# Optimizing DC cross-presentation

## Orchestrating the immune response

Thijs W.H. Flinsenberg

Cover and lay-out design: Michiel de Jong & Thijs Flinsenberg
Printing: Proefschriftmaken.nl || Uitgeverij BOXPress

ISBN: 978-90-8891-944-2

Printing of this thesis was financially supported by:
Immunity and Infection graduate school Utrecht, BD Bioscience, Stichting Afweerstoornissen Nederland, Eurocept, Luminex and Chipsoft

# Optimizing DC cross-presentation
# Orchestrating the immune response

Optimaliseren van DC cross-presentatie
Dirigeren van de immuunrespons

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op vrijdag 3 oktober 2014 des middags te 2.30 uur

door

**Thijs Willem Hendrik Flinsenberg**

geboren op 3 oktober 1984
te Beek

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This research was supported by KIKA, Vrienden WKZ, WKZ fonds en NGI

Voor mijn voorbeelden:
Martin & Yvonne
Raymond & Diny
Gerrit & Drika

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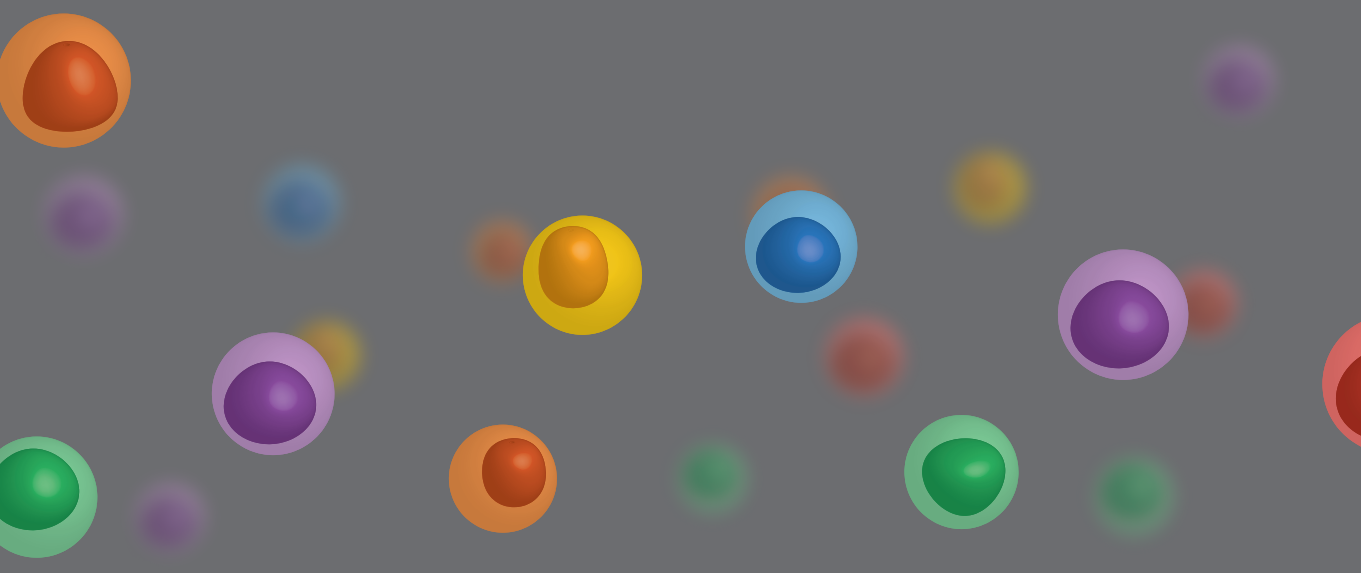
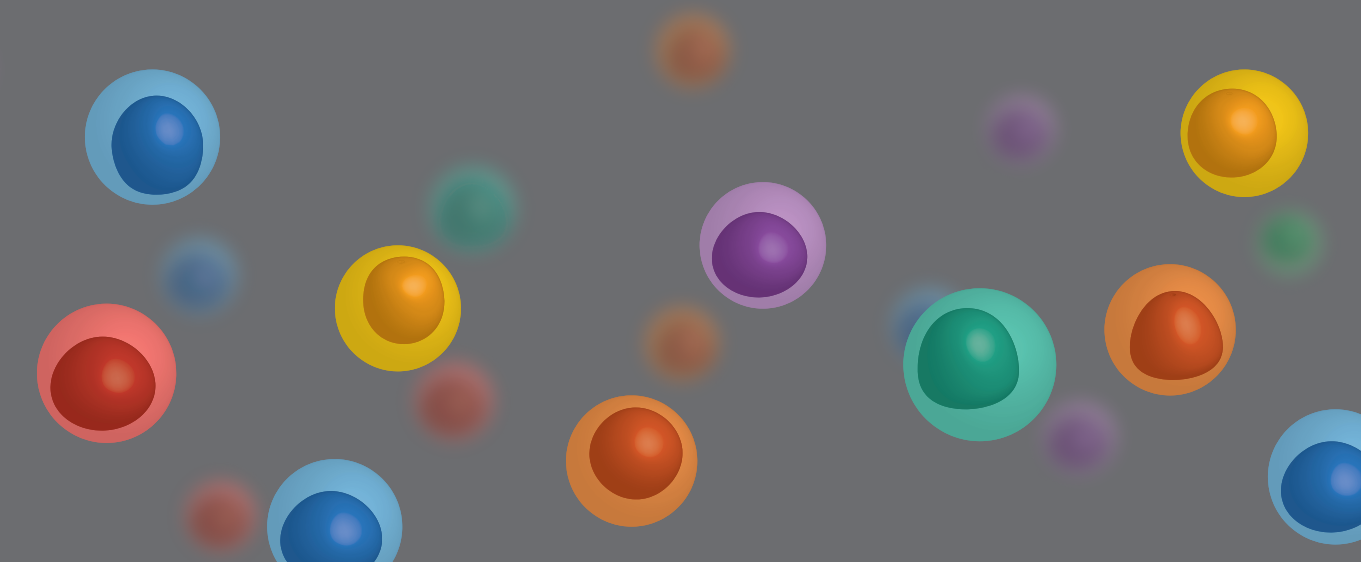


# General introduction



This thesis describes new insights in dendritic cell biology and contains disquisitions on antigen processing and presentation as well as the manipulation of these mechanisms using receptor mediated uptake. The work is aimed at better understanding dendritic cell (DC) biology, thereby contributing to pre-clinical work on DC vaccinations, which are being developed by the Utrecht Dendritic cells Against CancEr (U-DANCE) group. To scope the background of this thesis, in this 'general introduction' *chapter 1*, I will first elaborate on cord blood stem cell transplantation (CB-SCT) and the promise of DC-vaccinations. Subsequently, I will discuss basic DC background and elaborate on antigen presentation. Finally, I will summarize recent DC-based trials that were performed in SCT patients as anti-viral and anti-tumor treatment.

## Umbilical cord blood transplantation, historical perspective to current knowledge

### History

More than 25 years ago, the first umbilical CB-SCT was performed in a child with Fanconi Anemia*.[1;2] Although initially limited to transplantations in children using sibling CB, it is now used in both children and adults, with a wide range of indications. Moreover, the use of unrelated CB (first reported in: Kurtzberg et al.[3]) allowed for increased donor availability and boosted the field. In the last 25 years, more than 600.000 CB units have been stored worldwide and more than 30.000 CB-SCTs have been performed.[4]

Before CB was considered as a stem cell source, SCTs were predominantly being performed using aspired bone marrow (BM, *Figure 1*). Why develop CB as a source for stem-cells, when the outcome of BM-SCT was still evolving? To answer this question, one needs to consider that successful transplantation is dependent on multiple factors, that overall favors CB over BM. Some of those factors will be discussed next, and were effectively the foundation of the research discussed in this thesis.

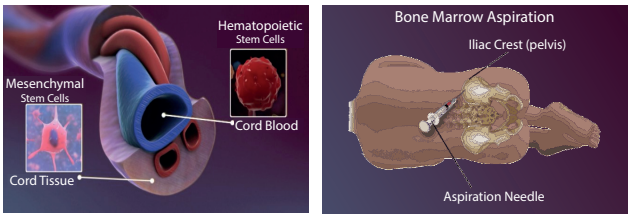



**Figure 1: Cord Blood and Bone Marrow as Stem Cell source**
Cord blood is aspired from the umbilical cord/plancenta after birth (left).
Donor stem cell collection requires a bone marrow aspiration (right) or peripheral apheresis.

### Availability

One of the major advantages of CB over BM is the availability. Where at the time of the first CB-SCT in 1988 the aspiration of CB of the patients newborn sibling was planned months in advance and the sibling CB unit had to be hand-delivered from Indiana USA to Paris, we are nowadays linked to multiple accredited CB banks worldwide having over 600.000 HLA-typed units uploaded to search-engines. Suitable units can be sent to transplant centers, making it possible to plan and perform the SCT within 1-2 weeks after HLA-typing and identifying a suitable donor. (informal discussion with JJ. Boelens).

* This person is now 1 year older than myself

### Outcomes

The evaluation of the effectiveness of CB over BM is made using serveral outcome criteria. The primary focus remains survival. In general it can be stated that in 25 years of CB-SCT, the survival has increased, although there remains ample of opportunity for improvement. *Figure 2* shows survival curves of patients treated with CB-SCT since 1996 in Europe and North America. In the UMC Utrecht >150 CB transplantations have been done since 2003 (data capture October 2012) with good clinical outcomes compared to the international registries (*Figure 2*). Of note, the indications for CB-SCT have expanded over the years. Diseases with high risk for mortality (mainly malignancies, eg. acute myeloid leukemia in children) hamper improvement of overall survival, while non-malignant indications (metabolic or immune-deficiencies, e.g. Hurler syndrome) can reach survival rates >90%.[5] Although more high-risk patients have been transplanted in recent years, the overall survival increased from approximately 60-65% to 65-70% for the overall group.

Several cell source studies comparing CB versus BM have been performed. In general, it can be concluded that CB-SCT has comparable overall survival (OS) rates in adults[6] and children.[7-9] When HLA-matching is optimal (CB 6/6), overall survival in pediatric patients treated with CB-SCT can even be much higher compared to BM-SCT (60% versus 38%).[8]

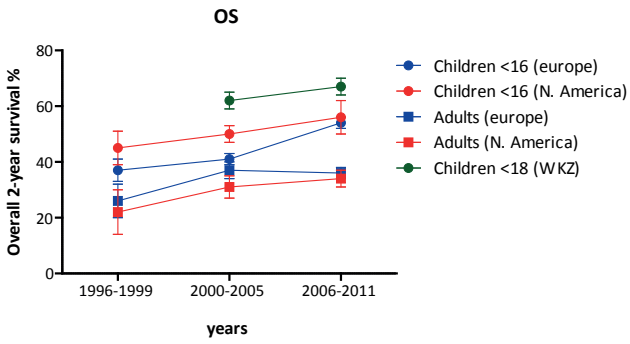



**Figure 2: OS at 2 years after CB-SCT for patients in Europe and North America** *(Adapted from Ballen et al, Blood 2013 [4]; data from WKZ adapted from patient outcome review 2012).*
Shown are average numbers of overall 2-year survival and standard deviations at specific time periods in children (dots) and adults (squares) in Europe (blue), WKZ Utrecht (green) and North America (red).

## Immune mediated complications after stem cell transplantation

### GvHD and Rejection

Transplantation with allogeneic hematopoietic cells can provoke immunological reactions against the graft (leading to rejection) or against the host. The latter leads to graft-versus-host disease (GvHD), a disease strongly associated with HLA-mismatching. HLA (Human Leukocyte Antigen) or MHC (Major Histocompatibility Complex) in animals, discovered in the late 1950s (van Rood, Nature 1958), functions as antigen presenting molecule, thereby fulfilling a role in orchestrating the immune system to fight "pathogens" (or non-self derived molecules). In case of SCT, HLA mismatching can result in an immune response targeted at the patient's body cells, leading to GvHD. This life threatening reaction is classically divided between acute GvHD (before day 100 post-SCT) and chronic GvHD (after day 100 post-SCT). Both seriously affect survival and quality of life.

There are 6 types of HLA (class I: A, B, C and class II: DR, DP, DQ) and every person carries 2 al-

leles of each, potentially leading to 12 different molecules that are highly polymorphic in protein sequence. Nowadays, patients are matched for alleles at 5 loci (A, B, C, DRB1 and DQB1) ultimately resulting in a 10/10 match (in case of BM). Higher matching in general results in better survival due to less morbidities (e.g. GvHD). [8;10;11]

For CB donors, the historically established matching criteria that are still being used, is matching for 3 loci (A, B and DRB1), with an optimal matching of 6/6. Despite these lower matching criteria, less GvHD is noted. The beneficial effect of 6/6 and 5/6 over 4/6 or less on aGvHD and cGvHD is overall accepted, where mismatched CB donors (less than 5/6) are more prone to develop GvHD.[8;12-14]
One consideration is that GvHD to some degree may be beneficial in case of malignant diseases, as low-level mismatch correlates with improved anti-tumor reactivity. On the other hand, GvHD grade II-IV elicits serious complications, hampering survival. The effect of graft matching on overall survival also remains point of discussion in literature. There are as many studies showing a positive effect of optimal graft matching on survival, as there are studies showing no effect (positive effect in: 8;14;15, no effect in: 12;13).

Cell source comparison studies revealed that CB-SCT patients have a decreased incidence and severity of GvHD [6;14-17], unpublished results from SCT unit WKZ 2012). This results in decreased use of immunosuppressive drugs which could benefit the immune response to fight opportunistic infections, as will be discussed in the next paragraph.

Finally, recent evidence reveals that matching 4 loci (including C) might improve overall survival. This reopens the discussion whether cord-blood behaves different than other stem-cell sources? And why should we stop with 8/8 and not match for 10/10 or even 12/12?[18;19]

### Relapse

As shown in *Figure 2*, the overall survival rate after CB-SCT has increased in the last decades. This is due to improvement on many different aspects of the procedure. One of the main remaining problems that prevent further improvement is disease relapse, for which incidence has remained stable over the past years (20-50% in pediatric patients, depending on type of malignant disease).[4;20] Especially in Acute Myeloid Leukemia (AML), relapse mortality remains high. Therefore, research predominantly focuses on novel treatment strategies for these diseases. One of those strategies involves dendritic-cell vaccinations, which will be discussed in more detail later in this chapter.

### Infectious complications

Infections after SCT is another major limitation of SCT associated with substantial morbidity and mortality. The combination of low or absent immune cells and mucosal barrier damage as a result of chemotherapy and immune-suppressants create an ideal situation for pathogens to surface and/or thrive. For optimal prevention, patients are treated at isolated wards and contact with potential pathogens is minimalized. However, the main threat does not reside in newly acquired pathogens, but with pathogens already present within tissues of the patient before the procedure was started. These pathogens are usually suppressed by our immune system and can only cause serious harm when a person is immunocompromised, a situation present in all SCT patients. For optimal prevention and treatment, patients receive

prophylactic antibiotics to prevent bacterial outgrowth. The presence of fungi is monitored weekly and upon detection, treatment is adjusted. Viral infections are both treated prophylactically and therapeutically (the risk of viral infections, its current treatment options and future perspectives will be discussed in the next paragraph). Although better monitoring, prophylactic measures and treatment improved overall survival, serious infections still affects 80-90% of SCT patients.[16]

Since cord-blood comprises mainly naïve cells, one would expect more opportunistic infections in patients undergoing CB-SCT compared to BM-SCT. However, a study performed in 136 children could not find a difference in incidence in the short (day 0-42 and day 43-100) nor the long term (day 100-180 and day 180-730). In fact, the only significant difference was found in T-cell depleted BM-SCT patients, where the risk for viral infections was higher before day 42.[16] Similar results were obtained in other cohorts, where no marked differences were found.[21;22] Data from the WKZ also show no difference in serious infection rates between CB and BM as SCT source (unpublished results form SCT unit WKZ 2012).

## Viral reactivations and infecti ons post SCT and treatment options

Viral reactivations remain a significant complication after CB-SCT and may have a significant impact on the overall outcome after transplantation. As stated before, virus reactivation can only thrive in an immunocompromized host. Timewise, these reactivations occur after the conditioning regime eradicates most of the patients innate and adaptive immune system. The timing of viral reactivation differs per virus (*Figure 3*). In addition to the direct effects of these infections, tissue-invasive diseases may be associated with increased risk of graft versus host disease, myelosuppression, and invasive bacterial and fungal infections.[23]

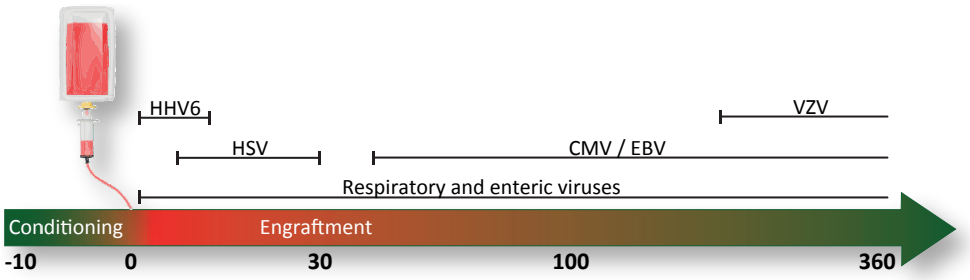



**Figure 3: Timeline for CB-SCT and viral reactivations** *(Adapted from Dykewicz; Trans Inf Dis 1999 [25], supplemented with data from de Pagter, BBMT 2013 [24]; vandenbosch, BBMT 2008 [28]; Ljungman, Infect Dis Clin N Am 2010 [27]; Mikulska, BBMT 2012 [21]; Gandhi, Lancet Inf Dis 2004 [26]).* Shown is the functionality of a patient's immune system (normal in green, poor defence in red). Frequent viral reactivations are shown in relation to time of reactivation/ clinical manifestation. CMV: Cyto Megalo Virus, EBV: Epstein-Barr Virus, HSV: Herpes Simplex Virus, HHV6: Human Herpes Virus 6, VZV: Varicella Zoster Virus.

Viruses of the Herpes family are studied most in the transplantation setting with the main focus on human Cytomegalovirus (CMV).[21;26;27] Other family members that are known to be opportunistic after CB-SCT are Epstein-Barr Virus (EBV)[29], Herpes Simplex Virus (HSV), Human Herpes Virus 6 (HHV6)[24] and Varicella Zoster Virus (VZV).[28] Then there are other viruses that can cause serious illness are for instance respiratoire virusen like Adenovirus, (para-) influenza

virus and Respiratory Sincytial Virus (RSV)30 and viruses associated with hemorrhagic cystitis like BK and JC virus.[31]

Viral infection or reactivation of dormant viruses within patients is associated with increased risk of GvHD, and in most studies, GvHD is a risk factor for viral reactivations.[32-37] This can be explained by the increased presence of immune cells at the site of viral reactivation, leading direct antigen presenting cell (APC) activation and indirect activation via tissue damage. As can be seen in *Figure 4*, these triggers can start off GvHD.[38] How GvHD can trigger viral reactivation is not exactly known. It could be that destruction of latent infected cells or activation of immune cells trigger viruses to proceed from latent into lytic state. Also, the treatment of GvHD includes immunosuppressants, further paving the way for opportunistic infections like these viruses.

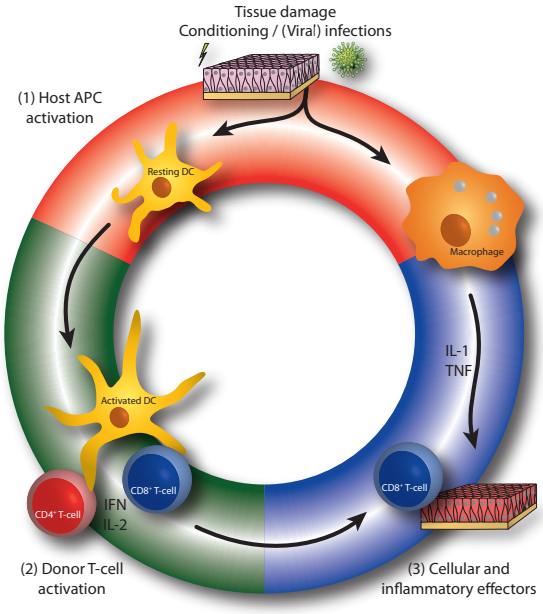



**Figure 4: Pathophysiology of GvHD** *(Adapted from Ferrara et al; Lancet 2009)*[38]
Tissue damage caused by active infections or the conditioning regime leads to host antigen presenting cell (APC) activation (1). These activated APCs can trigger donor T-cells (2) that will (via pro-inflammatory cytokines like interferon gamma (IFN-γ) and interleukin-2 (IL-2)) in the end trigger cellular and inflammatory effector functions (3) executed by cytotoxic T lymphocytes and macrophages (via pro-inflammatory cytokines like tumor necrosis factor (TNF) and interleukin-1 (IL-1)), together causing GvHD.

Treatment options are scarce and mainly focused on prevention of viral reactivation. Important are broad awareness on wards, hygiene measures and regular monitoring that enables early detection.[39] Furthermore, based on seropositivity, patients are prophylactically treated with antiviral drugs. Upon reactivation, optimalization of antiviral drugs can be helpful. EBV reactivation, which is specifically harboured in B cells, can be treated using the monoclonal antibody Rituximab against CD20. However, full clearance of viral reactivation is only achieved upon proper T-cell responses.[40] In order to achieve this, alterations in conditioning regimes are made involving reduced intensity chemotherapy, reduced immunosuppression and the administration of anti-thymocyte globuline (ATG) at an earlier time point. This allows for donor T-cells to proliferate earlier. Moreover, this decreases tissue damage, thereby decreasing the risk for GvHD and subsequently viral reactivations (*Figure 3*). Finally, new treatment strategies are being explored aimed at early T-cell reconstitution, either by infusing ex vivo cultured antigen specific T-cells or by infusion of ex vivo "trained/cultured" dendritic cells (DCs, discussed below).

