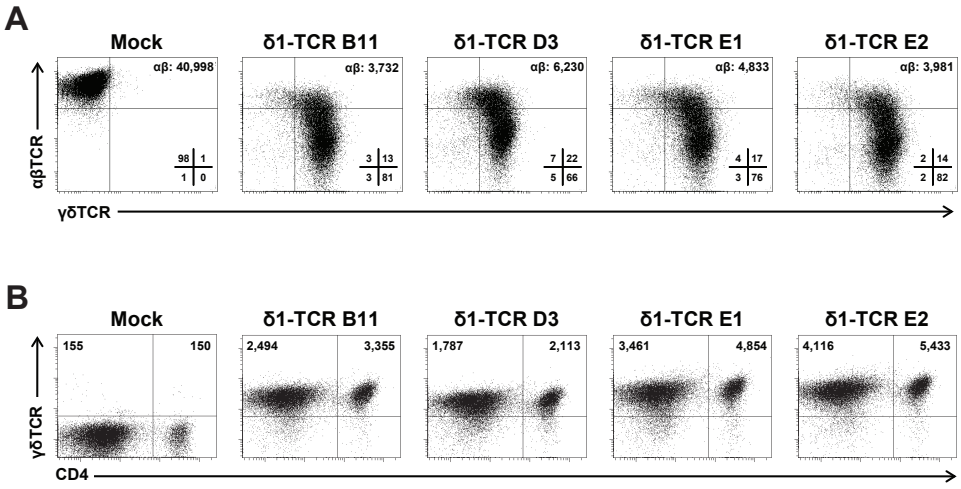
Supplementary Figure 1. Naïve γδT cells and total αβT cells after allo-SCT. (A) PBMCs of patients with conventional adult stem cell donors were collected weekly after allo-SCT, and the percentage of naïve $CD27^{pos}CD45RO^{neg}$ γδT cells was analyzed by flow cytometry. (B) Absolute counts of αβT cells after allo-SCT with conventional donors was measured by flow cytometry. A Mann Whitney U test was performed at all time points and significant differences are indicated (* $P < 0.05$).



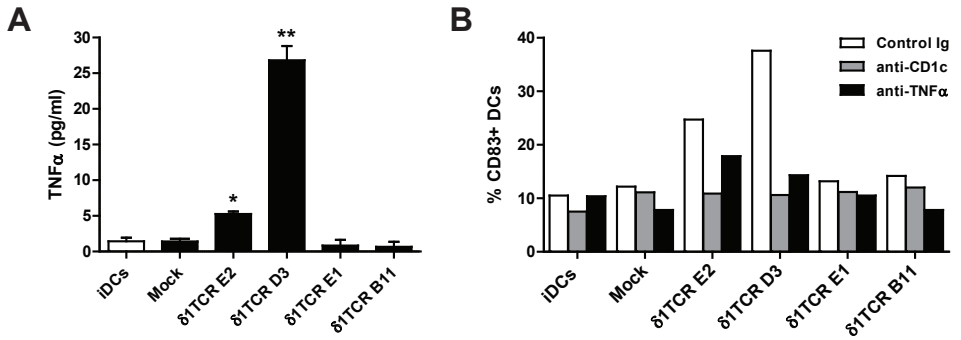
Supplementary Figure 2. γδTCR clonality analysis of γδT cells from CMV-reactivating patients. Representative spectratype analyses of Vδ1, Vδ2 and Vδ3 γδTCR clonality in blood samples of CMV-reactivating patients that received stem cells from conventional adult donors (A) or cordblood donors (B). All patients were analyzed during CMV-reactivation. The CDR3δ1 size of 11 amino acids, corresponding with the CDR3δ1 size of the public Vγ8Vδ1 TCR,(1) is indicated with arrows.



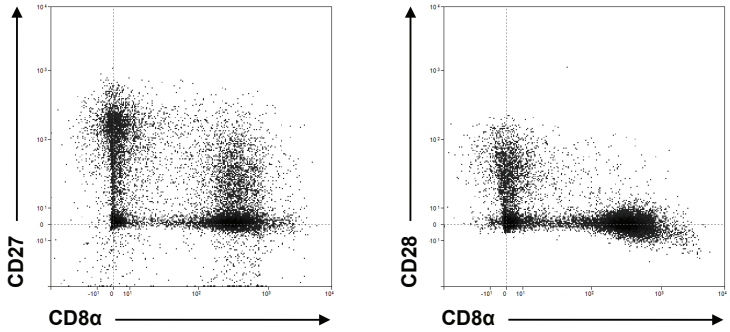
Supplementary Figure 3. Phenotyping of Vδ2^{neg} γδT cell clones. Vδ2^{neg} T cell clones were generated by limiting dilution and surface expression of indicated receptors was measured by flow cytometry. Gating was established based on appropriate isotype controls.



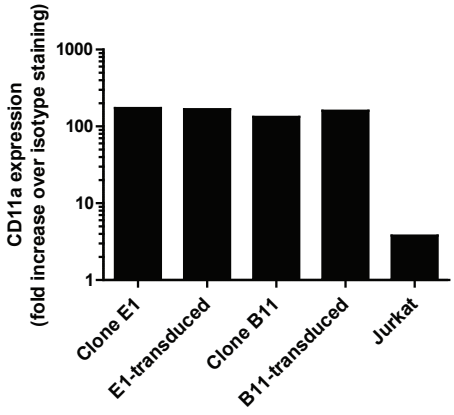
Supplementary Figure 4. Efficient retroviral expression of δ1-TCRs in CD4+ and CD8+ αβT cells. (A) Isolated δ1-TCRs were retrovirally transduced into αβT cells and surface expression of endogenous αβTCR and introduced γδTCR was determined by flow cytometry. Indicated in plots are percentages of quadrants and MFIs of γδTCR and αβTCR stainings. (B) Transduced αβT cells were costained for CD4 and expression levels (MFI) of γδTCRs on CD4- (i.e. CD8+) and CD4+ αβT cells is indicated in plots.



Supplementary Figure 5. Upregulation of DC maturation markers by γδTCR-transduced T cells involves TNFα and CD1c. (A) Immature DCs (iDCs) were cultured alone, with mock-transduced αβT cells, or with αβT cells expressing clone-derived γδTCRs for 48 hours and TNFα levels in culture supernatants were measured by ELISA (one-way ANOVA: * $P < 0.05$, ** $P < 0.01$). (B) iDCs were cultured as in (A) but now in the presence of control antibody or blocking antibodies against CD1c or TNFα. After 48 hours CD83 expression on DCs was measured as a representative marker of DC maturation.



Supplementary Figure 6. CD8α expression is associated with a differentiated effector phenotype (CD27neg/lowCD28neg) of γδT cells in CMV-infected newborns. Association of CD8α expression with CD27neg/low γδT cells (left panel) and CD28neg γδT cells (right panel). Stainings are representative for 11 CMV-infected newborns. Plots represent lymphocytes gated on CD3+γδTCR+ phenotype.



Supplementary Figure 7. Expression of CD11a on original clones, γδTCR-transduced αβT cells and Jurkat cells. Expression of CD11a is shown as a fold increase of MFI of the specific staining over MFI of the staining with control antibody.

Supplementary Table 1. Patient characteristics

	Patient groups	
	CMV-reactivation	No CMV-reactivation
Conventional graft cohort		
N	9	7
Median age (range)	56 (33-62)	49 (35-68)
Sex M/F (%)	89/11	57/43
Donor/recipient relation		
RD	5 (56)	4 (57)
MUD	4 (44)	3 (43)
Diagnosis		
AML	1 (11)	4 (57)
CLL	1 (11)	1 (14)
CML	1 (11)	0 (0)
MM	5 (56)	2 (29)
NHL	1 (11)	0 (0)
Conditioning		
NMA	9 (100)	9 (100)
MA	0 (0)	0 (0)
ATG	5 (56)	3 (43)
GVHD	8 (89)	3 (43)
CMV+ Patient	8 (89)	4 (57)
CMV+ Donor	5 (56)	1 (14)
OS at 2 years	5 (56)	5 (71)
Cordblood graft cohort		
N	6	4
Median age (range)	2 (1-10)	2 (1-15)
Sex M/F (%)	67/33	0/100
Diagnosis		
AML	2 (33)	0 (0)
ALL	2 (33)	3 (75)
JMML	0 (0)	1 (25)
NMID	1 (17)	0 (0)
NMMD	1 (17)	0 (0)
Conditioning		
NMA	0 (0)	0 (0)
MA	6 (100)	4 (100)
ATG	6 (100)	4 (100)
GVHD	2 (33)	1 (25)
CMV+ Patient	6 (100)	4 (100)
CMV+ Donor	0 (0)	0 (0)
OS at 2 years	5 (83)	3 (75)

ALL, acute lymphocytic leukemia; AML, acute myeloid leukemia; ATG, antithymocyte globuline; CLL, chronic lymfocytic leukemia; CML, chronic myeloid leukemia; CMV, cytomegalovirus; F, female; GVHD, graft-versus-host disease; JMML, juvenile myelomonocytic leukemia; M, male; MA, myeloablative; MM, multiple myeloma; NHL, non-Hodgkins lymphoma; NMA, non-myeloablative; NMID, non-malignant immunodeficiency; NMMD, non-malignant metabolic disease; OS, overall survival; RD, related donor; MUD, matched unrelated donor.

Supplementary Table 2. Comparison of γδT cells, αβT cells and NK-cells between patients with and without EBV-reactivation

	Patient groups		
	EBV-reactivation	No EBV-reactivation	P value
Conventional graft cohort			
N	6	10	
% αβT cells / lymphocytes	30.4	50.0	0.66
% γδT cells / lymphocytes	1.2	1.2	0.81
% $CD56^{pos}CD16^{pos}$ cells / $CD3^{neg}$ lymphocytes	34.1	74.6	0.01

EBV, Eppstein-Barr virus. P-values: Mann Whitney U test.

Supplementary Table 3. CDR3 sequences of $V\delta2^{neg}$ T cell clones

ImMunoGeneTics (IMGT®) JunctionAnalysis output (www.imgt.org)

TCRγ chains

Clone	V name	3'V-REGION	N1	P	D1-REGION	N2	D2-REGION	N3	P	5'J-REGION	J name	D1 name	D2 name
B11	TRDV1*01	tgtgctcttggggaac	aggtcg		ttccta	ttgatct	ggggat	tcc	gt	acaccgataaactcatcttt	TRDJ1*01	TRDD2*01	TRDD3*01
D3	TRDV1*01	tgtgctcttggg	aaaagtggca		gggggat	cacca				ataaactcatcttt	TRDJ1*01	TRDD3*01	
E1	TRDV1*01	tgtgctcttggggaact	cggacggggaggga	t	actgggggga	aatt				accgataaactcatcttt	TRDJ1*01	TRDD3*01	
E2	TRDV1*01	tgtgctcttggggaact			ctac	aca	tgggggatac	agcctt		ctttgacagcacaactctttctttt	TRDJ2*01	TRDD2*01	TRDD3*01

TCRδ chains

Clone	V name	3'V-REGION	N	5'J-REGION	J name
B11	TRGV4*02	tgtgccacctgggatgg	ccaggaagg	ttattataagaaactcttt	TRGJ1*01
D3	TRGV8*01	tgtgccacctgg	tccaggggggg	ccactggttggttcaagatattt	TRGJP1*01
E1	TRGV9*01	tgtgccttgtgggag	acttcctacctc	tattataagaaactcttt	TRGJ1*01
E2	TRGV9*01	tgtgccttgtgggag	ccc	aattattataagaaactcttt	TRGJ2*01

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CHAPTER 4

Multifunctional γδT cells and their receptors for targeted immunotherapy

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ABSTRACT

Human γδT cells possess broad anti-tumor reactivity and are involved in controlling viral infections. In our recent work we describe multifunctional γδT cells induced by CMV after allo-SCT, putting γδT cells and their receptors in the spotlight for novel immunotherapies.

MAIN TEXT

Over the last decades, CMV reactivation has been seen as a major life-threatening complication of allo-SCT. Nowadays, sensitive monitoring for early presence of CMV reactivation combined with the availability of effective antiviral treatment options has made CMV-related death post-transplantation a rare event. Fortuitously, this improved control of CMV disease has facilitated recent unexpected observations in studies with large cohorts of allo-SCT patients, where a surprising beneficial association was observed between CMV reactivation and a reduced risk of leukemic relapse (1). So far however it is unclear how viral reactivation could provide protection from leukemic relapse. It has been proposed that NK-cells may cross-react to CMV-infected cells and tumor cells by responding to CMV-infected residual AML blasts (1). In this year's January issue of Leukemia we propose an additional or even physiologically more relevant explanation for this paradoxical observation, namely that γδT cells may play a pivotal role in this CMV-induced clearance of tumor (2). We observed that these unconventional T cells expand in CMV-reactivating patients after allo-SCT, and moreover that these CMV-induced γδT cells not only react to CMV-infected cells but also cross-recognize leukemic cells. Thus, we propose that these multifunctional γδT cells could substantially contribute to CMV-associated protection from leukemic relapse after allo-SCT.

In humans, γδT cells are a minor population in peripheral blood where they mostly express TCRs containing Vδ2 and Vγ9 gene segments (so-called $Vδ2^{pos}$ γδT cells) (3). In contrast, γδT cells that reside in epithelial locations carry TCRs composed of mainly Vδ1 or Vδ3 chains ($Vδ2^{neg}$ γδT cells) and may express CD8αα. Over the last decade, many studies have implicated $Vδ2^{neg}$ γδT cells in the anti-CMV response and in tumor immunosurveillance, but the first report on $Vδ2^{neg}$ γδT cell cross-reactivity to CMV and cancer came from work on $Vδ2^{neg}$ γδT cell clones isolated from kidney transplant recipients (4). At least for some of these clones, this dual reactivity was mediated by the γδTCR recognizing a common stress antigen upregulated on CMV-infected and transformed intestinal epithelial cells (5), explaining why CMV-infection alone could induce an immune population with reactivity to both CMV and cancer. In our study however, gene transfer experiments using γδTCRs isolated from cross-reactive $Vδ2^{neg}$ γδT cell clones showed a crucial involvement of the γδTCR in tumor reactivity but not in recognition of CMV-infected cells, suggesting that for these clones CMV-reactivity was performed by receptors other than the γδTCR. Importantly, this brings up a major issue in the γδT cell field, namely that mechanisms of γδT cell activation and antigens of γδTCRs are still poorly defined. In this respect, one important finding of our study is the identification of CD8αα as a costimulatory molecule for activation of defined γδTCRs (2). Expression of

CD8αα on γδT cells has been long described, however so far there were no reports on its function. In αβT cells, the CD8αβ heterodimer serves as coreceptor for the αβTCR, restricting cytotoxic T cells to antigens presented by class I MHC molecules. γδTCRs however recognize antigens independently of MHC, suggesting that the coactivating function of CD8αα is likely to rely on alternative mechanisms. Although the precise functioning of CD8αα in this setting remains to be elucidated, we observed a striking increase in circulating CD8αα+ γδT cells in CMV-reactivating patients in our allo-SCT cohort as well as in an additional independent cohort of congenitally CMV-infected neonates, implying also in vivo relevance of CD8αα expression by γδT cells.

Our observations of γδT cells cross-recognizing CMV and a broad panel of hematological cancers make these cells particularly attractive for clinical application, such as adoptive

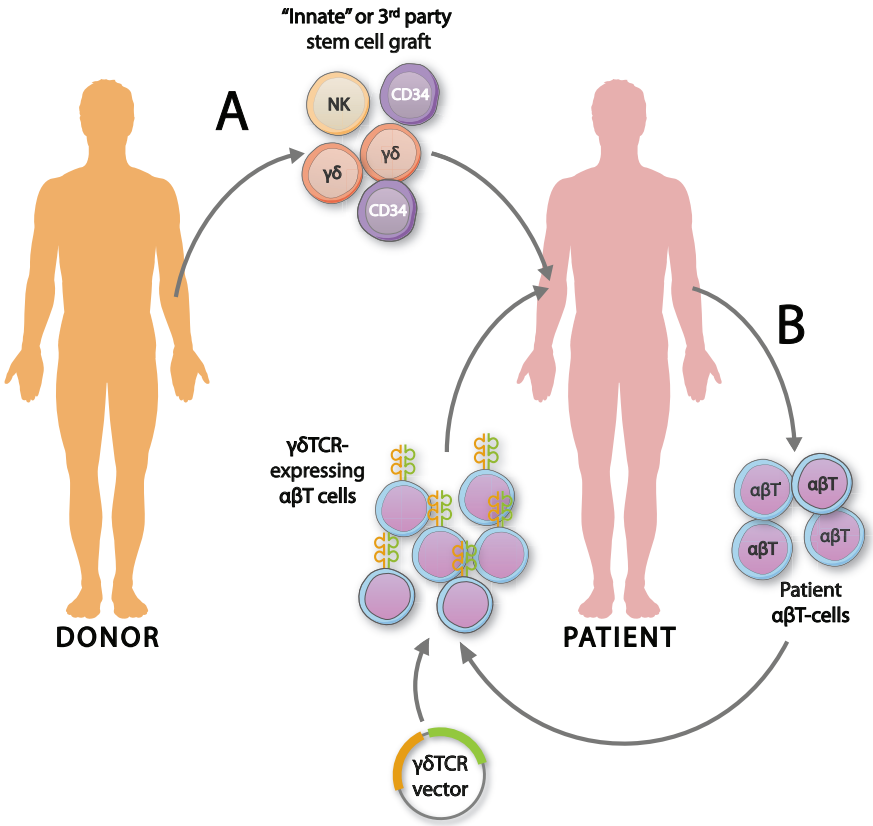

Figure 1. Application of antitumor strategies using γδT cells. In an "innate allo-SCT" (A) stem cell grafts from conventional or third party sources may selectively contain or be enriched for γδT cells to provide anti-CMV and anti-tumor protection in the absence of GVHD. In a complementary "autologous engineered transplantation" (B) T cells are isolated from cancer patients and are expanded and engineered to express (CTE-optimized) γδTCRs ex vivo. Reprogrammed T cells are subsequently reinfused into the patient, where they recognize and kill tumor.

immune therapies. In the context of an allo-SCT, a design favouring reactivity against CMV and leukemia in the absence of GVHD might be achieved with stem cell grafts enriched for γδT cells (Fig. 1). Clinical trials using stem cell grafts depleted for αβT cells and B-cells are therefore currently being conducted by us (trial registration NTR2463 and NTR3079) and others (6). Intriguingly, in particular αβTCR/CD19-depleted but usually not CD3/CD19-depleted transplantations show a very rapid reconstitution of a broad αβT cell repertoire (J. Kuball, unpublished observations), suggesting even a broader immune regulatory role for γδT cells, as suggested also recently by others (7). Alternatively, umbilical cord blood grafts could be used as a third party source of stem cells. These grafts typically contain high percentages of γδT cells and we demonstrate that CMV- and leukemia-reactive γδT cells can also be obtained from this CMV-naïve repertoire. Importantly, all GMP-grade clinical tools for preparation of enriched stem cells grafts are available. Finally, CD8αα+ γδT cells could be isolated as our results suggest a role for these cells in the anti-CMV response, although their precise involvement will first need to be investigated.

Complementary to this 'innate allo-SCT' approach, γδTCRs with broad tumor-reactivity could be characterized and used to reprogram patient-derived conventional αβT cells (8) (Fig. 1). Given the non-MHC-restricted antigen recognition by γδTCRs, defined γδTCRs could thus – in contrast to αβTCRs – be applied to a broad patient population in the absence of matched HLA types. Also, introduced γδTCR chains do not pair with endogenous αβTCR chains, preventing creation of novel TCRs with unpredictable

auto-reactivity. As we have shown previously, introducing defined γδTCRs effectively reprograms αβT cells to kill a broad collection of tumors both in vitro and in vivo (9). We furthermore introduced a technique called combinatorial-γδTCR-chain engineering (CTE), allowing design of γδTCRs with enhanced functional avidity towards tumors but not healthy tissues (10). By exploiting the abundance, potent cytotoxic machinery, and in particular the proliferation competence of αβT cells even in advanced stages of disease, an engineering of autologous immune cells with such receptors allows the generation of large numbers of tumor-reactive T cells while tackling the major limitations of current approaches using engineered αβTCRs. Taken together, we therefore advocate the application of γδT cells and their receptors as a promising new avenue in adoptive antitumor therapies.

ACKNOWLEDGEMENTS

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CHAPTER 5

CD8αα costimulation to γδT cell receptors involves diverse molecular interactions with ligands that include MHC class I-like molecules

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ABSTRACT

γδT cell receptors (TCRs) recognize a large variety of tumour-associated antigens in a manner that does not depend on classical antigen presentation, making these innate-like receptors valuable additions to currently pursued αβTCRs in gene-engineered tumour immunity. However, the molecular mechanisms of antigen recognition by γδTCRs are still poorly understood. We previously identified CD8αα on γδT cells as a coreceptor for tumour-reactive γδTCRs. Here, we demonstrate that CD8αα-dependent γδTCRs can redirect CD4+ T cells against multiple tumour targets after cotransfer of the CD8α gene. Mapping of molecular interaction requirements of CD8αα by site-directed mutagenesis and blocking experiments suggest diverse ligands, including classical MHC-like molecules, that mediate either signalling through CD8α or adhesion only. The here-described diversity of interaction partners of CD8αα and its opposing functional roles are in line with innate-like activation modes of γδT cells, and adds a novel level of complexity to the molecular demands for γδT cell activation. Thus, clinical efforts utilizing γδT cells or their individual receptors need to take such heterogeneity in activation mechanisms into consideration.

INTRODUCTION

γδT cells are innate-like lymphocytes with potent cytotoxicity against a broad variety of malignant cells (1,2). In contrast to conventional αβT cells, γδT cells do not depend on the recognition of processed tumour antigens presented by classical MHC molecules, but instead are activated by self molecules that are upregulated on transformed or otherwise stressed cells. As such, γδT cells represent attractive tools to complement immunotherapeutic strategies using conventional T cells, which have so far yielded promising results only in few types of cancer (3). In this line, recent work from our laboratory has recently demonstrated that broadly tumour-reactive γδT cell receptors (γδTCRs) can be isolated and used to genetically redirect αβT cells towards a broad array of tumours (4-6). Such clinical gene engineering strategies using MHC-unrestricted, broadly tumour-specific γδTCRs could be applied to patients with diverse tumours and HLA haplotypes, and thus could overcome major limitations of αβT cell-based clinical concepts (7-9). Nevertheless, the molecular mechanisms of antigen recognition by γδT cells and interaction partners of γδTCRs are largely unknown, and this poses a substantial hurdle to translational efforts using γδT cells and their receptors. We have recently identified a novel stimulatory function of CD8αα on γδT cells (6). Expansion of CD8αα+ γδT cells was a signature of cytomegalovirus infection and selected γδTCRs of such γδT cells were able to recognize primary leukemic blasts, but appeared to depend on the CD8αα coreceptor. The function of CD8 as a coreceptor has been widely studied on conventional αβT cells, where it is mainly expressed as a CD8αβ heterodimer and provides costimulation to αβTCR-mediated activation by binding to the peptide-MHC complex (10,11). CD8 expression can also be detected on subsets of innate immune cells, including NK cells, macrophages, dendritic cells and γδT cells, although on these cells CD8 is usually expressed

as a CD8αα homodimer (12). However, the precise function of CD8αα on innate-like cells, and γδT cells in particular, is poorly understood. Moreover, the significance of the dependence of defined γδTCRs on CD8αα for γδT cell-based immunotherapy, including γδTCR gene transfer strategies, is unclear. In the present study we therefore addressed the molecular requirements for CD8αα in the context of γδTCR-mediated tumour recognition and the implications of CD8αα-dependency of γδTCRs for gene engineering of tumour immunity.

MATERIALS & METHODS

Antibodies and flow cytometry
Antibodies used for flow cytometry included: γδTCR-PE (clone IMMU510, Beckman Coulter), CD4-PE-Cy7 (clone RPA-T4, BD), CD8α-APC (clone RPA-T8, BD), CD8α-PerCP-Cy5.5 (clone RPA-T8, Biolegend), CD8α-FITC (clone G42-8, BD), and CD8αβ-PE (clone 2ST8.5H7, BD). Samples were measured using a FACSCanto-II flow cytometer (BD) and analysed with FACSDiva software (BD).

Cell lines
Daudi, SW480 and Phoenix-Ampho cell lines were obtained from ATCC. EBV-LCL was kindly provided by Phil Greenberg (Seattle, WA). Phoenix-Ampho cells were cultured in DMEM supplemented with 1% Pen/Strep (Invitrogen) and 10% FCS (Bodinco), all other cell lines in RPMI with 1% Pen/Strep and 10% FCS. Healthy PBMCs were isolated by Ficoll-Paque (GE Healthcare) from buffy coats supplied by Sanquin Blood Bank (Amsterdam, The Netherlands).

Generation of γδT cell clone FE11
Clone FE11 was previously generated as described (6). In brief, bulk γδT cells were isolated from healthy donor PBMCs using the TCRγ/δ+ T Cell Isolation MACS Kit (Miltenyi Biotec), and expanded using a previously described rapid expansion protocol (13). Briefly, γδT cells were stimulated for two weeks with 1 μg/ml PHA-L (Sigma-Aldrich), 50 U/ml IL-2 (Novartis Pharma), 5 ng/ml IL-15 (R&D Systems), and irradiated allogeneic PBMCs, Daudi and EBV-LCLs. Fresh IL-2 was added twice a week. The γδT cell clone FE11 was generated from bulk γδT cells by limiting dilution, and expanded biweekly using the rapid expansion protocol.

Cloning of FE11 γδTCR and retroviral transductions
Total RNA of γδT cell clone FE11 was isolated using the Nucleospin RNA-II kit (Macherey-Nagel) and reverse-transcribed using SuperScript-II reverse transcriptase (Invitrogen). TCR γ and δ chains were amplified by PCR using Vδ1 (5'-GATCAAGTGTGGCCCAGAAG-3') and Vγ2-5 (5'-CTGCCAGTCAGAAATCTTCC-3') sense primers, and Cδ (5'-TTCACCAGACAAGCGACA-3') and Cγ (5'-GGGGAAACATCTGCATCA-3') antisense primers. PCR products were sequenced, and codon-optimized sequences of the FE11 γδTCR were subsequently synthesized by Geneart® (Life Technologies) and subcloned into the retroviral pBullet vector. pBullet constructs containing full-length human CD8α or CD8β were a kindly provided by Reno Debets

(Rotterdam, The Netherlands). Truncated versions of CD8α or CD8β (14) were kindly provided by Michael Nishimura (Chicago, US). Single amino acid mutants of CD8α (15) were kindly provided by Paula Kavathas (New Haven, US). NcoI and BamHI restriction sites were inserted up- and downstream of CD8α variant sequences by site-directed mutagenesis PCR, after which CD8α variants were subcloned into pBullet using the introduced NcoI and BamHI sites. Phoenix-Ampho packaging cells were transfected with gag-pol (pHIT60), env (pCOLT-GALV) and pBullet constructs containing TCRγ-chain-IRES-neomycine, TCRδ-chain-IRES-puromycin, CD8α, CD8β or CD8α mutant variants using Fugene6 (Promega). PBMCs preactivated with 30 ng/ml anti-CD3 (clone OKT3, Janssen-Cilag) and 50 U/ml IL-2 were transduced twice with viral supernatant within 48 hours in the presence of 50 U/ml IL-2 and 4 μg/ml polybrene (Sigma-Aldrich). Transduced T cells were expanded by stimulation with αCD3/CD28 Dynabeads (0.5x106 beads/106 cells) (Invitrogen) and 50 U/ml IL-2, and selected with 800 μg/ml geneticin (Gibco) and 5 μg/ml puromycin (Sigma) for one week. Where indicated, CD4+, CD8+, CD4+CD8αα+ and CD4+CD8αβ+ TCR-transduced T-cells were sorted using a FACSAria II (BD) flow cytometry to >99% purity. Following selection, TCR-transduced T-cells were stimulated biweekly using the rapid expansion protocol. Expression levels of CD8α mutants were measured by flow cytometry using two different anti-CD8α antibody clones (clones RPA-T8 and G42-8).