Several vaccination studies using donor derived antigen specific T-cells have been performed, mainly focusing on CMV reactivation. In vitro cultured antigen specific T-cells were administered in CMV reactivating patients in several centers, mainly in phase I/II trial settings. Conclusions in general were that infusion was safe and did not induce GvHD.[40-42] More importantly however, the specific T-cell transfer resulted in a persistent clearance of the CMV antigenemia, which allowed the patients to discontinue toxic antiviral drug therapy without further high-level reactivation of CMV.[43] T-cell transfers are now also being explored for other virus reactivations. First results in EBV and Adenovirus reactivation are promising.[44-46] Also for HHV6, this therapeutic option is being explored.[47] In conclusion, antiviral T-cell infusion is a promising therapeutic option for viral reactivation. However, culturing these T-cells takes time. Moreover, it is difficult to target multiple viruses.

Therefore, vaccination with antigen presenting DCs is being explored, aiming not only at viral reactivations, but also at cancer relapse. Using antigen-loaded DCs as vaccination could be used earlier in disease course, and could potentially be used against multiple targets. Moreover, since DCs can be cultured from stem cells, it is possible to generate larger amounts which will not only directly be efficient, but also allow for revaccinations.

## Dendritic Cell history

Dendritic cells (DCs) were first described by Ralph Steinman, postdoc at the time in Zanvil Cohn's laboratory in 1973.[48] They were named for their probing, branch-like or dendritic extensions (from the greek 'dendron', meaning tree) (*Figure 5*).

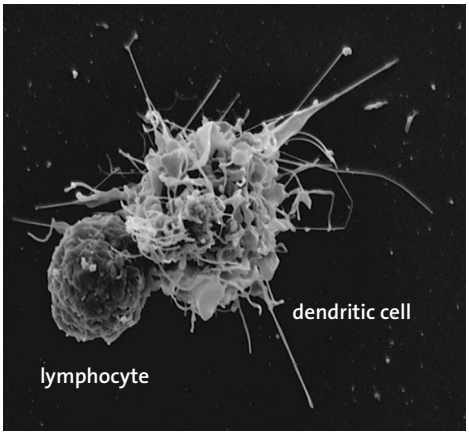



**Figure 5: Dendritic Cell** *(photo adapted from University of South Carolina, School of Medicine)*
A picture of a dendritic cell interacting with a lymphocyte. Note that the DC is relatively large compared to lymphocytes and has "branches" named dendrites to sample antigen.

In their paper, they described that DCs were distinct from other cells by several measures. Phase-contrast imaging showed a unique motility together with an irregular shape containing spiny processes (dendrites). Cytochemical stains showed sparse acid-phosphatase positive granules and a lack of membrane ATPase. Electron microscopy confirmed the absence of a well-developed lysosomal system. Although initially thought that DCs were of neural origin, later experiments using bone marrow chimeric mice revealed that DCs arise from bone marrow.[49] Later, it was shown that human DCs arise from $CD34^+$ hematopoietic stem cells. DCs were originally found in skin. We now know that DCs are present in almost all tissues of the

body. Comparing DCs in different tissues resulted in the recognition of several DC subsets (*Figure 6*). Most of this work has been done in mice, although recently more is revealed about human DCs. While not completely identical, there is a certain level of homology.[50]

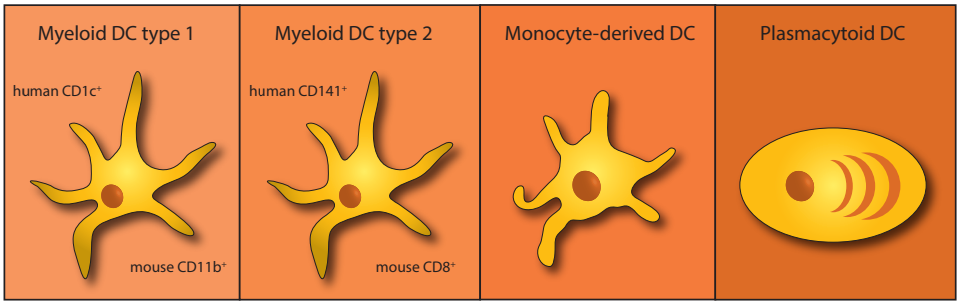



**Figure 6: Dendritic Cell subsets** *(Adapted from Collin et al; Nat Rev Imm 2011)*[50]
In both human and mice, four major dendritic cell subsets are found, classically devided in (1) myeloid DCs containing the classical $CD8^-$ DC (mouse) or $CD1c^+$ (human) and the cross-presenting $CD8^+$ DC (mouse) or $CD141^+$ DC (human), (2) plasmacytoid DCs and (3) the inducible monocyte-derived DC.

## Dendritic cells as master regulators of adaptive immune activation

Dendritic cells are key players in bridging the innate and adaptive immune system.[51-53] They can do this because of four major functions (*Figure 7*):

1. DCs reside in tissue lining body surface and continuously sample their environment. Once an antigen is being recognized, DCs can internalize it through a process called endocytosis.[54] This can happen by random sampling or by specific endocytosis after recognition by antigen binding receptors. The result of antigen capture through one of these receptors, the Fc gamma Receptor plays a central role in *chapters 3 and 4* of this thesis.
2. An endocytosed antigen will be degraded inside the DC. This will convert proteins to peptides that are subsequently presented on major histocompatibility complex (MHC) molecules which can be recognized by lymphocytes. The specific mechanism of antigen degradation and peptide loading has been intensely researched in the past decennia and at least for the classical presentation pathways, most of the mechanism is known. Concerning the mechanisms involved in the routes of antigen processing in cross-presentation, much is still unknown. This is the focus of many research groups around the globe. *Chapters 3, 5 and 7* involve experiments researching this part in DC biology.
3. DCs undergo maturation. During this maturation process, the intrinsic cell biology change the DC from a "sampling cell" to a "presenting cell". These features can also be used to distinguish immature from mature DCs. Examples are found in experiments concerning the maturation state or co-stimulatory molecule expression in *chapters 3, 5 and 7*.
4. DCs will migrate to the lymph nodes after antigen endocytosis. Here, they can encounter lymphocytes to which DCs can present the peptide-MHC complexes.[55]

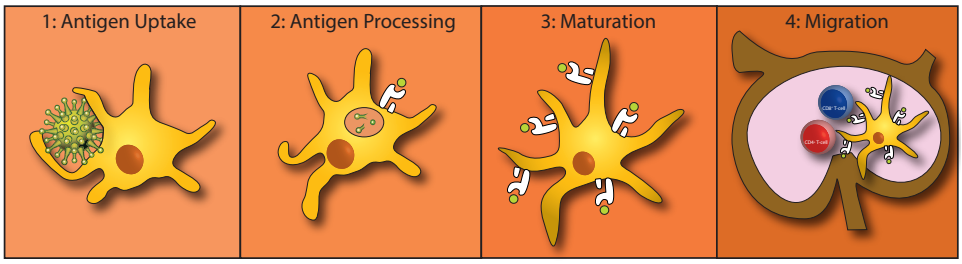



**Figure 7: Dendritic Cell functions**
Dendritic cells are key players in bridging the innate and adaptive immune system due to their four major functions: Antigen Uptake, Antigen Processing, Maturation and Migration (into the lymph nodes, shown in panel 4).

## Antigen presentation via Major Histocompatibility Complexes

Several of the dendritic cell functions are also carried out by other lymphocytes. However, their ability to present antigen on both classes of MHC in combination with essential co-stimulatory molecule expression, adhesion molecules and cytokines, crowns DCs as master regulators of adaptive immunity. To better understand these processes, I will first elaborate on MHC classes and its functions, before discussing antigen presentation.

As stated previously, there are 6 types of MHC (Major Histocompatibility Complex, in humans also known as HLA), divided into two classes. Class I MHC contains: A, B and C. Class II MHC contains: DR, DP and DQ. These two classes have evolved over time in order to be able to cope with pathogens that reside inside cells as well as in the extracellular milieu. Moreover, these molecules are differently distributed. MHC class I is expressed on all nucleated cells and its function mainly focuses on intracellular pathogens. *MHC I* molecules bind the T-cell receptor of *$CD8^+$ T-cells* (cytotoxic T-cells). MHC class II is found almost exclusively on professional antigen presenting cells and its function is aimed predominantly at extracellular pathogens. *MHC II* molecules bind the T-cell receptor of *$CD4^+$ T-cells* (helper and regulatory T-cells). The mechanisms of intracellular trafficking and loading are completely different between the two MHC classes.

### MHC class I

Most cells of our body are potential targets for intracellular infections (mainly by viruses) or could turn malignant during life. Our immune system thus needs a system to screen our own cells for presence of pathology, so that it is able to kill infected or malignant cells. Therefore, all cellular proteins (including viral or "malignancy" proteins) are regularly degraded into small parts called peptides that are subsequently presented to the immune system. The presentation of degraded intracellular proteins is done in the context of an MHC class I molecule (*Figure 8*). In the physiological situation, a circulating $CD8^+$ T-cell would recognize the presented peptides as "harmless". However, when "harmful" peptides are presented, the $CD8^+$ T-cell can force the target cell into apoptosis.

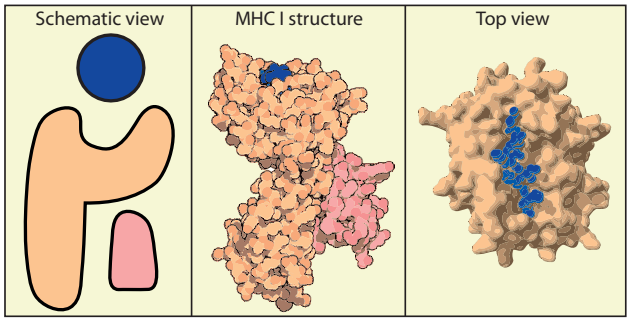



**Figure 8: MHC class I molecule** *(Adapted from: David Goodsell & RCSB Protein Data Bank and iayork.com)*
A schematic view of the MHC I molecule, consisting of 3 α-chains (yellow) and one β2-microglobulin domain (pink). The presented pepide is shown in blue. The most right illustration shows the molecule from top-view, where the peptide is situated in the binding groove formed by two of the α-chains.

Degradation of proteins is done by the (immuno-) proteasome. The peptides that are formed are transported from the cytosol into the endoplasmic reticulum (ER) and loaded onto the MHC I molecule by a loading complex involving the Transporter associated with Antigen Processing (TAP). The peptide-MHC I complexes are then transported to the cell-membrane via the Golgi network. Once the peptide-MHC I complex is situated on the cell-surface, it can be recognized by $CD8^+$ T-cells.[56] The MHC class I pathway is shown in *Figure 10*. Dendritic cells can present intracellular antigens on their MHC class I molecule just like all other nucleated cells. In contrast to most other cells, DCs can also present exogenous antigen on their MHC class I molecules. This process, referred to as cross-presentation, will be discussed below, and in more detail in *chapter 2*.

## MHC class II

Many pathogens exist in the extracellular space. Defense against these pathogens is carried out by different T-cells from both the innate and adaptive immune system, and many of those need "help" from $CD4^+$ T-cells (T-helper cells) to execute their function. For instance, macrophages can phagocytose extracellular pathogens, but sometimes require an extra "help" signal provided by T-helper cells for killing. Another important defense mechanism is provided by B cells and involves various isoforms of immunoglobulins (Ig). The production of certain types of Ig's, distinguishable by the heavy chain portions of each Ig molecule requires for the B-cells to interact with T-helper cells. $CD4^+$ T-cells have a slightly different T-Cell Receptor (TCR) complex compared to $CD8^+$ T-cells, which requires antigen presentation on MHC class II (*Figure 9*).

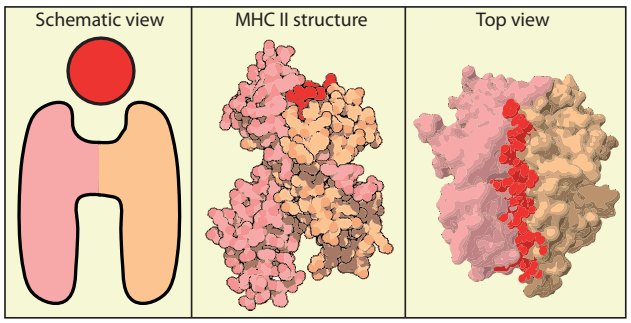



**Figure 9: MHC class II molecule** *(Adapted from: David Goodsell & RCSB Protein Data Bank and iayork.com)*
A schematic view of the MHC II molecule, consisting of 2 α-chains (yellow) and two β-chains (pink). The presented peptide is shown in red. The most right illustration shows the molecule from top-view, where the peptide is situated in the binding groove formed by an α-chain and a β-chain.

MHC class II can present endogenously produced proteins, but also deals with antigens that are derived from the extracellular space. These antigens need to be taken up, a process that is done by all phagocytes.[54] Antigens that are taken up from the extracellular space are contained in endosomes (lipid vesicles inside the cell). The uptake of antigens from the extracellular space and mechanisms involved with intracellular trafficking are discussed in more detail in *chapter 7 and 8*. Once the antigen is within an endosome, it can be degraded by several classes of proteases into peptides.[57] Peptides that are degraded in endosomes (and fit embedded in an MHC II molecule) are generally larger and of more variable sizes (13-25 amino acids) than peptides degraded by the proteasome (8-10 amino acids). These peptides can subsequently be loaded onto an MHC II molecule. The loading of peptides onto MHC class II molecules involves a series of events including the degradation or removal of the invariant chain[58;59], eventually leading to the exchange of an invariant chain peptide with the peptide of interest. The newly formed peptide-MHC II complex will then be transported to the cell surface where it can bind with a $CD4^+$ TCR (*Figure 10*).

### Antigen cross-presentation

The dichotomy of the classical MHC I and MHC II pathway and their functions lead to an important question: how are DCs able to present peptides on class I MHC if these peptides are not endogenously produced? This is mainly relevant in the case of viral infections that are not hosted inside the DCs, or in tumor immunity. In 1976 a third mechanism was identified, whereby exogenous class I MHC restricted antigens are captured by DCs, resulting in the induction of $CD8^+$ T-cell responses.[60] This process was termed 'antigen cross-priming' and was shown to be very important in anti-viral and anti-tumour immunity.[61;62] Cross-priming specifically describes the presentation to-, and priming of naïve T-cells. The general term for MHC class I presentation of exogenously acquired antigen is now referred to as cross-presentation.[40] The mechanisms involved in antigen cross-presentation and cross-priming are the main focus of this thesis. The current knowledge about these processes is discussed in more detail in *chapter 2*.

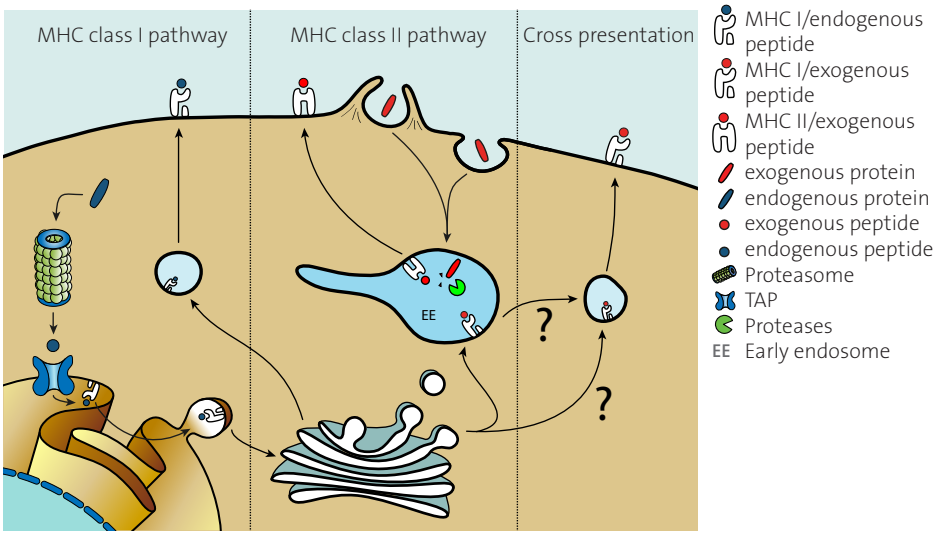


**Figure 10: The classical MHC class I and II pathway and cross-presentation**
*(Partly based on Villadangos et al, nature reviews 2007)*[63]
All dendritic cells (DCs) have functional MHC class I and MHC class II presentation pathways. MHC class I molecules (pathway shown in left panel) present peptides that are derived from proteins degraded mainly in the cytosol (blue proteins and peptides). MHC class II molecules acquire peptide cargo that is generated by proteolytic degradation in endosomal compartments (red proteins and peptides). DCs have a unique ability to deliver exogenous antigens to the MHC class I (cross-presentation) pathway, although the mechanisms involved in this pathway are still poorly understood.

## DC – T-cell interaction and required signals

Once an MHC molecule is loaded and transported to the cell membrane, it can interact with the TCR of patrolling T-cells. As described in the previous paragraphs, a successful DC – T-cell interaction requires a peptide/MHC – TCR match and binding of CD4 molecules to MHC II or CD8 molecules to MHC I. This is also referred to as *signal 1*. However, before T-cells can interact with DCs, they need to be attracted. DCs actively secrete chemokines that bind to naïve T-cells, which leads to T-cell entry into regions of interest where they can interact with DCs. Since this process occurs prior to DC – T-cell interaction, it is referred to as *signal 0*.[64] Proper stimulation of T-cells requires addition signaling through co-stimulatory molecules, mainly mediated by triggering of CD28 by CD80 and CD86, or CD40L by CD40. CD40, CD80 and CD86 are examples of co-stimulatory molecules that are expressed by DCs. The expression of these molecules is dependent on the maturation status of the DCs and/or ligation of pathogen recognition receptors to DCs. The signaling through co-stimulatory molecules is also referred to as *signal 2*. Upon interaction of DCs to T-cells, DCs can produce various cytokines and chemokines that can further direct T-cell development into various subsets. The release of these mediators, also known as *signal 3*, is also dependent on the maturation status of the DCs and/or ligation of pathogen recognition receptors to DCs.[65] Finally, the ability of DCs to direct T-cells to specific tissues by upregulating specific chemokine receptors is sometimes referred to as *signal 4*.[66]

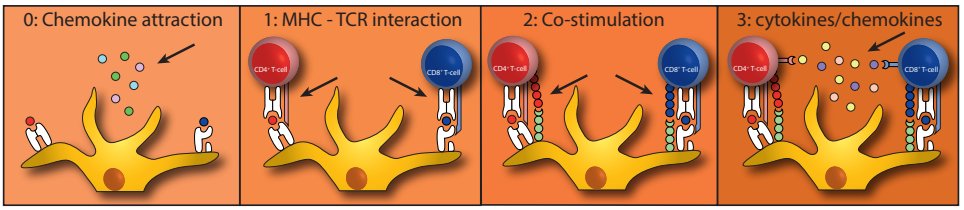



**Figure 11: Signal 0, 1, 2 and 3 in DC – TC interaction**
Dendritic cells (DCs) secrete chemokines to attract T-cells (signal 0). Peptide/MHC complexes can be recognized by antigen specific T-cells. This also requires CD4 - MHC II or CD8 – MHC I binding (signal 1). For optimal activation, co-stimulation via ao CD40, CD80 and CD86 is required (signal 2). DCs can secrete cytokines and chemokines to further instruct binding T-cells (signal 3).

## Dendritic cells for viral and tumor treatment

We now know that DCs are important players of the immune system. How can we use this knowledge to the benefit of patients?

As stated in the first part of this introduction, patients that receive an SCT are immunocompromised due to the lack of adaptive immunity. This severely decreases chances of survival since the adaptive immune system is crucial for antiviral immunity as well as prevention of relapse. In order to increase chances of survival, it is therefore desirable to aim the development of new therapies toward an effective adaptive immune system, preferably early after SCT. Since DCs are master regulators of T-cell immunity, a possible mode of action would be to "educate" the patients DCs, so that these DCs can prime naïve T-cells, thereby creating an effective memory T-cell compartment against the virus of interest, or against tumour cells. Once these T-cells circulate throughout the body, they could hypothetically prevent viral reactivation or tumour relapse.

While our knowledge of antigen presentation biology increases, the potential benefit of its function is being explored in patients. In the last 15 years, at least 50-100 phase I and phase II trials in humans were performed using DC vaccination, mainly aimed at eliciting anti-tumour responses and in some cases as anti-viral treatment. DC vaccination studies aimed at tumours were minimally successful in general, as reviewed by Rosenberg et al. and discussed in *chapter 2*.[40;67] Studies aimed at the induction of anti-viral immunity after allogeneic SCT targeted mainly human cytomegalovirus (HCMV). Vaccination with pp65-pulsed DCs induced a sustained antigen specific $CD4^+$ T-cell response; CTL responses were not assessed.[44;68] Overall, HCMV peptide-loaded DCs induced HCMV-specific CTL responses in five of 24 SCT patients at risk for HCMV after allogeneic SCT.[69] In one recent study aiming at the treatment of HIV (not after SCT), the infusion of DCs loaded with HIV-derived Tat, Nef and Rev antigens did not affect viral evolution or anti-viral $CD8^+$ T-cell responses.[69;70] In conclusion, anti-viral treatment with DCs is a promising therapy, especially after SCT, but the efficacy needs to be improved by understanding more of the underlying biology.

## Aim and outline of this thesis

As described above, there is an urgent need for novel immunotherapy options to overcome viral reactivations and relapse after CB-SCT, as they still account for more than 40% of post-SCT mortality. Preventing viral reactivation after CB-SCT will significantly impact patient survival. DC vaccination efforts, including those by the U-DANCE group at the UMCU, focus on the development of a dendritic cell (DC)-vaccine. We aim at a rapid induction of viral antigen-specific cytotoxic ($CD8^+$) T-lymphocytes, for it will provide better protection after HSCT.

As described more in detail in the next chapter, the generation of anti viral $CD8^+$ T-cells from the naïve T-cell pool requires the display of antigen by dendritic cells (DCs) to lymphocytes. Considering $CD8^+$ T-cell responses, antigen display via peptide/Class I MHC complexes requires the processing of whole antigen into peptide-size fragments, a process termed antigen cross-presentation.[40] Especially DCs are well-equipped with cell-biological mechanisms supporting antigen processing and presentation, rendering them potent APCs.[71] For vaccination strategies, full grasp on the uptake and processing mechanisms and preferentially a further

increase in $CD8^+$ T-cell stimulation potency are desired. Contributing to the understanding of these processes was the aim of our study.

In our studies, we aimed at better understanding and improving antigen (cross-) presentation by DCs, in order to generate more effective DC-vaccinations. This resulted in the following research questions:

1. Could we exploit Fc receptor (FcR) mediated uptake to improve antigen cross-presentation? And are there differences between subsets of DCs?
2. What mechanisms are involved in FcR mediated cross-presentation?
3. What is the role of $CD4^+$ T-cells in antigen cross-presentation?
4. What are the intracellular transport mechanisms involved in cross-presentation?

First, the principle of antigen cross-presentation is introduced and current insights in its use in DC vaccine development is discussed in *chapter 2*.[40] In *chapter 3*, we investigated the role of the FcγR in antigen cross-presentation in DCs. This was first done in MoDC and then translated to primary blood and lymphoid tissue BDCA-$1^+$ and BDCA-$3^+$ dendritic cells.[72] The main FcγR that was involved in this process was the subtype FcγRIIa. This lead to a screening for mutations in this receptor in a cohort of children with Primary Immune Deficiencies (PID). We found a novel variation of this receptor and researched its functional consequences in relation to the clinical symptoms in *chapter 4*. Also, one of the questions that remained unanswered in *chapter 3* was the role of $CD4^+$ T-cells in antigen cross-presentation. We showed that these cells are relevant in antigen cross-priming (the process of presenting to naïve T-cells) and cross-presentation in *chapter 5*. To conclude this part we correlated these findings to patients that had a CMV reactivation after CB-SCT, which is also described in *chapter 5*. The last two chapters are focused on intracellular trafficking within DCs. An overview of intracellular trafficking mechanisms and proteins involved is shown in *chapter 6*[71]. Finally, we looked more in detail into one of these transport mechanisms termed tubulation of the endosomal pathway. The requirements for this process and its relevance are discussed in *chapter 7*. How these findings can be placed in a bigger picture is discussed in *chapter 8 and 9*.

## Abbreviations used in this chapter:

CB: Cord Blood
SCT: Stem Cell Transplantation
BM: Bone Marrow
HLA: Human Leucocyte Antigen
OS: Overall Survival
GvHD: Graft-versus-Host Disease
aGvHD: Acute GvHD
cGvHD: Chronic GvHD
MHC: Major Histocompatibility Complex
CMV: Cyto Megalo Virus
EBV: Epstein-Barr Virus
HSV: Herpes Simplex Virus
HHV6: Human Herpes Virus 6
VZV: Varicella Zoster Virus
RSV: Respiratory Sincytial Virus
APC: Antigen Presenting Cell
ATG: Anti-Thymocyte Globuline
DC: Dendritic Cell
U-DANCE: Utrecht Dendric cells AgaiNst CancEr
PID: Primary Immune Deficiencies
ER: Endoplasmatic Reticulum
TAP: Transporter associated with Antigen Processing
Ig: Immunoglobuline
TCR: T-cell Receptor

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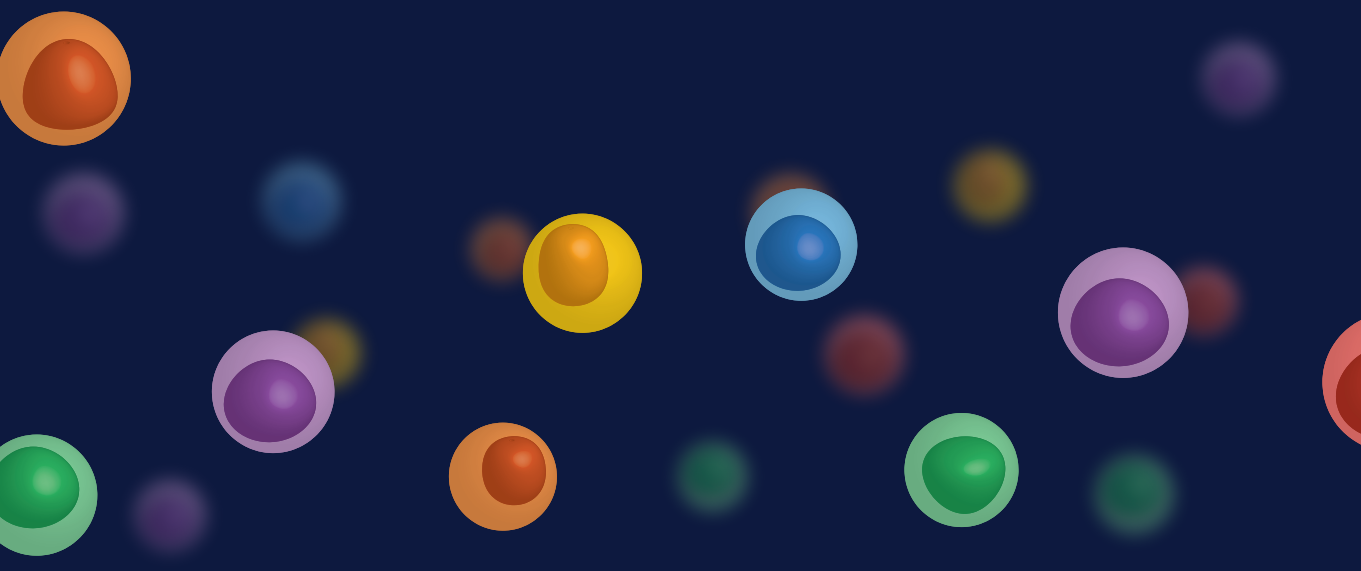
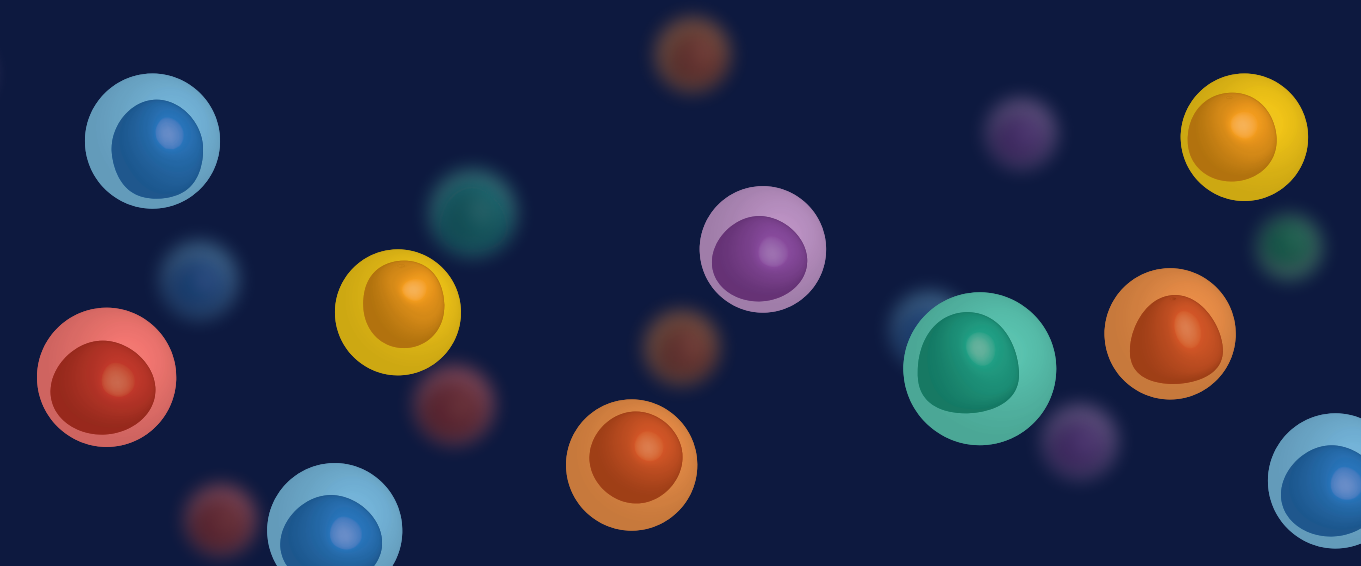
# Part A



# DC extrinsic mechanisms involved in cross-presentation



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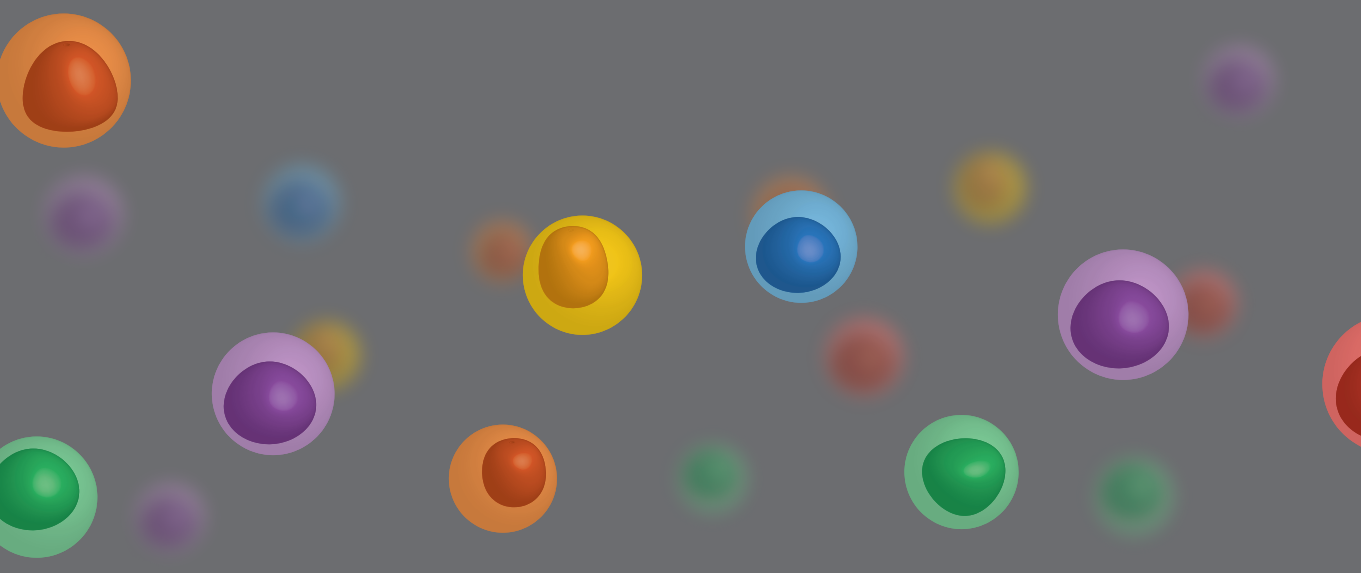
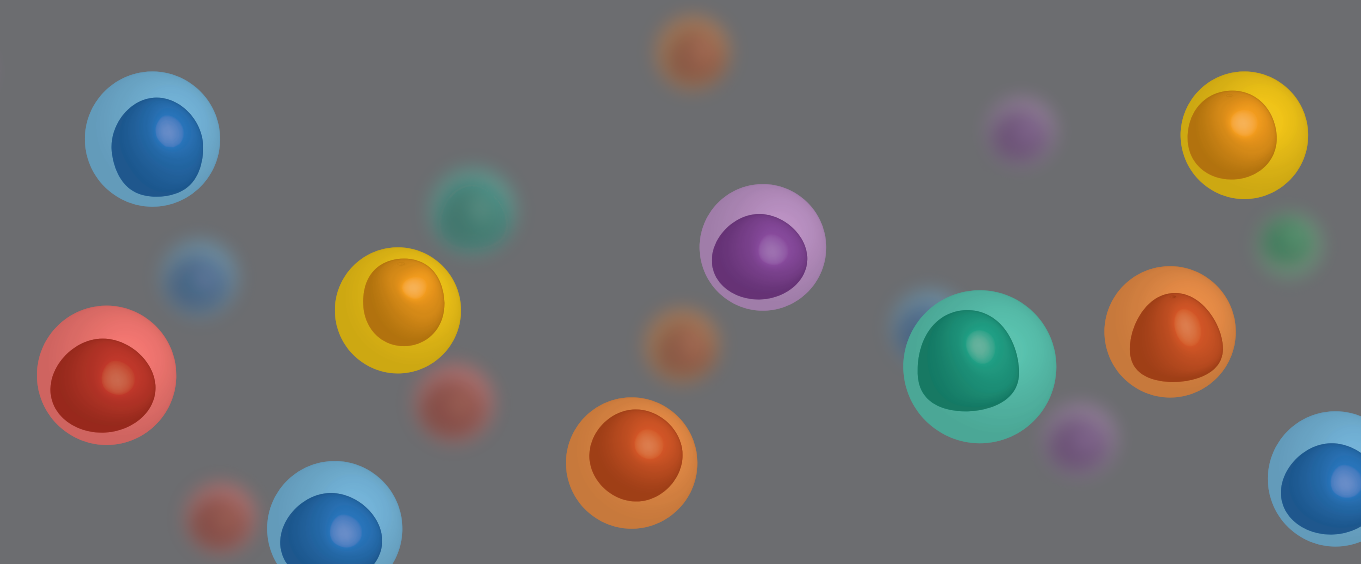


# Antigen cross-presentation: extending recent laboratory findings to therapeutic intervention

Thijs W.H. Flinsenberg, Ewoud B. Compeer,
Jaap-Jan Boelens, Marianne L. Boes

Clinical & Experimental Immunology, 2011 Jul;165(1)8-18



## Abstract

The initiation of adaptive immune responses requires antigen presentation to lymphocytes. Especially dendritic cells (DCs) are equipped with specialized machinery that promote effective display of peptide/MHC complexes, rendering them the most potent stimulators of naïve T-lymphocytes. Antigen cross-presentation to $CD8^+$ T-cells is an important mechanism for the development of specific CTL responses against tumours and viruses that do not infect antigen-presenting cells. Here, we review recent findings concerning antigen cross-presentation to $CD8^+$ T-lymphocytes. Specific subtypes of DCs in the mouse have been defined as being especially endowed for antigen cross-presentation, and a human homologue of these DCs has recently been described. DC vaccination strategies for the prevention and treatment of human diseases have in recent years been under investigation but have not generally reached satisfying results. We here provide an overview of new findings in antigen cross-presentation research and how they can be used for development of the next generation of human DC vaccines.

## Antigen cross-presentation to CD8$^+$ T-cells, a historical perspective

Dendritic cells (DCs) are key players in initiation and control of adaptive immune responses due to their exquisite ability to present antigenic fragments in the form of peptide/MHC complexes to T-cells.[1-3] Endocytosed antigens acquired from the outside environment are generally presented as peptide/Class II MHC complexes, while antigens acquired from within the cell are predominantly presented as peptide/Class I MHC complexes. This dichotomy raises one complication: how are DCs able to present viral or tumour peptides on Class I MHC if these peptides are not endogenously produced? In 1976 a third mechanism was identified, whereby exogenous Class I MHC-restricted antigens are captured by DCs, resulting in the induction of CD8$^+$ cytotoxic T-lymphocyte (CTL) responses.[4] This process was coined "antigen cross-priming". Cross-priming is important in anti-viral and anti-tumour immunity.[5;6] Mouse experiments in which non-hematopoietic cells were virally infected showed a requirement for cross-presentation by hematopoietic cells to elicit virus-specific CTL responses.[5;7] Secondly, antigen cross-presentation is relevant to the induction of central immune tolerance in the thymus[8] and peripheral tolerance in the draining lymph node[9], a process referred to as "cross-tolerance".[5;8] In the 1990s, the term "cross-presentation" was introduced to describe the antigen-presentation process underlying cross-priming and cross-tolerance. Experimental support again came from mouse model-based experiments, such as using the RIP-mOVA mice that express a membrane-bound form of ovalbumin [mOVA] on restricted tissues including pancreatic β cells.[9] Analysis of RIP-mOVA-thymus and control thymus grafted mice after being injected with OVA specific CD8$^+$ T-cells from OT-I transgenic mice, suggested that OVA-specific CD8$^+$ T-cells were lost and probably deleted after entry in the peripheral tissues.[9] Also, the inability of DCs to cross-present results in the accumulation of fully functional self-reactive CD8$^+$ T-cells that can cause auto-immune disease.[10]

DCs, B cells, monocytes and macrophages are classified as prototypic professional antigen presenting cells (APC) by virtue of their constitutive expression of Class II MHC molecules. Professional APCs are critically important for induction of protective CD8$^+$ T-cell responses against normal 'self'-antigens[11], tumour antigens[11;12] and viruses.[13] As was elegantly shown in 1996 already, the injection of OVA peptide-specific naïve CD8$^+$ T-cells into non-irradiated RIP-mOVA mice results in selective presence of these T-cells in the draining lymph nodes of OVA expressing tissues (i.e., pancreas and kidney) and not other lymph nodes.[11] These data supported the notion that cross-presentation is a constitutive mechanism, whereby T-cells can be primed to antigens that are present in non-lymphoid tissues that are normally not patrolled by circulating naive T-cells.

The activation of CTL upon recognition of infection- or tumour-associated peptides encompasses risk to autoimmune T-cell reactivity and is therefore under tight control. Under homeostatic as well as inflammatory conditions, tissue-specific DCs and to lesser degree macrophages, execute peripheral tolerance control by their ability to discriminate between cross-presentation and cross-tolerization.[14;15] Also liver sinusoidal endothelial cells are capable of cross-presenting soluble exogenous antigen to CD8$^+$ T-cells leading to tolerance.[16] Other cell types are not yet described to have the ability to induce cross-tolerance under those non-inflammatory conditions. During infection however, more cell types were recently identified as being able at cross-priming of foreign peptides and elicitation of CTL responses. Thus far, also B-cells[17;18], neutrophils[19;20], basophils[21], mast cells[22] and endothelial cells[23] were demonstrated to be capable of cross-presentation *in vitro*. Cross-presentation by basophils

was even shown to be relevant in an *in vivo* experimental autoimmune encephalitis model .[22] However, the involvement of the other cell types in cross-presentation *in vivo* has not yet been shown, and especially DCs appear pivotal for antigen cross-presentation in various circumstances, as for example demonstrated by a lack of CTL responses against cell-associated antigens after depletion of DCs *in vivo*.[24] The efficiency of DCs to cross-present exogenous antigens as peptide/Class I MHC *in vivo* was emphasized in a direct comparison study, where cross-presentation showed near equal efficiency as presentation of peptide/Class II MHC derived from the same antigen.[25]

Specific DC subsets are associated with antigen cross-presentation, and initial descriptions for these subsets are now reported in humans. Various mechanisms that facilitate cross-presentation by DC subsets were investigated in the last decade especially, mostly in mouse-based experiments. Human DC research that involves antigen cross-presentation is lagging behind. This review focuses on the mechanisms and cells that are known to be relevant for induction of effective $CD8^+$ T-cell responses to endocytosed antigens.

## Mechanisms in DCs that facilitate antigen cross-presentation

The ability of DCs to cross-present antigen to T-lymphocytes is not uniformly represented in all DC subsets. Some DCs types are more specialized in antigen transport from peripheral tissues to secondary lymphoid tissues, whereas others are non-migratory and are specialized at generation and display of peptide/MHC complexes to naïve T-cells that reside within lymph nodes. The role of the different subsets of DCs in antigen cross-presentation has been extensively studied in mice. DCs are characterized in literature as lineage-marker negative (CD3, 14, 15, 19, 20 and 56) and high expression of MHC Class II molecules. Mouse DCs are further marked by expression of the integrin CD11c, and additional delineation can be made using additional cell surface markers.[3;26-28]

Although some aspects of the human and mouse DC systems appear to be well conserved, other functions do not relate. In mice, a subset of resident DCs, characterized by high surface expression of $CD8\alpha$[29] is associated with the ability to cross-present exogenous (such as necrotic) antigens to $CD8^+$ T-lymphocytes.[30-36] The transcription factor Batf3 is crucial for the development of these $CD8\alpha^+$ DCs and absence of Batf3 in gene-targeted mice results in defective cross-presentation.[37] In 2010, the human equivalent of the mouse $CD8\alpha^+$ DCs was described. This human DC subset, characterized by the expression of BDCA-3 (CD141)[28], Clec9A[38;39] and the chemokine receptor XCR1[40] was present in human peripheral blood, tonsils, spleen and bone marrow and represents a major human DC subset expressing TLR3.[27;41] Results indicate a dominant role for $CD141^+$ DCs in cross-presentation of necrotic cell-derived antigens to $CD8^+$T-lymphocytes[27], as well as superior cross-presentation of soluble or cell-associated antigen to $CD8^+$ T-cells when directly compared with $CD1c^+$ DCs, $CD16^+$ DCs and plasmacytoid DCs cultured from blood extracted from the same donors.[40] The role of this DC subset can now be scrutinized in experimental setups in laboratories across the globe. Although culturing from hematopoietic precursors is possible, the low frequency of natural occurring $CD141^+$ DCs (1 in $10^4$ PBMCs) provides a further challenge before the ultimate goal of translation to clinical application using DCs to alter immune responses, can be achieved.

Mechanisms that promote antigen cross-presentation that are inherent to immature DCs include their ability to actively control alkalinization of their phagosomes[42], their low lysosomal

proteolysis[43], and expression of protease inhibitors[44], thereby increasing the propensity that exogenous antigens engulfed in the phagosome lumen are cross-presented to $CD8^+$ T-cells.[43] However, there are also mechanisms restricted to DC subsets or to DC maturation stages, resulting in variability in cross-presentation efficiency. In some instances, cross-presentation ability by DCs correlates with expression of specific uptake receptors or proteins associated with cross-presentation.[45;46] In addition, the nature of antigen itself also creates a bias towards presentation via Class I or Class II MHC molecules.[45]
Once exogenous antigen is internalized by DCs, distinct mechanisms take place by which antigen-derived peptides are cleaved from larger antigen fragments and loaded onto the Class I MHC molecules. To allow for display of exogenously acquired antigen in the form of peptide/Class I MHC complexes, the antigen undergoes proteolytic processing to create an appropriate size fragment. Further restriction to the formation of peptide/Class I MHC complexes involves the amino and carboxyl ends of the peptide, to harbor charged anchor residues that complement those of the peptide-binding groove of the Class I MHC molecule. Since the proteasome is demonstrated to be the main source of peptides in the classical MHC I pathway, it is not unexpected that proteasome activity is thought to be essential for cross-presentation.[13;45;47;48] However, other reports have shown proteasome-independent processing of the exogenous protein via specific proteases.[49;50] This controversy has led to two different models, the dominant cytosolic pathway and the vacuolar pathway.