Functional T cell assays

IFNγ ELISPOT was performed as previously described (16-19). 15,000 γδT cells (clone FE11) or γδTCR+ transduced αβT cells were coincubated with 50,000 target cells (ratio 0.3:1) for 18 hours in nitrocellulose-bottomed 96-well plates (Millipore) precoated with anti-IFNγ antibody 1-D1K (Mabtech). Plates were washed with phosphate-buffered saline (PBS) and incubated with biotinylated antibody 7-B6-1 (Mabtech) followed by streptavidin-HRP (Mabtech). IFNγ spots were visualized with TMB substrate (Sanquin) and the number of spots were quantified using ELISPOT Analysis Software (Aelvis). Where indicated, blocking of CD8α was performed using 10 μg/ml anti-CD8α antibody clone OKT8 (eBioscience), blocking of CD8β with 10 μg/ml anti-CD8β clone 2ST8.5H7 (Abcam), and blocking of MHC class I with 10 μg/ml anti-HLA-ABC clone W6-32 (Biologend).

Statistical analyses

Differences were analysed using the appropriate statistical tests in GraphPad Prism 5.0 (GraphPad Software Inc.).

RESULTS

Tumour recognition by the FE11 γδTCR requires costimulation by either CD8αα or CD8αβ

We previously generated the CD8αα+ γδT cell clone FE11 which displays CD8αα-dependent reactivity to the colon cancer cell line SW480 (6). To further characterize this clone and the involvement of CD8αα in tumour recognition, reactivity of clone FE11 was tested against not

only SW480 cells but also against EBV-LCLs (Epstein-Barr virus-transformed lymphoblastoid cell line) and Daudi cells in the absence or presence of CD8-blocking antibodies. As reported for reactivity against SW480, also reactivity against EBV-LCL and Daudi target cells was impaired after co-incubation with CD8α-blocking antibody (Fig. 1A), suggesting a general role for CD8αα within the context of tumour-recognition by CD8αα+ γδT cells. Blocking of CD8αβ had no effect on tumour recognition, as expected based on the CD8αα+ phenotype of clone FE11 (Supplementary Fig. 1). To test the involvement of the γδTCR of clone FE11 in the recognition of these tumour targets, TCR γ and δ chains of clone FE11 were sequenced (Supplementary Table 1) and retrovirally introduced into αβT cells from human PBMCs. γδTCR-

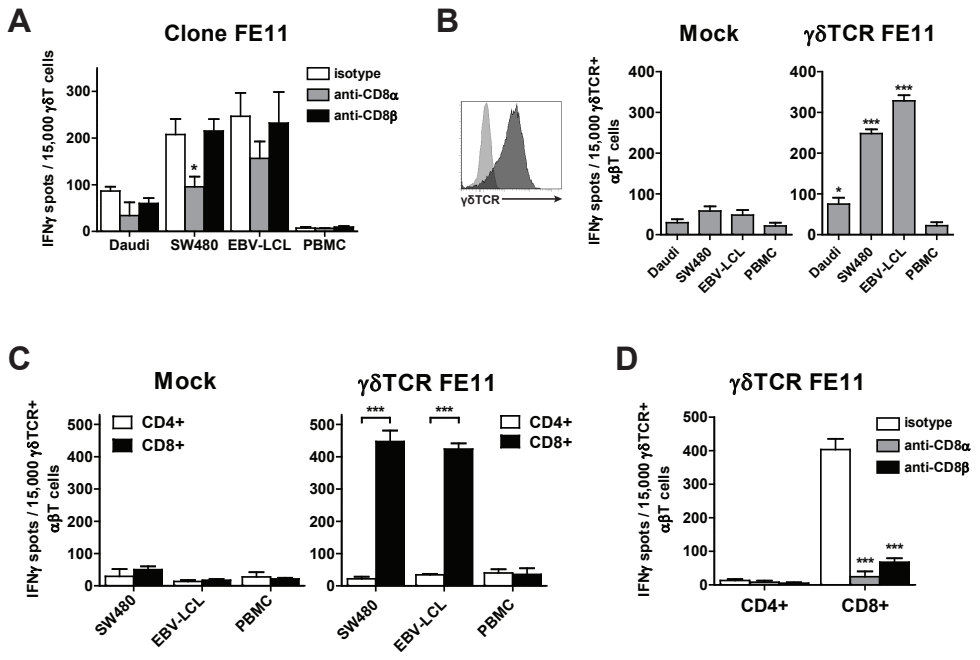

Figure 1. The γδTCR FE11 critically depends on the CD8 coreceptor for tumour recognition. (A) γδT cell clone FE11 was generated by limiting dilution. To assess tumour reactivity, FE11 cells were incubated with Daudi, SW480 or EBV-LCL tumour targets in the presence of control antibody or antibodies blocking CD8α or CD8β. IFNγ secretion was measured by ELISPOT. Healthy PBMCs served as negative control targets. (B) The TCR γ and δ chains of clone FE11 were sequenced and retrovirally transduced into αβT cells (left panel; γδTCR expression on mock-transduced (light curve) and γδTCR-transduced T cells is indicated). Transfer of γδTCR-mediated tumour-reactivity was tested by coincubating γδTCR- or mock-transduced T cells with indicated target cells in an IFNγ ELISPOT (right panel). (C) CD4+ and CD8+ αβT cells transduced with the FE11 γδTCR were sorted and cocultured with indicated target cells. T cell activation was assessed by IFNγ ELISPOT. (D) CD4+ and CD8+ αβT cells expressing the FE11 γδTCR were coincubated with SW480 target cells as in (C) but now in the presence of a control antibody or blocking antibodies against CD8α or CD8β. Data are representative of three (A,D), two (B), and five (C) separate experiments. Error bars represent S.E.M. (*P < 0.05; ***P < 0.001).

mediated reprogramming of transduced T cells against tumour targets was subsequently analysed by coculturing T cells and tumour targets in an IFNγ ELISPOT. T cells transduced with the FE11 γδTCR but not mock-transduced T cells recognized SW480, EBV-LCL and Daudi cells but not healthy PBMCs (Fig. 1B), strongly mimicking the tumour recognition pattern of the parental clone FE11.

A requirement for CD8αα in tumour recognition by the parental clone FE11 could imply a differential capacity of the FE11 γδTCR to redirect CD4+ and CD8+ T cells against cancer cells, as reported by us recently for a distinct γδTCR (clone B11) (6). To test differential reprogramming of CD4+ and CD8+ T cell subsets, γδTCR-transduced T cells were sorted into CD4+ and CD8+ populations and tested for recognition of the target cell lines SW480 and EBV-LCL. Similar to our previous observations with the CD8αα-dependent γδTCR B11, tumour cells elicited IFNγ production by CD8+ but strikingly not CD4+ T cells carrying the FE11 γδTCR (Fig. 1C). Differences in IFNγ secretion were not due to differences in expression levels of the FE11 γδTCR on both T cell subsets, as the γδTCR was generally expressed at even higher levels on CD4+ than on CD8+ T cells (data not shown). In contrast to the FE11 γδT cell clone, most CD8+ αβT cells express CD8αβ heterodimers rather than CD8αα ((20) and Supplementary Fig. 1). The implication of both CD8α and CD8β chains in providing costimulation to the FE11 γδTCR on transduced αβT cells was therefore tested using blocking antibodies. In contrast to previous observations with a different CD8αα-dependent γδTCR (6), not only CD8α but also CD8β blocking antibodies completely inhibited reactivity against SW480 cells (Fig. 1D), possibly reflecting diverse molecular dependencies on both CD8 chains by distinct γδTCRs. Taken together, these data demonstrate that tumour recognition by the γδT cell clone FE11, and importantly by its isolated γδTCR, depends on costimulation by either CD8αα or CD8αβ molecules.

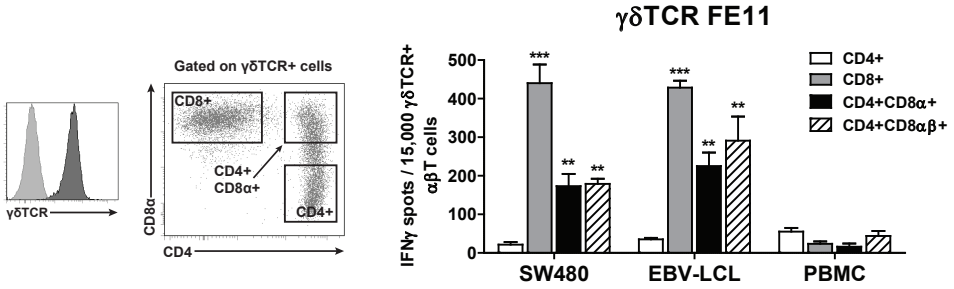

Figure 2. Transgenic CD8α rescues tumour-reactivity of FE11 γδTCR-transduced CD4+ T cells. αβT cells were retrovirally transduced with the FE11 γδTCR and either CD8α alone or CD8α in combination with CD8β. CD4+, CD8+, CD4+CD8α+ and CD4+CD8αβ+ subsets of T cells were subsequently sorted (left panel; indicated is a representative sorting plot for CD4+, CD8+ and CD4+CD8α+ cells, CD4+CD8αβ+ cells were sorted in a similar manner) and tested for recognition of SW480 and EBV-LCL target cells by IFNγ ELISPOT (right panel). Healthy PBMCs were included as negative control target cells. Data are representative of four separate experiments. Error bars represent S.E.M. (**P < 0.01; ***P < 0.001).

Cotransfer of CD8α alone is sufficient to re-establish tumour reactivity of CD4+ T cells expressing the FE11 γδTCR

So far, both CD8αα on the original clone FE11 and CD8αβ on transduced αβT cells are capable of providing costimulation to the FE11 γδTCR. We therefore questioned whether introduction of CD8αα or CD8αβ could also re-establish anti-tumour activity of the CD8-dependent γδTCR FE11 in CD4+ T cells. T cells were therefore cotransduced with the γδTCR and CD8α alone or CD8α together with CD8β. CD4+ T cells expressing the γδTCR as well as exogenous CD8αα (CD4+CD8α+) or CD8αβ (CD4+CD8αβ+) were subsequently sorted (Fig. 2). γδTCR-transduced T cells expressing endogenous CD4+ and CD8+ T cells were sorted as negative and positive controls for tumour recognition, respectively. Anti-tumour reactivity of the FE11 γδTCR on CD4+CD8αα+ and CD4+CD8αβ+ T cells was analysed by coculturing with SW480 and EBV-LCL target cells. Both CD4+CD8αα+ and CD4+CD8αβ+ T cells that express the FE11 γδTCR secreted significantly higher levels of IFNγ upon exposure to tumour targets than γδTCR+ CD4+ T cells (Fig. 2). IFNγ levels produced by these cells did not reach those of CD8+ T cells carrying the FE11 γδTCR however, despite comparable γδTCR and CD8 expression levels across the different cell populations (Supplementary Fig. 2). Reactivity of CD4+CD8αα+ and CD4+CD8αβ+ T cells could be blocked by CD8α and CD8β blocking antibodies (Supplementary Fig. 3), confirming the strict dependence of the γδTCR on introduced CD8 molecules. Thus, recognition of tumour targets by CD4+ helper T cells that express a CD8-dependent γδTCR can be re-established by the cointroduction of CD8α alone. Introduction of CD8β did not further enhance tumour recognition, but was apparently functionally involved in the molecular interaction with its target when present.

Opposing implications of the CD8αα signalling domain in γδTCR-mediated recognition of distinct tumour targets

The ability to rescue target recognition by a CD8-dependent γδTCR on CD4+ T cells after

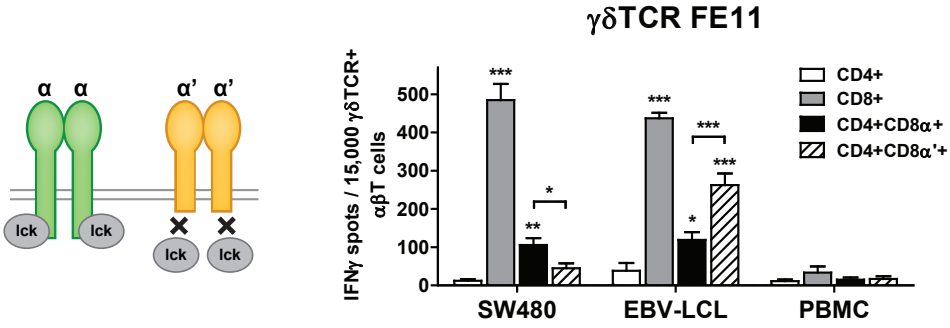

Figure 3. CD8αα signaling and adhesion functions are differentially involved in γδTCR-mediated recognition of distinct tumour target. αβT cells were transduced with wildtype CD8α or a truncated, signaling-deficient CD8α variant (CD8α'; left panel) alongside the FE11 γδTCR, after which CD4+, CD8+, CD4+CD8α+ and CD4+CD8α'+ T cells populations were sorted. Recognition of healthy PBMCs and SW480 and EBV-LCLs tumour targets was assessed by measuring IFNγ secretion using ELISPOT. Error bars represent S.E.M. (*P < 0.05; **P < 0.01; ***P < 0.001).

introduction of exogenous CD8αα not only substantiates the potential clinical value of such broadly tumour-reactive γδTCRs, but also provided a valuable platform to test the molecular requirements for CD8αα-mediated γδTCR help in the absence of endogenously expressed CD8 dimers. For costimulation of MHC class I-restricted αβTCRs, CD8αβ performs dualistic roles, serving as an adhesion molecule that stabilizes the TCR-MHC interaction (14,21) and providing intracellular activation signals by sequestering the signalling component Lck to the TCR/CD3 complex (22,23). We therefore questioned the importance of these two functions in the costimulation mediated by CD8αα to defined γδTCRs. Our results obtained with exogenous CD8 chains implied that the CD8α chain is sufficient for γδTCR help in CD4+ T cells (Fig. 2). A truncated, signalling-deficient version of CD8α only was therefore cotransduced with the FE11 γδTCR into CD4+ T cells (Fig. 3; left panel) and tumour recognition by transduced T cells was measured in an IFNγ secretion assay. CD4+γδTCR+ T cells carrying truncated CD8α secreted lower levels of IFNγ in response to SW480 cells than T cells cotransduced with full-length CD8α (Fig. 3; right panel), despite similar γδTCR and CD8α expression levels (Supplementary Fig. 4). However, this observation was strongly contrasted by the effects observed when T cells were coincubated with EBV-LCL target cells, as γδTCR+ T cells transduced with truncated CD8α produced more IFNγ than T cells with full-length CD8α. Thus, depending on the target cell recognized, intracellular CD8α signalling may be differentially involved in providing costimulation to defined γδTCRs.

CD8αα interacts with diverse ligands, including MHC class I-like molecules, using distinct structural domains

The striking difference in the effect of signalling-deficient CD8αα on the recognition of SW480 and EBV-LCL cells may suggest interactions with diverse CD8αα ligands on both target cell lines. As a first step, we therefore assessed the potential implication of classical MHC molecules, the prototypic CD8 ligand, in CD8αα-mediated γδTCR help by coincubating γδTCR+ T cells with SW480 and EBV-LCL target cells in the presence of an anti-MHC-I blocking antibody. Blocking MHC-I potently inhibited activation of CD8+ T cells transduced with the FE11 γδTCR in response to SW480 target cells, an effect that was also observed in CD4+ T cells transduced with the γδTCR as well as exogenous CD8α (Fig. 4A). In sharp contrast, activation of CD8+ T cells engineered to express the FE11 γδTCR was significantly increased when MHC-I blocking antibody was added to cocultures with EBV-LCL cells. Again, CD4+ T cells transduced with both the γδTCR and CD8α mimicked CD8+γδTCR+ T cells and showed an increase in IFNγ production upon coculture with EBV-LCL target cells in the presence of MHC-I blocking antibody. Thus, similar to our observations with truncated CD8α variants, blocking of MHC-I differentially impacts FE11 γδTCR+ T cell activation by distinct tumour targets. These data imply distinct molecular interactions and multiple ligands for CD8αα on SW480 and EBV-LCL cells in the context of γδTCR-mediated T cell activation. One class of these ligands may be classical MHC-I molecules.

Mutational studies addressing the role of CD8 in αβTCR-mediated target recognition have identified residues in the extracellular domain of CD8α that are critically involved in the molecular interaction with MHC class I (15,24). To corroborate the implication of MHC-I as

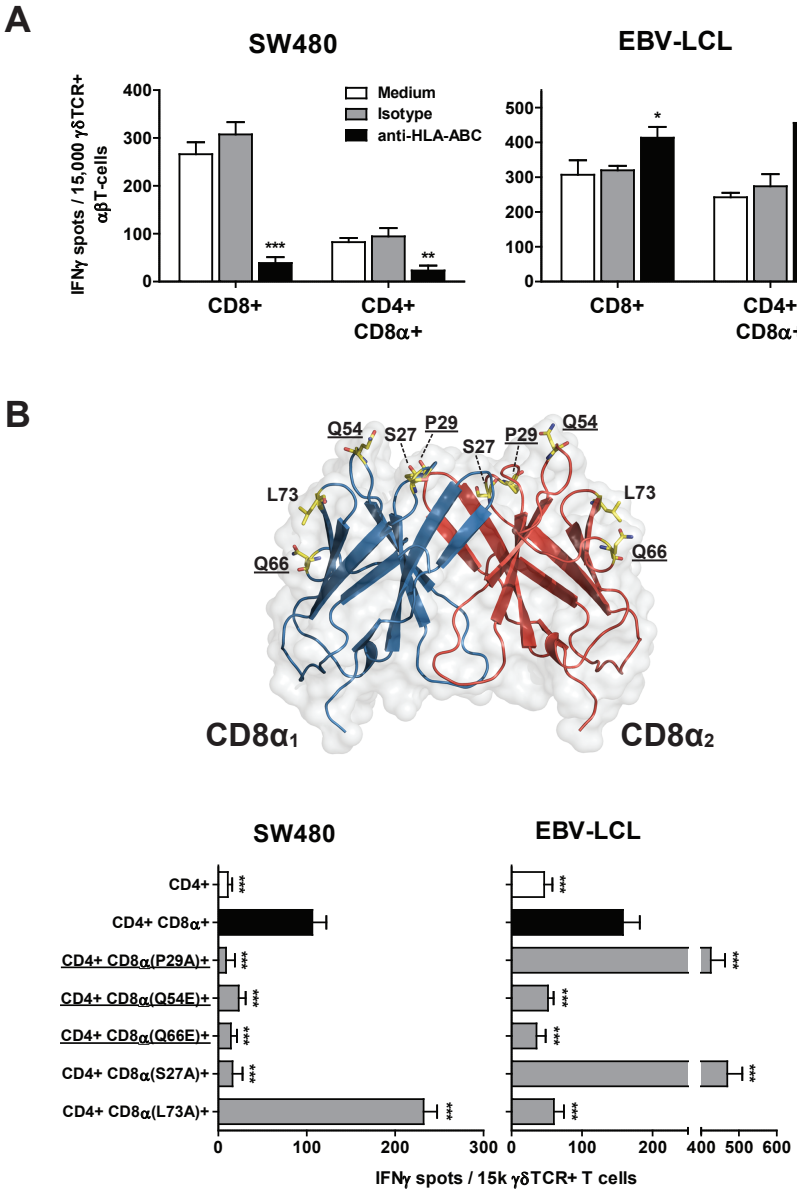

Figure 4. CD8αα binds to distinct ligands, including MHC-I-like molecules, on different tumour cells using diverse molecular domains. (A) CD8+ and CD4+CD8α+ T cells expressing the FE11 γδTCR were coincubated in an IFNγ ELISPOT with SW480 or EBV-LCL cells in the presence of no antibody, a control antibody or a pan-MHC-I blocking antibody. (B) CD4+ T cells were transduced with the FE11 γδTCR in combination with wildtype CD8α or with CD8α mutants. Included CD8α mutations are indicated in the CD8αα crystal structure (top panel). Underlined mutations indicate variants described to interfere with the interaction between CD8αα and MHC class-I molecules (15). The effect of CD8α mutations on γδTCR-

Figure 4. (continued) mediated tumour recognition was tested by measuring IFNγ production upon coincubation with SW480 and EBV-LCL cells (lower panel). Data are representative of two separate experiments. Error bars represent S.E.M. (***P < 0.001).

one of the possible CD8αα ligands in the context of FE11 γδTCR-mediated T cell activation, and to further investigate the molecular requirements for CD8α in this process, we analysed the effect of previously characterized CD8α mutations on the capacity of CD8α to provide costimulatory help to the FE11 γδTCR. To this end, CD4+ T cells were transduced with the FE11 γδTCR and wildtype CD8α or CD8α variants carrying single amino acid substitutions (Fig. 4B). Transduced T cells and tumour targets were subsequently cocultured, and T cell activation was analysed by measuring IFNγ production. When cocultured with SW480 target cells, T cells that expressed CD8α variants previously described to abrogate binding to classical MHC-I (P29A, Q54E and Q66E (15)) also potently inhibited γδTCR-mediated tumour recognition (Fig. 4B). However, also CD8α mutations known not to affect the interaction with MHC-I resulted in clear differences in the recognition of SW480 cells by the FE11 γδTCR. The S27A substitution strongly inhibited IFNγ secretion, while the L73A mutation induced an increase in γδTCR+ T cell activation. Strikingly, CD8α mutations partly had different effects on FE11 γδTCR-mediated recognition of EBV-LCL target cells. Although mutations Q54E and Q66E showed similar inhibition of γδTCR+ T cell activation against EBV-LCL as to SW480 target cells, the effect on EBV-LCL recognition by the S27A, P29A and L73A substitutions strongly contrasted their effect on recognition of SW480 cells (Fig. 4B). Although differences were observed in expression levels of transgenic γδTCR and CD8α variants across T cell samples (Supplementary Fig. 5), these differences could not explain the opposing effects of CD8α mutations on γδTCR-mediated recognition of SW480 versus EBV-LCLs. Taken together, the results obtained from experiments using truncated CD8α, MHC-I blocking, as well as single residue CD8α mutants strongly suggest distinct involvements of CD8αα in the recognition of different targets by the FE11 γδTCR, indicating interactions with diverse CD8α ligands on distinct target cells that for some targets mimic, but are not identical to, the interaction between CD8α and MHC-I.

DISCUSSION

The broad and MHC-unrestricted tumour-reactivity of γδT cells makes these innate-like cells and their receptors attractive tools for cancer immunotherapy. However, detailed knowledge on the molecular requirements for γδTCR-mediated T cell activation are still lacking. We have previously isolated clinically interesting γδTCRs that depend on CD8αα for recognition of diverse tumour targets (6). In the present study, we demonstrate that CD8αα-dependent γδTCRs are capable of redirecting CD4+ helper T cells against tumour cells after simultaneous introduction of CD8αα. Strikingly, the partly opposing effects of CD8α mutations and MHC class I blocking on recognition of different tumour targets pointed to MHC class I-like molecules

but also other ligands for CD8αα in combination with a single γδTCR. The implications of our findings are manifold. γδT cells are apparently more diverse than so far anticipated, and our observations on the very common coreceptor CD8αα add a new level of complexity to the immunobiology of innate-like γδT cells. Moreover, the implied recognition of multiple ligands on diverse target cells by CD8αα suggests innate-like activation modes of CD8αα in the context of γδTCR-mediated target recognition that resemble innate pattern recognition receptors. Finally, our findings have important consequences for therapeutic strategies using γδT cells, as the complexity of γδT cells likely requires a reconsideration of current concepts aimed at applying γδT cells or their individual receptors.

The data presented here oppose the well-described inhibitory functions of CD8αα on conventional αβT cells. CD8αα expression can be induced on CD4+ and CD8αβ+ αβT cells upon activation, and functions as a corepressor rather than a coreceptor by competing with CD8αβ for the Lck signalling molecule (20). On the other hand, CD8αα can also be detected on innate immune cells and appears to perform distinct functions on these cells compared to conventional T cells. For example, CD8αα expression has been reported on NK cells and macrophages, and these CD8αα+ cells have been shown to be more cytotoxic than their CD8αα- counterparts (25,26), implying an immune-stimulatory role for CD8αα on these cells that could be in agreement with the data presented in this study. Involvement of CD8αα+ macrophages in the anti-tumour response has furthermore been suggested by work in animal tumour models, demonstrating pronounced infiltrates of CD8αα+ macrophages at sites of tumour (27). Moreover, active roles for CD8αα+ but not CD8αα- γδT cells have been reported in controlling HIV infection by secreting cytokines that compete with HIV virions for binding to the CCR5 receptor (28). In line with this, we observed significant increases in circulating CD8αα+ γδT cells in human cytomegalovirus infection (6). Thus, CD8αα appears to have opposing functions on innate and adaptive immune cells, and our data indicate an innate-like, stimulatory role of CD8αα in the context of γδTCRs.

The tumour target-dependent effects of CD8α mutations and MHC class I blocking most likely suggest diverse ligands and functions for CD8αα on these cells. Even though the precise identity of these ligands so far remains elusive, we observed that reactivity to the colorectal cancer cell line SW480 could be blocked by pan-MHC-I antibodies, suggesting MHC class I molecules may serve as ligands for CD8αα on these target cells. However, the CD8α mutation data only partially corroborate an interaction with MHC-I molecules, since CD8α mutations had in part opposing effects on SW480 recognition compared to those described for class I MHC molecules (15). The non-classical MHC molecule HLA-G is a known ligand of CD8αα (29,30), and is expressed on some colorectal cancers (31). Moreover, the pan-MHC antibody used in our experiments also recognizes HLA-G (30), suggesting that HLA-G could be the CD8αα ligand on SW480 tumour cells. The ligand for CD8αα on EBV-LCLs remains elusive, but could involve the recently characterized CD8 ligand CEACAM5 (32). The differential effects of CD8α mutations on EBV-LCLs versus SW480 cells could agree with CEACAM5 as a ligand, since the CD8-CEACAM5 interaction has been demonstrated to depend on non-class I binding sites of CD8α (33). Identification of the ligand of the FE11 γδTCR will be most valuable in elucidating the molecular targets of CD8αα, although the combined implication of the γδTCR

and CD8αα in tumour recognition makes ligand identification of either receptor increasingly challenging. Increasing this complexity is the fact that CD8αα can apparently perform distinct functions in recognizing diverse tumour target, as removal of the CD8α signalling domain impaired γδTCR+ T cell activation in response to SW480 cells but not EBV-LCLs. This suggest that, depending on the recognized tumour target, CD8αα can perform either signalling or adhesion functions. In mice, CD8αα can bind thymus leukemia antigen (TL), a non-classical MHC homologue that is expressed on epithelial cells of the intestine and thymocytes (34). Intriguingly, the binding affinity of TL for CD8αα is higher than that for CD8αβ, and exceeds affinities measured between classical mouse MHC molecules and CD8αα or CD8αβ (34,35). While a human homologue of TL has not been identified, this suggests the potential existence of CD8 ligands with higher affinity to CD8αα than to CD8αβ in humans.

Our observations on the diverse involvement of CD8αα in helping a defined γδTCR recognize different tumour targets emphasize a tremendous diversity within γδT cells that has so far been largely underestimated, and point to an intricate interplay between receptors in the activation of γδT cells. These findings are in line with reports on other innate-like receptors such as NK receptors, which have revealed diverse involvements of such receptors in the activation of γδT cells. For example, NKG2D, a well-studied NK receptor that recognizes the MHC class I homologs MICA/B and ULPB1-4, can augment γδTCR-mediated responses but can also activate γδT cells irrespective of γδTCR signalling, depending on the antigenic context (36,37). Interestingly, NKG2D binds its multiple ligands using overlapping but non-identical antigen recognition domains (38), resembling our data obtained with CD8α mutants. Thus, in the context of γδTCR-mediated activation, the activating functions of CD8αα could mimic those of innate-like receptors, contributing to a coordinated and diverse interplay of receptors capable of sensing diverse multimolecular antigenic signatures.

Importantly, the newfound perspectives on γδT cell diversity, including the data presented here, call for a redesign of γδT cell-based therapeutic strategies. Efforts to use γδT cells in cancer immunotherapy have so far yielded limited results (9,39,40), and one likely explanation is the underappreciated diversity of γδT cells. Clinical trials using γδT cells to treat cancer have so far relied on the application of bulk populations of γδT cells that are likely to include cells with diverse specificities, functions and avidities. Clinical efficacy of γδT cells may therefore benefit from the selective application of defined γδT cell subsets rather than heterogeneous cell products. As an alternative, broadly tumour-specific, CD8αα-dependent γδTCRs reported by us here and elsewhere (6) may prove valuable tools for gene engineering of αβT cells, and broad redirected anti-tumour immunity may be achieved by cotransfer of CD8α into CD4+ T cells.

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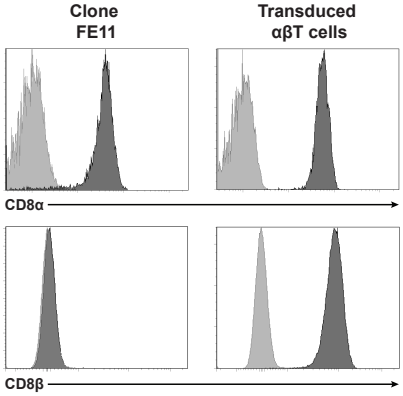
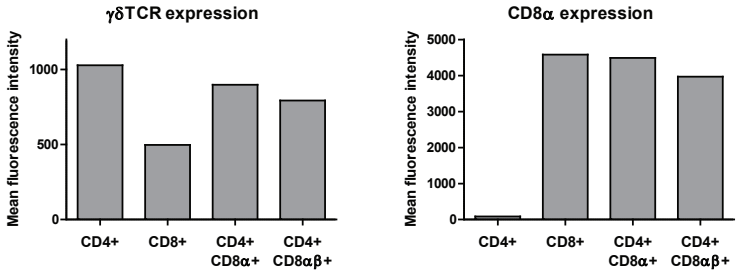
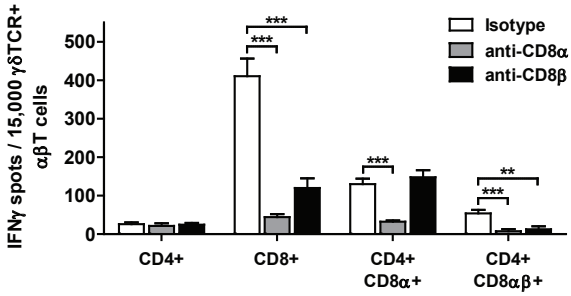
SUPPLEMENTARY DATA



Supplementary Figure 1. The γδT cell clone FE11 expresses CD8αα while αβT cells express CD8αβ. Surface expression of CD8α and CD8β on γδT cell clone FE11 and on γδTCR-transduced αβT cells was measured by flow cytometry. Light curves represent isotype controls, dark curves represent specific stainings.

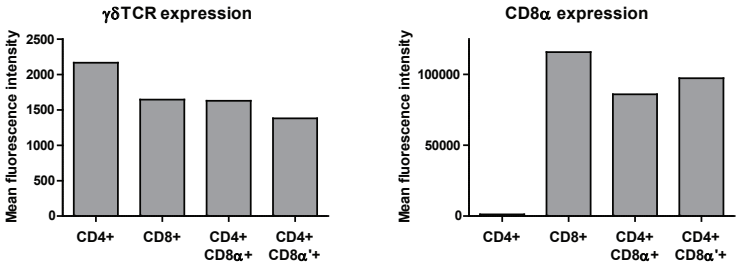


Supplementary Figure 2. Transgene expression levels on T cells transduced with the FE11 γδTCR in combination with CD8α alone or CD8α and CD8β. αβT cells were transduced with the FE11 γδTCR and either CD8α alone or CD8α combined with CD8β. Following, CD4+, CD8+, CD4+CD8α+ and CD4+CD8αβ+ T cells expressing the FE11 γδTCR were sorted and expression of γδTCR and CD8α was measured by flow cytometry.

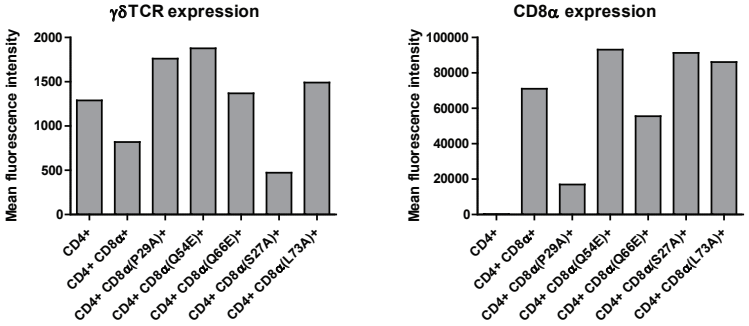


Supplementary Figure 3. CD8α and CD8β blocking on T cells transduced with the FE11 γδTCR and CD8α

Supplementary Figure 3. (continued) alone or CD8α with CD8β. αβT cells were transduced as in Supplementary Figure 2 and coincubated with SW480 target cells in the presence of control antibody or CD8α or CD8β blocking antibodies. IFNγ production was measured by ELISPOT.



Supplementary Figure 4. Transgene expression levels on T cells transduced with the FE11 γδTCR in combination with CD8α or truncated CD8α. αβT cells were transduced the FE11 γδTCR and with either wildtype CD8α or with a truncated, signaling-deficient CD8α mutant, and CD4+, CD8+, CD4+CD8α+ and CD4+CD8α'+ T cell populations were sorted. γδTCR and CD8 expression was subsequently assessed by flow cytometry.



Supplementary Figure 5. Transgene expression levels on T cells transduced with the FE11 γδTCR in combination with CD8α or CD8α mutants. As Supplementary Fig. 4, but now using CD8α single amino acid mutants instead of truncated CD8α.

Supplementary Table 1. CDR3 sequences of FE11 TCR γ and δ chains
ImMunoGeneTics (IMGT®) JunctionAnalysis output (www.imgt.org)

TCRγ

V name	3'V-REGION	P	N	5'J-REGION	J name
TRGV5*01	tgt gcc acc tgg gac agg	cct g	ag at	t tat tat aag aaa ctc ttt	TRGJ1*01

TCRδ

V name	3'V-REGION	N1	D1-REGION	P	N2	D2-REGION	N3	P	5'J-REGION	J name	D1 name	D2 name
TRDV1*01	tgt gct ctt ggg ga		t tcc tac	g	g	t ggg gga	ccc cta	t	ac acc gat aaa ctc atc ttt	TRDJ1*01	TRDD2*01	TRDD3*01

CHAPTER 6

Tumour recognition through Vγ9Vδ2 T cell receptors and BTN3A1 is critically mediated by the small GTPase RhoB

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ABSTRACT

Human Vγ9Vδ2 T cells respond to tumour cells by sensing elevated levels of small phosphorylated intermediates of the mevalonate pathway called phosphoantigens. Recent advances have pointed to BTN3A1 as a major player in Vγ9Vδ2 T cell activation. However, given the ubiquitous expression of BTN3A1 on healthy and transformed cells, it is unclear which molecular mechanisms link BTN3A1 to phosphoantigen accumulation in tumour cells. To identify additionally required mediators of Vγ9Vδ2 T cell activation, we developed a novel semi-high throughput screening method based on the hypothesis that genetic variation among target cells could segregate with their sensitivity to Vγ9Vδ2 T cell attack. Genome-wide correlation analysis using a library of genetically well-characterized cells and the capacity of these cells to activate Vγ9Vδ2 TCR+ T cells revealed several genetic loci that associated with Vγ9Vδ2 T cell activation. Subsequent investigation of candidate genes using RNAi highlighted a role for the small GTPase RhoB in modulating target cell recognition by Vγ9Vδ2 TCRs. Importantly, RhoB is a direct prenylation target of the mevalonate pathway, and inhibition of prenylation in tumour cells resulted in reduced recognition by Vγ9Vδ2 TCR+ T cells. Moreover, RhoB GTPase activity and subcellular localization associated with differential recognition of a wide variety of tumour cells. Crucially, we demonstrate that RhoB directly interacts with BTN3A1 only in recognized tumour cells, and as such determines the membrane mobility of BTN3A1. Together, these data identify RhoB as a crucial mediator of tumour recognition by Vγ9Vδ2 T cells, providing a "missing link" between the mevalonate pathway and BTN3A1 and novel therapeutic targets to improve Vγ9Vδ2 T cell-based cancer immunotherapies.

INTRODUCTION

γδT cells are unconventional T cells with strong reactivity towards a broad spectrum of tumours of diverse tissue origin. In contrast to conventional αβT cells, activation of γδT cells does not depend on the recognition of processed antigens presented by MHC molecules, but instead involves the sensing of generic stress molecules that are upregulated on transformed or otherwise stressed cells (1, 2). Activation of γδT cells by tumour cells results in the rapid release of cytotoxic molecules and the secretion of high levels of cytokines. Thus, γδT cells combine potent anti-tumour effector functions with the recognition of broadly expressed tumour antigens, and these features have put γδT cells into the spotlight for clinical application in cancer immunotherapy. However, the clinical success of γδT cells in cancer treatment is challenged by a poor understanding of the molecular requirements for γδT cell activation (3, 4). Moreover, a lack of prognostic markers makes it difficult to assess which patients may benefit from γδT cell therapy.

Vγ9Vδ2 T cells, the major γδT cell subset in human peripheral blood, express γδT cell receptors (TCR) composed of Vγ9 and Vδ2 chains and are specifically activated by small phosphorylated non-peptidic molecules called phosphoantigens (5, 6). These molecules are generated as intermediates of the mammalian mevalonate pathway, such as isopentenyl pyrophosphate

(IPP), or by the microbial 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway, such as (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP) (7). Intracellular phosphoantigen levels accumulate in tumour cells due to dysregulation of the mevalonate pathway or upon microbial infection, allowing the targeting of transformed or infected cells by Vγ9Vδ2 T cells. Similarly, intracellular phosphoantigen levels can be pharmaceutically increased by treating cells with mevalonate pathway inhibitors such as aminobisphosphonates (NBP), thus sensitizing cells towards recognition by Vγ9Vδ2 T cells. Even though the involvement of the Vγ9Vδ2 TCR in detecting elevated phosphoantigen levels was demonstrated as early as the 1990's (8-10), the molecular determinants on target cells required for activation of Vγ9Vδ2 TCRs have long remained elusive. Importantly, substantial progress has been made by recent breakthrough studies that identified the membrane-expressed butyrophilin BTN3A1 (CD277) as a key molecule in phosphoantigen-induced activation of Vγ9Vδ2 T cells (11-13). Binding of phosphoantigens to the intracellular domain of BTN3A1 correlated with the immobilization of BTN3A1 on the target cell surface (11, 12), and this has been suggested to contribute to an extracellular cue for recognition by Vγ9Vδ2 TCRs. However, BTN3A1 is expressed on transformed as well as healthy human cells (14), suggesting that additional molecules are involved in mediating selective recognition of tumour targets by Vγ9Vδ2 T cells. In particular, it is so far unclear which mechanisms link the accumulation of intracellular phosphoantigens to membrane alterations of BTN3A1.

Here, we used an unbiased, genome-wide screening method to identify the small GTPase RhoB as a critical mediator of Vγ9Vδ2 TCR activation. We show that the biochemical activity of RhoB correlates with the capacity of tumour targets to activate of Vγ9Vδ2 TCRs, and that this susceptibility to recognition by Vγ9Vδ2 TCRs is reflected by differential subcellular localization of RhoB. Importantly, RhoB activity regulated the membrane mobility of BTN3A1 through direct interactions between RhoB and BTN3A1. Together, these data provide novel insights into the molecular requirements for Vγ9Vδ2 T cell activation, which are highly required for the design of more effective, targeted γδT cell-based immunotherapies.

MATERIALS & METHODS

Cells and reagents

CEPH EBV-LCL lines (CEU population panel) were a kind gift from Tuna Mutis (UMC Utrecht, The Netherlands). Daudi, K562, SW480, HEK-293, HEK-293FT and Phoenix-Ampho cell lines were obtained from ATCC. LCL-TM (an EBV-LCL line separate from the CEPH panel) was kindly provided by Phil Greenberg (Seattle, U.S.A.). MZ1851RC was kindly provided by Barbara Seliger (University of Halle, Germany). Phoenix-Ampho cells were cultured in DMEM supplemented with 1% Pen/Strep (Invitrogen) and 10% FCS (Bodinco), all other cell lines in RPMI with 1% Pen/Strep and 10% FCS. Primary fresh PBMCs were isolated by Ficoll-Paque (GE Healthcare) from buffy coats supplied by Sanquin Blood Bank (Amsterdam, The Netherlands). Frozen primary acute myeloid leukemia (AML) samples were kindly provided by Matthias Theobald (Mainz, Germany) and were collected in compliance with GCP and Helsinki regulations.

The following reagents were used: pamidronate (Calbiochem), zoledronic acid monohydrate (zolidronate, Sigma-Aldrich), isopentenyl pyrophosphate (IPP) (Sigma-Aldrich), calpeptin (CN01; Cytoskeleton, Inc.), Rho Activator II (CN03; Cytoskeleton, Inc.), C3 transferase (Rho Inhibitor I CT04; Cytoskeleton, Inc.), farnesyl transferase inhibitor (FTI) (Sigma-Aldrich), and geranylgeranyltransferase inhibitor (Sigma-Aldrich).

Flow cytometry

Antibodies used for flow cytometry included: pan-γδTCR-PE (clone IMMU510, Beckman Coulter), CD4-FITC (eBioscience), CD8-APC (BD), unconjugated rabbit polyclonal RhoB (AbCam), goat-anti-rabbit AlexaFluor488 (Jackson ImmunoResearch). Mouse a-CD277 mAb (clone #20.1 and 103.2) were kindly provided by D. Olive (INSERM U891, Marseille, France). Samples were processed with FACSCalibur and FACSCanto-II flow cytometers (BD) and analyzed with FACSDiva software (BD). Primary leukemic stem cells and healthy progenitor cells were sorted according to phenotypic markers as previously described (15).

Retroviral transduction of TCRs

The Vγ9Vδ2-TCR clone G115 (16) and a HLA-A*0201-restricted $WT1_{126-134}$-specific αβTCR (17) were transduced into αβT cells as described (18, 19). In brief, Phoenix-Ampho packaging cells were transfected with gag-pol (pHIT60), env (pCOLT-GALV) and pBullet retroviral constructs containing TCRγ/β-chain-IRES-neomycine or TCRδ/α-chain-IRES-puromycin, using Fugene6 (Promega). PBMCs preactivated with αCD3 (30 ng/ml) (clone OKT3, Janssen-Cilag) and IL-2 (50 U/ml) were transduced twice with viral supernatant within 48 hours in the presence of 50 U/ml IL-2 and 4 μg/ml polybrene (Sigma-Aldrich). Transduced T cells were expanded by stimulation with αCD3/CD28 Dynabeads (0.5x106 beads/106 cells) (Invitrogen) and IL-2 (50 U/ml) and selected with 800 μg/ml geneticin (Gibco) and 5 μg/ml puromycin (Sigma-Aldrich) for one week. CD4+ TCR-transduced T cells were isolated by MACS-sorting using CD4-microbeads (Miltenyi Biotec).

Following transduction, transduced T cells were stimulated biweekly with 1 μg/ml PHA-L (Sigma-Aldrich), 50 U/ml IL-2 (Novartis Pharma), 5 ng/ml IL-15 (R&D Systems), and irradiated allogeneic PBMCs, Daudi and LCL-TM cells. Fresh IL-2 was added twice a week. Transgenic TCR expression and purity of CD4+ populations was routinely assessed by flow cytometry.

Functional T cell assays

IFNγ ELISPOT was performed as previously described (18, 20). Briefly, 15,000 Vγ9Vδ2 TCR-transduced or mock-transduced T cells and 50,000 target cells (ratio 0.3:1) were cocultured for 24 hrs in nitrocellulose-bottomed 96-well plates (Millipore) precoated with anti-IFNγ antibody (clone 1-D1K) (Mabtech). Plates were washed and incubated with a second biotinylated anti-IFNγ antibody (clone 7-B6-1) (Mabtech) followed by streptavidin-HRP (Mabtech). IFNγ spots were visualized with TMB substrate (Sanquin) and the number of spots was quantified using ELISPOT Analysis Software (Aelvis).

Alternatively, Vγ9Vδ2 TCR-transduced T cells and target cells were cocultured as above in round-bottom 96-well plates, and IFNγ levels in supernatants were measured by ELISA.

Where indicated, target cells were pretreated with pamidronate (100 μM), IPP (15 μM), FTI (10 μM), GGTI (50 μM), calpeptin (2 μg/ml) or C3 transferase (20 μg/ml) prior to coincubation. For testing stimulation of WT1 αβTCR-transduced T cells, the HLA-A2+ cell lines EBV-LCL 48 and MZ1851RC were pulsed with 10 μM $WT1_{126-134}$ peptide.

Zygosity/SNP correlation analysis

Recognition of CEPH EBV-LCL lines (pretreated with either medium, pamidronate (100 μM) or IPP (15 μM)) by Vγ9Vδ2 TCR-transduced CD4+ T cells was determined by ELISPOT. Mock-transduced T cells were included as effector controls, and any EBV-LCL line that elicited IFNγ production by mock-transduced cells were excluded from the analysis. Recognition of EBV-LCL lines by Vγ9Vδ2 TCR+ T cells in a single assay was defined as an at least two-fold increase in IFNγ spots compared to those produced in response healthy control target cells, irrespective of EBV-LCL pretreatment (i.e. medium, pamidronate or IPP). Assays were repeated five times, and a EBV-LCL line was defined as recognized only when recognized in at least three out of the five repeated assays. Hypothetical zygosities for candidate genetic loci were deduced using classical Mendelian inheritance patterns within CEPH family pedigrees, where the influence of candidate alleles on Vγ9Vδ2 TCR-mediated recognition was assumed to be dominant. Correlations of predicted zygosities with Hapmap SNP genotypes of CEPH individuals were subsequently calculated with the software tool ssSNPer, as previously described (21). Proxy SNPs within 500 kb of SNPs produced by ssSNPer were collected by querying the SNP Annotation and Proxy Search (SNAP) tool (22), using r2 = 0.8 as a threshold for linkage disequilibrium. eQTL analysis of ssSNPer SNP and their proxies was performed using the Genevar (GENe Expression VARiation) tool (23).

RNAi and CRISPR/Cas9 genome editing

HEK-293FT cells were transfected using Fugene 6 (Promega) with lentiviral constructs containing shRNAs (Sigma-Aldrich) against candidate genes of interest, together with lentiviral helper constructs VSVG and pspax2. EBV-LCL 48 cells were transduced with viral supernatants four days prior to functional T cell assays. Knockdown of targeted genes was confirmed using real-time Q-PCR, or in the case of RhoB, by flow cytometry.

RhoB knockout in MZ1851RC cells was achieved with the CRISPR/Cas system using a guide RNA that specifically targeted RhoB (GTGGTGGGCGACGGCGCGTG). A guide RNA with no homology to any human genome sequence was used as control. The efficiency of RhoB knockout was assessed using flow cytometry.

Western blot analysis

EBV-LCL lines 22, 48, 91 and 93 were treated with pamidronate overnight, and were lysed using lysis buffer containing NP-40. Lysates were centrifuged to remove cell debris and supernatants were separated by SDS-PAGE. Protein content was transferred to PVDF membranes (Millipore), blocked for 1 hr in blocking buffer (5% milk) and incubated overnight with rabbit polyclonal antibodies directed against RhoB (LifeSpan Biosciences) or β-tubulin (clone DM1A) (Sigma). Blots were subsequently incubated with HRP-conjugated secondary

antibodies, and bands were visualized using Pierce ECL substrate (Thermo Scientific).

Rho GTPase activity assay

EBV-LCL lines 48 and 93 were treated with pamidronate (100 μM) overnight, and lysed with NP-40-containing lysis buffer. Insoluble cell debris was separated using centrifugation and Rhotekin-coupled beads (Cytoskeleton, Inc.) was added to supernatants. Beads were pelleted by centrifugation, washed, and separated using SDS-PAGE. Total protein content was visualized using silver staining.

Confocal microscopy and data analysis

For intracellular immunofluorescence staining of RhoB, cells were treated with pamidronate overnight (where indicated) and were allowed to attach to coverslips precoated with poly-L-lysine (Sigma-Aldrich). Cells were subsequently permeabilized with Permeabilizing solution 2 (BD), blocked with blocking serum (50% pooled normal human serum in PBS), and stained with a rabbit polyclonal anti-RhoB antibody (AbCam) followed by a secondary Goat anti-Rabbit IgG AlexaFluor488-conjugated antibody (Jackson ImmunoResearch). Cells were washed with blocking serum, fixed with 4% paraformaldehyde, stained with DAPI (where indicated), and mounted onto microscopy slides using Mowiol. Images were acquired using a Zeiss confocal laser scanning microscope LSM 700. Ratios between nuclear and extranuclear signal of RhoB were determined using Volocity software (PerkinElmer), where DAPI staining was used, when available, to mark nuclei.

To determine colocalization between BTN3 molecules and the actin cytoskeleton, HEK-293 cells were cultured on poly-L-lysin-coated coverslips and pretreated with pamidronate or Rho Activator II (CN03) overnight. Cells were blocked, permeabilized and BTN3 and F-actin were stained with DyLight 680-conjugated BTN3 antibody (clone BT3.1, Novus Biologicals) and fluorescein-coupled phalloidin (Sigma), respectively. The correlation coefficient between BTN3 and F-actin signal was determined using using Volocity software and was taken as a measure of colocalization.

FRAP microscopy

FRAP analysis was performed as previously described (11, 12). In brief, HEK293FT cells expressing either EmGFP-fused CD277 were laid on m-slides (Ibidi) and analyzed using a Nikon A1 RS confocal microscope (60xNA 1.40 oil immersion objective). Selected rectangular areas were photobleached for 500 ms by using full power of laser intensity (> 90% of loss of fluorescence). Images were collected every 5 s, before (30 s) and after (120 s) bleaching using low laser intensity. Images were analyzed with Metamorph 7.5 (Molecular Devices, Universal Imaging) and NIS (Nikon) imaging software. The resulting curves were fitted using one-phase exponential equations.

Flow cytometry FRET

HEK-293 cells were permeabilized using Permeabilization solution 2 (BD), blocked with blocking serum (50% pooled normal human serum in PBS) and labeled with rabbit polyclonal

anti-RhoB antibody (AbCam). After washing with PBS, samples were labeled with either Alexa594-conjugated goat anti-rabbit IgG (acceptor) (Jackson ImmunoResearch) and CD277-PE (donor) (BT3.1, Biollegend). Donor fluorescence was measured using a FACSCanto-II flow cytometer (BD), where donor fluorescence of the double-labeled samples was compared with that of samples labeled only with donor antibody. FRET efficiency between RhoB and CD277 was calculated as the fractional decrease of donor fluorescence in the presence of the acceptor.

To assess homodimerization of CD277 molecules, HEK-293 cells were co-stained with equal amounts of PE-conjugated anti-CD277 (donor) and Dylight 680-conjugated anti-CD277 (acceptor) and samples were measured using a FACSCanto-II flow cytometer (BD). FRET efficiency was calculated as previously described (24), where the donor fluorophore was excited at 488 nm and detected at 576 ± 26 nm, the acceptor fluorophore was excited at 635 nm and detected at 780 ± 60 nm, whereas FRET intensity was measured using excitation at 488 nm and detection at 780 ± 60 nm. Correction factors for the spectral overlap between the different fluorescence channels were obtained from data measured on unlabeled and single-labeled cells.

Conformational changes of BTN3 molecules were assessed as previously described (25). In brief, cells were labeled with 5 μg/ml BODIPY-FL DPHE (donor) (Life Technologies) for 10 minutes on ice followed by 10 minutes at 37 °C. Cells were subsequently washed extensively with ice-cold PBS, and labeled with mouse anti-CD277 mAbs (either clone #20.1 or #103.2) and Alexa594-conjugated goat anti-mouse Fab fragments (acceptor) (Jackson ImmunoResearch). After washing, cells were resuspended in ice-cold PBS and measured immediately using a FACSCanto-II flow cytometer (BD). FRET efficiency was calculated from the fractional decrease of the donor fluorescence in the presence of the acceptor.

Proximity ligation assay

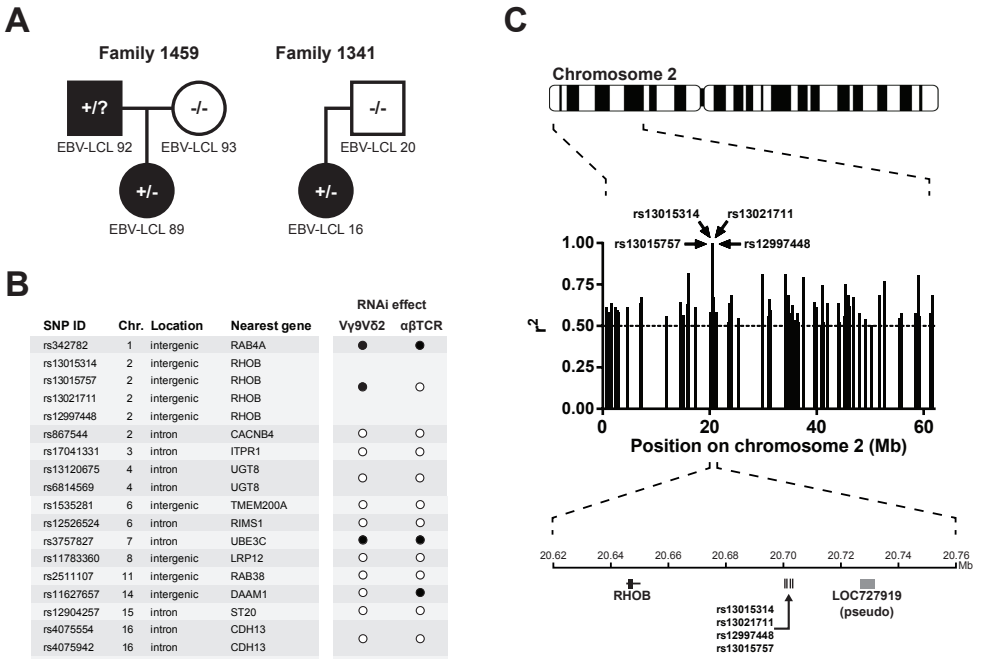
HEK-293FT cells were cultured on poly-L-lysine-coated coverslips and treated with 100 μM pamidronate overnight prior to permeabilization with Permeabilization buffer 2 (BD) for 15 minutes. Subsequently, cells were washed with PBS and blocked with blocking serum (50% pooled normal human serum in PBS). After blocking, cells were labeled with rabbit anti-RhoB (AbCam) and mouse anti-CD277 (Novus Biologicals) in blocking serum. Cells were washed with PBS-T (0.05% Tween) and incubated with secondary mouse PLUS and rabbit MINUS antibodies. Cells were washed in PBS-T before detection of the probe with the in situ PLA detection kit (Abnova). Cells were analyzed with a 63× objective on a Zeiss LSM 710 fluorescence microscope.