The cytosolic pathway proposes that antigen is transported into the cytosol after internalization where proteasome degradation ensues, prior to transportation to the location of peptide assembly into peptide/Class I MHC molecules. Based on the mechanism used by DCs corroborated by the size-restriction of the antigen, internalization of antigens occurs by receptor-mediated endocytosis, pinocytosis (components < estimated 0.5 μm) or phagocytosis (components > estimated 0.5 μm). Upon internalisation, antigens are initially located in phagosomes. These phagosomes fuse with early endosomes (characterized by a near neutral, slightly acidic pH) and later with late endosomes (pH approx. 5.5). Accordingly, ultimate degradation into single amino acids takes place after fusion with acidic lysosomes, a route that is more prevalent in macrophages than in DCs.[51] Degradation within lysosomes occurs by proteases and hydrolases that have their enzymatic optimum close to the acidic pH found in lysosomes (pH 4.8), for antigen degradation[52], as well as degradation of cellular constituents as part of the normal cell homeostasis. The changes that occur to phagosomes in the endocytosis pathway is termed phagosome maturation. Phagosome maturation is important in regulation within the immune system, in the decision process whether an immune response is triggered, or tolerance is established. The importance of this route is exemplified by changes in cell degradation that have been shown to result in autoimmune disease[52;53] For example: in a DNase II-/- IFN-IR-/- mouse model, where macrophages were unable to degrade mammalian DNA and started the production of TNF, activation of synovial cells was observed resulting in chronic polyarthritis symptoms.[53]

It can be deducted that the process of protein degradation following phagosome maturation must be tightly regulated. In DCs, the pH in phagosomes is kept near neutral (pH 7.5) for the first few hours after phagocytosis.[54] This is in stark contrast to rapid acidification that is seen in neutrophils and macrophages, where the pH drops to 5 within 30 minutes after phagocytosis.[55] Acidification of the phagosome, thereby increasing the lysosomal protease activity, has

been shown to counteract cross-presentation in mice in a NADPH oxidase complex 2 (NOX2) dependent manner.[54] This indicates that DCs have a unique ability to regulate proteolytic activity in phagosomes, therefore controlling the amount of peptide destined for cross-presentation. Rab27a-dependent inhibitory lysosome related organelles are involved in this pathway. These organelles are continuously recruited to phagosomes and limit acidification and degradation of ingested particles in DCs, thus promoting antigen cross-presentation.[56;57] Being able to interfere with phagosome acidification, thereby executing control of the rate of antigen cross-presentation could provide new opportunities in increasing the efficacy of CTL targeting in DC vaccination.

The Insulin-Regulated AminoPeptidase (IRAP) was implicated in antigen cross-presentation in peptide cleavage for generation of peptide substrates for Class I MHC molecules.[58] IRAP was found in the early endosome of human monocyte-derived DCs and murine bone marrow-derived DCs, where it co-localized with MHC class I molecules and the mannose receptor (MR), but not with endoplasmic reticulum aminopeptidases (ERAPs). IRAP-deficient mice were capable of phagocytosis of antigen as well as presenting endogenously produced peptides, but cross-presented exogenous antigens with 50-70% decreased efficiency compared to wild-type mice.[58] IRAP-dependent cross-presentation requires active proteasome and function of the ATP-binding cassette transporter family member TAP (transporter associated with antigen processing), but not lysosomal proteases. Therefore, this route of antigen cross-presentation involves cytosolic antigen degradation that is followed by peptide transport via TAP into IRAP+ endosomes. However, IRAP as well as MR appear dispensable for cross-presentation in murine splenic CD8α$^+$ DCs but not mouse monocyte-derived DCs induced by inflammation, suggesting a role for these two molecules in inflammatory DCs, but not in steady-state CD8α$^+$ DCs.[59]

To allow for generation of peptides by the proteasome and cytosolic peptidases, antigen must traverse from the phagosome into the cytosol. Recent reports demonstrate that peptide transfer across the phagosomal membrane occurs via a selective, size-specific, reduction, unfolding, (partial proteolysis) and Sec61-dependent process.[60-62] Conversely, TAP transporters appear essential for peptide transport from the cytosol into a Class I MHC loading compartment, since TAP knock-out mice are not capable of cross-presenting exogenously-acquired viral peptides.[7] Moreover, efficient cross-presentation required TLR4- and signaling molecule myeloid differentiation factor 88 (MyD88)-dependent relocation of TAP[63], essential for peptide loading of Class I MHC, to early endosomes/phagosomes.[64] After processing in the cytosol, the generated peptides are transported via TAP either into the ER, thereby entering the canonical Class I MHC presentation pathway[13;47], or back into the phagosomal pathway.[64-66] The latter situation is likely to contribute to a rapid cross-presentation, since all necessary components are in a separate Class I MHC loading-competent compartment that is distinct from the ER. Peptides generated locally in the phagosomal pathway would not undergo rigorous competition with the large pool of endogenous peptides, for association with newly assembled Class I MHC complexes as would occur in the ER. It has indeed been shown that all relevant components of Class I MHC loading complexes are present in early phagosomes and that these are functional.[47] How the necessary components are transported from the ER to the phagosomes is not clear. Phagosome-ER fusion was proposed[67], but other groups were unable to confirm these findings.[64;68] The vacuolar pathway is an alternative model that is based on notions of proteasome- and TAP transporter-independent cross-presentation, enabled by proteases that

reside in late endosomes and lysosomes.[49;69-71] Most antigen cross-presentation studies performed in human DCs to date focus on this pathway, and less on proteasome/TAP-dependent mechanisms.[58;59;71] Since only peptide-bound Class I MHC molecules are transported to the plasma membrane, peptide-exchange should be able to occur in the endosomal encountered Class I MHC molecules. Earlier *in vitro* experiments already suggested that peptide-receptive Class I MHC molecules can be generated under late endosomal/lysosomal pH conditions.[72] Multiple pathways can coexist in the same cell type, indicating that these pathways are compartmentalized and require sorting and specific antigen targeting to specialized endosomal compartments.[49]

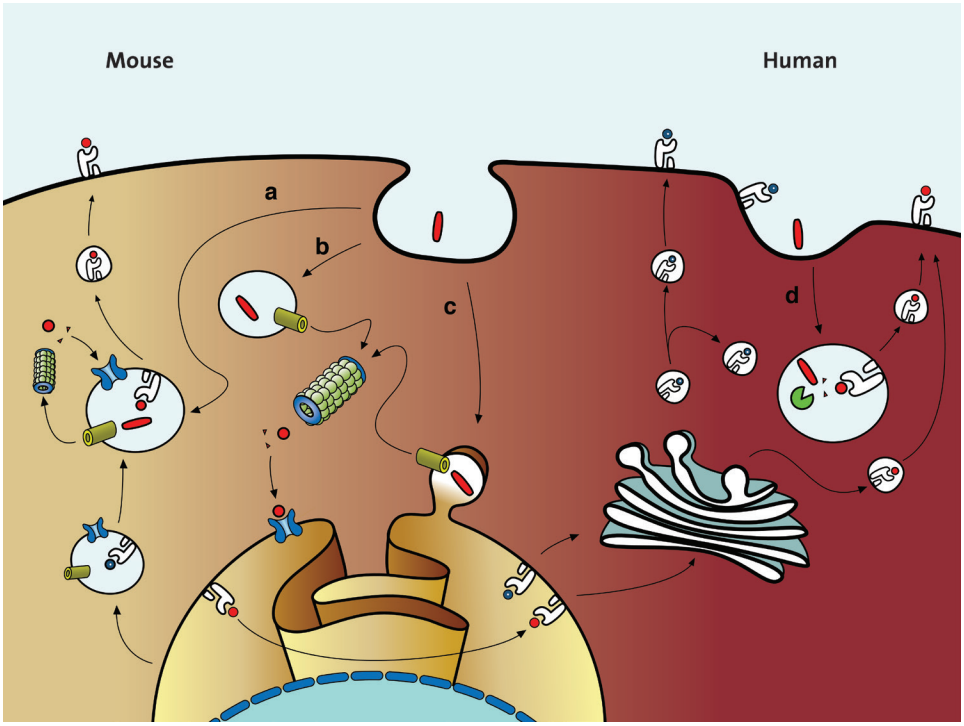



**Figure 1: Proposed pathways of antigen cross-presentation.**
Yellow area (left side) relates to mechanisms described only in mice so far, whereas the gradient towards the red area (right side) depicts the transient increase in knowledge of antigen cross-presentation mechanisms in human cells.
By receptor-mediated endocytosis, the antigen (red rod shape) is engulfed into a phagosome and subsequently processed in a cytosolic proteasome- (a, b, c) or endosomal protease-dependent (d) manner. For proteasome-mediated degradation the antigen is transported across the endosomal membrane into the cytosol by Sec61, accessed by the proteasome via ER-endosome fusion (c) or a delivery vesicle derived from the ER (a, b). After processing by the proteasome, possibly assisted by cytosolic peptidases, peptides either re-enter the endosomal compartment via TAP where loading on Class I MHC may occur (a), or the canonical Class I MHC presentation pathway in the ER (b, c). After proteolytic processing by endosomal pathway-resident proteases, peptides are loaded onto Class I MHC molecules by replacing either exogenous peptide- (recycling) or endogenous peptide (classical) loaded on Class I MHC complexes (d).

: antigen; : exogenous peptide; : endogenous peptide; : Sec61; : TAP; : MHC class I molecules
: proteasome; : proteases.

## Antigen uptake routes by DCs control antigen cross-presentation efficiency

To allow for the induction of specific adaptive immunity, pathogens or antigenic components that are pathogen-derived must be internalized by DCs for antigen processing and display as peptide/MHC complexes at the DC surface. It is clear that DCs can (cross-)present exogenous antigen without being infected.[13] Antigen can be acquired directly from the surrounding milieu, or can be received by a cross-presenting DC from a distant site through transport by migratory DCs. It has been shown that skin-derived migratory DCs transfer antigen to lymph node resident DC for efficient cross-presentation.[73] Secondly, it was shown that tumours secrete exosomes that contain proteins, which can be taken up by DCs. This system can facilitate anti-tumour immunity.[12] Thirdly, DCs use gap junctions to gain peptide antigens from adjacent cells. These peptides can thereafter be used for cross-presentation.[74]

To allow for antigen internalization, DCs are equipped with a variety of receptors that can either directly recognize pathogen associated molecular patterns (PAMPS) or indirectly via plasma complement (activated large proteolytic fragments of complement proteins, C3b, C4b, iC3b and C3d - collectively called C') that binds to complement receptors (CR1/CD35 and CR2/CD21). Immunoglobulins (Ig) present in plasma bind the immunoglobulin receptors (activating receptors FcRI, II and III and FcRIIA, and the inhibitory FcRIIB). Both complement fragments and Ig are soluble receptors present in plasma that bind structures on pathogen surfaces to facilitate pathogen opsonization, internalization and destruction. Second, small proteolytic complement protein fragments (i.e., C3a, C4a, C5a) act as chemoattractants to recruit and activate new phagocytes. CRs and FcRs allow for internalisation after binding C'- or Ig-opsonized antigens. Antigen opsonization with Ig rather than C' facilitates antigen cross-presentation[57], thus supporting a role for Ig rather than C' in tailoring appropriate antigen-specific adaptive immune responses.

DCs use multiple additional membrane-expressed receptors for the internalization of antigens. The presence and dominance of these receptors differs between DC subpopulations within species and between DC subpopulations, as compared between mouse and humans.[26] Targeting specific receptors can drive the immune response either towards Class II MHC-restricted $CD4^+$ T-helper cell responses or to Class I MHC-restricted $CD8^+$ cytotoxic T-cell responses via cross-presentation and can therefore be an effective method for inducing antiviral or anti-tumour CTL responses.[46]

In both mice and human, the presence of many different uptake receptors has been shown (*Table 1*).

Uptake via distinct endocytic receptors controls the efficiency of cross-presentation of peptide/Class I MHC complexes to $CD8^+$ T-cells. The effects of individual uptake receptors on antigen targeting to the Class I or Class II MHC presentation route seem to be roughly conserved between mice and men, but opposing effects of some receptor related to endosomal targeting and processing of antigens have been found. In mice, antigen cross-presentation is promoted when antigen uptake occurs via MR[45], DEC205[80;83;88;92;93], dectin-2[89], DNGR-1[94], FcγR[57;76-80] and LOX-1.[90] Also in human cells, antigen cross-presentation is promoted upon antigen uptake via DEC-205[83;84], DC-SIGN[85;86] and FcγR[81] in vitro as well as in vivo MR targeting in a humanised mouse model.[95] However, in contrast to the results in mice, MR-mediated an-

tigen uptake induced CD4+ T-cell responses by human DCs.[82] Antigen targeting to dectin-1[88], DCIR-2[46;80] and CD40[96] induce CD4+ T-cell responses in the mouse. Langerin (CD207) targeted uptake induces both CD4+ and CD8+ T-cell responses.[80] In humans, antigen cross-presentation is favoured by uptake via DCIR.[87] These examples underscore that knowledge on receptor mediated cross-presentation in mice cannot always immediately be translated to the human system. Since enhancing cross-presentation can be an effective means to improve CTL responses in diverse DC vaccination programs, more research about receptor targeting in the human system is needed.

**Table 1.** Receptors involved in targeting antigen to the Class I or Class II MHC antigen presentation pathway in mouse and human dendritic cells [26;38;39;41;75]

| | Mouse | Human |
|---|---|---|
| **Activating Fc Receptors** | | |
| ***Fcγ Receptors (FcγR)*** | | |
| Fcγ I (CD64) | | |
| Fcγ II (CD32) | MHC I [57;76-80] | MHC I [81] |
| Fcγ III (CD16) | | |
| **Pathogen Recognition Receptors (PRRs)** | | |
| ***C-type lectin receptors (CLRs)*** | | |
| *Type I* | | |
| Mannose receptor (MR/CD206) | MHC I [45] | MHC II [82] |
| DEC205 (CD205) | MHC I [23;80] | MHC I [83;84] |
| *Type II* | | |
| DC-SIGN (CD209) | n.a. | MHC I [85;86] |
| Langerin (CD207) | MHC II/ MHC I [80] | n.d. |
| DCIR (CLEC 4A) | MHC II [46;80] | MHC I [87] |
| Dectin I | MHC II [88] | MHC I (CLEC 9A)[27] |
| Dectin II | MHC I [89] | n.d. |
| LOX-1 | MHC I [90] | n.d. |
| ***Scavenger receptors (SR)*** | | |
| SR-A1 and SR-A2 | n.d. | n.d. |
| SR-B1 (CD36) | n.d. | MHC I [91] |

The involvement of Complement-receptors and Toll-like receptors has not been determined.
n.d., not determined; n.a., not applicable

## Antigen uptake routes in DCs can be decisive in induction of immunity or tolerance

Cytotoxic CD8⁺ T-cells directed to virus-infected cells are considered crucial for efficient anti-viral responses. In parallel, the elicitation of tumour-directed CTLs is considered crucial for effective anti-tumour responses to occur. Considering tumour-associated antigens, dead tumour cells are a major antigen source for APCs.[91] For example, Asano *et al* showed that dead tumour cells traffic via the lymph vessels to the tumour-draining lymph node where dead tumour cell-associated antigens are internalized by APCs and cross-presented to CD8⁺ T cells.[97] In cancer therapy, many investigators have taken advantage of the immunogenicity of tumour-associated antigens for tumour vaccination, either by direct injection of dead tumour cells[98] or using DCs loaded with dead tumour cells.[99]

For pathogen-associated antigens, a large pool of antigens is also available in cells that are dead or dying as a consequence of the pathogen infection, forming a rich source for antigens for loading into the cross-presentation pathway. It is demonstrated that cells dying from infection are engulfed by APCs for CD8⁺ T-cell activation by cross-presentation. For example, virally infected dying cells like influenza A[91], Epstein-Barr virus[100], and Canarypox virus[101], or bacterially-infected dying cells from S. typhimurium[102] induce CD8⁺ T-cell responses. It is clear from these examples that providing antigen in the form of dead cells can be a powerful tool to favour cross-presentation of the antigen.

However, not all dead cells are immunogenic and induce cross-presentation. In humans, it is estimated that under homeostatic conditions around one million cells turn over each second, which generally does not result in autoreactivity[103] However, deficiencies in the clearance of these dead cells can results in autoimmune disorders (i.e., systemic lupus erythematosus, in individuals lacking early components of the complement cascades), indicating its role in maintenance of self-tolerance. Clearly, this example shows that the immune system is able to process dead cells in a tolerogenic or immunogenic manner, depending on several factors. As reviewed by Green *et al*., these factors are related to the type of cell death, the cell death pathway, how the dead cells are engulfed, the engulfing cell, where the engulfment takes place and which cells of the immune system eventually encounter the antigens presented along with the dead cells[104] Clarification (and possibly modulation) of these processes should provide a venue for development of efficient cross-presentation routes that can be exploited in DC vaccination strategies. Several of such attempts are exemplified in experiments on heat shock protein (HSP)-associated antigen uptake. HSPs are intracellular chaperone molecules that readily associate with neighbouring proteins, such as with antigen inside tumour cells. Injection with HSP, e.g. HSP 70, HSP 90 and glycoprotein (gp)96 induces CTL responses against the cells from which the HSPs were isolated (reviewed in [105]). While HSPs may not be essential for antigen cross-presentation, they have been shown to promote antigen cross-presentation using *in vitro* assays employing multiple cell-lines as well as primary mouse and human immune-cells.[105-109] The myeloid differentiation factor 88 (MyD88) is essential in the developmental maturation of DCs that allows them to prime CD8⁺ T-cells through cross-presentation after uptake of HSP-coupled antigen.[63]

## Clinical experience with anti-tumour and anti-viral DC-vaccines

In recent years, multiple insights were obtained in mechanisms that underlie cross-presentation in mouse as well as human cells. The primary cross-presenting CD8α⁺ DC in mice was identified, and groups around the world are currently investigating what seems to be a human homologue, the human CD141⁺ DC. Distinct pathways are shown to be present in the murine

system, and more and more knowledge is gathered about the cross-presentation pathways in the human system. These mechanisms are not always conserved between species, which alerts us that knowledge gathered in mouse systems cannot without risk be extrapolated to the human system.

While our knowledge in antigen presentation biology increases, the potential benefit of its function is being explored in patients. In the last 15 years, at least 50 phase-I and phase-II trials in humans were performed using DC vaccination as anti-tumour[45;110-118], or anti-viral treatment[119;120] related to allogeneic stem cell transplantation (SCT) in both adult and pediatric settings (references [113;115-118] and [110-112;114;120], respectively). The main focus of these phase I/II studies was safety and none reported serious direct side effects. Although the studies were not set up for evaluation of effectiveness of induction of CTL responses, disease regression/prevention and immune responses were measured. In general, anti-tumour responses were minimal[110;112;114;117], as reviewed by Rosenberg *et al.*[121] One recent study performed in 10 AML patients after at least one anti-leukaemic chemotherapeutic regimen but not end-stage disease, showed more promising results. In this study, vaccination with DCs loaded with mRNA encoding Wilms' tumor 1 (WT1) protein induced complete remission in three out of 10 patients and temporary remission in two additional patients. Moreover, an increased frequency of WT1-specific $CD8^+$ T-cells was found in two of five tested HLA-$A0201^+$ patients, correlating significantly with long-term response.[118]

There are more examples of DC vaccination studies in which the induction of T-cell responses was measured. For example, in studies in stage IV melanoma patients, peptide-pulsed $CD34^+$ progenitor cell-derived DCs induced both $CD4^+$ and $CD8^+$ T-cell responses.[115;116] Also, in patients suffering from breast-cancer, DCs exogenously loaded with peptides derived from the HER-2/neu proto-oncogene and the epithelial mucin MUC1 lead to an induction of peptide-specific CTLs and decrease in serum amount of tumour markers.[113] DC vaccination in 35 non-Hodgkin B cell lymphoma patients targeting tumour-specific immunoglobulin resulted in a tumour regression rate of 31.6%.[122] In studies aimed at the induction of anti-viral immunity after allogeneic SCT, mainly targeted at human cytomegalovirus (HCMV), vaccination with pp65-pulsed DCs induced a sustained antigen-specific $CD4^+$ T-cell response; CTL responses were not assessed.[120] HCMV peptide-loaded DCs induced HCMV-specific CTL responses in 5 out of 24 SCT patients at risk for HCMV after allogeneic SCT.[119]

As described, the results on anti-tumour and anti-viral clinical trials show a modest immunological response, which may not yet result in an increase in patient survival. Since most of these studies were designed as phase-I and phase-II safety studies the included patient groups tend to be late in disease progression (with a relative high residual tumour load or already virus associated disease). Patients with a substantial lower tumour load and absent viral disease may be more likely to show a benefit of induced specific anti-tumour- or anti-viral activity, as also suggested by recent results.[118] On the other hand, the potency of immunologic responses was far from optimal and provides opportunities for improvement. New information on human DC subsets and education of DCs allow for the optimization and improvement of current DC vaccination strategies. Distinct DC subsets offer unique possibilities in DC vaccination strategies.[123] In addition, priming DCs in a specific way determines the immunological outcome, which can be accomplished by inclusion in vaccine formulations of ligands to TLRs[124] and NLRs[125] and may work through modulation of the DCs migratory and T-cell stimulatory capacity. Secondly, in reported studies thus far, the elicitation or reactivation of

CD4+ T-cell responses can often be observed and even humoral anti-tumour responses can be measured. Class I MHC-restricted CD8+ T-cell responses are the focus in only the minority of papers, while it is CTLs that are key players in anti-tumour and anti-viral immunity. Therefore, the induction of potent antigen cross-presentation should be especially explored in current human immunology studies. Clarification of the mechanisms that increase the amount of peptide/Class I MHC complexes is pivotal for development of next generation DC-based anti-tumour and anti-viral intervention strategies.