RESULTS

Identification of genetic loci associated with target cell recognition by Vγ9Vδ2 TCRs

To identify molecules involved in the recognition of tumour cells by Vγ9Vδ2 T cells, we hypothesized that a differential capacity of target cells to elicit Vγ9Vδ2 T cell responses might

be reflected by genetic variation between those cells. In this case, genetic information of recognized and non-recognized target cells could be used to identify genetic loci of molecules involved in the activation of Vγ9Vδ2 TCRs. To test this, we took advantage of the Centre d'Etude du Polymorphisme Humain (CEPH) library of cell lines, which contains a large collection of EBV-transformed B cell lines (EBV-LCLs) obtained from several family pedigrees (26). These cell lines have been genotyped for millions of SNPs by the International Hapmap Project (27), and could therefore represent a powerful tool to associate recognition of individual CEPH cell lines by Vγ9Vδ2 T cells to genetic loci. Activation of primary Vγ9Vδ2 T cells is substantially influenced by their diverse γδTCR repertoire (28) and by the expression of a variety of NK receptors (3, 29), and we suspected that these factors could strongly confound the outcome of the genetic association analyses. Recognition of different CEPH cell lines by Vγ9Vδ2 TCRs was therefore tested using αβT cells transduced with a single, well-studied Vγ9Vδ2 TCR (clone G115) (16), of which we previously demonstrated its capability to reprogram αβT cells towards a variety of cancers (18, 28). Only CD4+ G115-transduced αβT cells were used, as CD4+ T cells express fewer NK receptors than CD8+ T cells ((30); our unpublished observation). Recognition of a total of 42 CEPH EBV-LCLs by Vγ9Vδ2 TCR-transduced T cells was tested by measuring IFNγ production. The resulting recognition phenotypes (Supplementary Fig. 1) combined with information on family pedigrees of the used CEPH cell lines allowed the prediction of zygosities of candidate loci for 12 CEPH individuals (Fig. 1A). Direct correlation between predicted zygosities and Hapmap SNP genotypes was subsequently evaluated using the ssSNPer software tool (21), and 22 SNPs were identified of which genotypes correlated perfectly (100%) with predicted zygosities (Fig. 1B, Supplementary Fig. 2). As the ssSNPer software tool includes genotype information for many, but not all SNPs of CEPH individuals, the ssSNPer output was complemented with proxy SNPs that are in high linkage disequilibrium ($r^2 > 0.8$) with the 22 SNPs. None of the original 22 SNPs nor their proxies directly affected genes by causing changes in protein coding sequences. Thus, we hypothesized that here-identified SNPs could represent surrogate markers for genetic regions associated with susceptibility to Vγ9Vδ2 TCR recognition rather than playing direct roles, and queried the genomic vicinity of the 22 SNPs for neighboring candidate genes (Fig. 1C). To test the relevance of these genes for Vγ9Vδ2 TCR-mediated target recognition, cells of a CEPH EBV-LCL line that is recognized by Vγ9Vδ2 TCR+ T cells (EBV-LCL 48) were transduced with vectors encoding shRNAs against all 17 SNP-adjacent genes, and the effect of knockdown on activation of Vγ9Vδ2 TCR+ T cells was assessed by measuring IFNγ. To ensure that potential knockdown effects pointed to genes implicated specifically in Vγ9Vδ2 TCR-dependent activation, rather than general T cell-target interaction mechanisms, such as cell adhesion, knockdown assays were conducted in parallel with T cells engineered to express a defined αβTCR (specific against the Wilm's tumour 1 [WT1] antigen) in response to EBV-LCL 48 cells pulsed with cognate WT1 peptide antigen. Significantly reduced IFNγ production was observed with the knockdown of several candidate genes (Supplementary Fig. 3). However, knockdown of only the small GTPase RhoB selectively affected the activation of Vγ9Vδ2 TCR+ T cells (Fig. 1B), suggesting that RhoB could play a role in Vγ9Vδ2 TCR-mediated target recognition. To confirm the involvement of RhoB in the stimulation of Vγ9Vδ2 TCR+ T cells by target cells other than EBV-LCLs, RhoB was subsequently



SNP ID	Chr.	Location	Nearest gene	RNAi effect Vγ9Vδ2	RNAi effect αβTCR
rs342782	1	intergenic	RAB4A	●	●
rs13015314	2	intergenic	RHOB		
rs13015757	2	intergenic	RHOB	●	○
rs13021711	2	intergenic	RHOB		
rs12997448	2	intergenic	RHOB		
rs867544	2	intron	CACNB4	○	○
rs17041331	3	intron	ITPR1	○	○
rs13120675	4	intron	UGT8	○	○
rs6814569	4	intron	UGT8		
rs1535281	6	intergenic	TMEM200A	○	○
rs12526524	6	intron	RIMS1	○	○
rs3757827	7	intron	UBE3C	●	●
rs11783360	8	intergenic	LRP12	○	○
rs2511107	11	intergenic	RAB38	○	○
rs11627657	14	intergenic	DAAM1	○	●
rs12904257	15	intron	ST20	○	○
rs4075554	16	intron	CDH13	○	○
rs4075942	16	intron	CDH13		
rs37038	16	intron	NDRG4	○	●
rs11084914	19	intergenic	SCAMP4	○	○
rs12150939	19	intron	ZNF383	○	○
rs9978228	21	intron	NCAM2	○	○

Figure 1. Zygosity/SNP correlation analysis to identify genetic loci associated with Vγ9Vδ2 TCR stimulation. (A) The recognition of CEPH EBV-LCL lines by Vγ9Vδ2 TCR+ T cells provided the basis for deducing hypothetical zygosities of candidate loci in each cell line (black: recognized; white: not recognized; square: male; circle: female; +/-: heterozygous; -/-: homozygous negative; +/?: undetermined). Members of two CEPH families are shown as examples. For CEPH ID numbers of cell lines, see Supplementary Fig. 1. (B) Genetic association analysis revealed 22 SNPs of which genotypes correlated 100% (r2 = 1) with between predicted zygosities of cell lines. Locations and nearest neighboring genes of SNPs are indicated. The effect of knocking down candidate genes on recognition of EBV-LCL 48 by T cells transduced with either Vγ9Vδ2 TCR clone G115 or an HLA-A*0201-restricted WT1126-134-specific αβTCR are indicated by black circles (significant effect on T cell activation) and white circles (no effect). For testing recognition by WT1 αβTCR+ T cells the EBV-LCL 48 line was pulsed with WT1126-134 peptide. (C) Associating SNPs resulting from association analysis with candidate genes. The genetic region of the SNPs neighboring RhoB is shown as an example. Each bar represents one SNP and r2 values represent correlation between predicted zygosities and SNP genotypes.

knocked down in the prototypic Vγ9Vδ2 T cell target cell line Daudi. Also in Daudi target cells, knockdown of RhoB resulted in reduced activation of Vγ9Vδ2 TCR-transduced T cells (Fig. 2A). As EBV-LCLs and Daudi target cells both represent B cell lineage-derived tumours, the involvement of RhoB in the recognition of transformed cells from other tissue origin was tested by knocking out RhoB in the MZ1851RC renal carcinoma cell line using the CRISPR/Cas9 system. Again, interfering with RhoB expression resulted in reduced activation of

Vγ9Vδ2 TCR-transduced, but not WT1 αβTCR-transduced T cells (Fig. 2B), demonstrating that the RhoB GTPase is implicated in the recognition of diverse tumour targets by Vγ9Vδ2 TCRs. Changes in expression levels of Rho GTPases (which include RhoA, RhoB and RhoC isoforms) are associated with development of various cancers (31, 32), and thus could explain the involvement of RhoB in the activation of Vγ9Vδ2 T cells. RhoB protein levels were therefore determined by western blot analysis in two recognized and two non-recognized CEPH EBV-LCL

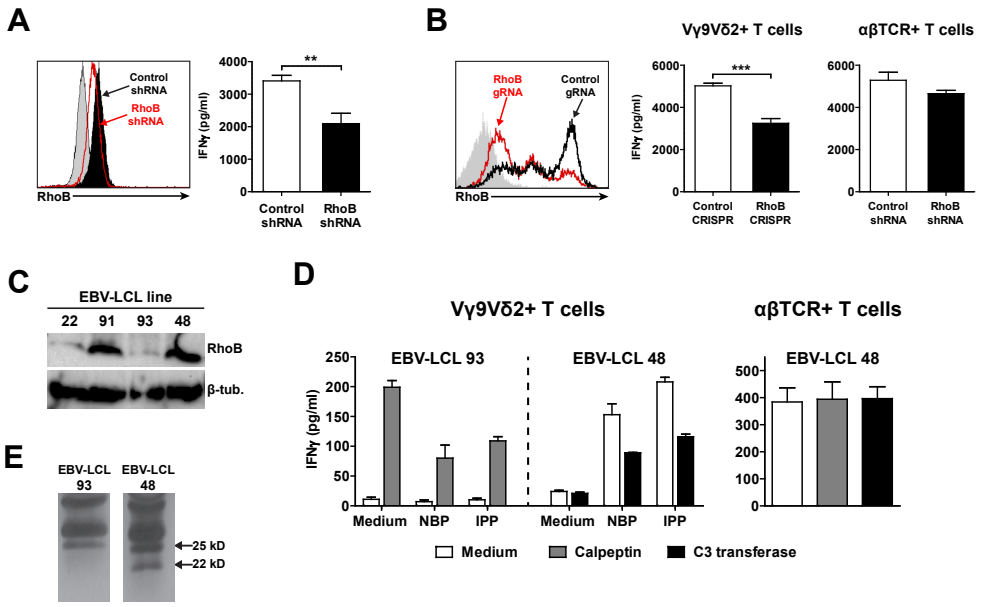


Figure 2. RhoB expression and activity correlates with target cell recognition by Vγ9Vδ2 TCR+ T cells. (A) Daudi cells were lentivirally transduced with shRNA targeting RhoB, and the effect of RhoB knockdown on recognition by Vγ9Vδ2 TCR+ T cells was assessed by measuring IFNγ. A vector encoding an irrelevant shRNA served as negative control. (B) RhoB was knocked out in the renal cancer cell line MZ1851RC using the CRISPR/Cas9 system. MZ1851RC cells were subsequently pretreated with either pamidronate or HLA-A*0201-restricted WT1126-134 peptide and the effect on target cell recognition by Vγ9Vδ2+ and WT1 αβTCR+ T cells, respectively, was determined by measuring IFNγ. A guide RNA targeting an irrelevant sequence was used as control. (C) RhoB protein levels were measured in the recognized EBV-LCL lines 48 and 91 and the non-recognized line 22 and 93 by western blot analysis. β-tubulin served as loading control. (D) The non-recognized EBV-LCL line 93 was pretreated with the Rho GTPase activator calpeptin in combination with the NBP pamidronate, with soluble IPP or with medium and the effect on stimulation of Vγ9Vδ2+ T cells was assessed by measuring IFNγ. The recognized EBV-LCL line 48 was treated as EBV-LCL 93 cells, only here the Rho GTPase inhibitor C3 transferase was used instead of calpeptin. The effect of Rho-modulating compounds on recognition of WT1126-134 peptide-pulsed EBV-LCL 48 cells by WT1 αβTCR+ T cells was measured in parallel. (E) Cell lysates of pamidronate-treated EBV-LCL 48 and 93 cells were coincubated with bead-coupled Rhotekin, after which Rhotekin-bound precipitates were separated via SDS-PAGE electrophoresis and protein content was visualized using silver staining. Error bars represent S.E.M. (**P < 0.01, ***P < 0.001).

lines. Recognized cell lines 48 and 91 expressed higher levels of RhoB than non-recognized cell lines 22 and 93 (Fig. 2C), suggesting that RhoB protein levels correlated with recognition by Vγ9Vδ2 TCRs.

Target cell recognition by Vγ9Vδ2 TCRs depends on Rho GTPase activity

Small GTPases such as RhoB function as molecular switches by cycling between active, GTP-bound and inactive, GDP-bound conformations (33). We therefore questioned whether observed differences in RhoB protein expression reflected also differential GTPase activity of RhoB in recognized versus non-recognized target cells. CEPH EBV-LCL lines were therefore pretreated with calpeptin or C3 transferase, compounds that activate or inhibit the enzymatic activity Rho GTPases, respectively (34, 35), and tested for recognition by Vγ9Vδ2 TCR+ T cells. Even though the EBV-LCL line 93 is not recognized by Vγ9Vδ2 TCR-transduced T cells, even after stimulating cells with the NBP pamidronate or exogenous phosphoantigen, pretreatment with calpeptin markedly sensitized EBV-LCL 93 cells for recognition by Vγ9Vδ2 TCR+ T cells (Fig. 2D). Conversely, inhibition of Rho GTPase activity by pretreating the NBP- and IPP-sensitive EBV-LCL line 48 with C3 transferase resulted in substantially reduced activation of Vγ9Vδ2 T cells. Importantly, modulation of Rho GTPase activity did not affect the recognition of WT1 peptide-pulsed EBV-LCL 48 cells by WT1 αβTCR-transduced T cells. To test whether enzymatic activity of Rho GTPases correlated with recognition by Vγ9Vδ2 T cells also in untreated EBV-LCL lines, lysates of non-recognized EBV-LCL 93 cells and recognized EBV-LCL 48 cells were treated with bead-coupled recombinant Rhotekin, an interaction partner of Rho GTPases that selectively binds to the active, GTP-bound state of Rho GTPases. Rhotekin-bound precipitates were subsequently separated on polyacrylamide gels and protein content was analyzed by silver staining. Interestingly, comparison of both precipitates revealed the differential presence of a band at ~22 kD, the expected molecular size of Rho GTPases (Fig. 2E). Thus, these data suggest that the biochemical activity of Rho GTPases is a critical factor defining the recognition of target cells by Vγ9Vδ2 T cells.

The intracellular distribution of RhoB in target cells marks susceptibility for Vγ9Vδ2 TCR-mediated recognition

The regulation of Rho GTPase activity is in part regulated by sequestering to distinct subcellular locations, a process that strongly depends on the posttranslational incorporation of prenyl groups (either farnesyl pyrophosphate or geranyl-geranyl pyrophosphate) to the C-terminus of small GTPases (36, 37). Farnesyl and geranyl-geranyl pyrophosphates are end products of the same mevalonate pathway that produces Vγ9Vδ2 T cell-activating phosphoantigens (7, 38), and thus could provide a link between dysregulation of the mevalonate pathway in tumour cells and the differential Rho GTPase activity in recognized versus non-recognized target cells observed above. Intracellular RhoB immunofluorescence staining was therefore used to investigate whether recognition of target cells by Vγ9Vδ2 T cells could correlate with distinct subcellular localization of RhoB. Importantly, the use of a RhoB-specific monoclonal antibody allowed the distinction between RhoB and the other two Rho isoforms RhoA and RhoC, which was not possible using the Rho GTPase modulating compounds and Rhotekin-

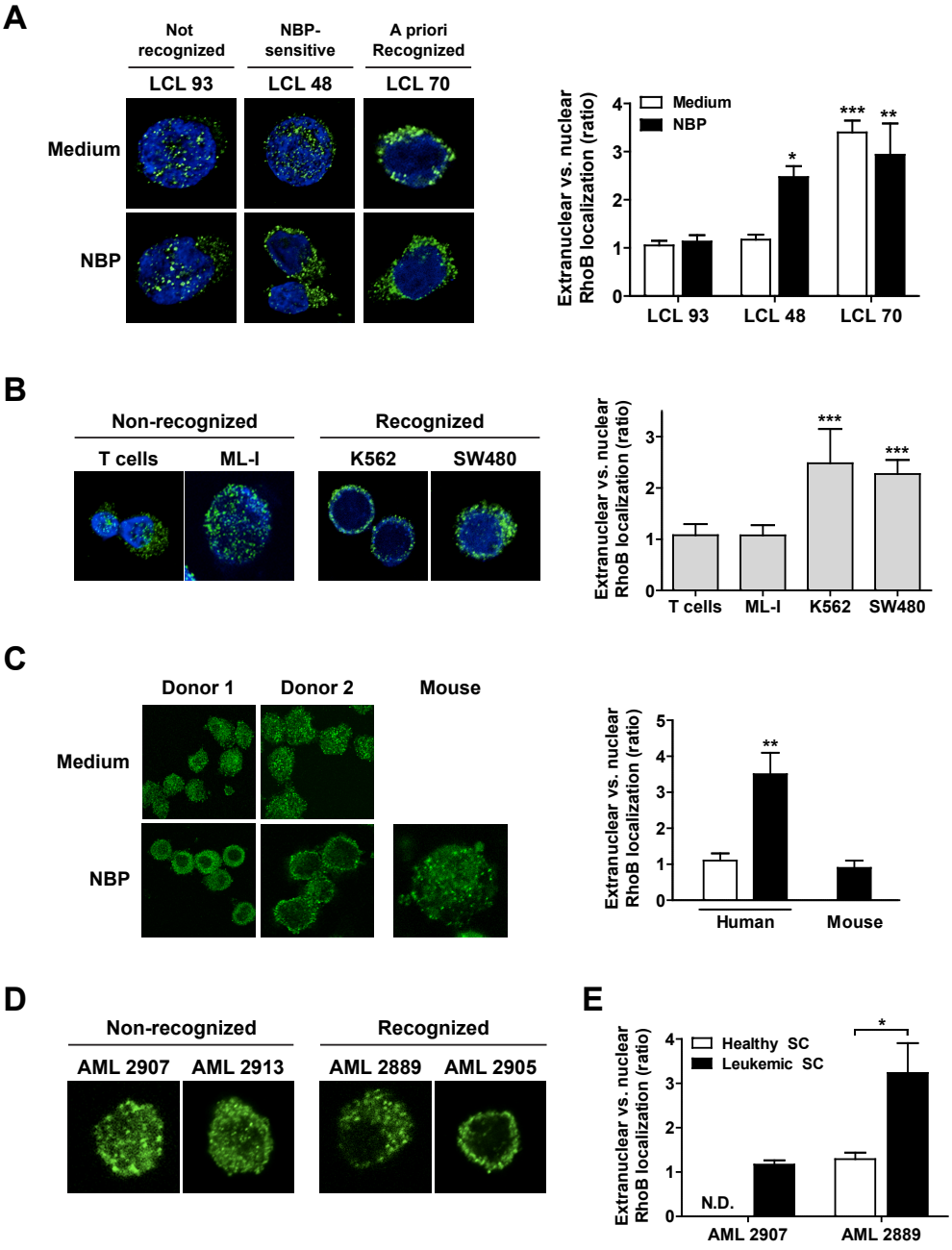


Figure 3. Intracellular distribution of RhoB correlates with the recognition of target cells by Vγ9Vδ2 TCR+ T cells. (A) Left panel: non-recognized EBV-LCL 93 cells, NBP-sensitive EBV-LCL 48 cells and a priori recognized EBV-LCL 70 cells were treated with medium or with pamidronate and loaded onto poly-L-lysine-coated coverslips. Attached cells were fixed and permeabilized,

Figure 3. (continued) and stained using RhoB-specific antibody followed by an AlexaFluor488-conjugated secondary antibody. RhoB distribution was subsequently analyzed by confocal microscopy and representative images are shown (green: RhoB; blue: nucleus [DAPI]). Right panel: the RhoB signal ratio between nuclear and extranuclear cellular compartments was measured using Volocity image analysis software. Graphs show average ratios of at least 10 different cells. Statistically significant differences were determined relative to medium-treated EBV-LCL 93 cells. (B) The intracellular RhoB distribution and extranuclear/nuclear RhoB signal ratios for the non-recognized leukemic cell line ML-I cell line and healthy T cells, and the recognized leukemia cell line K562 and colon carcinoma cell line SW480 were analyzed as in (A). Statistically significant differences were determined relative to healthy T cells. (C) Intracellular RhoB distribution was determined in monocyte-derived human dendritic cells from two different donors in the presence or absence of pamidronate. Bone marrow-derived murine dendritic cells (>95% CD11c+) were treated with pamidronate and used for intracellular labeling of RhoB. (D) The intracellular distribution of RhoB in non-recognized and recognized primary AML samples was determined as in (A), but here in the absence of nuclear DAPI staining. (E) CD34+CD38- leukemic stem cells were sorted from two patients of which leukemic blasts were recognized (AML 2889) and not recognized (AML 2907), respectively, and the ratios between extranuclear and nuclear RhoB signal was measured. CD34+CD38+ healthy progenitor cells from patient 2889 were sorted as negative control. Confocal imaging in all experiments was set up for optimal visualization of RhoB distribution, without taking into account potential expression differences of RhoB between cell samples. Error bars represent S.E.M. (*P < 0.05, **P < 0.01, ***P < 0.001).

coupled beads. Confocal imaging revealed a pronounced intracellular patch-like staining pattern of RhoB in all cells tested (Fig. 3A-D), in line with the previously described localization of RhoB primarily to membranes of intracellular vesicles such as endosomes (39, 40). Strikingly however, distribution of RhoB patches segregated strongly with the susceptibility of different EBV-LCL lines to Vγ9Vδ2 TCR-mediated recognition, being homogeneously distributed in non-recognized cells (i.e. EBV-LCL 93 and EBV-LCL 48 without NBP pretreatment) while being excluded from nuclear areas in recognized cells (i.e. NBP-treated EBV-LCL 48 and EBV-LCL 70) (Fig. 3A). These differences were highly reproducible, as assessed by quantification of nuclear versus extranuclear signal ratios across cell samples. Of note, prevention of protein prenylation by treatment with farnesyltransferase inhibitor (FTI) and geranyl-geranyltransferase inhibitor (GGTI) resulted in decreased recognition of NBP-stimulated EBV-LCL 48 cells by Vγ9Vδ2 TCR+ T cells (Supplementary Fig. 4). These data are in line with previous observations showing that FTI and GGTI treatment induces a nuclear localization of RhoB (41), resembling the signature RhoB distribution of non-recognized tumour cells observed here. Importantly, similar correlations between subcellular RhoB distribution and recognition by Vγ9Vδ2 TCRs were seen in alternative target cells previously tested for Vγ9Vδ2 TCR triggering (18), including the recognized myeloid leukemia cell line K562 and colon carcinoma cell line SW480 and the non-recognized leukemic cell line ML-I cell line and primary T cells (Fig. 3B). Interestingly, and in line with our previous observations that NBP-treated monocyte-derived dendritic cells are recognized by Vγ9Vδ2 TCR+ T cells (18), RhoB was excluded from nuclei of dendritic cells that were pretreated with NBP but not in untreated controls (Fig. 3C). Moreover, mouse cells are not recognized by human Vγ9Vδ2 T cells (7), and nuclear exclusion of RhoB was not observed in murine dendritic cells, even after pretreatment with NBP (Fig. 3C).

We have previously shown that leukemic blasts obtained from a proportion of patients

suffering from acute myeloid leukemia are susceptible to Vγ9Vδ2 TCR-mediated killing (18), and therefore questioned whether RhoB distribution associated also with recognition of these target cells. Indeed, also in primary leukemic blasts, RhoB localization correlated with the recognition by Vγ9Vδ2 TCRs, with RhoB being excluded from nuclei in recognized patient samples (Fig. 3D). One important determinant of the efficacy of cancer treatment is the capacity to target cancer stem cells. Thus, CD34+CD38- leukemic stem cells and CD34+CD38+ healthy progenitor cells from patient samples were sorted and evaluated for intracellular RhoB distribution. Strikingly, leukemic stem cells of a patient sample recognized by Vγ9Vδ2 T cells (AML 2889), but not healthy progenitor cells of the same patient, displayed RhoB distribution patterns associated with recognition by Vγ9Vδ2 TCRs (Fig. 3E), in line with our previous data showing that Vγ9Vδ2 T cells can target cancer stem cell compartments (18). Leukemic stem cells from a non-recognized patient sample (AML 2907) displayed a RhoB distribution pattern associated with lack of Vγ9Vδ2 TCR activation (extranuclear/nuclear RhoB localization ration of ~1), suggesting that the sensitivity of patient leukemia samples to Vγ9Vδ2 T cell attack depends primarily on recognition of leukemic stem cells. Taken together, the intracellular distribution of the small GTPase RhoB strongly correlates with recognition of diverse tumour targets, including cancer stem cells, and exclusion of RhoB from the nucleus is a signature of tumour cells susceptible for targeting by Vγ9Vδ2 TCR+ T cells.

RhoB regulates membrane mobility of BTN3A1 on target cells

The transmembrane protein BTN3A1 has recently been identified as a critical determinant of Vγ9Vδ2 T cell activation (11-13). Activation of Vγ9Vδ2 T cells correlated with decreased mobility of BTN3A1 on the target cell surface, suggesting that BTN3A1 immobilization is required for stimulating Vγ9Vδ2 T cells (11, 12). However, the mechanisms linking intracellular accumulation of phosphoantigens to changes in BTN3A1 membrane mobility are unclear. RhoB performs important functions in cytoskeletal reorganization and formation of actin stress fibers (42), suggesting that the implication of RhoB in Vγ9Vδ2 T cell activation could be via cytoskeletal rearrangements that impact BTN3A1 membrane mobility. In line with previous reports (11, 12), treating cells with the NBP zoledronate resulted in decreased BTN3A1 membrane mobility (Fig. 4A). Strikingly, treatment with the general Rho GTPase activator CN03 induced immobilization of BTN3A1 to similar levels as those of NBP treatment. To specifically test the implication of RhoB but not other Rho GTPases, the induction of BTN3A1 immobility in response to NBP was assessed in cells in which RhoB was knocked out using CRISPR/Cas9. Importantly, depletion of RhoB inhibited the NBP-induced immobilization of BTN3A1 to levels comparable to those of medium controls (Fig. 4A), suggesting that NBP-mediated changes in BTN3A1 mobility depend on RhoB.

To next assess a role for RhoB-induced cytoskeletal rearrangements in mediating the observed changes in BTN3A1 mobility, the relation between BTN3 molecules and F-actin was investigated by colocalization experiments. HEK cells were stained with fluorescently labeled anti-BTN3 and phalloidin and colocalization coefficients were determined in response to treatment with NBP and the Rho activator CN03. Without treatment, a variable but considerable colocalization between BTN3 and F-actin was observed. However, treatment

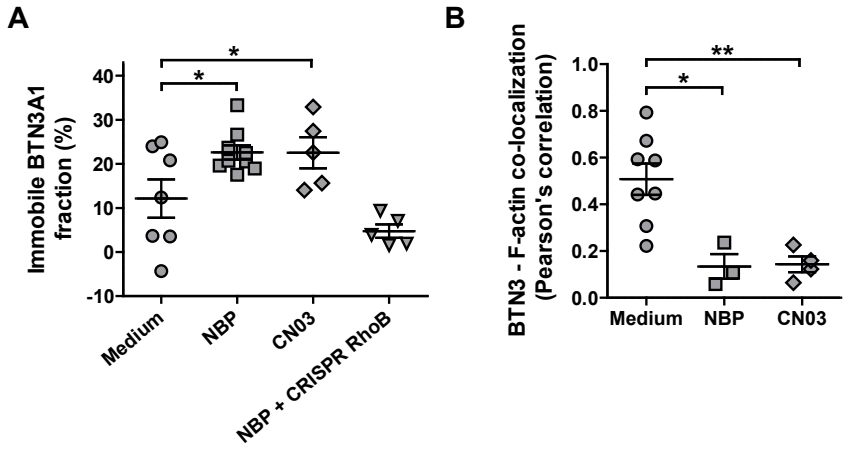


Figure 4. RhoB activity modulates BTN3A1 membrane mobility and its association with the actin cytoskeleton. (A) HEK-293 cells were transfected with BTN3A1-emGFP fusion constructs and treated with medium, zoledronate, or the Rho activator CN03. Zoledronate treatment was also applied to HEK-293 BTN3A1-emGFP+ cells in which RhoB was knocked out by CRISPR/Cas9. Selected membrane regions of interest were subsequently photobleached and FRAP was measured as described in the Materials & Methods section. (B) HEK-293 cells were pretreated with pamidronate or with the Rho activator CN03, and BTN3 molecules and filamentous actin (F-actin) were stained using a fluorescently labeled anti-BTN3 antibody and fluorescent phalloidin, respectively. The colocalization of BTN3 and F-actin was subsequently assessed by determining the localization correlation of both signals, as described in the Materials & Methods section. Center lines and error bars represent average and S.E.M., respectively (*P < 0.05, **P < 0.01).

with NBP resulted in a marked reduction in BTN3-actin colocalization (Fig. 4B). Strikingly, and similar to its effect on BTN3A1 membrane mobility, the Rho activator CN03 reduced colocalization between BTN3 and F-actin to comparable levels observed with NBP treatment, suggesting that both phosphoantigen accumulation and Rho activation may induce the formation of cytoskeleton-depleted membrane domains in which BTN3 molecules are immobilized, as reported for the antibody receptor FcεRI (43). Together, these data suggest that RhoB activity contributes to target recognition by Vγ9Vδ2 TCRs by modulating BTN3A1 membrane mobility through cytoskeletal rearrangements.

RhoB interacts with BTN3A1 homodimers in target cells recognized by Vγ9Vδ2 TCRs

Given the strong requirement for RhoB activity in the membrane immobilization of BTN3A1, we questioned whether regulation of BTN3A1 involved direct interactions with RhoB. A potential interaction between the two proteins was therefore investigated using an in situ proximity ligation assay (PLA), which allows the detection of proteins in <40nm proximity. Using PLA, RhoB and BTN3A1 were observed to be in close proximity in recognized EBV-LCL 48 cells pretreated with pamidronate (Fig. 5A). Interestingly, PLA signals were typically excluded from

the nuclear area and distributed close to the plasma membrane, in line with our hypothesis that RhoB could be involved in Vγ9Vδ2 TCR-mediated recognition by regulating membrane-expressed BTN3A1. To determine whether potential RhoB-BTN3A1 interactions could be detected at even higher resolution, interactions were examined using fluorescence resonance energy transfer (FRET), which allows detection of proximity distances of less than 10nm. HEK-293 cells, which are only recognized by Vγ9Vδ2 T cells after NBP treatment ((11) and data not shown), were labeled with RhoB and BTN3 antibodies conjugated to FRET-compatible fluorochromes and FRET levels were measured by flow cytometry. Close association between RhoB and BTN3A1 was negligible in untreated HEK cells, however increased markedly after treating cells with the NBP pamidronate (Fig. 5B), suggesting that RhoB and BTN3 molecules closely interact at the surface membrane of Vγ9Vδ2 TCR-recognized target cells.

Crystallization studies have revealed that the extracellular domain of BTN3A1 exists as homodimers in solution (44), however whether BTN3A1 molecules dimerize when expressed in a cellular context has not been reported. Thus, HEK-293 cells were transfected with two variants of BTN3A1 fused to distinct, FRET-compatible fluorochromes and FRET efficiency was again measured by flow cytometry. In line with the crystallographic data using soluble BTN3A1, these experiments confirmed that BTN3A1 molecules are expressed as homodimers on the cell surface of target cells (Fig. 5C), however the pairing of BTN3A1 molecules was insensitive to NBP-induced phosphoantigen accumulation. Taken together, these data demonstrate that, selectively in a Vγ9Vδ2-stimulatory context, RhoB is capable of interacting with surface-

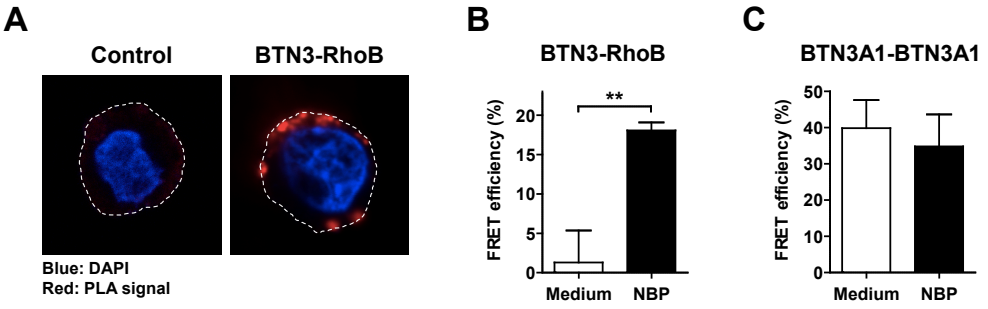


Figure 5. RhoB interacts with BTN3 molecules in response to phosphoantigen accumulation. (A) EBV-LCL 48 cells were treated with pamidronate, loaded onto poly-L-lysine-coated coverslips and permeabilized. The interaction between RhoB and BTN3 was subsequently assessed by Duolink PLA using anti-RhoB and anti-CD277 antibodies. Duolink PLA without antibodies against RhoB and CD277 served as negative control (red: PLA signal; blue: nucleus [DAPI]). (B) HEK-293 cells were pretreated with medium or pamidronate, trypsinized, permeabilized and stained with anti-RhoB-Alexa488 (FRET donor) and anti-CD277-DyLight 680 (FRET acceptor) antibodies. FRET efficiency was subsequently measured by flow cytometry as described in the Materials & Methods section. (C) HEK-293 cells were treated as in (B) and costained with equal amounts of anti-CD277-PE (donor) and anti-CD277-Dylight 680 (acceptor) antibodies, and FRET efficiency was subsequently measured. Error bars represent S.E.M. (**P < 0.01).

expressed BTN3A1 homodimers, suggesting direct modulation of BTN3A1 by RhoB.

Phosphoantigen accumulation associates with conformational changes of BTN3A1 dimers
Conformational changes of BTN3A1 in response to elevated levels of phosphoantigens have been proposed to serve as a stimulatory signature for Vγ9Vδ2 TCRs (11, 12, 44), however no experimental data have so far been reported to substantiate this hypothesis. The crystal structures of the extracellular domain of BTN3A1 in complex with the functionally well-characterized BTN3-specific antibodies 20.1 and 103.2 (11) have recently been resolved, and revealed that both antibodies bind to distinct epitopes on the membrane-distal Ig-V domains of BTN3A1 dimers (44). To study BTN3A1 conformational changes in response to increased phosphoantigen levels, surface membranes of HEK-293 cells were stained with the fluorescent lipid conjugate BODIPY FL, and BTN3 molecules were labeled with either 20.1 or 103.2 antibodies followed by fluorescently labeled secondary Fab fragments. Without NBP stimulation, potent FRET efficiencies between stained membrane and both antibodies were observed (Fig. 6A), suggesting that the BTN3 Ig-V domain is in close proximity to the cell membrane. Strikingly however, treatment of cells with NBP resulted in a marked reduction in FRET signals (Fig. 6A), demonstrating that intracellular phosphoantigen accumulation associates with a conformational change of BTN3 molecules that involves a pronounced distancing of the Ig-V domain from the cell membrane (Fig. 6B). These data provide support for the hypothesis that increases in intracellular phosphoantigen levels can induce extracellular changes in BTN3A1 dimers that may act as or contribute to an antigenic signature that is recognized by Vγ9Vδ2 TCRs.

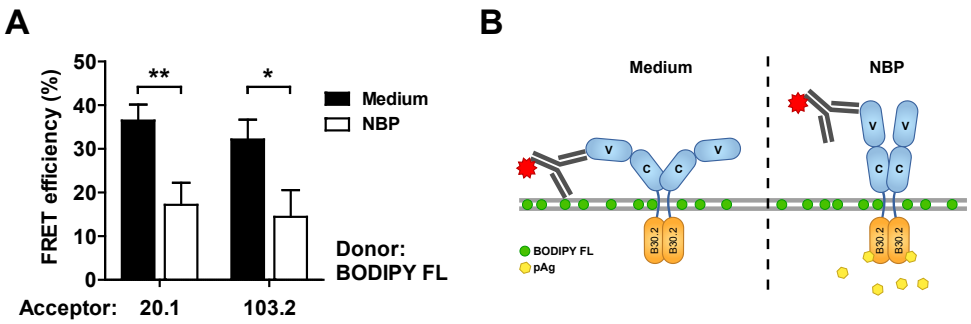


Figure 6. Intracellular phosphoantigen accumulation induces extracellular conformational changes in BTN3. (A) HEK-293 cells were pretreated with medium or pamidronate and trypsinized. The surface membrane of HEK-293 cells was subsequently stained with the fluorescent lipid conjugate BODIPY FL (FRET donor) and BTN3 molecules were labeled with either the 20.1 or the 103.2 antibody followed by staining with secondary Alexa594-conjugated Fab fragment (FRET acceptor). FRET efficiency was measured by flow cytometry. Error bars represent S.E.M. (*P < 0.05, **P < 0.01). (B) Model of conformational changes of BNT3 molecules in response to NBP treatment. pAg: phosphoantigen.

DISCUSSION

The translation of tumour-reactive Vγ9Vδ2 T cells to clinical application has proven challenging due to a poor understanding of the molecular mechanisms by which Vγ9Vδ2 T cells recognize their target cells. Important progress has been made in this respect by recent elegant reports identifying BTN3A1 as a phosphoantigen sensor that plays a key role in the activation of Vγ9Vδ2 T cells (11-13). Nevertheless, it is still unclear how intracellular phosphoantigen accumulation translates into extracellular signatures that can be recognized by Vγ9Vδ2 TCRs. In this study, we provide novel insights into these mechanisms by identifying the small GTPase RhoB as a critical player in phosphoantigen-induced Vγ9Vδ2 TCR activation. Our data demonstrate that RhoB interacts with BTN3A1 upon NBP stimulation and that the biochemical activity of RhoB strongly correlates with membrane rearrangements of BTN3A1 and stimulation of Vγ9Vδ2 TCRs. Together, these data put RhoB forward as a novel actor in the cellular mechanisms that couple phosphoantigen accumulation to BTN3A1-mediated triggering of Vγ9Vδ2 T cell responses.

Like other GTPases, RhoB has intrinsic GTPase activity that allows it to cycle between biologically active GTP-bound and inactive GDP-bound conformations, which in turn enables the differential association with components of downstream signaling pathways. As such, RhoB functions as a molecular switch in diverse cellular processes, ranging from gene transcription to the regulation of cytoskeletal changes and vesicle transport (42). Importantly, RhoB function is intimately coupled to the mevalonate pathway by posttranslational prenylation, which involves the addition of the mevalonate end products farnesyl pyrophosphate or geranyl-geranyl pyrophosphate to the C-terminus of RhoB (37). Moreover, dysregulation of RhoB prenylation has been associated with changes in cellular proliferation and transformation (45, 46), suggesting the relevance of these modifications of RhoB for tumourigenesis. Our data provide several lines of evidence that support a direct link between the mevalonate pathway-dependent changes in RhoB activity and recognition of tumour cells by Vγ9Vδ2 TCRs. First, we show that the subcellular distribution of RhoB in tumour cells strongly correlates with their capacity to elicit Vγ9Vδ2 TCR responses, and that the sensitization of unrecognized cells for Vγ9Vδ2 TCR targeting by NBP-mediated modulation of the mevalonate pathway causes a redistribution of RhoB and the concomitant recognition of cells by Vγ9Vδ2 TCRs. Second, treating tumour cells with farnesyl- and geranyl-geranyltransferase inhibitors results in reduced recognition by Vγ9Vδ2 TCRs. Similar treatments have been shown to change the subcellular localization of RhoB to include the nucleus (41), which is in line with our data showing that nuclear localization of RhoB is a hallmark of cells not recognized by Vγ9Vδ2 TCRs. Third, the well-described effect of NBP-induced accumulation of intracellular phosphoantigens on the membrane mobility of BTN3A1 (11, 12), which is suggested to provide the activation cue for Vγ9Vδ2 TCRs, is mimicked by the pharmaceutical activation of RhoB. Finally, the direct interaction between RhoB and BTN3A1 occurs selectively when cells are treated with NBP. Taken together, these results provide new evidence connecting intracellular changes induced by the accumulation of phosphoantigens to the recognition of target cells by Vγ9Vδ2 TCRs. Of note, even though inhibition of the mevalonate pathway and

hence prenylation of RhoB by NBP treatment could argue for reduced recognition of target cells by Vγ9Vδ2 TCR+ T cells, our data demonstrates increased expression levels of RhoB in tumour cells recognized by Vγ9Vδ2 TCRs. Thus, the increased basal expression of RhoB in recognized target cells may overcome the suppressive effect of mevalonate inhibition on RhoB prenylation and activity.

Our data demonstrating a direct, phosphoantigen-elicited interaction between intracellular RhoB and transmembrane BTN3 molecules suggests that RhoB could bind to the cytoplasmic B30.2 domain of BTN3. This interaction is supported by preliminary results from biolayer interferometry experiments using RhoB and the intracellular domain of BTN3A1 (E. Adams, personal communication). Indeed, B30.2 domains have been reported to function as scaffold modules by spatiotemporally sequestering signaling proteins to distinct subcellular locations (47). Nevertheless, the precise mechanisms by which RhoB impacts BTN3A1 to accommodate Vγ9Vδ2 TCR activation remain unclear. One possibility is that RhoB acts as a chaperone molecule actively transporting phosphoantigens to BTN3A1 at the plasma membrane. Alternatively, or in addition, RhoB may mediate the immobilization of BTN3A1 at the cell surface by locally modulating the submembranous actin cytoskeleton, similar to its reported role in modulating membrane structures such as focal adhesions (46, 48). Moreover, our data demonstrate a clear conformational change in the extracellular domain of BTN3A1 upon treatment with NPB, suggesting that this structural change could contribute to the extracellular signature that is recognized by Vγ9Vδ2 TCRs. This observation corroborates data from crystallographic experiments using soluble BTN3A1 domains, which revealed a pronounced flexibility of BTN3A1 dimers that correlated with treatment with the agonist 20.1 antibody that is able to mimic phosphoantigen-induced stimulation of Vγ9Vδ2 TCRs (44). However, whether RhoB directly mediates conformational changes of BTN3A1 remains to be determined. Nevertheless, these observations provide additional evidence for the proposed "inside-out" mechanism of phosphoantigen signaling to Vγ9Vδ2 TCRs (12, 44), whereby intracellular phosphoantigen accumulation is translated into surface changes of BTN3A1 by RhoB.

Even though our data point to an important role for RhoB in the recognition of tumour cells by Vγ9Vδ2 TCRs, recent data by others suggest that additional factors are likely to be involved. In particular, transfer of human BTN3A1 alone to rodent cells, which are not recognized by Vγ9Vδ2 T cells (7), was not sufficient to sensitize cells towards recognition by Vγ9Vδ2 TCRs (12). Instead, this required cotransfer of a substantial proportion of the human chromosome 6 (13, 49), suggesting that the combined presence of BTN3A1 and additional human genes located on this chromosome is essential for facilitating target cell recognition by Vγ9Vδ2 TCRs. The human RhoB gene locates to chromosome 2, and moreover, protein sequences of RhoB are fully conserved between humans and rodents. Thus, other human-specific genes that mediate activation of Vγ9Vδ2 T cells are yet to be identified. The molecular switch function of Rho GTPases is tightly controlled by GTPase activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs), the concerted actions of which regulate the balance between the active, GTP-bound state and inactive, GDP-bound state of Rho GTPases (50, 51). Even though such enzymes regulating RhoB activity could thus represent interesting candidates, none of

the known regulators of Rho GTPase signaling locate to the region on chromosome 6 required to transfer Vγ9Vδ2 TCR susceptibility to rodent cells. Identification of these missing factors linking RhoB and BTN3A1 to target cell recognition by Vγ9Vδ2 TCRs is therefore an important challenge for further elucidating the molecular activation mechanisms of Vγ9Vδ2 T cells.

The identification of RhoB as a mechanistic link between transformation-associated dysregulation of the mevalonate pathway and BTN3A1-dependent activation of Vγ9Vδ2 T cells has important implications for cancer immunotherapeutic concepts using Vγ9Vδ2 T cells or their TCRs. First, the divergent intracellular distribution of RhoB among recognized and non-recognized tumour cells may be used as a biological marker for cells susceptible to Vγ9Vδ2 TCR-mediated attack, and thus could serve a criterion for the selection of cancer patients that may benefit from Vγ9Vδ2 T cell-based therapy. Second, our data put RhoB forward as a novel therapeutic target for improving immunotherapy using Vγ9Vδ2 T cells. In contrast to BTN3A1, which is ubiquitously expressed on healthy as well as malignant cells, the data presented here and elsewhere (42) demonstrate that RhoB possesses transformation-selective characteristics and could therefore represent a more sensible therapeutic target compared to BNT3A1. Moreover, the mobilization of Vγ9Vδ2 T cells in cancer patients using in vivo or ex vivo stimulation protocols with NBPs such as zoledronate (3, 4, 52) is likely limited by the poor pharmacokinetic profile of NBPs (53) and their toxicity to T cells upon prolonged exposure (54). Agents targeting RhoB could therefore represent attractive opportunities to complement currently pursued Vγ9Vδ2 T cell-based therapies.

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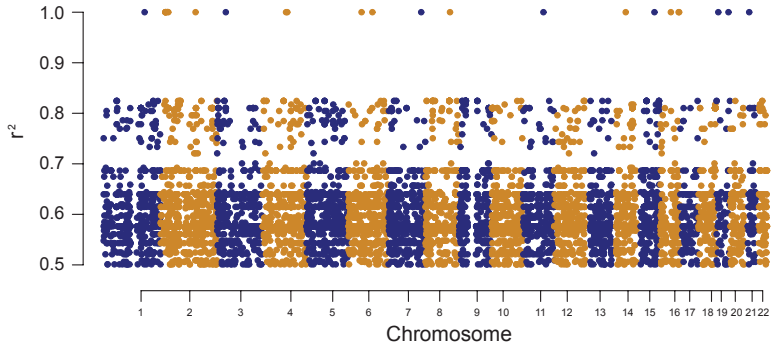
SUPPLEMENTARY FIGURES

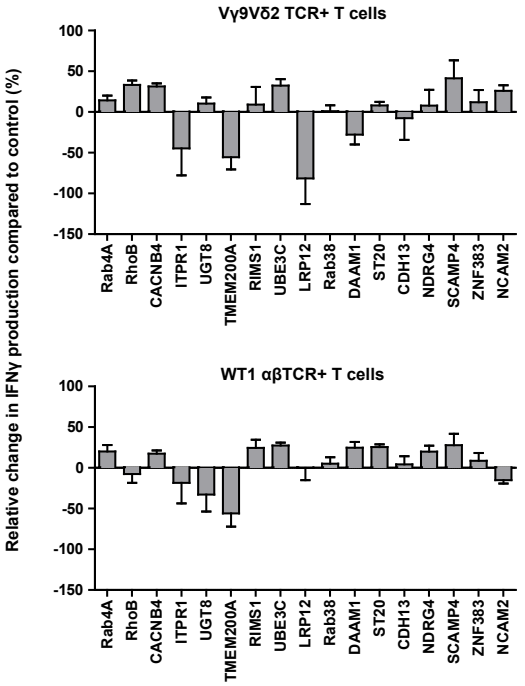
CEPH line Corriel nr.	10846	12145	12146	12239	7029	7019	6994	7022	6991	7034	6993	10851	11839	11840	10860	10861	11993	11994	11995	12155
Numbering used in this manuscript	1	4	5	6	7	8	9	12	16	18	20	22	36	37	48	49	51	52	53	61
Recognition phenotype	+	+	+	+	+	+	-	+	+	+	-	-	+	-	+	+	+	+	+	+

CEPH line Corriel nr.	12156	10838	12003	12004	12005	12006	12753	12762	12763	12801	12802	12812	12814	12864	12865	12872	12873	12875	12878	12892
Numbering used in this manuscript	62	66	68	69	70	71	76	79	80	81	82	83	86	88	89	90	91	93	94	96
Recognition phenotype	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	+	+

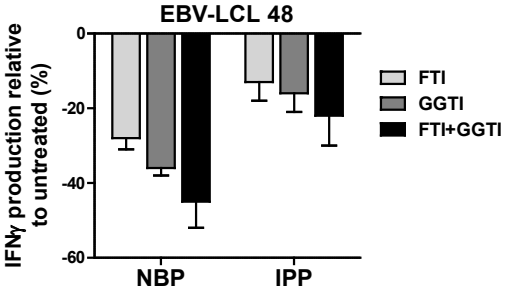
Supplementary Figure 1. CEPH EBV-LCL lines used for identifying genetic loci associated with Vγ9Vδ2 TCR-mediated recognition. Recognition phenotype indicates whether EBV-LCL lines are recognized (+) or not (-) by Vγ9Vδ2 TCR+ T cells.



Supplementary Figure 2. Genome-wide correlation analysis between predicted zygosities of candidate loci and Hapmap SNP genotypes. Each dot represents one Hapmap SNP, and the correlation (r^2) of each SNP is depicted.



Supplementary Figure 3. Candidate genes were knocked down in EBV-LCL line 48 using shRNA, and the effect of gene knockdown on recognition by either Vγ9Vδ2 TCR- or WT1 αβTCR-transduced T cells was determined by measuring IFNγ production. Data are represented as relative change in IFNγ production compared to EBV-LCL 48 target cells transduced with control shRNA. In cocultures with WT1 αβTCR-transduced T cells, EBV-LCL 48 cells were pulsed with cognate $WT1_{126-134}$ peptide antigen.



Supplementary Figure 4. EBV-LCL line 48 was pretreated with FTI and/or GGTI prenylation inhibitors and the recognition of cells by Vγ9Vδ2 TCR+ T cells in the presence of pamidronate or IPP was assessed by measuring IFNγ. Data are represented as the relative decrease in IFNγ production compared to IFNγ levels produced by Vγ9Vδ2 TCR+ T cells in response to pamidronate-treated EBV-LCL 48 cells.

CHAPTER 7

Partial murinization of therapeutic T cell receptors allows efficient enrichment of untouched, high-avidity TCR-engineered T cells

Wouter Scheper, Elselien Frijlink, Guido Kierkels, Daniel Sommermeyer, Wolfgang Uckert, Trudy Straetemans, Jürgen Kuball

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ABSTRACT

Despite encouraging clinical data obtained with T cell receptor (TCR) gene-modified T cells in cancer immunotherapy, the clinical translation of engineered T cells is challenged by the lack of GMP-compatible methods that facilitate the purification of T cells expressing optimal levels of introduced TCRs. In this study, we demonstrate that the substitution of the human αβTCR constant domains with their murine counterparts prevents recognition by a clinical grade antibody directed against human αβTCRs currently used to deplete T cells from stem cell products. Introduction of selected murine residues into only the TCRβ constant domain is sufficient to completely prevent recognition by the anti-human αβTCR antibody, thus minimizing the content of foreign sequences. By taking advantage of the natural competition between introduced and endogenous TCRs, this allows the selective depletion of T cells which poorly express or lack introduced TCRs, resulting in the efficient enrichment of untouched cells that express high levels of therapeutic TCRs. This efficient method utilizes readily available GMP-grade tools and is therefore both cost-effective and readily translatable to clinical use. Moreover, this concept will be applicable to virtually any clinical TCR, in autologous as well as allogeneic TCR gene therapy.

INTRODUCTION

Adoptive T cell therapy has shown great clinical promise in the treatment of cancer, particularly in patients suffering from melanoma (1-4). For most cancers however, the generation of sufficient numbers of tumour antigen-specific T cells from a patients polyclonal repertoire remains a major challenge. Recent efforts have therefore increasingly focused on the genetic engineering of T cells by transfer of genes encoding defined tumour-reactive T cell receptor (TCR) α and β chains (5). Such reprogramming of T cell specificity allows for the creation of vast numbers of T cells reactive against any tumour antigen of interest, also from patient T cell repertoires that lack naturally occurring tumour-reactive T cells. Although clinical trials have established the value of adoptive transfer of TCR-engineered cells in cancer patients, clinical benefit is generally observed only in a proportion of patients (6-9). One likely explanation for the observed limited efficacy of TCR-engineered T cells is a suboptimal surface expression of therapeutic TCRs, caused by competition for CD3 components between introduced and endogenous TCRs and the mixed pairing of TCR chains to form unwanted TCR dimers (10). To prevent TCR competition and mispairing, and to improve transgenic TCR expression, site-directed genome editing techniques such as TALENs and zinc finger nucleases (ZNF) have been explored to permanently knock-out endogenous TCR α and β loci in T cells (11-13). Clinical applicability of such elegant techniques has yet to be established however, since rather low knock-out efficiencies and off-target editing (14) raise important limitations to the rapid generation of sufficient numbers of safely edited T cells within the time constraints of acute treatment. Thus, there is a pressing need for readily translatable strategies that use

clinically established tools to generate cell products expressing high levels of tumour-specific TCRs.

We have previously shown that genetic engineering of T cells using broadly tumour-reactive TCRs isolated from unconventional γδT cells provides an interesting tool to cope with these limitations. γδTCR chains do not pair with αβTCR chains (15) and can robustly replace endogenous αβTCRs from the surface of engineered T cells (15-17). Importantly, these features allow the generation of untouched T cell products expressing high levels of therapeutic γδTCRs by depleting untransduced T cells using clinical grade anti-αβTCR antibodies (Straetemans & Kuball, unpublished observation). However, analogous concepts for conventional αβTCRs are so far lacking.

Similar to human γδTCRs, fully murine as well as partially murinized human αβTCRs genetically transferred into human T cells are capable of displacing endogenous αβTCRs from the cell surface, most likely due to superior competition for CD3 components by murine TCRs (18-20). Moreover, partly murinized TCR chains do not pair with human αβTCR chains (18,21), making such hybrid TCRs interesting for application in clinical TCR gene-engineering concepts. In contrast to human γδTCRs, which structurally resemble immunoglobulins rather than αβTCRs (22), murine αβTCRs are highly homologous to human αβTCRs (23), and human-mouse hybrid TCRs are therefore preferential candidates for designing isolation strategies for T cells engineered to express defined αβTCRs.