## Acknowledgements

We acknowledge for financial support of the Wilhelmina Research Fund (to M.B.). We thank Michiel de Jong (www.TomatoesandPotatoes.com) for help with design figure 1. We additionally thank Sytze de Roock and Annemieke Laarhoven for critically reading of the manuscript, and the members of the Boes laboratory and Center for Molecular and Cellular Intervention (CMCI) for helpful discussions.

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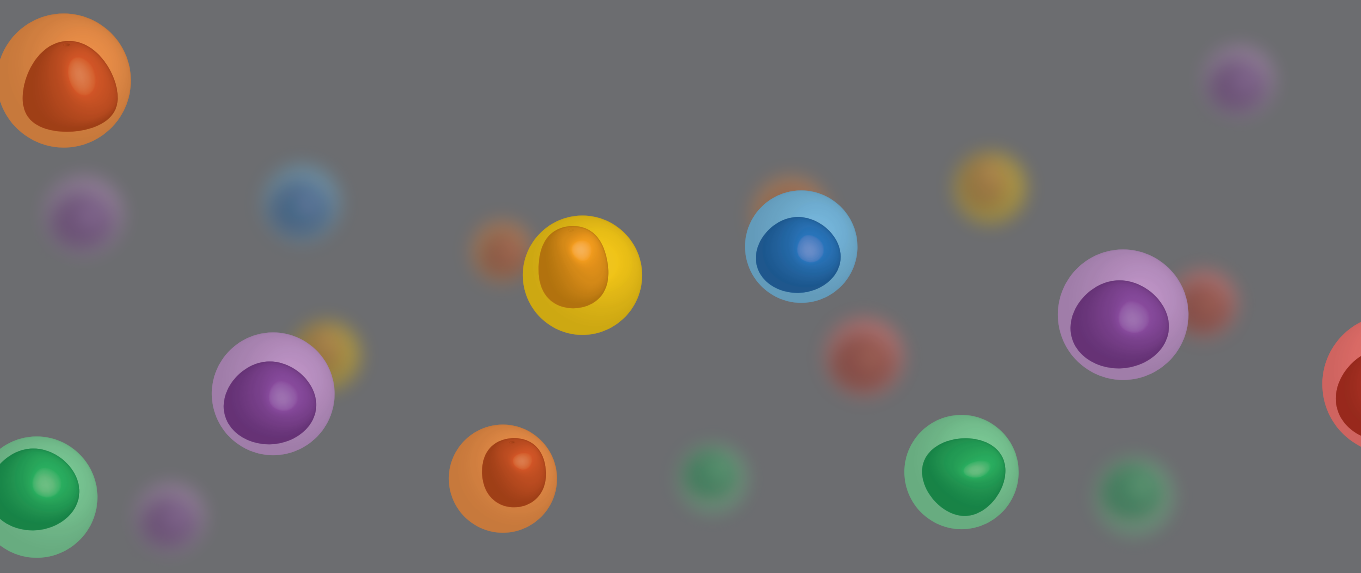

# Fcγ receptor antigen targeting potentiates cross-presentation by human blood and lymphoid tissue BDCA-3$^{+}$ dendritic cells

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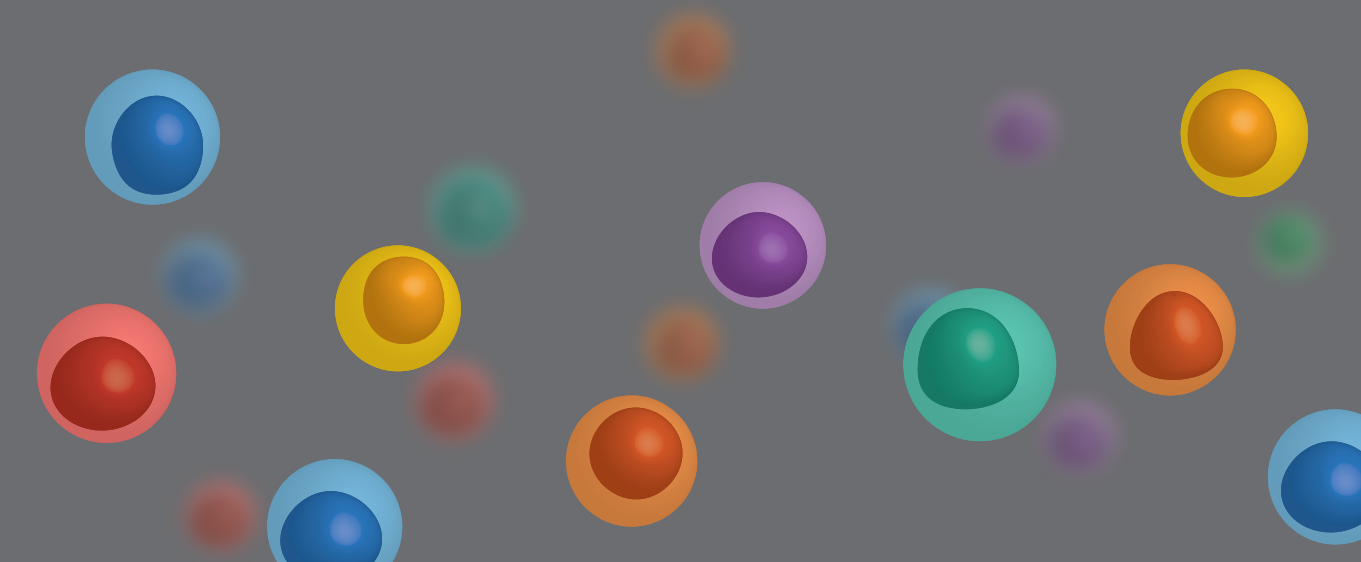
Blood, 2012 Dec 20;120(26)5163-72



## Abstract

The reactivation of human cytomegalovirus (HCMV) poses a serious health threat to immune compromised individuals. As a treatment strategy, dendritic cell (DC)-vaccination trials are ongoing. Recent work suggests that BDCA-3$^+$ (CD141$^+$) subset DCs may be particularly effective in DC vaccination trials. BDCA-3$^+$ DCs had however been mostly characterized for their ability to cross-present antigen from necrotic cells. We here describe our study of human BDCA-3$^+$ DCs in elicitation of HCMV-specific CD8$^+$ T-cell clones. We show that Fcgamma-receptor (FcγR) antigen targeting facilitates antigen cross-presentation in several DC subsets, including BDCA-3$^+$ DCs. FcγR antigen targeting stimulates antigen uptake by BDCA-1$^+$ rather than BDCA-3$^+$ DCs. Conversely, BDCA-3$^+$ DCs and not BDCA-1$^+$ DCs show improved cross-presentation by FcγR targeting, as measured by induced release of IFNγ and TNF by antigen-specific CD8$^+$ T-cells. FcγR-facilitated cross-presentation requires antigen processing in both an acidic endosomal compartment and by the proteasome, and did not induce substantial DC maturation. FcγRII is the most abundantly expressed FcγR on both BDCA-1$^+$ and BDCA-3$^+$ DCs. Furthermore we show that BDCA-3$^+$ DCs express relatively more stimulatory FcγRIIa than inhibitory FcγRIIb in comparison to BDCA-1$^+$ DCs. These studies support the exploration of FcγR antigen targeting to BDCA-3$^+$ DCs for human vaccination purposes.

## Introduction

Viral reactivation, for example of human cytomegalovirus (HCMV), poses a major threat in patients receiving hematopoietic and solid organ transplantation, decreasing 3-year survival rates from an estimated 80-90% to 50%.[1-3] Strategies that increase early anti-viral adaptive immune responses after transplantation are therefore under exploration, which could ultimately help to establish full clearance of, and long-term immunological memory against viruses. The induction of adaptive immune protection against for example HCMV requires the display of antigen by professional antigen presenting cells (APCs) to lymphocytes. Considering $CD8^+$ T-cell responses, antigen display via peptide/Class I MHC complexes requires the processing of whole antigen into peptide-size fragments. Especially dendritic cells (DCs) are equipped with cell-biological mechanisms supporting antigen processing and presentation, rendering them potent APCs.[4] In mice, DC subsets can be divided alongside their various specialties, including antigen uptake, processing and presentation to T-cells, and migratory or resident properties in the body.[5-7] Mouse DCs expressing CD8α are specialized at antigen cross-presentation, the process by which exogenous antigen is presented as peptide/Class I MHC complexes.[8] This process is pivotal in anti-viral and anti-tumor immune responses. In the human setting, tumor cell-based trials are underway, geared towards cross-presentation and activation of tumoricidal $CD8^+$ T-cells.[9]

Human BDCA-$3^+$ ($CD141^+$) DCs are considered a human counterpart of mouse $CD8\alpha^+$ DCs, and are found at low frequencies in peripheral blood, lymph nodes, bone marrow and spleen.[10-12] Human BDCA-$3^+$ DCs internalize dead cell material and cross-present exogenous soluble or cell-associated proteins to $CD8^+$ T-cells.[11-13] Recombinant soluble HCMV pp65 antigen was cross-presented with increased efficiency by BDCA-$3^+$ DCs to antigen-specific $CD8^+$ T-cells.[12] For vaccination strategies, full grasp on the uptake and processing mechanisms and preferentially a further increase in $CD8^+$ T-cell stimulation potency is desired, which was the aim of our study.

In whole organisms, blood-borne soluble antigen is readily opsonized by serum opsonins, most prominently components of the complement pathway and Ab, which facilitates antigen exposure to DCs as IgG-antigen immune complexes. Immune complexes are particularly formed for antigens that are community-borne and to which antibody titers are routinely present, including HCMV.[14] Antigen targeting to specific receptors, including Fcgamma-receptors (FcγR) directs antigen presentation towards the Class I MHC or the Class II MHC presentation pathway.[3,15-17] We here addressed the possible role of FcγR-mediated antigen targeting as a mechanism within human BDCA-$3^+$ DCs. FcγR-mediated antigen targeting in BDCA-$3^+$ DCs, as we show, is particularly effective at potentiating antigen cross-presentation ability in these DCs, a process that is fully blocked when FcγR function is absent. FcγR-mediated cross-presentation of HCMV antigen involves proteolysis in both the endosomal pathway and the proteasome. Thus, FcγR antigen targeting in BDCA-$3^+$ DCs could be exploited in HCMV vaccination strategies to counteract viral reactivations related to organ and stem cell transplantation.

## Material and methods

*MoDC culture*
Peripheral blood mononuclear cells (PBMC) from healthy HLA-A2 positive donors were separated from peripheral blood by ficoll isopaque density gradient centrifugation (GE Healthcare Bio-Sciences AB) and were used either directly, or frozen until further experimentation. For DC induction, PBMCs were incubated at 37°C and 5% $CO_2$ for 1 hour to plastic in order for the monocytes to adhere, in X-vivo 15 medium (Lonza) containing 2% human serum (Invitrogen). Cells were washed 3 times with PBS (RT) and subsequently cultured for 5 days at 37°C and 5% $CO_2$ in X-vivo 15 medium containing 450 U/ml GM-CSF (Immunotools) and 300 U/ml IL-4 (Immunotools). Cytokines were refreshed after 3 days. DCs were collected for experiments on day 5 by incubation in PBS (4°C) for one hour.

*Primary DCs*
Primary blood DCs were isolated from HLA-A2 positive PBMC buffy-coats. PBMC were separated by ficoll isopaque density gradient centrifugation (GE Healthcare Bio-Sciences AB) and were subsequently depleted for CD3, CD14, CD19 and CD20 with magnetic-based cell sorting (Miltenyi MACS). Next, cells were labeled with anti-human antibodies (Ab) (CD1c, CD3, CD11c, CD14, CD19, CD20, CD56, CD141, HLA-DR) and sorted by the FACS aria II (BD Bioscience) into a 96-wells plate (Thermo) (*Figure S1a*: gating strategy).
Tonsillar DCs were extracted from tonsils originating from anonymous patients that underwent a tonsillectomy. Mononuclear cells were separated by ficoll isopaque density gradient centrifugation (GE Healthcare Bio-Sciences AB) and were subsequently sorted for CD141 positivity by magnetic cell sorting (Miltenyi MACS).

*T-cell cloning*
An HLA-A*0201–restricted, HCMV pp65–specific $CD8^+$ T cell clone was prepared. In brief, T cells from an HLA-A*$0201^+$ donor were stained with HLA-A2/$pp65_{495\text{-}503}$ tetramers, and subsequently single-cell sorted in a 96-wells plate (Thermo) containing irradiated B-LCL feeder cells ($1 \times 10^5$ cells/ml, irradiated with 70 Gy) and PBMCs from three healthy donors ($1 \times 10^6$ cells/ml, irradiated with 30 Gy). 1 µg/ml leucoagglutinin PHA-L (Sigma-Aldrich) and 120 U/ml of recombinant IL-2 (Immunotools) were added. T-cell clones specific to $pp65_{495\text{-}503}$ were selected using tetramer staining. Positive clones were restimulated and expanded during several stimulation cycles and frozen in aliquots that were freshly thawn before each use in an assay.
HLA-A2/pp65495-503 tetramer generation
HLA heavy chains and ß2-microglobulin were constructed in pET plasmids and expressed in BL21 Escherichia coli strains. Heavy chain, ß2-microglobulin and peptides were refolded by dialysis. HLA-tetramers with specificity for the CMV-derived peptide NLVPMVATV were complexed with HLA-A2 protein. Monomeric complexes were concentrated, biotinylated, HPLC purified on a BioSep SEC-S 3000 column (Phenomenex) and bound to either APC- or PE-streptavidin (Sigma). Tetrameric product was HPLC purified.

*Cross-presentation assay*
DCs were loaded with either soluble HCMV pp65 protein (Miltenyi Biotec, purity >95%, low endotoxin (<10EU/ml)) or pp65:Ab IC (Abbiotec rabbit polyclonal anti-pp65) and incubated overnight at 37°C and 5% $CO_2$ for processing. Blocking of FcRs was done by adding 10 µg/

ml human IgG-Fc fragments (ITK), or 1µg/ml FLIPr-like (kind gift from J.A. van Strijp and K.P. van Kessel). When endosomal or proteasomal antigen processing was assessed, DCs were incubated in the presence of 2 µM MG132, 100 µM Lactacystin, 10 µM Epoxomicin (Cayman chemical), 50 µM Chloroquine, 15 µM Leupeptin or 50 µM Primaquine (all from Sigma, except for Epoxomycin). After incubation, DCs were washed and HCMV pp65–specific $CD8^+$ T-cells were co-cultured with pp65-loaded DCs for 4-6 hours in the presence of Golgistop (1/1500; BD Biosciences). Cells were subsequently stained for surface markers and presence of intracellular IFN-γ and TNF, followed by flow cytometry-based analysis.

*DC maturation assay*
Primary BDCA-$1^+$ and BDCA-$3^+$ or day 4½ MoDCs were incubated O/N in the presence of medium, pp65 antibodies, pp65 (3µg/ml), pp65-IC (3µg/ml pp65 complexed with 4-fold increased molar amount rabbit polyclonal anti-pp65 IgG) or Poly(I:C) (30 µg/ml (Sigma-Aldrich) and LPS (100 ng/ml (Sigma-Aldrich)). Cells were subsequently harvested and analyzed for co-stimulatory marker expression using flow-cytometry.

*Flow cytometry*
For staining, cells were first washed twice in PBS containing 2% FCS (Invitrogen) and 0.1% sodium azide ($NaN_3$, Sigma-Aldrich). Next, antigen non-specific binding was prevented by prior incubation of cells with 10% mouse serum (Fitzgerald). Cells were next incubated with combinations of pacific blue-, Phycoerythrin (PE)-, fluorescein isothiocyanate (FITC)-, allophycocyanin (APC)- and PE-Cy7-conjugated mouse anti-human Ab (CD1c, CD3, CD8, CD11c, CD14, CD16, CD19, CD20, CD32, CD40, CD64, CD80, CD83, CD86, CD107a, CD141 and HLA-DR). Where indicated, after surface staining, T-cells were washed twice in PBS/2% FCS/0,1% $NaN_3$) and fixed, permeabilized, and intracellularly stained using mAb to IFN-γ and TNF. Cells were acquired on FACSCanto II and analyzed using FACS Diva Version 6.13 software (BD Biosciences) or FlowJo (7.6.5). Data was analyzed using Graphpad Prism 5.

*Antigen uptake assay*
To analyze DC uptake capacity, we incubated primary BDCA-$1^+$ and BDCA-$3^+$ DCs or day-5 MoDCs for 10 minutes (pulse) with 6 µg/ml eGFP (BioVision) or eGFP-IC, subsequently washed and chased for 50 minutes (37°C/ 5% $CO_2$, in X-vivo 15 medium). Immune complexes were made using 4-fold increased molar amount compared to eGFP of rabbit anti-eGFP polyclonal IgG (Pierce Biotechnology). Next, cells were washed in PBS/2% FCS/0,1% $NaN_3$ and eGFP fluorescence was measured by flow cytometry. To determine the effect of proteasome and endosomal blockers used in our cross-presentation experiments on uptake, we incubated DCs with MG132, Lactacystin, Epoxomicin, Chloroquine, Leupeptin or Primaquine during both the pulse and chase period (concentrations of inhibitors are described above). Data shown are MFI values of eGFP treated DCs corrected for background MFI (MFI of non-eGFP treated DCs).

*Confocal microscopy*
MoDCs were cultured for 5 days in Lab-Tek II chambered coverglass dishes (Thermo), as described in the MoDC culture section. We coated slides with alcian blue 8GX (Klinipath) prior to addition of monocytes, and added eGFP or eGFP-IC for 1 or 4 hours. MoDCs were washed (PBS), fixed (3.7% paraformaldehyde/PBS, 10 minutes) and washed (PBS, 3 times). Next, MoDCs were permeabilized by adding saponin solution (1% BSA, 0.5% saponin (Sigma) in PBS, 30 minutes)

and blocked (10% whole donkey or goat serum/PBS, 30 minutes, RT). EEA-1 was detected using goat anti-EEA-1 polyclonal IgG (Santa Cruz Biotechnology Inc., in PBS, 45 minutes, RT), washed and stained with donkey-anti-goat-Dyl647 (Jackson, in PBS/0.1% Tween, 45 minutes, RT). LAMP-1 was detected using mouse-anti-LAMP-1 polyclonal IgG (Biolegend, in PBS, 45 minutes RT), washed and stained with goat-anti-mouse-Dyl647 (Biolegend in PBS/0.1% Tween, 60 minutes, RT). After washing, we added 8µl/well of MOWIOL (Calbiochem) supplemented with 1 µg/ml DAPI (Sigma). Cells were coverslipped and analyzed at RT using a LSM 710 confocal microscope and "Plan-Apochromat" 63x1.40 Oil DIC M27 objective (Zeiss, Jena Germany). Image analysis was performed using Zen2009 (Zeiss) and ImageJ software including JACoP.[18]

*Real-time PCR*
Primary human BDCA-1$^+$ and BDCA-3$^+$ DCs were acquired as described above. After cell-sorting, total RNA was isolated using tripure (Roche) according to manufacturer's instructions. cDNA was synthesized from up to 1 µg of total RNA using the iScript cDNA synthesis kit (Biorad). Real-time PCR was performed as described[19] using IQ SYBR Green PCR Supermix (Biorad) and the CFX96 Touch Real-Time PCR Detection System (Bio-Rad), according to manufacturer's instructions. PCR assays were done in triplicate. Data was calculated as values relative to GAPDH and further analyzed using Graphpad Prism 5.

## Results

*Potentiation of viral antigen cross-presentation by FcγR targeting*
DC vaccination therapy to date utilizes monocyte-derived DCs (MoDCs) at large.[3] We therefore first established our FcγR antigen targeting work in MoDCs, before moving into primary BDCA-3$^+$ DCs. We proposed that FcγR antigen targeting may potentiate DC vaccination-induced CD8$^+$ T-cell responses as in mice[15-17], and therefore assessed FcγR expression on MoDCs, cultured in the presence of GM-CSF and IL-4 for 5 days. FcγRII (CD32) was highly expressed, while expression of FcγRIII (CD16) and FcγRI (CD64) were low (*Figure 1A, Figure S1A*), confirming published data.[19-21] MoDCs expressed the maturation markers CD40, CD80, CD83 and CD86 (*Figure S1B*), but could be further upregulated after stimulation with LPS (100 ng/ml, O/N), classifying them as intermediately matured DCs. We thereby corroborate data shown in mice that intermediately matured DCs cross-present immune-complexed antigen.[22]

To test for cross-presentation ability, we cultured MoDCs in the presence of 0.3, 3 or 30 µg soluble HCMV pp65 protein or left MoDCs untreated (50.000 DCs/100 µl culture, 12-16 hours). We then added to DC cultures $pp65_{495\text{-}503}$–specific CD8$^+$ T-cells recognizing HLA-A2/NLVPMVATV complexes (50.000 cells/well). T-cells used were freshly thawn from frozen stock, which we had previously expanded from healthy donor blood T-cells and characterized (*Figure S1C, S1D, S1E*). We measured T-cell stimulation (4-6 hours co-culture), by induced cytokine production and LAMP-1 surface expression (*Figure 1B, 1C*). Background levels of IFN-γ-producing T-cells were always between 0.1 and 2%. We observed the induced production of IFN-γ and TNF and surface displayed LAMP-1 in the majority of T-cells: DC exposed to 0.3 µg pp65 protein induced IFN-γ at background levels, whereas 3 and 30 µg pp65 induced IFN-γ in 20% and 58% of T-cells, respectively. TNF and LAMP-1 expression showed comparable results. Based on these data, we performed all following experiments using 3 µg pp65 protein. Next, we cultured MoDCs u

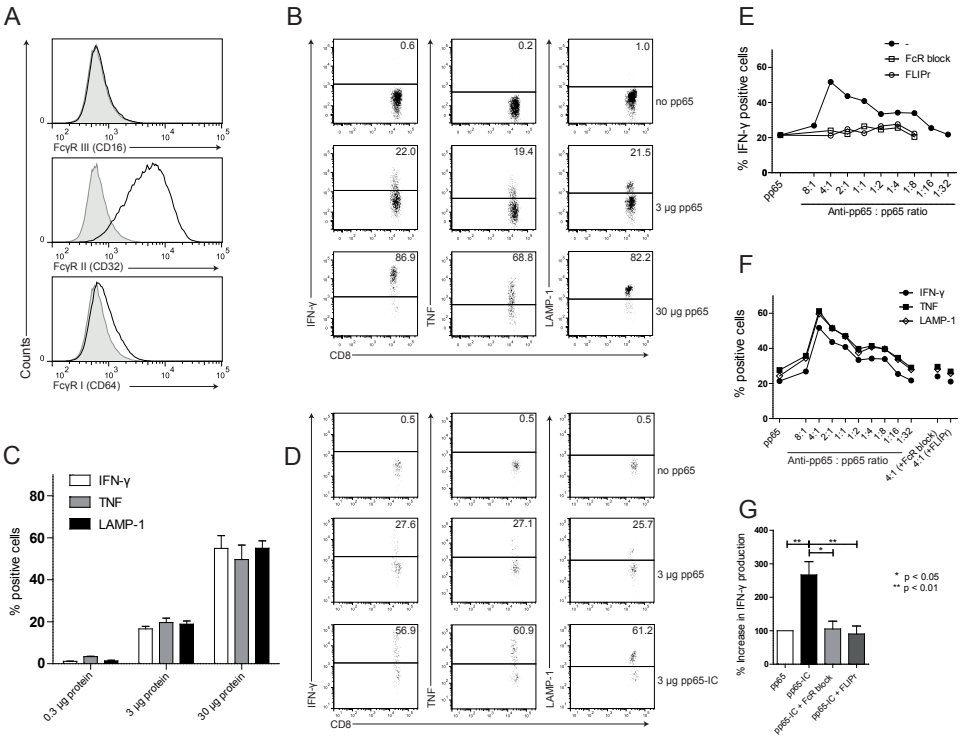



**Figure 1: Human MoDCs express FcγRs that facilitate antigen cross-presentation**
(A) FcγRI, II and III expression on human MoDCs, cultured under serum-free conditions (n=6). (B and C) Cross-presentation of $pp65_{495-503}$ to $CD8^+$ T-cells (n=6). (B) Representative plots of $CD8^+$ T-cell activation. MoDCs were loaded with HCMV derived pp65 and co-cultured with A2/NLVPMVATV specific T-cells. Freshly thawn T-cells were gated based on CD3 en CD8 expression and analyzed for activation-induced production of IFN-γ (left) and TNF (middle) and LAMP-1 surface expression (right). (C) Summary (mean + SEM) of HCMV $pp65_{495-503}$ cross-presentation. Bars represent production of IFN-γ (white) and TNF (grey) and LAMP-1 (black) surface expression after co-culture with MoDCs loaded with 0.3, 3 and 30 µg pp65 (n=5-8). (D-G) Increased cross-presentation by FcγR targeting of pp65. (D) MoDCs were loaded with pp65 (top plots) or pp65-IC (lower plots) and analysed as in B. (E) HCMV pp65 was added across a range of Ab:Ag ratio's and production of IFN-γ was analysed (closed circles). Contribution of FcγR in IC-mediated cross-presentation by inclusion of FcγR-blocking reagents: purified IgG-Fc-fragments (open squares) or recombinant S. aureus-FLIPr-like (open circles). (F) IFN-γ (closed circles), TNF (closed squares) and surface display of LAMP-1 (open diamonds) on $CD8^+$ T-cells. (G) Summary (mean + SEM) of IFN-γ production after IC-mediated cross-presentation in absence (black bar, n=4) or presence of FcR blocking reagents (IgG-Fc-fragments, light grey; FLIPr-like, dark grey; n=3).

der serum-free conditions, and added pp65 across a range of Ab:Ag ratio's, allowing immune complex (IC) formation between pp65 and antigen-specific anti-pp65 IgG. We used polyclonal rabbit anti-pp65 since cross-reactivity with human FcγR is described.[23] We confirmed optimal potentiation of cross-presentation when pp65 was administered in complex with 4-fold more molar amount of anti-pp65 IgG, as measured by production of IFN-γ in 52% of T-cells. Again, TNF and surface display of LAMP-1 on $CD8^+$ T-cells showed comparable results. (*Figure 1D, E, F, G, n=4*). Ab alone did not induce cytokine production (*Figure S1G*). The induced cross-pres-

entation of anti-pp65:pp65 IC (hereafter referred to as pp65-IC) by MoDCs was mediated by FcγR targeting, as cross-presentation was completely blocked when FcγR blocking agents were included in the culture (FcγR block: inclusion of purified IgG Fc-fragments; FLIPr-like: staphylococcus aureus derived formyl peptide receptor like1 inhibitor protein (FLIPr-like).[49] Of note, FcγR targeting of viral antigen also enhanced cross-presentation significantly in MoDCs cultured in serum-sufficient medium, that contains polyclonal IgG (*Figure S1F*). Taken together, the cross-presentation of viral soluble antigen by human MoDCs is potentiated by antigen targeting to FcγRs, at least in this culture system. Recent work in mice confirms that circulating antigen-specific IgG can potentiate systemic cross-presentation in mice.[17]

*Increased cross-presentation of pp65-IC is not due to increased antigen uptake or maturation*
Increased cross-presentation could be explained by increased uptake of immune-complexed antigen, in analogy to mouse DCs in which IgG opsonization of *E. coli* stimulates pathogen internalization.[24] To determine whether ICs are better endocytosed when compared to soluble antigen, we performed pulse-chase experiments using 5-day MoDCs and soluble eGFP protein. We compared uptake of eGFP (6 μg/well) with eGFP-IC (the same amount of eGFP pre-complexed with 4-fold increased molar amount of anti-eGFP rabbit IgG) and determined eGFP uptake by DCs using flow-cytometry. We found comparable uptake of soluble eGFP and eGFP-IC by MoDCs (*Figure 2A*). We confirmed the presence of internalized soluble eGFP in 5-day MoDCs using confocal microscopy by Z-stack analysis (1 hour of MoDC culture in the presence of eGFP) (*Figure 2C*).

Earlier work showed that efficient pp65 cross-presentation by DCs derived from HCMV-infected fibroblasts requires soluble factors secreted by the infected fibroblasts, causing their maturation.[25] To investigate whether pp65-IC also induces DC maturation, we analysed for induced upregulation of maturation markers CD40, CD80 and CD86 (*Figure 2B*). To this end, we incubated MoDCs overnight with medium, anti-pp65, pp65, pp65-IC or a combination of LPS (100 ng/ml) and poly(I:C) (30 μg/ml). Of note, these experiments were performed without re-plating DCs to 96 wells plates, since the latter showed maturation independent of pp65 or toll-like receptor (TLR) stimulation, indicating mechanically induced maturation (*Our unpublished data*,[26,27]). Incubation of pp65 or pp65-IC did not result in overt increase of the co-stimulatory markers CD40, CD80 or CD86, with only a minor increase in CD86 expression in pp65-IC treated MoDCs. Therefore, we concluded that increased cross-presentation of pp65-IC is not caused by increased co-stimulatory molecule expression. Finally, do endocytosed immune complexes localize to early and late endosomal compartments? To this end, we performed confocal microscopy analyses using MoDCs that were allowed to internalize eGFP-IC for one hour and were chased for one and four hours (37°C). While over time, eGFP-IC presence decreased in $EEA1^+$ early endosomal compartments ($p<0.01$, comparing eGFP fluorescence at 1 hour and 4 hours), eGFP fluorescence remained high in $LAMP\text{-}1^+$ late endosomal compartments. Thus, immune complexes localize to early and late compartments of the endosomal pathway in human MoDCs.

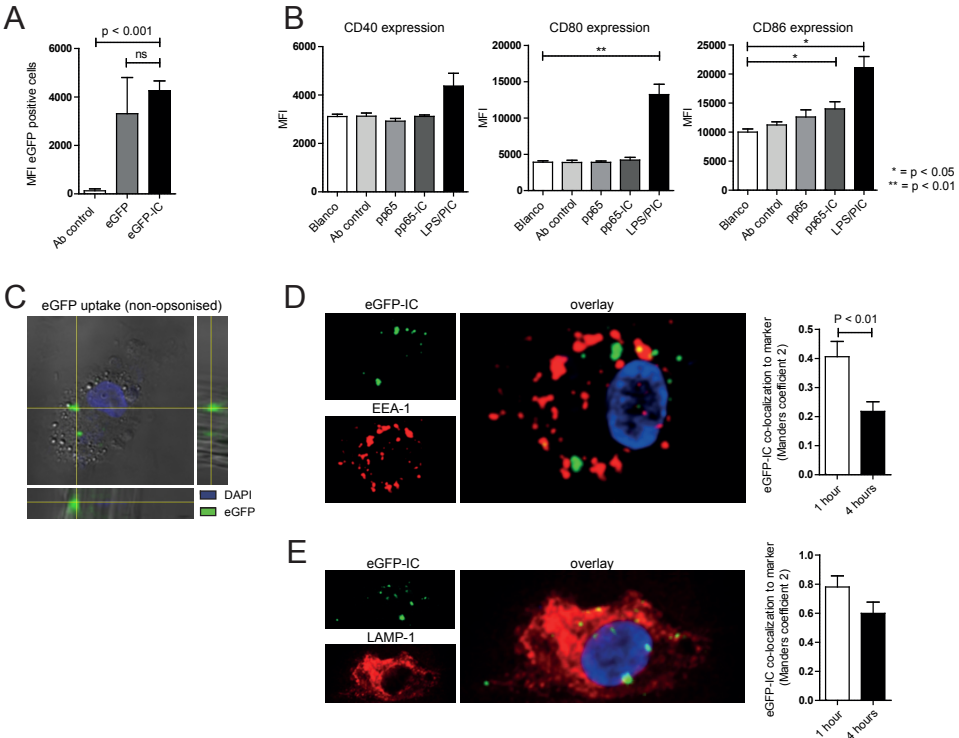



**Figure 2: Enhanced cross-presentation of immune-complexes is not due to increased antigen uptake or DC maturation**

(A) Day-5 human MoDCs were cultured in the presence of eGFP (grey bar) or eGFP-IC (black bar) for 10 minutes (pulse), washed three times and cultured for 1 hour (chase) to assess uptake efficiency (mean + SEM, n=3). Data shown are MFI values corrected for background MFI (DCs cultured without eGFP). (B) Day 4½ human MoDCs were cultured overnight in the presence of medium (white bars), pp65 antibody (light grey bars), pp65 alone (grey bars), pp65-IC (dark grey bars) or 100ng LPS and 30µg poly(I:C) (PIC) to assess maturation status. Data shown are MFI values (mean + SEM, n=3) of CD40 (left graph), CD80 (middle graph) and CD86 (right graph). (C and D) MoDCs were cultured on confocal slides and incubated with eGFP (C) or eGFP-IC (D). (C) Non-opsonized eGFP (green) is internalized by human MoDCs (bright field, nucleus visualized using DAPI). (D and E) MoDCs were allowed to internalize eGFP-IC for one hour and were chased for one and four hours (37°C, analysis of 10-25 slides containing multiple DCs for each condition in 2 separate experiments). Cells were fixed and stained for confocal microscopy. Distribution of internalized eGFP-IC was quantified as percentage of vesicles positive for EEA-1 (D) or LAMP-1 (E).

*Cross-presentation of FcγR-targeted viral antigen requires antigen processing in both the endosomal pathway and by the proteasome*

Increased antigen cross-presentation can also be attained by restraining the endosomal processing of IgG-coupled antigen, as was shown in mouse DCs.[28] For immune complexes, the requirements for endosomal processing and proteasome-mediated peptide generation are not fully clear, particularly for human myeloid DCs.[3,29] We therefore assessed in human MoDCs the role of antigen processing in the endosomal pathway and by the proteasome. We assessed cross-presentation of HCMV pp65 antigen to pp65-specific $CD8^+$ T-cell clones, as reported[12,30], but in our case administered pp65 antigen as IgG-pp65 immune complexes to facilitate FcγR-mediated uptake. We treated human MoDC with inhibitors of the proteas-

ome (MG132, lactacystin and epoxomicin), or with endosomal inhibitors that target either acidification (chloroquine), proteolysis (leupeptin) or surface-directed transport of recycling endosomes (primaquine). We first assessed the ability of MoDCs to stimulate pp65-specific $CD8^+$ T-cell clones after inhibitor treatment, by addition of exogenous peptide. At inhibitor concentrations used, the MoDCs were still able to stimulate pp65-specific $CD8^+$ T-cells (*Figure 3A-G, S2A*). We next confirmed that these inhibitors at concentrations used do not affect antigen uptake. We treated MoDCs with inhibitors by incubating eGFP-IC for 10 minutes (pulse) in the presence of inhibitors. DCs were then washed and incubated for one hour (chase), again in the presence of inhibitors, and analysed for eGFP uptake by flow cytometry. Incubation of DCs in the presence of MG132, lactacystin, epoxomicin, chloroquine, leupeptin or primaquine did not alter the capacity to internalize eGFP-IC (*Figure S2B*).

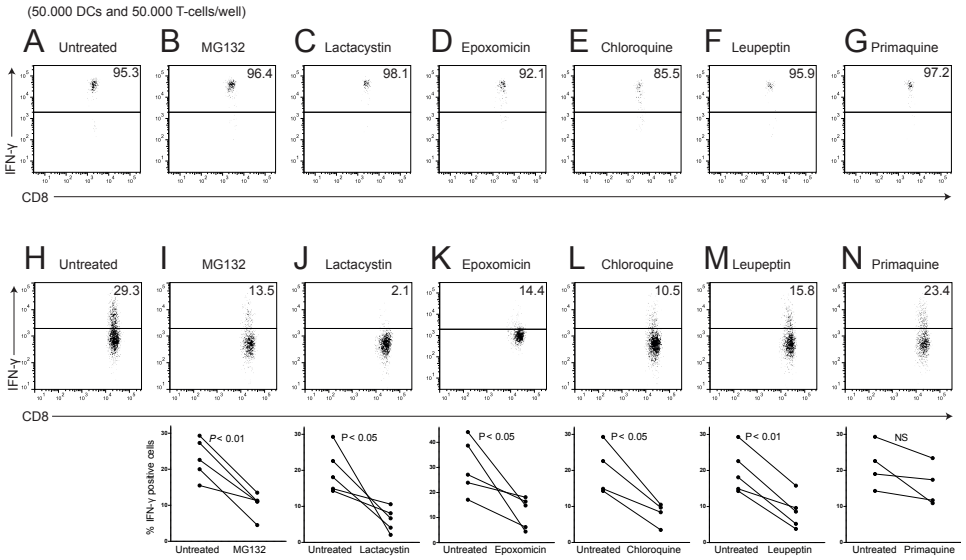



**Figure 3: Cross-presentation of FcγR-targeted viral antigen requires antigen processing in both the endosomal pathway and by the proteasome**

Human MoDCs were allowed to process 3μg pp65-IC in absence (A, H) or presence of proteolysis inhibitors indicated (O/N, 37°C; B-G, I-N). To ascertain that inhibitors do not counteract HLA-A2 mediated presentation indiscriminately, DCs were loaded with $1^{-6}$ M NLVPMVATV peptide and T-cell activation assessed after 4 hours in the presence of Golgi-stop (A-G). Shown are representative plots of IFN-γ production by A2/NLVPMVATV-reactive T-cells (n > 3 independent experiments, summarized in supplemental figure 2) for peptide control experiments (A-G) and representative plots (top) and summarizing graphs for each inhibitor (H-N, n=4-5). (B, I) MG132 proteasome inhibitor, 2 μM. (C, J) lactacysin proteasome inhibitor, 100 μM. (D,K) epoxomicin proteasome inhibitor, 10 μM. (E, L) chloroquine endosomal acidification inhibitor, 50 μM. (F, M) leupeptin lysosomal cysteine protease inhibitor,15 μM. (G, N) primaquine recycling endosome inhibitor, 50 μM.

To test antigen-processing requirements, we performed cross-presentation experiments, now adding pp65-IC to DCs in the presence of relevant inhibitors (O/N). Pre-treatment of MoDC with proteasome inhibitor MG132 (2 μM), lactacysin (100 μM) or epoxomicin (10 μM) significantly reduced cross presentation of A2/NLVPMVATV complexes, as measured by IFNγ pro-

duction by HCMV $pp65_{495-503}$–specific $CD8^+$ T-cells (*Figure 3H-K*). We considered that endosomal antigen proteolysis may facilitate transfer of antigenic fragments across the endosomal membrane. Accordingly, proteasome digestion may perform final cleavage, to allow for generation of peptide cargo and assembly into peptide/HLA Class I complexes. To clarify a possible role for endosomal antigen processing, we pre-treated MoDCs with chloroquine (50 μM) to inhibit endosomal acidification. Addition of chloroquine significantly reduced stimulation of $pp65_{495-503}$–specific $CD8^+$ T-cells (*Figure 3L*). To confirm the need for endosomal proteolysis to cross-presentation in MoDCs, we pre-treated MoDCs with the cysteine protease inhibitor leupeptin (15 μM), which again resulted in diminished antigen stimulation of $pp65_{495-503}$–specific $CD8^+$ T-cells (*Figure 3M*). Finally, inhibition of recycling endosome to cell surface transport using primaquine (50 μM) did not diminish cross-presentation in this system (*Figure 3N*). These data together support that transition of antigen from endosome to cytosol is required for cross-presentation, while negating the possibility that all antigen processing occurs within endosomal constraints. Indeed, in human plasmacytoid DCs, cross-presentation of viral antigen is independent of proteasome digestion, with all processing being performed in the endosomal pathway.[31] In ovalbumin cross-presentation by mouse DC systems, data collectively suggests that processing may occur in either the endosomal or cytosolic/proteasomal pathway, depending on the endocytic route taken (i.e., choice of binding to endocytic receptors) and configuration of the antigen (i.e., soluble or particulate).[15,28,32,33] Our data in human MoDCs now shows that for HCMV pp65, FcγR-mediated uptake potentiates cross-presentation in a manner that requires processing both in the endosomal pathway and by the proteasome.

*Human primary BDCA3⁺ DCs express Fcγ Rs that contribute to antigen cross-presentation*

Thus far, we have demonstrated that FcγR antigen targeting potentiates the cross-presentation of HCMV antigen by MoDCs. Are these findings applicable to human BDCA-$3^+$ DCs? BDCA-$3^+$ DCs are a recently described myeloid DC subset that exhibits superior cross-presentation abilities, but their possible application in trials commands preclinical analysis for favorable antigen targeting for yielding effector $CD8^+$ T-cell activation. Affirmation that FcγR antigen targeting further potentiates cross-presentation efficiency by BDCA-$3^+$ DCs should facilitate their application in DC vaccine clinical trials, as matched pairs of recombinant viral antigens and monoclonal Ab are available and can be generated in clinical grade quality.

We therefore first assessed the presence of FcγRs on both BDCA-$3^+$ and BDCA-$1^+$ DC subsets that we extracted from human PBMCs and tonsils. Our flow cytometry gating strategy involved the selection for lineage negative, HLA-DR and CD11c positive, BDCA-3 or BDCA-1 positive cells (*Figure S3A*) in human PBMCs (*Figure 4A*) and human tonsils (*Figure 4B*). In peripheral blood and tonsil DCs, both BDCA-$3^+$ and BDCA-$1^+$ expressed FcγRs. Similar to MoDCs, FcγRII (CD32) was expressed on BDCA-$1^+$ and expressed at intermediate levels on BDCA-$3^+$, while FcγRIII (CD16) expression was low on either subset. FcγRI (CD64) was expressed only on BDCA-$1^+$ DCs (*Figure 4A, 4B, S3B*). We then confirmed FcγRII expression by determining mRNA levels. FcγRII mRNA expression levels were again present in both DC subsets, and much higher in BDCA-$1^+$ DCs compared to BDCA-$3^+$ DCs (*Figure 4C*, RT-PCR amplification of FcγRII isoforms, relative to GAPDH). The relative expression of inhibitory FcγRIIb compared to stimulatory FcγRIIa appeared FcγRIIb-dominated in BDCA-$1^+$ DCs, with comparable expression of stimulatory FcγRIIa and inhibitor FcγRIIb in BDCA-$3^+$ DCs, possibly contributing to increased cross-presentation ability by BDCA-$3^+$ DCs (*Figure 4D, $p<0.01$, $n=5$*).