Here, we demonstrate that minimal murinization of human αβTCR constant domains can be used not only to replace endogenous human TCRs from the T cell surface, but also to interfere with recognition by a clinical grade antibody directed against human αβTCRs, thus allowing the selective depletion of untransduced T cells using straightforward antibody-based depletion methods. In contrast to enrichment approaches pursued so far, the here-presented selection strategy can generate untouched, high-avidity cell products in a cost-effective manner and is based on GMP-compliant tools, making the method readily translatable into the clinic. Moreover, by depleting T cells that express high levels of endogenous TCRs, this strategy can be applied to a broad patient population in autologous as well as allogeneic settings, using virtually any therapeutic αβTCR.

MATERIALS & METHODS

Flow cytometry

Cells were stained with CD3-eFluor450 (clone OKT3, eBioscience), Vβ4-FITC (clone WJF24, Beckman Coulter), Vβ21-FITC (clone IG125, Beckman Coulter), murine TCRβ-PE (clone H57-597, BD), pan-αβTCR-PE (clone BW242, Miltenyi Biotec), and PE-conjugated NY-ESO157-165/HLA-A*02:01 pentamers (ProImmune). To avoid binding competition between antibodies directed against components of the CD3/TCR complex, all antibodies were used in separate stainings. Samples were measured on a FACSCanto-II flow cytometer (BD) and analyzed using FACSDiva (BD) and FlowJo (FlowJo, LLC) software.

Cells

Phoenix-Ampho cells were obtained from ATCC and cultured in DMEM with 1% Pen/Strep (Invitrogen) and 10% FCS (Bodinco). The human CD4+ TCRβ-deficient (TCRβ-/-) Jurma cell line was kindly provided by Erik Hooijberg (VU Medical Center, Amsterdam, The Netherlands) and cultured in RMPI with 1% Pen/Strep and 10% FCS. Human PBMCs were isolated by Ficoll-Paque (GE Healthcare) from buffy coats supplied by Sanquin Blood Bank (Amsterdam, The Netherlands).

Cloning and retroviral transductions

Wild-type and murinized domain variants of NY-ESO-1157-165/HLA-A*0201 TCR α and β chains cloned into the clinical grade retroviral vector pMP71 were generated as previously described (19). A fully murine nonsense TCR was composed of the TCRα chain of a MDM281-88-specific TCR (24) and the TCRβ chain of a p53-specific TCR (25). Single amino acid substitutions in the NY-ESO-1 TCRβ constant domain were generated by site-directed PCR mutagenesis using the "minimally murinized" human TCRβ chain (19) as backbone. Successful mutagenesis was confirmed by sequencing.

Phoenix-Ampho packaging cells were transfected with gag-pol (pHIT60), env (pCOLT-GALV) and pMP71 constructs containing TCRα(MDM2)-T2A-TCRβ(p53) or NY-ESO-1157-165/HLA-A2-specific TRC α or β chains using Fugene6 (Promega). For transduction of a $WT1_{126–134}$-specific αβTCR (26), pBullet constructs containing TCRα-IRES-puromycine or TCRβ-IRES-neomycine were transfected into Phoenix-Ampho cells. PBMCs (preactivated with 30 ng/ml anti-CD3 (clone OKT3, Janssen-Cilag) and 50 U/ml IL-2) or Jurma cells were transduced twice within 48 hours with viral supernatant in 6-well plates (± 3 x 106 cells/well) in the presence of 4 μg/ml polybrene (Sigma-Aldrich). After transduction, Jurma cells transduced with the $WT1_{126–134}$-specific TCR were selected with 800 μg/ml geneticin (Gibco) and 5 μg/ml puromycine (Sigma-Aldrich) for one week. Following transduction, cells were cultured in RPMI supplemented with 1% Pen/Strep and 10% FCS. Expression of transduced TCRα and TCRβ chains was routinely analyzed by flow cytometry using anti-Vβ4, or anti-CD3 antibodies (untransduced TCRβ-/- Jurma cells do not express CD3 on the cell surface). Expression of WT1-specific αβTCRs was analyzed using a Vβ21-specific antibody. Where indicated, TCR-transgenic cells were sorted using a FACSAria II (BD) flow cytometry to >95% purity.

Magnetic-activated cell sorting

Selective depletion of murinized NY-ESO-1157-165 TCR-expressing Jurma cells was performed by first mixing NY-ESO-1 TCR+ Jurma cells with human WT1 TCR+ ¬Jurma cells in a 1:1 ratio, followed by incubation with biotinylated pan-αβTCR mAb BW242 and anti-biotin microbeads (Miltenyi Biotec). Cell fractions were subsequently separated using MACS LD columns (Miltenyi Biotec).

TCR crystal structure visualization

TCR structure visualizations were performed using PyMol version 1.3 (Schrödinger).

Statistical analysis

Statistical analysis was performed using Graphpad Prism version 5.0 (Graphpad Software Inc.). P values <0.05 were considered significant.

RESULTS

Murinization of human TCR α and β constant domains abrogates binding by the anti-αβTCR antibody BW242

The clinical grade anti-human αβTCR antibody clone BW242 is currently being used by us (trial registration NTR2463 and NTR3079) and others (27,28) for the depletion of αβT cells from clinical hematopoietic stem cell products. Clone BW242 recognizes αβTCR heterodimers irrespective of TCR α and β variable gene usage (29), suggesting that the epitope recognized by BW242 locates to the constant region of the αβTCR. Human and murine TCRα and TCRβ chains are structurally highly homologous, but still differ substantially in the sequences of their constant regions (30) (32% and 18% difference in α and β chains, respectively). We therefore questioned whether partial murinization of a human αβTCR abrogates binding by clone BW242, thereby making murine TCR domains useful for strategies aimed at the untouched isolation of engineered immune cells expressing engineered αβTCRs, as observed by us with exogenous γδTCRs (Straetemans et al, submitted manuscript). In order to allow a detailed binding study analysis of the BW242 antibody, the TCRβ-deficient Jurma T cell line was transduced with a fully murine αβTCR, or with two variants of an HLA-A2-restricted αβTCR specific for the well-described tumour antigen NY-ESO-1 (31), one fully human and one in which the human TCR constant regions were substituted by the corresponding murine sequences. Transduced cells were sorted to >95% purity based on Vβ4 or mouse TCRβ expression and recognition of cells by the BW242 αβTCR antibody was measured by flow

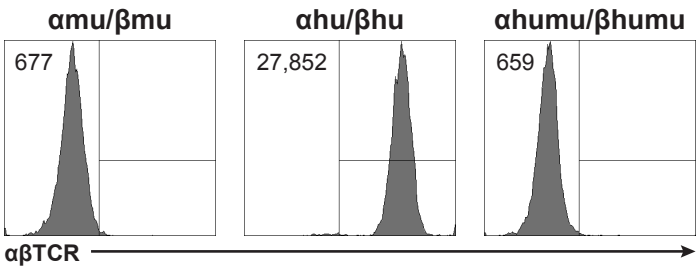

Figure 1. Murinization of human αβTCR constant regions disrupts binding by the anti-human αβTCR antibody BW242. TCRβ-/- Jurma cells were retrovirally transduced with a fully murine αβTCR (αmu/βmu), the fully human NY-ESO-1-specific αβTCR (αhu/βhu), or the NY-ESO-1 αβTCR of which the TCR α and β constant domains were replaced with their murine equivalents (αhumu/βhumu), and staining of TCRs by the BW242 antibody was assessed by flow cytometry. Numbers included in plots indicate mean fluorescence intensities (MFIs).

cytometry. As expected, Jurma cells expressing the fully human NY-ESO-1 αβTCR (from here on referred to as αhu/βhu) were efficiently stained by clone BW242 (Fig. 1). In contrast, replacing human TCR α and β constant domains by their murine equivalents (from here on referred to as αhumu/βhumu) abrogated binding by the BW242 antibody to levels resembling those of the fully murine TCR (αmu/βmu), despite similar expression levels of αhu/βhu and αhumu/βhumu TCRs (Supplementary Fig. 1). Thus, the sequence differences between murine and human TCR α and β constant regions are sufficient to fully prevent binding of the anti-human αβTCR antibody BW242.

Epitope mapping of antibody clone BW242 by swapping selected sequences of murine and human constant regions

The data above suggest that differences in homology between constant domains of murine human αβTCR chains can provide an opportunity for the design of antibody-based selection strategies for the generation of untouched T cells expressing engineered αβTCR chains. However, replacing extensive regions of human αβTCRs by murine sequences may increase the risk of immunogenicity of such TCRs when used in patients (32). In order to minimize this risk we set out to determine the minimal molecular requirements critical to disrupt binding of the BW242 antibody. By taking advantage of three TCRα chain variants (designated αM1, αM2 and αM3) and four TCRβ chain variants (designated βM1, βM2, βM3 and βM4) of the NY-ESO-1 αβTCR, covering all amino acid differences between the constant regions of human and mouse αβTCRs (19) (Fig. 2A), the individual contribution of each segment to the BW242 epitope was assessed. First, TCRβ chain variants βM1-4 were transduced into TCRβ-deficient Jurma cells along with an unmodified, fully human TCRα chain (αhu), and binding of the BW242 antibody was subsequently analyzed by flow cytometry. The unmodified human NY-ESO-1 TCR (αhu/βhu) as well as the αhumu/βhumu variant were included as controls. All variant TCRs were expressed at similar levels as measured by staining with anti-Vβ4 antibody (Supplementary Fig. 2A). Selectively murine sequence substitutions in domain 3 of the TCRβ constant region (αhu/βM3) substantially reduced BW242 antibody binding when compared to all other segments (Fig. 2B). However, reductions of staining by BW242 never reached the level of total staining abrogation as observed for αhumu/βhumu, suggesting that sequences in this TCRβ domain strongly contribute to the binding epitope of the αβTCR antibody BW242, however were alone not sufficient to completely prevent binding.

Next, the contributions of different segments of the TCRα constant domain to the binding epitope of antibody BW242 were assessed by introducing the three hybrid TCRα chains αM1, αM2 and αM3 into Jurma cells together with the fully human TCRβ chain counterpart. All hybrid TCR variants were expressed at similar levels (Supplementary Fig. 2B), and comparable to the fully human NY-ESO-1 TCR (data not shown). Only minor differences between samples were observed in staining with the BW242 antibody however, suggesting that the TCRα constant domains alone do not critically contribute to the binding epitope of the BW242 antibody (Fig. 2C).

In order to assess whether partial murinization selectively inhibits BW242 antibody binding while not affecting the specificity of given TCRs for their cognate antigens, cells expressing the

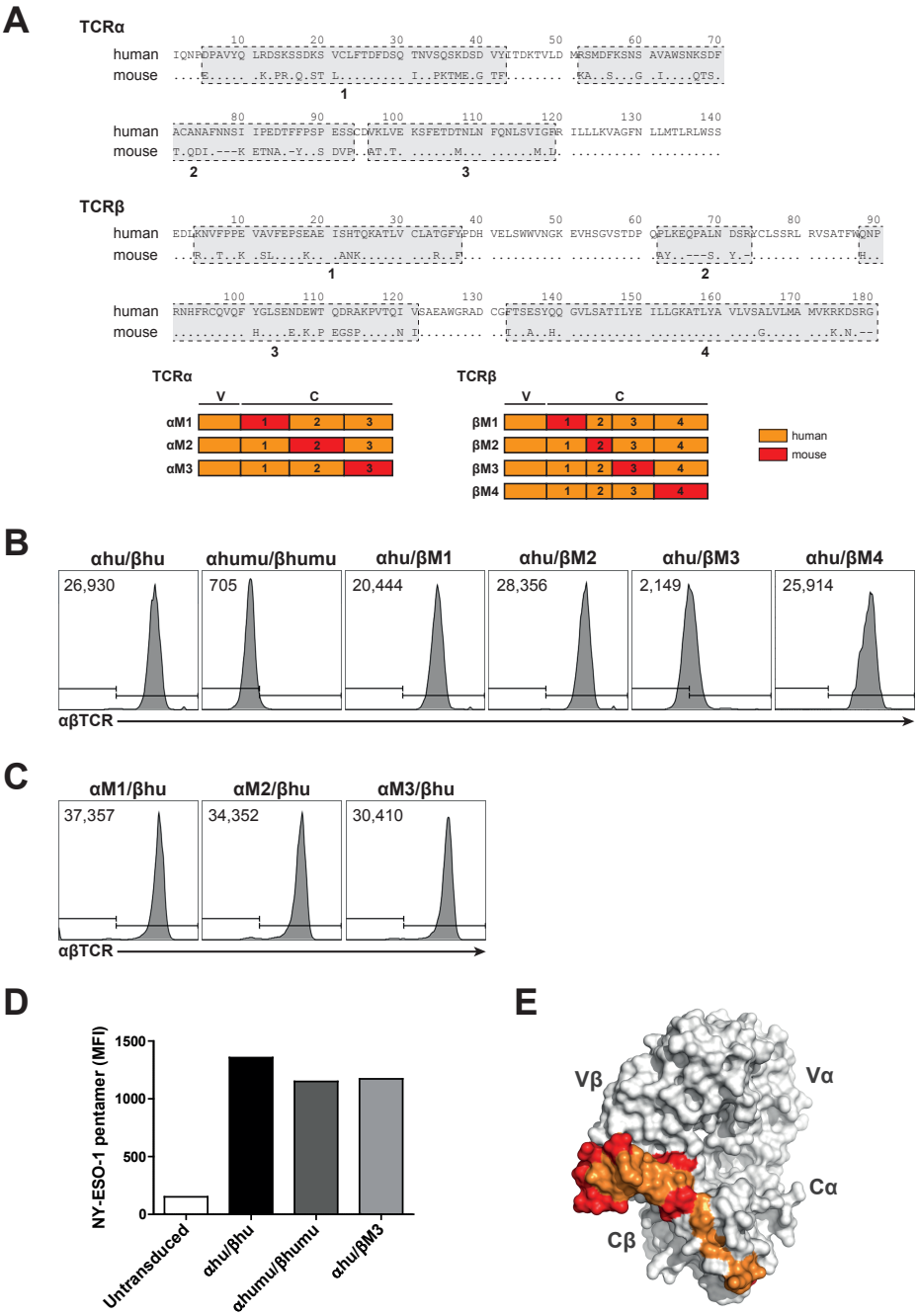

Figure 2. Partial murinization of only the human TCRβ constant domain is sufficient to abolish binding by the anti-human αβTCR antibody BW242. (A) Top panel: sequence alignments human and mouse TCRα and TCRβ constant regions. Gray boxes represent three and four domains covering all amino

Figure 2. (continued) acid differences of TCR α and β chains, respectively, as defined by (19). Homologous residues are indicated by periods (.). Lower panel: schematic representation of the three TCRα and four TCRβ gene variants used for transductions (V: variable domain; C: constant domain). (B) Jurma cells were transduced with the fully human NY-ESO-1 αβTCR (αhu/βhu), the NY-ESO-1 αβTCR with murinized TCRα and TCRβ constant regions (αhumu/βhumu), or with four different NY-ESO-1 TCRβ hybrid variants combined with the fully human TCRα chain (αhu/βM1, αhu/βM2, αhu/βM3 and αhu/βM4). Recognition of TCR variants by the anti-αβTCR antibody BW242 was subsequently tested by flow cytometry. (C) Identical to (B), but here three different NY-ESO-1 TCRα chain variants were combined with the fully human TCRβ chain (αM1/βhu, αM2/βhu and αM3/βhu). (D) The capacity of Jurma cells transduced with αhu/βhu, αhumu/βhumu or αhu/βM3 NY-ESO-1 TCR variants to bind cognate antigen was assessed by staining cells with NY-ESO$_{157-165}$/HLA-A*02:01 pentamers. Untransduced Jurma cells served as negative control. (E) Crystal structure of a human αβTCR (PDB: 3GSN) depicting domain 3 of the TCRβ constant domain, as defined in (A). Human-mouse non-homologous residues in this domain are indicated in red, homologous residues in orange.

αhumu/βhumu and αhu/βM3 TCR variants were stained with NY-ESO-1$_{157-165}$/A2 pentamers and revealed similar levels as the fully human NY-ESO-1 TCR (Fig. 2D). Thus, murinization of a limited number of residues of the human TCRβ constant domain is sufficient to diminish binding by the BW242 anti-αβTCR antibody without altering TCR specificity. Inspection of the αβTCR crystal structure substantiates these observations, as domain 3 of the TCRβ constant region is highly surface-exposed and heterologous domains do not substantially interfere with the variable domains of the TCR (Fig. 2E).

Combined murinization of selected TCRα constant domains and TCRβ domain 3 reduces TCR stability

Even though murinization of domain 3 of the human TCRβ constant region strongly disrupts BW242 antibody binding, recognition of the αhu/βM3 TCR variant by BW242 is not completely reduced to levels comparable to TCR variants in which the complete human constant domain has been exchanged with murine sequences (αhumu/βhumu). Although not dominant for inhibiting antibody BW242 binding to αβTCRs (Fig. 2B), we hypothesized that murinization of TCRα constant domains could still impact binding by the BW242 antibody in combination with the TCRβ chain variant murinized in domain 3 of the constant region. Jurma cells were therefore transduced with the TCR βM3 variant in combination with the three TCRα chain variants (αM1, αM2 and αM3), and sorted to 95% purity based on Vβ4 expression. However, consistently lower Vβ4 expression levels of αM1/βM3, αM2/βM3 and αM3/βM3 TCR chain combinations were observed compared to the αhu/βM3 TCR (Supplementary Fig. 3), indicating that combined murinization of these particular TCR α and β chain sequences interferes with the overall stability of the TCR.

Efficient enrichment of untouched cells expressing murinized αβTCRs using a BW242 antibody-based depletion strategy

The differential binding of the BW242 antibody to human versus partially murinized αβTCRs could theoretically allow the separation of cells engineered to express modified TCRs from cells expressing natural human αβTCRs. To test this, cell populations containing cells expressing

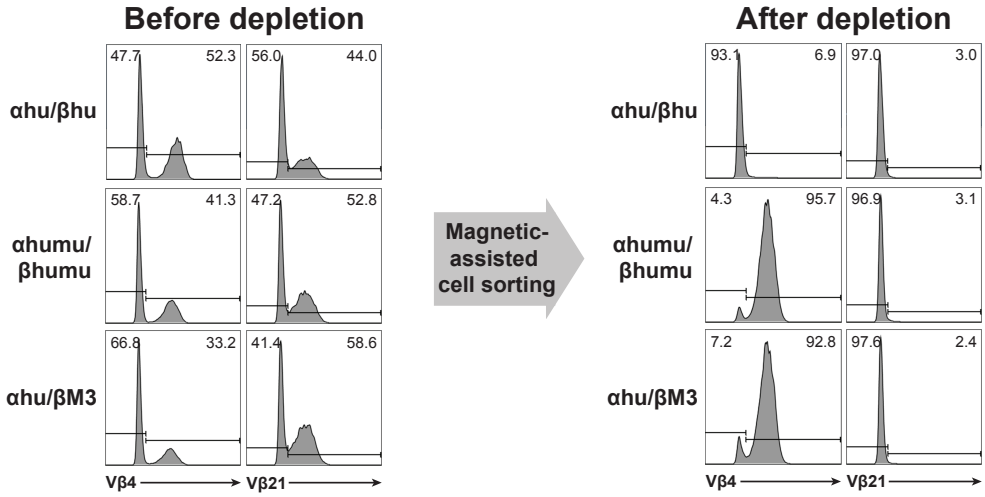

Figure 3. Partial murinization of the human TCRβ chain permits the efficient depletion of cells expressing fully human αβTCRs using the anti-human αβTCR BW242. Cell populations containing a mixture of human and murinized αβTCRs were generated by mixing Jurma cells transduced with the fully human NY-ESO-1 TCR or with the NY-ESO-1 TCR variants αhumu/βhumu or αhu/βM3 with Jurma cells transduced with a fully human, Vβ21+ WT1-specific αβTCR in a 1:1 ratio (left). Depletion of cells expressing only human αβTCRs was subsequently tested by incubating cell populations with magnetic bead-coupled anti-αβTCR antibody BW242 and separating cells over magnetic columns. Flowthrough fractions were analyzed for presence of cells expressing NY-ESO-1 TCR variants by staining for Vβ4 and for WT1 TCR+ cells by staining for Vβ21 (right).

endogenous human TCRs and cells expressing transgenic murinized TCRs were mimicked by mixing variants of NY-ESO-1 TCR-transduced Jurma cells in a 1:1 ratio with Jurma cells that expressed a fully human Vβ21+ αβTCR directed against a Wilm's tumour (WT1) antigen. Cells were incubated with BW242 antibody-coated magnetic beads, MACS-sorted, and unlabeled flow-through fractions were stained with anti-Vβ4 and anti-Vβ21 antibodies to measure content of NY-ESO-1 TCR+ cells and WT1 TCR+ cells, respectively. Cells modified with human-mouse chimeric αβTCRs or αhu/βM3-TCRs were efficiently enriched from mixed populations, while cells expressing either fully human NY-ESO-1-specific or WT1-specific αβTCRs were depleted from the cell preparations (Fig. 3). These data demonstrate that partial murinization of αβTCR constant regions permits the depletion of cells expressing high levels of natural αβTCRs, resulting in the efficient enrichment of untouched αβTCR gene-engineered cells.

Towards minimal murinization of human αβTCRs to disrupt the BW242 antibody binding epitope

So far, our data consistently show that murinization of domain 3 of the TCRβ constant region, containing eleven amino acids that are non-homologous between the human and mouse TCRβ constant region (Fig. 2A), most substantially inhibits binding of the BW242 antibody to

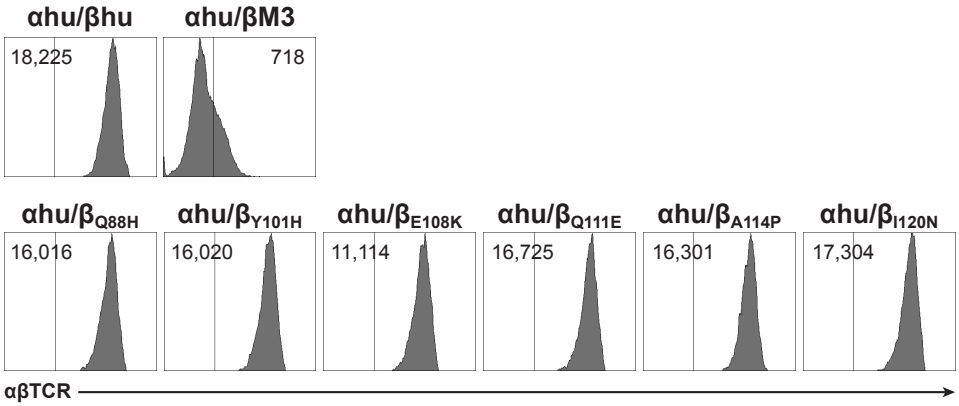

Figure 4. Fine-mapping of the binding epitope of the BW242 antibody on human αβTCRs by single amino acid mutant TCR variants. Jurma cells were transduced with the human NY-ESO-1 TCR, with the αhu/βM3 variant, or with six different NY-ESO-1 TCR variants each containing a single human-to-mouse amino acid substitution in domain 3 of the TCRβ chain. Staining of cells by the anti-αβTCR antibody BW242 was subsequently tested by flow cytometry.

human αβTCRs. In order to reduce the risk of immunogenicity of partially murinized TCRs, we questioned whether this number could be further reduced without affecting the reduction in BW242 antibody binding. Thus, we set out to generate single amino acid mutants at each of the eleven non-homologous positions. To take advantage of established strategies to improve pairing of genetically introduced αβTCR chains, a NY-ESO-1 TCRβ variant was used that contained five murine amino acid substitutions in domains 1 and 4 that have been shown to be critical and sufficient to induce preferential TCR chain pairing (19). Six out of eleven TCRβ mutants have been generated so far and were transduced into Jurma cells together with the human TCRα chain. TCR transduction efficiency was evaluated by staining for CD3 and all mutants expressed similar levels of mutant TCRs (Supplementary Fig. 4). Of these available mutants, the substitution of glutamic acid at position 108 of the human TCRβ chain with the "murine" lysine (E108K) most notably resulted in a reduction of staining by the BW242 αβTCR antibody (Fig. 4), suggesting that murinizing this residue is crucial, but alone not sufficient, for blocking binding of antibody BW242 in the context of the human αβTCR backbone. The additional five mutants are currently being generated in our laboratory and will need to be tested to identify additional residues that contribute to the disruption of binding by the anti-αβTCR antibody BW242 to human αβTCRs.

Replacement of human TCRs from the T cell surface by transgenic murine TCRs facilitates the enrichment of untouched T cells expressing high levels of transgenic TCRs

In order to test the validity of the proposed strategy to purify untouched αβTCR-engineered cells in the context of physiological competition with endogenous αβTCR chains, compared to the setting where endogenous human αβTCR chains are completely absent (e.g. through

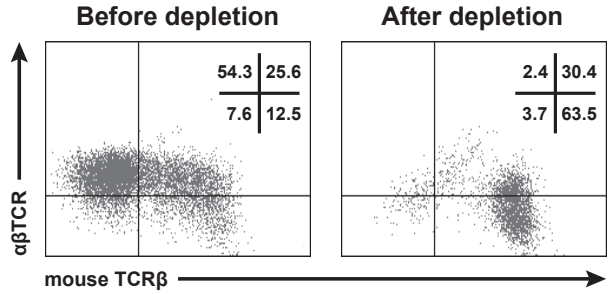

Figure 5. Efficient negative selection of human T cells engineered to express a murine αβTCR using an antibody directed against human αβTCRs. Primary human αβT cells were retrovirally transduced with a murine αβTCR and expression of endogenous human αβTCRs and transgenic murine TCR was measured by flow cytometry (left panel). Non-transduced T cells were subsequently depleted by magnetic-assisted cell sorting using anti-human αβTCR antibody clone BW242 coupled to magnetic beads (right panel). Indicated in plots are percentages of quadrants and MFIs of γδTCR and αβTCR stainings.

genome-engineering techniques), primary human αβT cells were transduced with the clinical grade retroviral pMP71 vector containing a fully murine αβTCR. Expression of both human and mouse TCRs was analyzed by flow cytometry. As reported before (21), and similar to our previous observations using γδTCRs (15-17), transgenic murine αβTCRs (muTCR) are strong competitors for components of the CD3 complex and can substantially inhibit expression of endogenous human αβTCR chains, resulting in muTCRhi/huTCRlo and muTCRlo/huTCRhi T cells (Fig. 5, left panel). This preferential downmodulation of huTCR allowed us to test whether untouched muTCRhi/huTCRlo cells could be efficiently enriched by negative selection using the clinical grade BW242 antibody coupled to magnetic beads. Indeed, also in the presence of high expression levels of endogenous human αβTCRs, non- and poorly engineered immune cells could be very efficiently separated from engineered immune cells (Fig. 5, right panel), which are the preferred fraction for adoptive transfer. Current efforts in our laboratory aim to elucidate whether also minimal exchange of human and murine residues is sufficient to distinguish suboptimally engineered cells from the therapeutic fraction using the here-presented strategy.

DISCUSSION

One important limitation to the anti-tumour efficacy of TCR-engineered T cells is the suboptimal and heterogeneous expression of therapeutic TCRs on clinical cell products, and enrichment strategies to selectively produce pure populations of high-avidity T cells are highly needed. In the present study, we therefore investigated whether murinization of selected domains of human αβTCRs would not only lead to replacement of endogenous human αβTCRs from T cells, but could furthermore interfere with recognition by antibodies directed against human

αβTCRs, as the combination of these features would allow a selective antibody-mediated enrichment of TCR-engineered cells. Using murinized variants of a well-studied tumour-reactive αβTCR, we demonstrate that partial murinization of human αβTCR constant domains abrogates recognition by a clinical grade anti-human αβTCR antibody. In contrast to so far explored methods to generate pure populations of TCR gene-engineered T cells, such as introduction of additional gene markers (33,34) and site-directed genome editing techniques (11-13), the here-described method enables the efficient enrichment of untouched T cells that express high levels of engineered αβTCRs. Importantly, this concept is transferrable to virtually any type of TCR, and the use of established GMP-compliant techniques and reagents makes it rapidly applicable to TCR gene-engineered T cell therapies for various cancers and infectious diseases.

Previously reported TCR murinization strategies have exploited the capacity of murine αβTCRs to efficiently displace human αβTCRs from the cell surface (18,21) and to prevent the formation of mixed TCR dimers (19,21) by substituting human TCR sequences with their murine counterparts. Even though these murinization techniques have yielded increased functional avidity of engineered cells and reduced the formation of mixed TCR dimers at the cell surface (19,20,35,36), substantial proportions of untransduced T cells or T cells that express only low levels of the transgenic TCR still remain in cell products engineered with these TCRs. Importantly, preclinical animal studies have demonstrated that cotransfer of such therapeutically irrelevant bystander cells may limit clinical efficacy of TCR gene-engineered cells, for example by competing for growth factors and cytokines in vivo (37,38). The here-described murinization of therapeutic TCRs takes advantage of established TCR mutations that induce preferential pairing of transgenic TCR chains (19), and combines these with additional minimal murinization in order to permit selective depletion of cells with low transgenic TCR expression. Thus, this method permits the production of highly enriched clinical gene-engineered T cells that express minimal levels of mixed TCRs with unpredictable specificity.

Our epitope mapping experiments using human-mouse hybrid domain substitutions point to a dominant role for the TCRβ chain in making up the binding epitope for the anti-αβTCR antibody BW242. This is not surprising, since truly TCRα-specific antibodies are rare due to more extensive glycosylation of the TCRα chain compared to the TCRβ chain (39). The results obtained with TCRβ chain variants carrying single amino acid mutations demonstrate that five out of six residues tested so far do not critically contribute to the BW242 binding epitope. Thus, substitution of these amino acids may be omitted to reduce the risk of murine residue-induced immunogenicity. One mutation (E108K) was observed to impact binding of the BW242 antibody to the TCR. The effect was modest however, suggesting that other residues are involved in forming the BW242 epitope. Evaluation of the remaining five single amino acid mutants will be required to identify the minimal number of murine substitutions sufficient to diminish BW242 antibody binding, and these experiments are currently in progress in our laboratory. Of note, the combined expression of the TCRβ variant murinized in domain 3 (βM3) and any of the partially murinized TCRα chains αM1, αM2 or αM3 resulted in reduced surface expression of αβTCR variants, suggesting that these TCRs suffered from

inherent instability. Surprisingly, the same TCRα variants do not cause stability issues when coexpressed with the unmodified human TCRβ chain. Moreover, no interactions between residues of the βM3 domain and any of the αM1, αM2 or αM3 domains are apparent upon inspection of the crystal structure (Supplementary Fig. 5), nor are predicted using protein chain interaction software such as SPPIDER (http://sppider.cchmc.org) (data not shown). This suggests that the particular combination of murinized residues in the βM3 and αM1, αM2 or αM3 domains causes rather long-range structural alterations that are incompatible with stable surface expression of TCRs.

The capacity to generate pure, high-avidity cell products that are devoid of endogenous TCR expression has important implications for clinical concepts using TCR gene-engineered cells not only in autologous immunotherapy but importantly also in allogeneic settings. For example, in leukemia patients that receive allogeneic stem cell transplantation, donor-derived αβT cells can mediate not only a beneficial anti-leukemic reactivity but also detrimental graft-versus-host disease, an autoimmune response against healthy tissues of patients due to αβTCR-mediated alloreactivity against mismatched HLA molecules (40). Cell products engineered to express leukemia-specific immune receptors that lack endogenous αβTCRs could allow a valuable separation of the anti-leukemic and anti-self reactivity of engineered cells applied to these patients. Indeed, using αβT cells engineered with broadly tumour-specific γδTCRs, we have previously demonstrated that the downmodulation of the endogenous αβTCR upon genetic transfer of γδTCRs results in reduced alloreactivity of engineered cells against HLA-mismatched healthy target cells (15,16). Moreover, reactivations of viruses such as cytomegalovirus and Epstein-Barr virus cause considerable morbidity in patients after stem cell transplantation, and the murinization method presented here could also have value for clinical strategies aimed at engineering T cells with TCRs directed against antigens of these viruses (41,42).

Taken together, the data presented here provide proof-of-principle for a novel method for the rapid generation of untouched TCR-engineered T cells of high-avidity. A number of remaining issues will need to be addressed to put clinical application of the technique within reach. First, even though primary T cells gene-modified with fully murine αβTCRs could be efficiently purified using our proposed selection strategy, this concept needs to be demonstrated using T cells engineered with partially murinized TCRs as well. Also, a further fine-mapping is required of the murine sequences on the TCRβ chain critically involved in disrupting BW242 antibody binding, as minimal murinization of human TCRs will reduce the risk of immunogenicity. The actual immunogenicity of such engineered TCR remains to be evaluated, for example using in silico prediction models (35,43) or ultimately in clinical trials. Importantly, encouraging data in this regard comes from a clinical trial in which cancer patients treated with T cells engineered to express fully murine αβTCRs developed antibody responses against the variable, but importantly not the constant domain of murine TCRs (32). Despite these standing issues, the strategy of partial murinization presented in this study lays the basis for the generation of more efficacious TCR-engineered cell products, not only in the context of autologous cancer immunotherapy but in virtually any anti-tumour or anti-viral TCR gene-engineered therapy.

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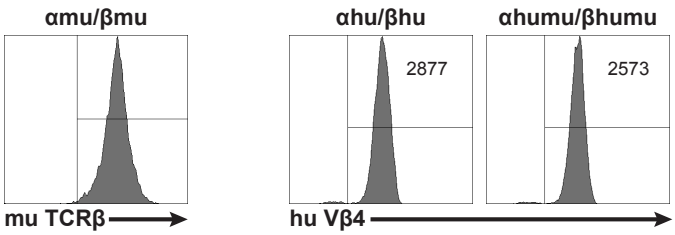
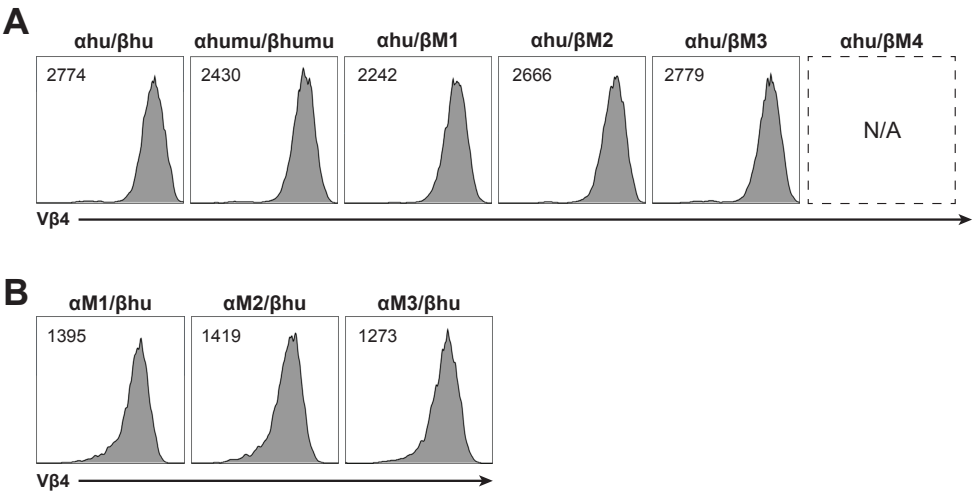
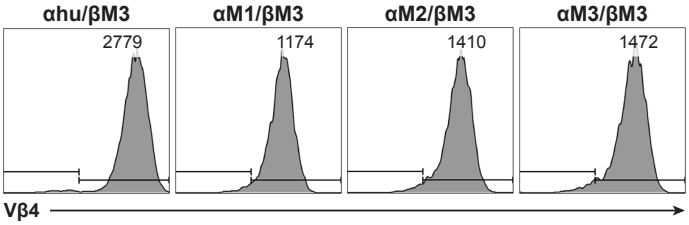
SUPPLEMENTARY FIGURES



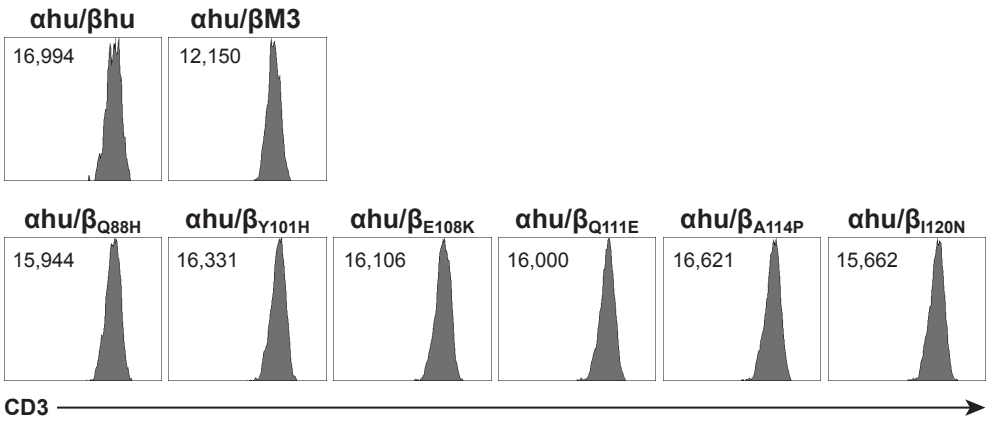
Supplementary Figure 1. Jurma cells were transduced with with a fully murine αβTCR (αmu/βmu), the fully human NY-ESO-1-specific αβTCR (αhu/βhu), or the NY-ESO-1 αβTCR of which the TCR α and β constant were murinized (αhumu/βhumu), and expression of TCRs was analyzed using antibodies directed against the murine TCRβ chain or against the human Vβ4 chain by flow cytometry. Numbers in plots indicate mean fluorescence intensities (MFIs).



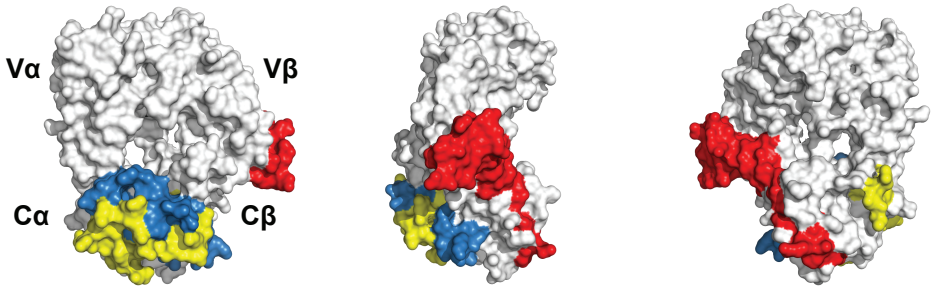
Supplementary Figure 2. (A) Jurma cells were transduced with the fully human NY-ESO-1 αβTCR (αhu/βhu), the NY-ESO-1 αβTCR with murinized TCRα and TCRβ constant regions (αhumu/βhumu), or with four different NY-ESO-1 TCRβ hybrid variants combined with the fully human TCRα chain (αhu/βM1, αhu/βM2, αhu/βM3 and αhu/βM4). TCR expression was subsequently analyzed using an anti-Vβ4 antibody. (B) Identical to (A), but here three different NY-ESO-1 TCRα chain variants were combined with the fully human TCRβ chain (αM1/βhu, αM2/βhu and αM3/βhu). Numbers in plots indicate MFIs. N/A: not available.



Supplementary Figure 3. Jurma cells were transduced with the NY-ESO-1 βM3 chain variant in combination with the fully human NY-ESO-1 TCRα chain (αhu) or with one of the partly murinized TCRα chains (αM1, αM2 or αM3). TCR expression was subsequently analyzed using an anti-Vβ4 antibody. Indicated numbers represent MFIs.



Supplementary Figure 4. Jurma cells were transduced with the human NY-ESO-1 TCR, with the αhu/βM3 variant, or with six different NY-ESO-1 TCR containing single human-to-mouse amino acid substitutions in domain 3 of the TCRβ chain. TCR expression was subsequently analyzed using an anti-CD3 antibody. Numbers in plots indicate MFIs.



Supplementary Figure 5. Front (left panel), side (middle panel) and back (right panel) views of a human αβTCR crystal structure (PDB: 3GSN). Sequences of αM1 and αM2 regions of the TCRα chain are indicated in blue and yellow, respectively. The βM3 domain is represented in red. The αM3 domain is not included in the crystal structure.

CHAPTER 8

General discussion

This chapter, and parts of Chapter 1, have been published:

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A REMARKABLE DIVERSITY HAMPERS APPLICATION OF γδT CELLS IN CANCER IMMUNO-THERAPY

Recent years have seen important progress in the understanding of γδT cell immunobiology and have uncovered a striking diversity in γδT cell functions and subsets. These new insights have important implications for the use of γδT cells in the treatment of cancer. To date however, a profound appreciation of this γδT cell diversity has lacked from γδT cell-based clinical concepts and this is likely to contribute to the limited clinical results observed so far. At least three levels of γδT cell heterogeneity can be distinguished (Figure 1), including (a) a multitude of immune functions mediated by γδT cells, (b) a diverse γδTCR repertoire that, also for similar antigen-specificities, mediates different affinities, and (c) the complex and diverse molecular needs for target recognition within the same and across different γδT cell populations. A thorough consideration of these features will be of central importance to improving the clinical efficacy of γδT cells in treating cancer.

γδT cell functions: the more the better?

γδT cells have, as discussed above, been attributed important and valuable functions in tumour immunosurveillance, but reactivity towards tumours is far from the only part that γδT cells play in immunity. By now, it is evident that γδT cells perform a plethora of functions that underline their involvement in diverse pathophysiological conditions other than cancer, including host defence against infectious pathogens such as bacteria, viruses and parasites, the modulation of the activity of other immune cells, and promoting tissue regenerating after injury (1,2).

Rapid expansions of γδT cells are observed in humans infected with a variety of viruses or bacteria and γδT cells possess a potent capacity to directly kill infected cells (3). Moreover, a proportion of γδT cells contribute to pathogen clearance by the secretion of antimicrobial peptides such as granulysin and cathelicidin (4-6). Intriguingly, the recognition of pathogens may have important implications for γδT cell-mediated cytotoxicity against cancers, as subsets of γδT cells that respond to cytomegalovirus (CMV) infection have been reported to cross-recognize solid (7) as well as haematological (8) tumour cells in vitro. A role for virus-induced γδT cells in the protection from cancer in vivo is supported by observations that CMV infection in kidney transplant recipients was observed to associate with increased levels of γδT cells and concomitantly a reduced risk of developing cancer (9). Also in leukemia patients treated with hematopoietic stem cell transplantation, CMV infection associates with lower incidence of leukemic relapse after transplantation (10,11) and work from our laboratory has demonstrated that tumour surveillance by CMV-induced γδT cells is likely to play a major role in this (8), emphasizing the clinical value of such dual-reactive γδT cells in immunotherapy.

In addition to their strong reactivity to a wide variety of tumours and pathogens, a valuable feature of γδT cells is their capability to broaden immune responses by recruiting and activating additional immune cell populations. For example, activated γδT cells have the potential to orchestrate adaptive αβT cell responses, both directly by functioning as antigen-presenting cells (12-14) as well as indirectly via the interaction with dendritic cells (8,15,16). In addition,

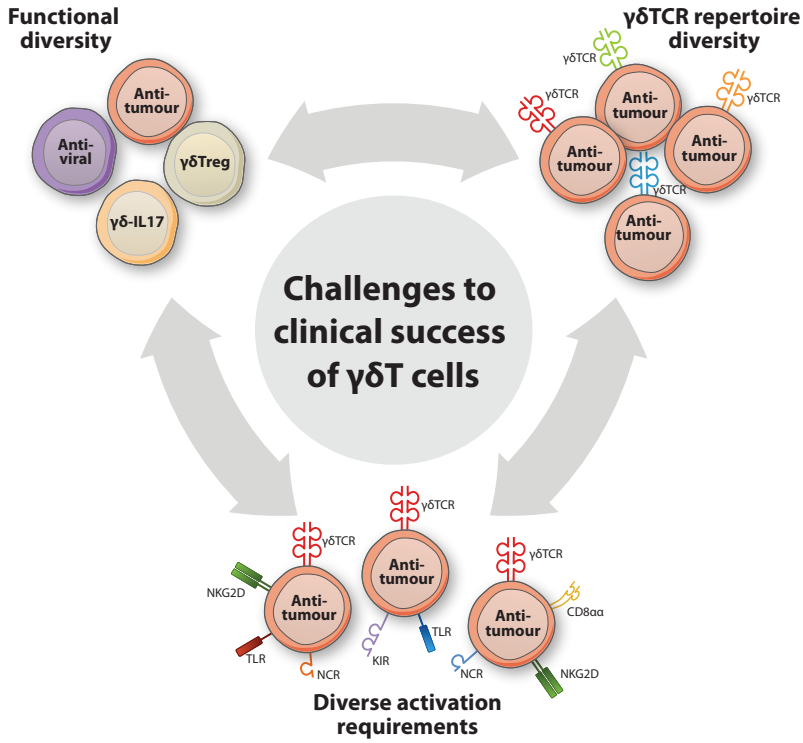

Figure 1. A broad functional and clonal diversity challenges the clinical success of γδT cells in cancer immunotherapy. New insights into γδT cell biology have pointed to at least three levels of diversity that each have a major impact on the design of successful γδT cell-based interventions to treat cancer. A striking functional diversity has come to light by the identification of new γδT cell subsets, such as regulatory (γδTreg) and IL17-producing (γδ-IL17) γδT cells, that now complement the well-established subsets with antiviral or anti-tumour functions. Within γδT cell populations that perform identical functions, another level of diversity is created by the extraordinarily diverse γδTCR repertoire that results in considerable variation in functional avidities of individual γδT cells. Additional diversity within and across γδT cell populations is represented by variable expression patterns of and complex activation requirements for additional immune receptors, including TLRs, CD8αα, and NK cell receptors such NKG2D, the natural cytotoxicity receptors (NCR) NKp30, NKp44 and NKp46, and activating and inhibitory killer cell immunoglobulin-like receptors (KIRs).

γδT cells have been reported to secrete cytokines to provide B cell help in the production of antibodies (17,18), to prime NK cells to kill tumour cells (19), to rapidly recruit neutrophils via the secretion of IL-17 (20,21) and to synergize with monocytes to mount anti-microbial αβT cell responses (22). However, in addition to the immunostimulatory roles of γδT cells, their modulatory function may be of regulatory nature as well, suggesting complex implications of γδT cells in mediating broader immune responses. For example, depending on antigenic exposure, γδT cells may suppress rather than promote antibody production by B cells (23,24).

Similarly, γδT cells can strongly inhibit the proliferation of activated αβT cells (25,26), and a suboptimal maturation of DCs by γδT cells (8) may induce tolerogenic rather than cytotoxic αβT cell responses. Importantly, human and mouse IL17-producing γδT cells have recently been demonstrated to facilitate tumour growth by recruiting myeloid-derived suppressor cells to tumour sites (27,28). With the recent identification of bona fide Foxp3-expressing regulatory γδT cell subsets (29), it is thus becoming clear that, depending on their local or temporal cytokine milieu, activated γδT cells may suppress instead of activate local immune responses (30). Indeed, even though the presence of γδT cells may correlate with increased survival of cancer patients in some studies (see above), their infiltration into tumour sites may also associate with worse clinical outcome of patients due to a immunosuppressive phenotype of local γδT cells (31-33).

A very diverse γδTCR repertoire produces receptors with variable anti-tumour affinities

Like αβTCRs and B cell receptors, γδTCRs are generated during T cell maturation through the somatic recombination of germline-encoded variable (V), diversity (D) and joining (J) gene segments. Despite the fact that the number of germline Vγ and Vδ genes is far more limited than the repertoire of Vα and Vβ genes, more extensive junctional diversification processes during TCR γ and δ chain rearrangement leads to a potential γδTCR repertoire that is roughly 105-fold larger than that of αβTCRs (34). Despite this extensive γδTCR repertoire, the diversity of antigens that are recognized by γδTCRs appears to be surprisingly limited. The vast majority of Vγ9Vδ2+ TCRs on circulating γδT cells are restricted to sensing elevated levels of phosphoantigens (35,36), a process that has recently been demonstrated to involve the butyrophilin family member BTN3A1 (37,38). Similarly, all antigens of $V\delta2^{neg}$ γδTCRs identified so far, including MICA/B (39), CD1 (40,41) and EPCR (42), belong to the family of non-classical MHC homologues, although additional antigens are likely to still be identified and may include MHC-unrelated molecules.

An important question is why this rather narrow antigen restriction of γδT cells is confronted with such a broad γδTCR diversity, instead of a rather oligoclonal or invariant repertoire as expressed by for example NKT cells (43). One possible explanation may be that the extensive γδTCR repertoire of γδT cells allows an important fine-tuning of γδTCR-mediated target cell recognition. Indeed, we have shown recently that phosphoantigen-responsive Vγ9Vδ2+ γδT cell clones differed widely in their functional avidity towards tumour cells (44). γδTCR transfer and mutation experiments showed that this variability in the ability to respond to tumour cells was mediated primarily through diverse sequence compositions that dictate the affinities of individual clone-derived Vγ9Vδ2+ TCRs. A similar γδTCR-mediated heterogeneity in anti-tumour specificity can be observed in the $V\delta2^{neg}$ subset of γδT cells, as we recently demonstrated that individual Vδ1+ γδT cell clones display γδTCR-mediated reactivity against diverse arrays of tumour cells (8). Moreover, γδTCRs of other Vδ1+ clones were not involved in tumour recognition but mediated interactions with dendritic cells, demonstrating that a diverse γδTCR repertoire can mediate not only a fine-tuning of anti-tumour avidity but also different functions. Accordingly, diverse γδT cell functions that segregate with γδTCR composition have been observed for the human Vγ9Vδ2+ and $V\delta2^{neg}$ subsets, as Vγ9Vδ2 γδT

cells have been generally ascribed potent cytotoxic effector functions, while Vδ2^{neg} γδT cells rather have immunomodulatory roles (45,46). However, these observations are contrasted by reports showing a superior tumour-homing and -killing capacity of Vδ2^{neg} γδTILs over Vγ9Vδ2 γδTILs in some cancers (47,48), further underlining the heterogeneous and context-dependent nature of both γδT cell subsets.

γδT cell activation: a complex interplay between receptors

Alongside the γδTCR, γδT cells can be activated through a variety of activating and inhibitory NK receptors (49,50) and toll-like receptors (TLR) (51), emphasizing the innate-like nature of these unconventional T cells. Depending on the pathophysiological context, these receptors can provide costimulation to γδTCR-mediated activation signals or can activate γδT cells independent of γδTCR triggering, adding yet another level of heterogeneity and complexity to γδT cell biology. The best-studied receptor with dualistic roles in γδT cell activation is NKG2D, a natural cytotoxicity receptor (NCR) that is expressed on NK cells, most γδT cells and CD8+ αβT cells. NKG2D recognizes the non-classical MHC homologues MICA/B and ULBPs, the expression of which is upregulated on many different tumours (52,53). On Vγ9Vδ2+ γδT cells, NKG2D can amplify γδTCR-mediated effector functions in response to MICA/B-positive target cells (54,55). In other cases however, sole signalling through NKG2D has been proposed to be sufficient for activating γδT cells, without requiring γδTCR engagement (56,57). However, as most of these studies have used TCR blocking antibodies and not receptor gene transfer experiments, the impact of TCR affinity and signalling in NKG2D-triggered γδT cell activation might have been underestimated (Gründer & Kuball, unpublished observation). Factors that determine the directly stimulatory versus costimulatory function of NKG2D are not known, but may involve signalling by polymorphic receptors such as inhibitory NK receptors (56). Apart from serving as ligand for NKG2D, MICA/B is also recognized by selected Vδ1+ γδTCRs (39). In fact, overlapping binding epitopes for NKG2D and γδTCRs on MICA/B result in competitive binding of both receptors for MIC ligands, suggestive of complex, temporally regulated interactions of both receptors for MIC ligands (58). Similarly, engagement of the NCRs NKp30, NKp44 and NKp46 on γδT cells can be sufficient for eliciting anti-tumour cytotoxicity, but interestingly only after expression of these receptors on γδT cells has been induced via triggering of the γδTCR (59). Differential involvement of the γδTCR and additional receptors has also been reported in pathophysiological processes other than cancer, as work by us and others has demonstrated that reactivity of γδT cells against CMV-infected cells may involve γδTCR-dependent (7,60) and -independent (8) pathways, suggesting multimodal pathogen-sensing mechanisms that may involve NK receptors (49).

Recently, we have found additional evidence for a complex interplay between receptors in the response of γδT cells against tumour cells by demonstrating that CD8αα, that serves as coreceptor for selected γδTCRs as reported by us recently (8), mediates γδTCR costimulation in a manner that depends on the particular tumour cell target (Scheper & Kuball, unpublished observation). Expression of CD8αα on T cells engineered to express a tumour-reactive γδTCR was a prerequisite for recognition of all tested tumour cell lines, but coexpression of signalling-deficient CD8α variants or mutants with single residue substitutions in the extracellular

domain of CD8α alongside the γδTCR differentially impacted T cell reactivity towards the different tumour targets. Even though CD8αα+ γδT cells were first identified over 20 years ago, when CD8αα was found to be commonly expressed on Vδ1+ γδT cells in the intestine but not circulating Vγ9Vδ2+ T cells (61,62), the functional implications of CD8αα expression on γδT cells have remained rather controversial. A number of studies have reported regulatory functions for CD8αα+ γδT cells, being capable of for example inhibiting inflammatory responses in celiac disease (63) but also to suppress αβT cell-mediated responses against tumour cells (32). On the other hand, and in line with our data (8), stimulated CD8αα+ γδT cells have been reported to be as capable as CD8αα- γδT cells of secreting high levels of Th1 cytokines such as IFNγ (64). Moreover, cytokines produced by CD8αα+ but not CD8αα- γδT cells have been implicated in the controlling of R5-tropic HIV replication and persistence (65). Thus, CD8αα+ γδT cells appear to perform diverse functions depending on the context in which they are activated.

Taken together, the emerging insights into the molecular requirements for γδT cell activation and the interplay between different receptors in this process have substantially furthered our understanding of the response of γδT cells against cancer cells, but also unveil substantial challenges to the design of uniform γδT cell-based strategies for cancer immunotherapy.