Does FcγR antigen targeting potentiate the cross-presentation of HCMV pp65 by BDCA-3$^+$ DCs? We administered either soluble HCMV pp65 protein or pp65-IC to BDCA-3$^+$ and as comparison to BDCA-1$^+$ DCs (O/N), washed DCs and added HCMV pp65-specific CD8$^+$ T-cells for co-culture. We confirmed that cross-presentation was superior in BDCA-3$^+$ DC compared to BDCA-1$^+$ DC (*Figure 4E*).[11] This was not caused by differences in MHC class I expression (*Figure S3C*), neither did anti-pp65 antibody alone cause IFNγ secretion by $pp65_{495\text{-}503}$–specific CD8$^+$ T-cells (*Figure S3D*). Similar as in *Figure 1*, HCMV antigen cross-presentation experiments were performed using serum-free medium, to avoid interference with serum-derived IgG. BDCA-3$^+$ primary DCs extracted from PBMC and tonsils induced 2 to 3-fold increased stimulation of pp65-specific T-cells as measured by induced IFNγ production (*Figure 4F, 4G*). Also in primary BDCA-3$^+$ DCs, pp65-IC-facilitated cross-presentation was fully mediated by FcγR, as pp65 T-cell stimulation was blocked by using human IgG Fc fragments and recombinant FLIPr-like (*Figure*

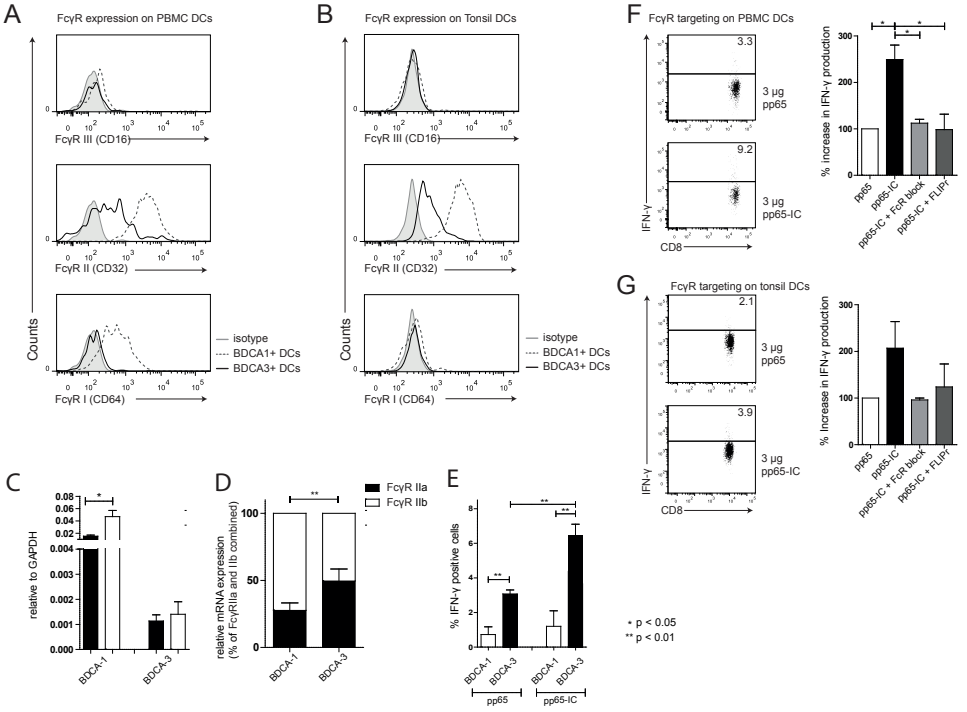



**Figure 4: FcγRs on human BDCA-3$^+$ contribute to antigen cross-presentation**
FcγRI, II and III expression on BDCA-1$^+$ and BDCA-3$^+$ DCs extracted from human blood (A) and tonsils (B) (BDCA-1$^+$, dashed line) and (BDCA-3$^+$, solid line) (n=3). (C) FcγRIIa (black bars) and FcγRIIb (white bars) mRNA expression in BDCA-1$^+$ and BDCA-3$^+$ DCs extracted from human blood (mean + SEM, n=5). (D) relative FcγRIIa (black bars) and FcγRIIb (white bars) mRNA expression in BDCA-1$^+$ and BDCA-3$^+$ DCs (mean + SEM, n=5). (E) BDCA-1$^+$ (white bar) and BDCA-3$^+$ (black bar) were FACS-sorted (at least 98% purity) and cultured in the presence of 3µg pp65 (left bars) or pp65-IC (right bars) (O/N, 37°C). Next, A2/NLVPMVATVCD8$^+$ T-cells were added to DC cultures and cross-presentation was analyzed as in figures 1 and 2 (mean + SEM, n=3). (F and G) Representative plots and summary of IFN-γ production (mean + SEM) after IC-mediated cross-presentation in the absence (black bar) or presence of FcR blocking reagents (IgG-Fc-fragments (light grey), recombinant S. aureus-FLIPr-like (dark grey; n=3 figure F; n=2 figure G).

*4F and G*). In a recent paper, lymphoid organ-resident DCs were able to cross-present MelanA long peptide antigen without prior *in vitro* activation, whereas blood DCs fail to do so.[34] Others had shown earlier that human blood DCs do not cross-present antigen unless previously activated via TLR ligation.[10,35] We here focused on the ability of BDCA-3$^+$ DCs to cross-present immune complexed pp65 antigen, and show that both lymphoid organ- and blood-derived BDCA-3$^+$ DCs cross-presented $pp65_{495\text{-}503}$/HLA-A2 complexes without prior stimulation.

*Differential antigen uptake does not explain increased cross-presentation by BDCA3$^+$ DCs*
It is unclear whether human DC subtypes differ in their capacity to take up soluble proteins. To solve this question, we sorted BDCA-1$^+$ and BDCA-3$^+$ DCs and examined their ability to internalize eGFP immune complexes. Cells were incubated for 10 minutes with eGFP-IC, washed, and incubated for 1 additional hour (37°C). Unexpectedly, BDCA-1$^+$ DCs endocytosed at least 10-fold more eGFP-IC protein compared to BDCA-3$^+$ DCs (*Figure 5A, representative histograms and 5B, n=3*). Taken into account that BDCA-3$^+$ DCs exhibit decreased efficiency in IC uptake, yet stimulate $pp65_{495\text{-}503}$/HLA-A2-specific CD8$^+$ T-cells more efficiently than BDCA-1$^+$ DCs, these results support the observation that BDCA-3$^+$ DCs are superior in their antigen cross-presentation capacity.

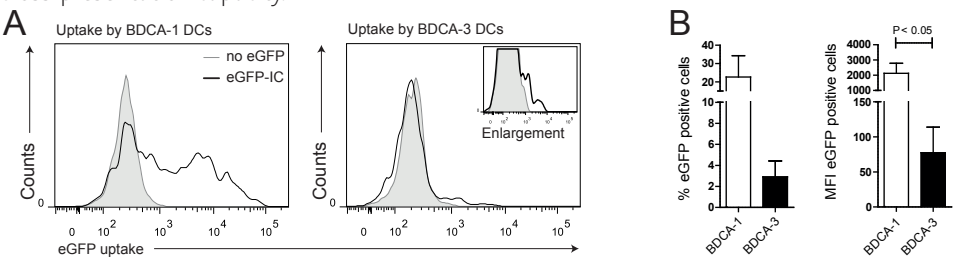



**Figure 5: BDCA-1$^+$ rather than BDCA-3$^+$ DCs internalize immune-complexed antigen**
Human BDCA-1$^+$ and BDCA-3$^+$ DCs were cultured in the presence of eGFP-IC for 10 minutes, washed 3 times, and incubated for 1 hour (37°C). Uptake was analysed using flow-cytometry (A and B). Shown are representative plots of eGFP-IC (black line) uptake (A) and mean uptake (% of positive cells (left) or MFI (right)) of BDCA-1$^+$ (white bars) and BDCA-3$^+$ DCs (black bars).

Various processing routes are shown to be relevant in cross-presentation biology. Involvement of these pathways seems distinctive between species, cell types, antigen and route of antigen-uptake.[3] Evidence for cytosolic entry of (partly) processed protein, and subsequent proteasome involvement is abundant[33,36-38], but proteasome-independent routes have been described as well[31,32]. In case of proteasome-dependent cross-presentation, newly formed peptides could enter the ER[39] or possibly be transported back into early endosomes[33,40], late endosomes or endo/lysosomes.[41-43] These phagosomes contain MHCI loading complex components.[36,37,40,44,45] To clarify mechanisms involved in BDCA-3$^+$ DC cross-presentation, we tested the involvement of endosomal and proteasomal processing, analogous to experiments described in *Figure 2*. We cultured freshly isolated BDCA-3$^+$ DCs in the presence of inhibitors (O/N) and confirmed antigen presentation ability by adding peptide after O/N treatment after we washed cells, followed by co-culture with pp65-specific CD8$^+$ T-cells. (*Figure S4A*). Treatment with pp65 or pp65-IC alone did not stimulate BDCA-3$^+$ DC maturation, as levels of CD40, CD80 and CD86 were not increased compared to untreated or anti-pp65 antibody-treated BDCA-3$^+$

DCs (16 hours of stimulation, positive control LPS/poly(I:C) *Figure S4B*). Cross-presentation in BDCA-3$^+$ DCs required proteasome activity, as presentation of A2/NLVPMVATVcomplexes to HCMV pp65$_{495\text{-}503}$–specific CD8$^+$ T-cells was diminished after proteasome inhibitor MG132 (2 μM), lactacysin (100 μM) or epoxomycin (10 μM) treatment (*Figure 6A, B, C*). However, endosomal processing was also required, since addition of chloroquine (50 μM) and leupeptin (15 μM) resulted in significantly reduced stimulation of pp65$_{495\text{-}503}$–specific CD8$^+$ T-cells (*Figure 6D, E*). Finally, inhibition of recycling of MHC class I molecules to the plasma membrane using primaquine (50 μM) resulted in diminished cross-presentation, suggesting re-entry of peptides into the endosomes and subsequent loading onto recycling MHC-class I molecules. Taken together, FcγR targeting enhances the cross-presentation capability of BDCA-3$^+$ DCs, that are already known as superior cross-presenting DCs. Mechanistically, we have clarified some of the intracellular pathways that support this exquisite cross-presentation capability. Our study supports the exploration of BDCA-3$^+$ DCs for human vaccination strategies, in particular aimed at prevention of viral reactivation complications seen in immune compromised individuals.

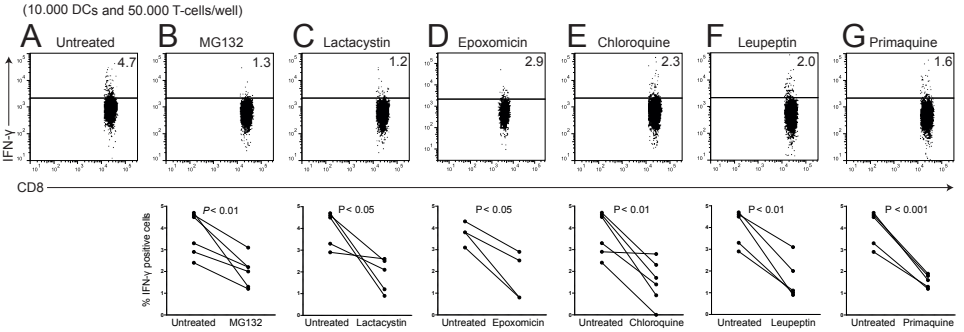



**Figure 6: Cross-presentation of FcγR-targeted antigen by human BDCA-3$^+$ DCs requires antigen processing in both the endosomal pathway and by the proteasome**

Human BDCA-3$^+$ DCs were allowed to process 3μg pp65-IC in the absence (A) or presence of proteolysis inhibitors indicated (O/N, 37°C; B-G). Next, DCs were washed and co-cultured with A2/NLVPMVATVCD8$^+$ T-cells (4 hours in the presence of Golgi-stop), and IFN-γ production by T-cells was measured as a read-out for cross-presentation. Shown are representative plots (top) and summarizing graphs for each inhibitor (n=4-5). (B) MG132 proteasome inhibitor, 2 μM. (C) lactacysin proteasome inhibitor, 100 μM. (D) epoxomicin proteasome inhibitor, 10 μM. (E) chloroquine endosomal acidification inhibitor, 50 μM. (F) leupeptin lysosomal cysteine protease inhibitor, 15 μM. (G) primaquine recycling endosome inhibitor, 50 μM.

## Discussion

Most FcγR-mediated antigen uptake and cross-presentation studies were performed in mouse systems.[15-17] It was our aim to show the possible applicability of such studies to human DC vaccination, in the context of viral reactivation post stem cell transplantation. In human DC trials, most DC vaccination therapies currently utilize monocyte-derived DCs (MoDCs). We therefore started our studies in MoDCs. As antigen cross-presentation is crucial in anti-viral responses, we extended our studies to BDCA-3$^+$ DCs which were recently described as expert antigen cross-presenting DCs. Our second objective was therefore to test whether BDCA-3$^+$ DC application in human DC vaccination therapy may be enforced by FcγR-targeted antigen loading. Our data collectively show that FcγR antigen targeting enhances the cross-presentation of immune-complexed HCMV-derived antigen that we employed as a model viral antigen. Our work extends recent work in mice, that circulating antigen-specific IgG can potentiate systemic cross-presentation in mice.[17]

Mouse-based work suggests that in general, cross-presentation may involve NADPH-oxidase NOX2-mediated reduction of endosomal proteolysis[46], and the fusion of endoplasmic reticulum (ER) vesicles to endosomal counterparts by SNARE Sec22b[40]. Clarification of cross-presentation mechanisms within human DCs might reveal how to improve their effective applicability towards T-cell stimulation in the clinic. We considered that DC vaccination as a treatment would be helped by optimizing the potency of DC subsets at stimulating antigen-specific CD8$^+$ T-cell responses. We here show for MoDCs and BDCA-3$^+$ DCs that processing by the proteasome, located in the cytosol, is required for cross-presentation, thus negating the possibility that all antigen processing occurs within endosomal constraints. In contrast, in human plasmacytoid DCs, cross-presentation of viral antigen is independent of proteasome digestion, with all processing being performed in the endosomal pathway.[31] In ovalbumin cross-presentation by mouse DC systems, data collectively suggests that processing may occur in either the endosomal or cytosolic/proteasomal pathway, depending on the endocytic route taken (i.e., choice of binding to endocytic receptors) and configuration of the antigen (i.e., soluble or particulate)[4,15,28,32,33] . Our data in human MoDCs now shows that for HCMV pp65, FcγR-mediated uptake potentiates cross-presentation in a manner that requires processing both in the endosomal pathway and by the proteasome. Earlier flow cytometry-based work suggested that FcγR are not expressed on BDCA-3$^+$ DCs found in PBMC.[47,48] Our work using inhibitors to FcγR-mediated antigen binding and signalling however show their functional relevance to cross-presentation. We corroborate our FcγR expression data by RT-PCR and by isolation of BDCA-3$^+$ DCs from both human tonsils and PBMC.

Our study has some limitations. Immune complexes did not stimulate antigen cross-presentation at all ratios of antibody:antigen. The optimal ratio we found for anti-pp65 IgG:pp65 was 4-fold molar excess compared to pp65 protein. For application in human DC vaccination, it will therefore be necessary to test individual antibody: antigen ratio's for each pair employed. Using the experimental setup we show in *Figure 1E and 1F*, such an endeavour should be relatively uncomplicated. Further, we showed that both antigen processing in the endosomal pathway and by the proteasome is instrumental to FcγR-mediated antigen cross-presentation. Additional enhancement of the CD8$^+$ T-cell stimulatory activity by particularly BDCA-3$^+$ DCs could be reached by modulation of the endosomal pathway, restraining superfluous antigen degradation. Such manipulations we considered may be less applicable

to translation into the clinical setting, and therefore fell outside the scope of our current study. Finally, our BDCA-3$^+$ DCs work was performed using primary DCs extracted from blood and tonsils. For application into clinical settings of these DCs, it will be necessary to optimize the culture conditions of these DCs from stem cell precursors, to generate a standardized and consistent supply of these cells for vaccination purposes.

Taken together, FcγR targeting enhances the cross-presentation capability of BDCA-3$^+$ DCs, that are already known as superior cross-presenting DCs. Mechanistically, we have clarified some of the intracellular pathways that support this exquisite cross-presentation capability. Our study supports the exploration of BDCA-3$^+$ DCs for human vaccination strategies, in particular aimed at prevention of viral reactivation complications seen in immune-compromised individuals.

## Acknowledgements

The authors like to thank members of the Boes laboratory and Dr M. Ressing and Dr. S. Nierkens (both at the University Medical Centre, Utrecht) for helpful discussions. We thank A. Stoppelenburg for help with qPCR experiments. We also thank Dr. J.A. Van Strijp and Dr. K.P. Van Kessel for providing FLIPr-like recombinant protein. We thank T. van den Broek and members of the ENT-department for help in obtaining surplus human tonsil samples from anonymous donors. This study was supported by a grant from the Netherlands Genomics Initiative (NGI)/ Netherlands Organization for Scientific Research (NWO) to M.B. and by a KiKa grant to J.B.

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## Supplemental Figures

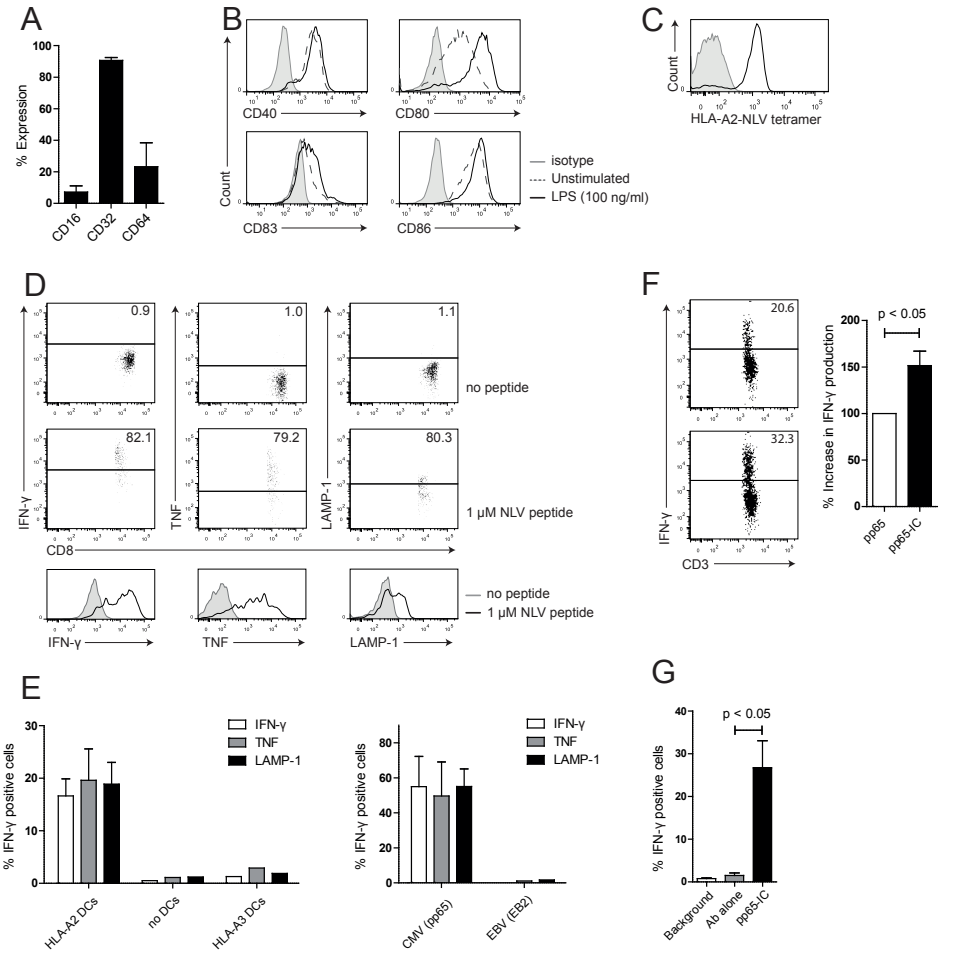



**Figure S1: Human MoDCs are of intermediately matured phenotype and express FcγRs that facilitate antigen cross-presentation to A2/NLVPMVATV-specific CD8⁺ T-cell clones**

(A) FcγRI, II and III expression on human MoDCs (mean + SEM). (B) MoDCs (day 5 cultures) expressed the maturation markers CD40, CD80, CD83 and CD86 (dashed line), that could be further upregulated after stimulation with 100 ng/ml LPS (black line), classifying them as intermediately matured DCs (n=4). (C-E) Characterization of A2/NLVPMVATV-restricted T-cell clones used. (C) CD8⁺ T-cells wereA2/NLVPMVATV tetramer positive and (D) recognized A2/NLVPMVATVcomplexes when co-cultured for 4-6 hours with NLVPMVATV-peptide loaded DCs. (E) T cells did not respond to non-HLA-A2 DCs or HLA-A2 DCs loaded with the EBV derived protein EB2. (F) Antigen cross-presentation assay in 10% human serum. Human MoDCs were cultured for 5 days in the presence of 10% human serum and were subsequently allowed to internalize pp65 or pp65-IC (O/N, 37°C). Next, DCs were washed and CD8⁺ T-cells were added to DC cultures. Shown are percentages (mean + SEM) of IFN-γ producing T-cells after co-culturing with pp65 (white bar) or pp65-IC (black bar) loaded DCs. (G) T-cell response to DCs loaded with anti-pp65 alone.

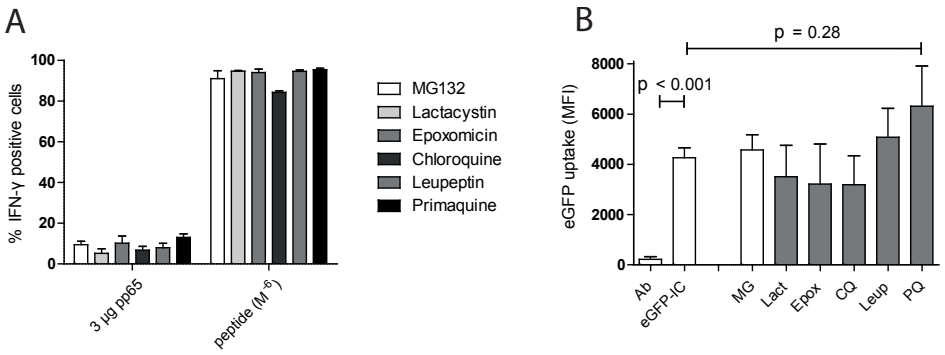



**Figure S2: Human MoDCs retain antigen presenting capacity after culture in the presence of selective inhibitors**

(A) MoDCs were loaded with 3µg pp65 and cultured O/N in the presence of MG132, lactacystin, epoxomicin, chloroquine, leupeptin or primaquine, analogous to figure 3. 1 µM of NLVPMVATV peptide was added for 1 hour and cells were washed prior to addition of CD8$^+$ T-cells. Bars show percentages of IFN-γ producing CD8$^+$ T-cells as a measure of antigen presentation capacity after culture of DCs in the presence of inhibitors indicated (mean + SEM, n=3). (B) MoDCs were loaded with 6µg eGFP-IC in the presence of MG132, lactacystin, epoxomicin, chloroquine, leupeptin or primaquine, analogous to figure 2. Uptake capacity of MoDCs was assessed using flow cytometry. Data shown are MFI values (mean + SEM, n=3) after correction for background MFI (DCs cultured in presence of inhibitors without eGFP).