SUCCESSFUL TRANSLATION USING γδT CELLS: PICKING THE RIGHT ONES

Beyond doubt, the implications of the functional and clonal heterogeneity of γδT cells for their application in the treatment of cancer are substantial, and a failure to fully recognize this diversity in clinical concepts and trial designs is likely the most important contributing factor in the limited clinical results observed with γδT cells to date. Current clinical protocols based on the broad activation of unselected γδT cells are likely to induce γδT cell populations with diverse specificities, avidities and functions, including regulatory. Consequently, high-avidity γδT cells with strong tumour-reactivity and a desired functional profile may represent only a relatively minor population of such cell products. In addition, stimulation of γδT cells using agents that primarily depend on strong γδTCR-mediated activation, such as the use of aminobisphosphonate and phoshoantigen compounds to expand Vγ9Vδ2+ γδT cells in trials pursued to date, most likely selects for γδT cells with low affinity Vγ9Vδ2+ γδTCRs and thus low activity on primary tumour cells. Moreover, γδTCR-based activation strategies do not necessarily mobilize γδT cells that express a repertoire of NK and toll-like receptors required to potently respond to the multimolecular stress signature of tumour cells. Thus, the selection of optimally tumour-reactive γδT cell populations will likely be a critical parameter in the design of improved cancer immunotherapeutic concepts. In principal, this would favour strategies aimed ex vivo rather than in vivo expansion of γδT cells, since the first allows a careful monitoring and culture-dependent skewing of γδT cell phenotype and functionality that is far more challenging to accomplish using in vivo stimulation protocols. With the clinical data available so far, it is difficult to corroborate this by comparing clinical responses observed in both types of trials, as studies using adoptive transfer of ex vivo generated γδT cells have

so far relied on similar stimulation protocols (aminobisphosphonate or phosphoantigen in combination with IL-2) and the potential for extended in vitro manipulation for enhanced antitumor efficacy has not yet been investigated (66-72). Importantly, ex vivo manipulation of patient γδT cells could also include a valuable enrichment of tumour-specific γδT cells with high functional avidity, for instance using selection techniques based on the upregulation of activation markers or the production of cytokines such as IFNγ by γδT cells after in vitro coculture with autologous tumor cells. Nevertheless, γδTCR repertoires vary widely among individuals (73,74), and generating sufficient numbers of γδT cells that recognize tumours with high avidity may therefore be challenging in certain patients. Similarly, NK receptor and TLR repertoires as well as CD8α expression levels differ considerably between γδT cell subsets (8,59,61,75) and between individuals (51,76,77), putting additional constraints on the generation of γδT cell products potently capable of rejecting cancer.

To overcome the limitations of patient γδT cell repertoires, γδTCRs with broad tumor-specificity could be identified in vitro and genetically introduced into patient-derived immune cells. Recent work by our group has demonstrated that gene transfer of tumor-specific Vγ9Vδ2+ and Vδ1+ γδTCRs can be used to efficiently reprogram conventional αβT cells to recognize a wide variety of tumor cells (8,44,53). By exploiting the abundance and superior proliferation potential of αβT cells, large numbers of autologous γδTCR-engineered T cells with defined tumor-specificity can be generated ex vivo and subsequently reinfused into cancer patients. In contrast to αβTCR gene transfer strategies, introduced TCR γ and δ chains do not dimerize with endogenous αβTCR chains (53) and therefore do not lead to the formation of unwanted TCRs with unpredictable, and potentially dangerous, specificities. Moreover, since antigen recognition by γδTCRs does not depend on classical MHC molecules, well-characterized γδTCRs that mediate superior anti-tumour functional avidities can be applied to a broad patient population without the requirement for HLA matching. Additionally, transgenic expression of γδTCRs downregulates surface expression of endogenous αβTCR chains (8,44,53), enabling the use of engineered cell product even in an allogeneic "off-the-shelf" fashion. The ex vivo generation of γδTCR-engineered T cells furthermore allows additional manipulation of cell products, such as the selection of T cells with highest γδTCR expression levels or T cells which express beneficial TLRs or NK receptors. Importantly, such strategies can take advantage of the valuable lessons that have been learned from efforts to apply conventional αβT cells and their receptors in cancer immunotherapy, such as evidence for the effect of the differentiation status on in vivo persistence and function of clinical T cells (78). Our group has initiated the first clinical trial using γδTCR-gene modified T cells to treat cancer patients (scheduled to start in 2015). Donor T cells engineered with a well-characterized tumor-reactive Vγ9Vδ2+ γδTCR (44) will be administered to leukemia patients after allogeneic stem cell transplantation as part of an engineered donor lymphocyte infusion. Ex vivo manipulations of gene-modified T cell products will include the depletion of cells that express only low levels of the clinical γδTCR and adapted culturing conditions to prevent terminal differentiation of engineered T cells before infusion into patients.

CLOSING REMARKS

Even though γδT cells have traditionally been regarded as a homogeneous immune population, important advances in the understanding of γδT cell immunobiology have revealed a striking diversity in functionality and molecular activation modes. These new insights are generally met with great enthusiasm as they give acclaim to γδT cells for their nonredundant involvement in so many pathophysiological and homeostatic processes. However, this pleiotropy of γδT cells is likely an important factor that stifles the clinical success of their application to treat cancer. As for adaptive immune interventions, it may be absolutely mandatory to carefully consider the plethora of γδT cell functions, the diversity in γδTCR specificities and affinities as well as the complex requirements for proper γδT cell activation. At the end, such broadly tumour-reactive γδT cells might be highly effective only under very defined molecular and pathophysiological conditions and therefore less broadly applicable as initially thought, though a valuable addition to current therapeutic options. This new concept represents a major challenge in the design of next generation γδT cell-based immunotherapies, and clinical trials that incorporate these exciting insights will need to be pursued to confirm the clinical potential of γδT cells in the treatment of cancer.

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APPENDIX

Summary

Nederlandse samenvatting

Dankwoord

List of publications

Curriculum vitae

SUMMARY

Immunotherapy to treat cancer holds the promise of generating targeted and durable anti-tumour responses, and immune-based therapies using antibodies and conventional αβT cells have already yielded impressive clinical results over the last decade. Innate-like γδT cells are unconventional T cells with characteristics that make them valuable additions to current cancer immunotherapeutic concepts based on adaptive immunity: γδT cells are activated by unique types of antigen and display potent cytotoxicity against tumour cells of surprisingly diverse tissue origin. However, results of clinical trials using γδT cells to treat cancer have so far fallen short. Efforts of recent years have led to important new insights into γδT cell biology and revealed a striking diversity in γδT cell functions and their molecular activation modes. Nevertheless, a profound understanding of the immunobiology and diversity of these "swiss army-knives" of immunity is still lacking, and this represents an important obstacle for the clinical success of γδT cell-based cancer immunotherapies pursued so far. In this thesis, we aimed to investigate the diverse functional and molecular mechanisms by which γδT cells interact with cancer cells, in order to lay the groundwork for the design of more efficacious γδT cell-based cancer immunotherapies.

In **Chapter 2**, we review current literature on the ways by which innate-like immune cells, including γδT cells and natural killer (NK) cells, recognize their target cells. Mechanisms of γδT cell and NK cell activation are discussed in the context of allogeneic stem cell transplantation (allo-SCT), the preferred treatment for patients with poor-prognosis hematological cancers. Allo-SCT provides a valuable framework to study the multitude of functions of innate-like cells due to (a) the naïve immune state of patients, allowing a careful tracking of changes in immune populations, (b) the diverse non-self exposure to not only cancer cells but frequently also viral and bacterial infections, and (c) the opportunity to study the education – and failure thereof – of immune cells *in vivo*. γδT cells and NK cells display a degree of overlap in their immunological functions, including a valuable capacity to cross-recognize cancer cells and virus-infected cells (see also Chapter 3), and the receptor-antigen interactions involved in mediating these reactivities are outlined. Next, results of clinical trials using γδT cells and NK cells to treat hematological cancers are discussed. The outcome of such trials have been promising but also quite heterogeneous, likely owing to a large diversity in bot only the methods used for preparing cellular products, but also in the patient preconditioning regimes, and in the cell populations that have been administered to patients so far. As one way to address these limitations, we propose to move towards a "engineered" innate-like anti-tumour approach. Options for such manipulation of innate immunity to better target cancers include enriching stem cell grafts for innate-like immune cells, reprogramming patient immune cells with tumour-reactive innate receptors, and the generation of soluble anti-tumour chimeric compounds based on innate-like receptors.

In **Chapter 3**, we investigated the response of distinct γδT cell subsets to cytomegalovirus (CMV) infection in leukemia patients that received allo-SCT. Although harmless in most

healthy individuals, CMV infections remain a major problem in immunocompromised patients after allo-SCT and can lead to life-threatening complications. Paradoxically, recent data suggest a favourable association between CMV reactivation after allo-SCT and reduced leukemic relapse. By comparing leukemia patients with and without an active CMV infection after allo-SCT, we observed that expansion of specifically the Vδ2^{neg} γδT cell subset, but not other subsets, was induced in these patients by CMV. Importantly, when tested *ex vivo*, these CMV-induced γδT cells were capable of reacting not only to CMV-infected cells but also to a variety of cancer cells. We therefore propose that γδT cells that expand in response to CMV infection in allo-SCT patients may explain the association between CMV infection and reduced risk of leukemic relapse observed by others. In addition, we found that CMV infection induces γδT cells with a rather diverse functional profile, as CMV- and leukemia-reactivity could be restricted to the same clonal population, whereas other γδT cells interacted with dendritic cells. Cloned γδT cell receptors (γδTCRs) mediated leukemia-reactivity and interactions with dendritic cells, but surprisingly not CMV-reactivity, suggesting diverse molecular interactions of these γδT cells with their target cells. Finally, we demonstrate in this chapter for the first time a direct costimulatory role of CD8αα for distinct tumour-reactive γδTCRs. We summarize these findings in **Chapter 4** and discuss their implications for improving adoptive cellular therapies, both in the context of allo-SCT as well as cancer immunotherapy in general.

The molecular mechanisms of antigen recognition by γδTCRs are still poorly understood, and this represents a major hurdle for truly targeted γδT cell-based immunotherapies. In **Chapter 5** we therefore further characterized the coreceptor function that CD8αα performs for selected γδTCRs, as identified by us in Chapter 3. We demonstrate that genetic transfer of CD8αα-dependent γδTCRs can redirect CD4+ T cells against multiple tumour targets only after cotransfer of the CD8α gene. Surprisingly, detailed mapping of the molecular interaction requirements of CD8αα by site-directed mutagenesis and blocking experiments suggested diverse ligands for CD8αα on distinct tumour targets, including MHC class I-like molecules. Moreover, cotransfer of a signaling-deficient CD8α mutant led to opposing effects on γδTCR-mediated activation by different tumour targets, suggesting diverse requirements for CD8αα adhesion and signaling functions depending on the target cell that is encountered. Based on these data, we strongly advocate that clinical efforts utilizing γδT cells or their individual receptors take such heterogeneity in activation mechanisms into careful consideration.

In **Chapter 6**, we developed a semi-high throughput screening method to identify the molecular determinants of activation of Vγ9Vδ2+ γδT cells, a major circulating γδT cell subset in humans that responds to elevated levels of small intermediates of the mevalonate pathway called phosphoantigens. Genome-wide correlation analysis using a genetically well-characterized cell library and subsequent candidate follow-up revealed the small GTPase RhoB as a key modulator of tumour cell recognition by Vγ9Vδ2+ TCRs. We demonstrate that RhoB expression, activity, and intracellular localization associates with differential recognition of tumour cells by Vγ9Vδ2+ TCRs. Moreover, RhoB is a direct prenylation target of the mevalonate pathway, and inhibition of prenylation in tumour cells resulted in reduced recognition by Vγ9Vδ2 TCR+

T cells. We furthermore show that RhoB can directly interact with BTN3A1, a major player in Vγ9Vδ2+ γδT cell activation recently identified by others. We demonstrate that this interaction occurs exclusively in recognized tumour cells, resulting in changes in the conformation and membrane mobility of BTN3A1 that may serve as the antigenic signature on tumour cells that is recognized by Vγ9Vδ2 TCRs. Together, these data put RhoB forward as an eagerly awaited "missing link" between mevalonate pathway dysregulation in tumour cells, elevated phosphoantigen levels and membrane alterations of BTN3A1 molecules.

The previous chapters of this thesis addressed the surprising functional diversity of γδT cells and their molecular modes of activation, and the challenges that these issues create for the design of effective cancer immunotherapies with γδT cells. An additional challenge to the clinical application of in particular TCR gene-engineered T cells is a lack of GMP-compatible techniques that allow for the selective isolation of T cells that express high levels of introduced TCRs. In **Chapter 7**, we introduce an isolation strategy – here based on a well-defined tumour-specific αβTCR – that facilitates the efficient depletion of cells that express only low levels of transgenic TCRs. We demonstrate that the substitution of the human αβTCR constant domains with their murine counterparts prevents recognition by a clinical grade antibody directed against human αβTCRs currently used to deplete T cells from stem cell products. Moreover, we found that introduction of selected murine residues into the TCRβ constant domain is sufficient to completely prevent recognition by the anti-human αβTCR antibody, thus minimizing the content of foreign sequences. By taking advantage of the natural competition between introduced and endogenous TCRs, this allows the selective depletion of T cells which poorly express or lack introduced TCRs, resulting in the efficient enrichment of untouched cells that express high levels of therapeutic TCRs. One important advantage of this approach is the fact that it is based fully on the use of readily available GMP-grade tools, making translation to the clinic straightforward and cost-effective.

Without doubt, γδT cells possess attractive features for cancer immunotherapy. However, the data described in this thesis and valuable recent insights gained by others in the field have uncovered a surprising diversity within the γδT cell population, including a myriad of γδT cell-mediated immune functions, diverse specificities and affinities within the γδT cell repertoire, and a multitude of complex molecular requirements for γδT cell activation. Although this has delivered these cells increasing fame and interest, it also poses new challenges to the design of effective γδT cell-based immunotherapies. In fact, the so far underappreciated heterogeneity within the γδT cell population is likely to be a major cause for the rather limited results of γδT cell-based cancer therapies pursued to date. A careful consideration of the diversity of components of adaptive immunity, such as αβT cells and antibodies, has delivered great progress to their clinical success; addressing also the extraordinary diversity among γδT cells will therefore hold the key to improving the efficacy of γδT cell-based strategies to battle cancer.

NEDERLANDSE SAMENVATTING

De klinische successen die de laatste jaren zijn behaald met kankerbehandelingen op basis van antilichamen en conventionele T cellen toont de potentie van immuuntherapieën om specifieke en duurzame responsen tegen kankercellen bewerkstelligen. γδT cellen zijn een ongebruikelijk type T cellen met eigenschappen van zowel het aspecifieke (aangeboren) als het adaptieve (verworven) immuunsysteem. Deze mix van eigenschappen maakt γδT cellen erg interessant voor gebruik in immuuntherapie tegen kanker: γδT cellen reageren op unieke typen antigenen op tumorcellen en zijn in sterke mate in staat om verrassend veel verschillende typen tumorcellen te doden. Toch is de klinische effectiviteit van kankertherapieën met γδT cellen tot nu toe teleurstellend gebleken. Onderzoek van de laatste jaren heeft uitgewezen dat er een enorme diversiteit bestaat in de immunologische en moleculaire eigenschappen van γδT cellen. Echter, gedetailleerde kennis over de functies en biologie van deze "Zwitserse zakmessen" van het immuunsysteem ontbreekt nog en dit staat de effectieve toepassing van γδT cellen in de strijd tegen kanker in de weg. Het doel van dit proefschrift is het onderzoeken van de diverse functies van γδT cellen en de mechanismen via welke zij geactiveerd kunnen worden, teneinde bij te dragen aan de ontwikkeling van meer effectieve therapieën met γδT cellen tegen kanker.

Hoofdstuk 2 geeft een overzicht van de huidige stand van de literatuur over de manier waarop γδT cellen en natural killer (NK) cellen – een tweede type immuuncel op het grensvlak van aspecifieke en adaptieve immuniteit – kankercellen en infecties kunnen herkennen. De activatiemechanismen van deze cellen wordt besproken in de context van allogene stamceltransplantatie (allo-SCT), de aangewezen behandeling voor patiënten met vergevorderde bloedkanker. Allo-SCT is een erg waardevol en bruikbaar platform om de diverse functies van γδT cellen en NK cellen te bestuderen, aangezien (a) veranderingen in het "nieuwe" immuunsysteem van patiënten nauwkeurig gevolgd kunnen worden, (b) dit nieuwe immuunsysteem blootgesteld wordt aan een verscheidenheid aan activatiestimuli van niet alleen kankercellen maar bijvoorbeeld ook virale en bacteriële infecties, en (c) het de mogelijkheid biedt de ontwikkeling van immuuntolerantie – of het ontbreken hiervan – te bestuderen. γδT cellen en NK cellen kunnen deels dezelfde immunologische functies vervullen, waaronder het herkennen van zowel kankercellen als virale infecties (zie ook Hoofdstuk 3), en de receptoren en hun liganden die bij deze functies betrokken kunnen zijn worden in detail uiteengezet. Vervolgens worden de prikkelende, maar tot op heden beperkte, klinische resultaten van kankerbehandelingen met γδT cellen en NK cellen besproken. De resultaten van deze klinische studies verschillen in sterke mate tussen individuele patiënten en volledige genezing komt tot op heden helaas maar weinig voor. Belangrijke oorzaken hiervoor zijn hoogstwaarschijnlijk de diversiteit in zowel de manier waarop celproducten gegeneerd worden, als in de preconditionering van patiënten, en in de celpopulaties die worden toegediend. Om de effectiviteit van deze therapieën te verbeteren en om kankercellen beter en specifieker te bestrijden stellen we een benadering van nauwkeurig "gemanipuleerde" immuuntherapie met γδT cellen of NK cellen voor. Mogelijkheden voor zulke manipulaties

zijn bijvoorbeeld de verrijking van γδT cellen en NK cellen in stamcelproducten en het gebruiken van tumorspecifieke receptoren van γδT cellen en NK cellen voor het genetisch herprogrammeren van immuuncellen of het producteren van therapeutische eiwitten.

In **Hoofdstuk 3** onderzochten we de activiteit van verschillende γδT cel subpopulaties tegen cytomegalovirus- (CMV-)infecties in leukemiepatiënten die een allo-SCT hebben ondergaan. Hoewel een CMV-infectie vaak geen gezondheidsproblemen oplevert bij gezonde personen, vormt infectie met het virus een grote bron van complicaties in immuundeficiënte patiënten na een allo-SCT. Toch wijzen recente onderzoeksresultaten verrassend genoeg op een beschermend effect van CMV-infectie als het gaat om het terugkeren van leukemie na allo-SCT. Door patiënten te vergelijken die na allo-SCT wel of geen actieve CMV-infectie vertoonden, konden we vaststellen dat specifiek de Vδ2^{neg} subpopulatie van γδT cellen substantieel expandeerde als reactie op infectie. Bovendien observeerden we dat deze geëxpandeerde γδT cellen *ex vivo* niet alleen CMV-geïnfecteerde cellen konden herkennen, maar ook verscheidene typen kankercellen. We stellen daarom voor dat γδT cellen die expanderen in patiënten na allo-SCT als reactie op CMV-infecties een van de verklaringen kunnen zijn voor het voordelige verband tussen CMV-infectie en de verlaagde kans op terugkeer van de leukemie. Hiernaast stelden we vast dat CMV-infecties γδT celpopulaties induceren met verschillende immunologische functies, aangezien bepaalde populaties reactiviteit vertoonden tegen kankercellen en CMV-geïnfecteerde cellen terwijl andere populaties interacties aan konden gaan met dendritische cellen. Geïsoleerde γδT cel receptoren (γδTCR) waren verantwoordelijk voor de herkenning van kankercellen en dendritische cellen, maar verrassend genoeg niet voor herkenning van CMV-infectie, hetgeen een diversiteit aan moleculaire interacties van deze γδT cellen met andere cellen suggereert. Tot slot hebben we in dit hoofdstuk voor het eerst vastgesteld dat CD8αα een costimulatoire functie kan hebben voor bepaalde γδT celreceptoren. In **Hoofdstuk 4** vatten we de bevindingen van Hoofdstuk 3 samen en bediscussiëren we de implicaties van deze inzichten voor het verbeteren van cellulaire immuuntherapieën, in de context van zowel allo-SCT als kanker in het algemeen.

De moleculaire mechanismen die verantwoordelijk zijn voor de herkenning van kankercellen door γδT cellen zijn nog maar in beperkte mate bekend. In **Hoofdstuk 5** onderzoeken we daarom de functie van CD8αα als coreceptor van bepaalde γδTCRs, zoals geïdentificeerd in Hoofdstuk 3, in meer detail. We laten zien dat CD4+ T cellen geherprogrammeerd kunnen worden om kankercellen te kunnen herkennen na genetische introductie van zowel een CD8αα-afhankelijke, tumorspecifieke γδTCR als het CD8α-gen. Door de moleculaire vereisten voor CD8αα in de context van deze γδTCR te onderzoeken met behulp van blokkerende antilichamen en geïntroduceerde mutaties in het CD8α-gen, blijkt CD8αα diverse liganden te herkennen op verschillende typen kankercellen, waaronder moleculen gerelateerd aan MHC klasse I. Bovendien had de introductie van bovengenoemde γδTCR in combinatie met een CD8α mutant welke deficiënt is in intracellulaire signalering tegengestelde effecten op de herkenning van verschillende kankercellen. Deze observaties suggereren dus verscheidene moleculaire functies voor CD8αα in de costimulatie van bepaalde γδTCRs, afhankelijk van het

type kankercel dat wordt herkend. We stellen daarom nadrukkelijk voor dat deze diversiteit in moleculaire vereisten voor activatie via γδTCRs nauwkeurig in overweging dient te worden genomen bij de ontwikkeling van therapeutische strategieën die gebaseerd zijn op γδT cellen of individuele γδTCRs.

In **Hoofdstuk 6** hebben we een nieuwe screeningsmethode ontwikkeld om de moleculaire vereisten voor activatie van Vγ9Vδ2+ γδT cellen te identificeren. Vγ9Vδ2+ γδT cellen vormen de grootste subpopulatie van γδT cellen in het bloed en reageren op verhogingen in de concentratie van kleine phosphoantigenen die geproduceerd worden als onderdeel van de mevalonaat-route. Genoombrede correlatieanalyse met behulp van een genetisch gekarakteriseerde celbibliotheek wees op de GTPase RhoB als een belangrijke speler in de herkenning van kankercellen door Vγ9Vδ2+ γδT cellen. We tonen aan dat de expressie, de activiteit en de intracellulaire lokalisatie van RhoB in verband staan met de mate waarin tumorcellen herkend kunnen worden door Vγ9Vδ2+ TCRs. Bovendien is bekend dat de mevalonaat-route RhoB moduleert door middel van prenylatie en we observeerden dan ook dat het remmen van prenylatie in kankercellen hun herkenning door Vγ9Vδ2+ TCRs vermindert. Hiernaast tonen we aan dat RhoB directe interacties aan kan gaan met BTN3A1, een molecuul met een recentelijk ontdekte, belangrijke rol in de activatie van Vγ9Vδ2+ TCRs. Deze interactie vindt enkel plaats in kankercellen die herkend worden door Vγ9Vδ2+ TCRs en leidt in die cellen tot veranderingen in zowel de membraanmobiliteit als de conformatie van BTN3A1. Deze observaties presenteren RhoB als “missende link” tussen de mevalonaat-route, verhoogde concentraties van phosphoantigenen en membraanveranderingen van BTN3A1 moleculen, waarvan de laatste zou kunnen dienen als een moleculair antigeenprofiel dat herkend kan worden door Vγ9Vδ2+ TCRs.

De vorige hoofdstukken van dit proefschrift beschreven de verrassend diverse functies en activatiemechanismen van γδT cellen en de uitdaging die deze diversiteit vormt voor de effectieve behandeling van kanker met op γδT cellen gebaseerde immuuntherapieën. Een andere uitdaging, vooral voor therapeutische strategieën met T cel receptor-gemodificeerde T cellen, bestaat uit het feit dat er nog maar weinig klinische middelen beschikbaar zijn voor de verrijking van cellen die de geïntroduceerde T cel receptor in hoge mate tot expressie brengen. In **Hoofdstuk 7** introduceren we daarom een isolatiemethode – in dit geval voor een eerder beschreven tumor-specifieke αβTCR – die cellen die de geïntroduceerde TCR in slechts lage mate tot expressie brengen op een efficiënte manier uit het celproduct kan verwijderen. We tonen aan dat het vervangen van de sequentie van het menselijke αβTCR constante domein door de corresponderende sequenties uit de muis αβTCR de binding tussen de TCR en een reeds klinisch toegepast anti-αβTCR antilichaam belemmert. Vervolgens demonstreren we dat de introductie van slechts enkele van de muis afgeleide aminozuren in de β-keten van de menselijke αβTCR was voldoende om binding van het antilichaam compleet te remmen en tegelijkertijd de hoeveelheid niet-humane sequenties tot een minimum te beperken. Door gebruik te maken van de natuurlijke competitie tussen endogene en geïntroduceerde, therapeutische TCRs staat deze methode toe om selectief

die cellen te depleteren die geïntroduceerde TCRs slechts in beperkte mate tot expressie brengen. Op deze manier kunnen TCR-gemodificeerde celproducten op een efficiënte manier verrijkt worden voor cellen met een hoge expressie van de therapeutische TCR. Een belangrijk voordeel van deze benadering is dat hij volledig is gebaseerd op middelen die reeds goedgekeurd zijn voor gebruik in de kliniek, hetgeen de vertaling van deze methode naar klinische toepassing simpel en kosteffectief maakt.

Zonder twijfel beschikken γδT cellen over eigenschappen die hen aantrekkelijk maken voor gebruik in immuuntherapie tegen kanker. Echter, de bevindingen die beschreven worden in dit proefschrift in combinatie met waardevolle recente observaties door anderen in dit veld duiden op een enorme diversiteit binnen de γδT celpopulatie, zowel wat betreft hun diverse immunologische functies, als de verschillende specificiteiten van hun γδTCRs en de verscheidenheid aan complexe moleculaire mechanismen van hun activatie. Hoewel deze immunologische verscheidenheid γδT cellen veel onderzoeksinteresse heeft opgeleverd, vormt het ook nieuwe uitdagingen voor de ontwikkeling van succesvolle immuuntherapieën met γδT cellen. Sterker nog, de tot op heden ondergewaardeerde diversiteit binnen de γδT celpopulatie is een waarschijnlijke oorzaak voor het gebrek aan effectiviteit van de huidige kankerbehandelingen met deze cellen. Het zorgvuldig rekening houden met de diversiteit binnen componenten van het adaptieve immuunsysteem, zoals αβT cellen en antilichamen, heeft sterk bijgedragen aan hun klinische succes; het onderkennen van ook de enorme diversiteit van γδT cellen zal daarom cruciaal zijn voor het verbeteren van kankertherapieën met deze veelzijdige cellen.

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CURRICULUM VITAE

Wouter Scheper was born on the 14th of August in 1980 in Oldenzaal, The Netherlands. In 1998, he completed secondary education at "Thij college" in Oldenzaal. That same year, he started studying Technology Management at the University of Groningen, and obtained his master's degree in 2004. Due to a strong interest in medical biology, he decided to study Life Science & Technology at the same university and received his master's degrees *cum laude* in 2010. As part of this study, he wrote a thesis on induced pluripotent stem cells which resulted in his first first-author publication in a peer-reviewed journal in 2009. In March of 2010, he started his PhD training in the departments of Immunology (now Laboratory of Translational Immunology) and Hematology of the UMC Utrecht under supervision of Dr. Jürgen Kuball. During this time he studied the immunobiology of innate-like γδT cells and their immune receptors, and their value for cancer immunotherapy. The results are presented in this thesis. Continuing in the field of tumor immunology, he started working as a postdoc in the group of Ton Schumacher at the Netherlands Cancer Institute (Nederlands Kanker Instituut) in November of 2014.