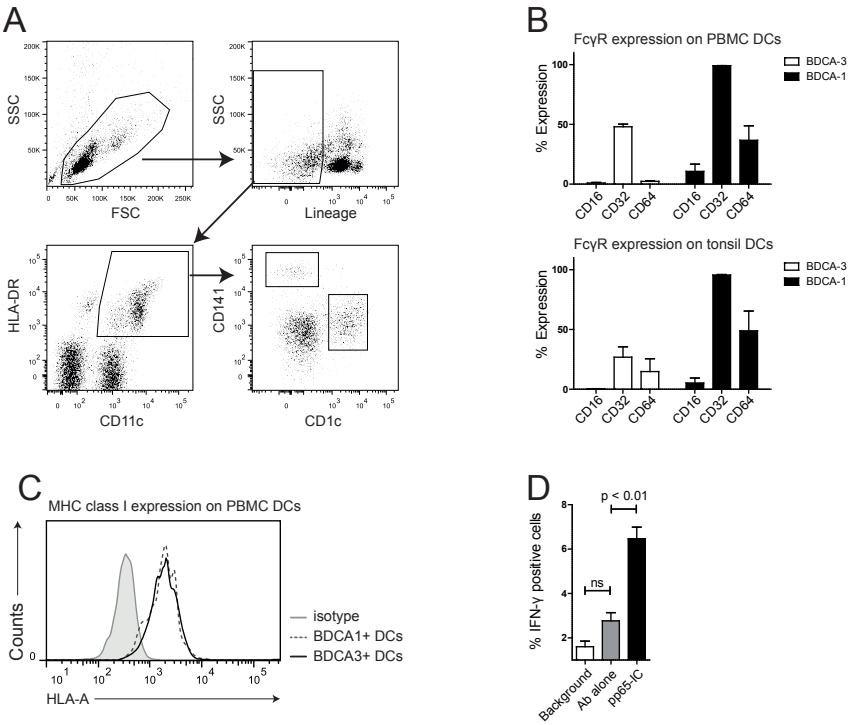



**Figure S3: Human BDCA-3⁺ and BDCA-1⁺ DCs express FcγRs that contribute to antigen cross-presentation**
(A) Human peripheral blood or tonsil mononuclear cells were isolated and depleted for CD3, CD14, CD19 and CD56 by magnetic cell sorting. Next, primary BDCA-1⁺ and BDCA-3⁺ DCs were FACS-sorted by gating on lineage negative cells, followed by positive sort for CD11c and HLA-DR and expression of either CD141⁺ or CD1c⁺ cells. (B) FcγRI, II and III expression on human blood (upper graph) and tonsil (lower graph) derived BDCA-1⁺ and BDCA-3⁺ DCs (mean + SEM). (C) HLA I expression on human blood derived BDCA-1⁺ (dashed line) and BDCA-3⁺ (solid line) DCs. (D) T-cell response to DCs loaded with anti-pp65 alone.

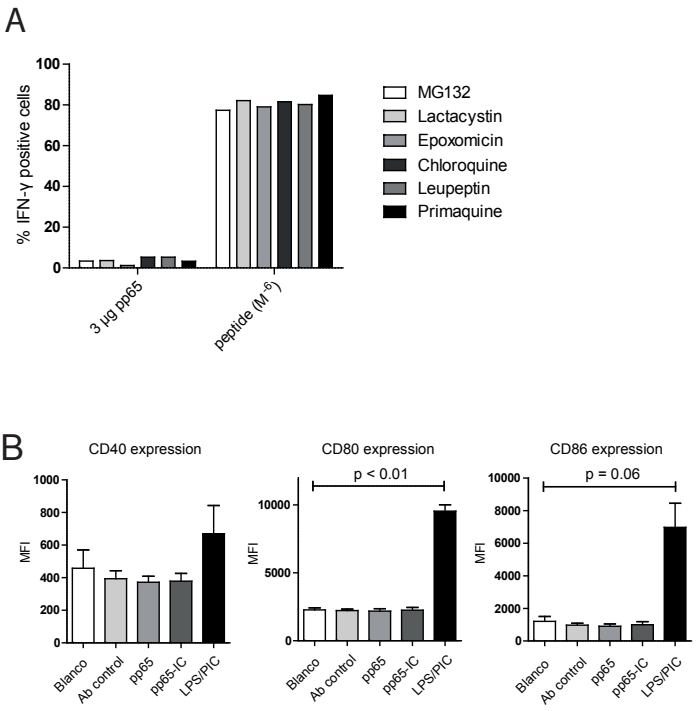
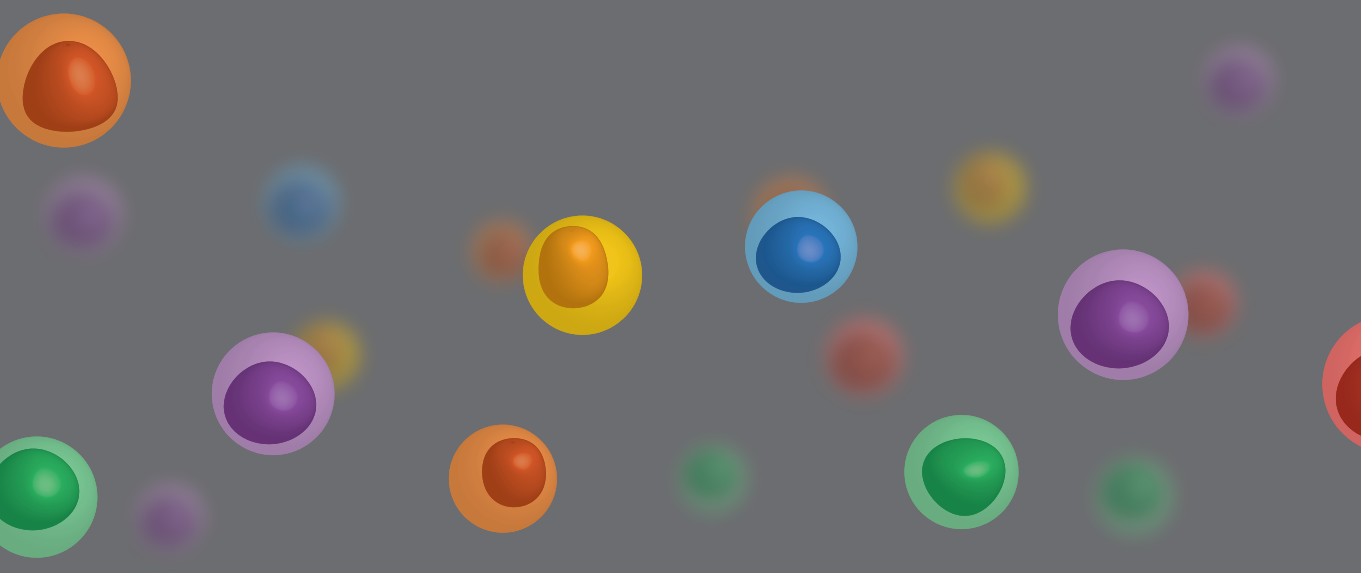



**Figure S4: Human primary BDCA-3⁺ DCs retained antigen presenting capacity after culturing in the presence of specific blockers**

(A) Human primary BDCA-3⁺ DCs were allowed to internalize and process pp65 (3µg) in the simultaneous presence or absence of MG132, lactacystin, epoxomicin, chloroquine, leupeptin or primaquine (O/N, 37°C). As a positive control, to some cultures, 1 µM of NLVPMVATV peptide was added for 1 hour and cells were washed prior to addition of CD8⁺ T-cells. Bars show percentages of IFN-γ producing CD8⁺ T-cells as a measure of antigen presentation capacity after DC-culturing in the presence of inhibitors indicated. (B) BDCA3⁺ DCs were cultured overnight in the presence of medium (white bars), pp65 antibody (light grey bars), pp65 alone (grey bars), pp65-IC (dark grey bars) or 100ng LPS and 30µg poly(I:C) (PIC) to assess maturation status. Data shown are MFI values (mean + SEM, n=3) of CD40 (left graph), CD80 (middle graph) and CD86 (right graph).

# 4



# A novel Fcγ-receptor IIa Q27W gene variant associates with common variable immune deficiency through defective FcγRIIa downstream signaling

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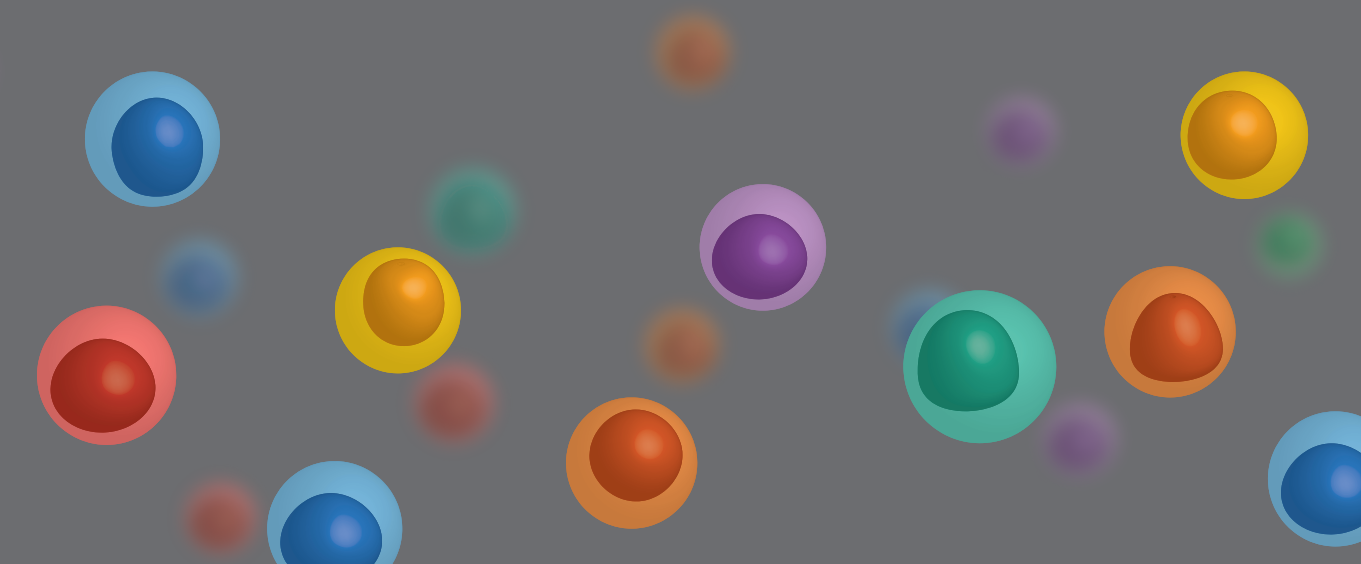
Clinical Immunolgy, 2014, accepted for publication



## Abstract

We identified a novel Q27W FcγRIIa variant that was found more frequently in common variable immunodeficiency (CVID) children. We analysed the possible functional consequence of the Q27W FcγRIIa mutation in human cells. We used peripheral blood mononuclear cells from Q27W FcγRIIa patients and healthy controls, and cultured cells that overexpress the Q27W and common FcγRIIa variants. The Q27W FcγRIIa mutation does not disrupt FcγRIIa surface expression in peripheral blood mononuclear cells. Mononuclear cells express multiple FcγR, precluding careful analysis of Q27W FcγRIIa functional deviation. For functional analysis of FcγRIIa function, we therefore overexpressed the Q27W FcγRIIa and common FcγRIIa variant in IIA1.6 cells that are normally deficient in FcγR. We show that FcγRIIa triggering-induced signaling is obstructed, as measured by both decrease in calcium flux and defective MAPK phosphorylation. In conclusion, we here describe a novel Q27W FcγRIIa variant that causes delayed downstream signaling. This variant may contribute to CVID.

## Abbreviations

FcγR: Fc gamma Receptor
CVID: Common variable immunodeficiency
ITAM: immunoreceptor tyrosine-based activation motif
BCR: B cell receptor
DC: dendritic cells
MAPK: mitogen-activated protein kinase
PBMC: peripheral blood mononuclear cells
ADCC: antibody-dependent cellular cytotoxicity
EGFR: epidermal growth factor receptor
WT: wild type

## Introduction

Common variable immunodeficiency (CVID) is a heterogeneous disease characterized by defective antibody production.[1,2] Although more gene mutations are recently being described,[3] the disease origins remain unknown in 90% of CVID patients.[4-6] Most of the mutations described directly involve the B cell receptor (BCR, CD19) complex involving additional molecules like CD20.[7] However, other receptors or pathways expressed on B-cells and other immune cells are also associated with CVID. For example, TNF receptor defects such as the transmembrane activator and CAML interactor (TACI)[7,8] and the B-cell activating factor receptor (BAFFR)[9-11] or Fc receptor (FcR) defects are described to be associated with CVID.[12] FcR defects or polymorphisms are of particular interest, since these receptors bind immunoglobulins, or antibodies, which are typically reduced in CVID.[1]

Blood-borne soluble antigens are readily opsonized by circulating immunoglobulins, thereby forming antigen immune complexes (IC). The capture and internalization of IC by immune cells is a well-studied process, and exerts through the family of Fc Receptors (FcR) that are widely expressed throughout the immune system. IC-FcR engagement elicits a range of cell type-specific effector mechanisms that include antibody-dependent cell-mediated cytotoxicity in neutrophils and peptide/MHC presentation by dendritic cells. Several isoforms for FcγRs exist that are often co-expressed on immune cell subsets, hampering functional studies of individual FcγR types. FcγRI, FcγRII (a,b, and c) and FcγRIII (a and b)[13] are expressed in varying numbers and ratio's on neutrophils,[14] monocytes/macrophages,[14] B cells,[15,16] dendritic cells (DCs)[17] and thrombocytes.[14,18] Our study focuses on the FcγR member FcγRIIa. FcγRIIa is a unique FcγR, since it harbors a signaling immunoreceptor tyrosine-based activation motif, or ITAM, within its ligand binding chain. Elicitation of signaling therefore does not require multimerization with signaling chains such as the γ-chain, known to associate with other FcR isoforms.[19] Moreover, studies performed in transgenic mice pinpoint the FcγRIIa as central mediator of inflammation in humans.[20,21] In this current study, we explore the function of FcγRIIa in immune activation, assisted by analysis of an FcγRIIa gene variant found in two unrelated immunodeficiency patients.

The interaction of Ig immune complexes with FcγRIIa induces signal transduction via its ITAM-motif, which appears to be triggered by receptor dimerization.[13,19] Genetic variants in FcγRIIa have been described[22] that link FcγRIIa to several immune diseases, including SLE, atherosclerosis and ulcerative colitis.[12, 23-27] One of these FcγRIIa polymorphisms is found at position 131 and results in altered affinity to mouse IgG1 or human IgG2 (FcγRIIa 131H and 131R). FcγRIIa-131H is the only receptor that binds human IgG2.[22] These polymorphisms result in variations in susceptibility to the development of auto-immune diseases. Structural studies on FcγRIIa predict that ligand binding induces FcγRIIa signaling through dimeric complexes.[28,29] Indeed, a dimer of FcγRIIa would place the two ITAM-containing chains in close proximity, suitable to propagate downstream signals. Studies in live cells from animals or patients to confirm the FcγRIIa crystallography studies had however not yet been performed.

From a genetic screen in a cohort of common variable immunodeficiency (CVID) patients we identified two patients a homozygous FcγRIIa gene variant resulting in a Q27W change in the extracellular domain of FcγRIIa (NM 001136219.1). These two patients do not suffer from autoimmune diseases, suggesting a different mode of FcγRIIa immune deviation than IgG2-

related disease-predisposition seen in FcγRIIa-131H patients. The FcγRIIa Q27W variant falls near the residues T23 and R30, which were shown to be important in receptor pairing within crystals and in their native context in membranes.[29] This FcγRIIa variant allowed us to study FcγRIIa signaling in human cells. We here describe functional consequences of the FcγRIIa Q27W variant in a model system. We show that the FcγRIIa Q27W variant yields reduced calcium mobilization and delayed mitogen-activated protein kinase (MAPK) phosphorylation.

## Material and Methods

*Next generation sequence and patient selection*
Next generation sequencing was performed as previously described.[30] In short: sample preparation was done using SOLiD compatible truncated adapters and barcoded primers. Enrichment was performed using the Agilent SurePrint G3 1M Custom CGH Microarray. Subsequently, emulsionPCR, bead enrichment and sequencing were performed according to manufacturers' instructions using an AB SOLiD 5500XL sequencer (Applied Biosystems, Bleiswijk, the Netherlands). Sequencing reads were mapped against the hg19 reference genome using Burrows-Wheeler Aligner. The study was approved by the local institutional review board.

All known and newly diagnosed CVID (-like) patients as well as multiple healthy controls were tested using NGS. At the time of writing this manuscript, 48 CVID or CVID-like patients were analyzed using NGS. CVID was diagnosed according to the European Society for Immunodeficiencies criteria. Several patients did not completely meet these CVID criteria and were therefore given a diagnosis of CVID-like disorder, which was defined as selective antibody deficiency (defined as the inability to produce protective titers of specific antibodies on vaccination to polysaccharide, to recall antigens, or both) combined with low IgA, low IgM, and/or low IgG subclass levels or a solitary decreased IgG level. These patients had recurrent infections and an inadequate response to prophylactic antibiotic treatment, which was defined by more than 4 breakthrough infections per year. Secondary immunodeficiencies (eg, iatrogenic or caused by enteral protein loss) were ruled out in all patients. Previous studies have shown that pediatric patients with CVID-like disorders, as described above, are clinically and immunologically (eg, B- and T-cell phenotype characterization) comparable with patients with definite CVID.[5]

*FcγRIIa mRNA by real-time PCR*
Primary human neutrophils, monocytes, B cells, BDCA1$^+$ and BDCA3$^+$ DCs blood DCs were isolated from Q27W patient or healthy donor blood. PBMC were separated by ficoll isopaque density gradient centrifugation (GE Healthcare Bio-Sciences AB). Next, cells were labeled with anti-human antibodies (Ab) (CD1c, CD11c, CD14, CD19, CD141, HLA-DR) and sorted by the FACS aria II (BD Bioscience) into 5 ml tubes (Thermo). After flow cytometry-assisted cell-sorting, total RNA was isolated using tripure (Roche) according to manufacturer's instructions. cDNA was synthesized from up to 1 μg of total RNA using the iScript cDNA synthesis kit (Biorad). Real-time PCR was performed as described (*Liu JI 2006*) using IQ SYBR Green PCR Supermix (Biorad) and the CFX96 Touch Real-Time PCR Detection System (Bio-Rad), according to manufacturer's instructions. PCR assays were done in triplicate. Data was calculated as

values relative to GAPDH and further analyzed using Graphpad Prism 5.

*Flow cytometry*
Whole blood staining were performed after erythrocyte lysis according to manufacturers instructions (Lysing buffer by BD Bioscience). For staining, cells were first washed twice in PBS containing 2% FCS (Bodinco, Alkmaar) and 0.1% sodium azide (Sigma-Aldrich). Next, antigen non-specific binding was prevented by prior incubation of cells with 10% mouse serum (Fitzgerald). Cells were next incubated with combinations of pacific blue-, Phycoerythrin (PE)-, Peridinin chlorophyll/cyanide dye (PerCP5.5)-, fluorescein isothiocyanate (FITC)-, allophycocyanin (APC)- and PE-Cy7-conjugated mouse anti-human Ab (CD1c, CD11c, CD14, CD19, CD32, CD32A, CD141 and HLA-DR). Cells were acquired on FACSCanto II and analyzed using FACS Diva Version 6.13 software (BD Biosciences) or FlowJo (7.6.5). Data was analyzed using Graphpad Prism 5.

*Antibody dependent cell-mediated cytotoxicity*
ADCC assays using $^{51}$Cr-labeled target cells were performed as described previously.[33] Briefly, A431 human epidermoid carcinoma cells having high surface expression of EGFR were used as target cells (5000 cells / well) and were labeled with 100 mCu $^{51}$Cr for 2 hours at 37 °C, washed three times in complete medium. Ficoll-Histopaque-separated PMNs and the chimeric anti-EGFR mAb cetuximab in medium were added to round-bottom microtiter plates (Corning Incorporated) at an effector-to-target (E:T) ratio of 80:1. Cells were incubated together for 4 hours in 200 mL RPMI1640 + 10 % FCS and $^{51}$Cr release was measured using a g-scintillator and expressed as counts per minutes (cpm). Percentage of specific lysis was calculated as follows: (experimental cpm–basal cpm)/(maximal cpm–basal cpm) x 100, with maximal lysis determined in the presence of 5% triton and basal lysis in the absence of antibody and effectors. To block specific FcgRs, PMNs were pre-incubated with 1 mg/ml F(ab')2 fragments of the FcgRIII-specific mAb 3G8 and with 5 mg/ml Fab fragments of FcgRII-specific mAb IV.3 for 15 minutes on room temperature before the addition of tumor cells.

*Site-Directed Mutagenesis*
The mutant form of CD32 was generated by site-specific mutagenesis of the wild type CD32 (Genecopoeia EX-T2966-M68). First, the vector was grown in DH5a in order to obtain methylated DNA. We then purified cDNA using maxiprep according to manufacturer's instruction (Macherey-nagel). The mutant was entered using PCR reaction according to the guidelines of Phusion DNA polymerase (Finnzymes, F-5302S) using primers as shown in *Figure S2a*. Template (methylated) DNA was degraded by adding 1 µl DpnI (NEB, R0176s) and incubated overnight at 37°C. Wild type and mutant vector were transformed in commercial DH5a (NEB, C2987I), and cDNA was purified. The variant was confirmed by sequencing.

*Transfection of IIA1.6 cells*
B cell lymphoma IIA1.6 cells were cultured and transfected with a protocol adapted from.[12] In short: IIA1.6 cells were electroporated at 250 V and 975 mF (Genepulser II from Bio-Rad Laboratories, Hercules, Calif) with an EX-T2966-M68 vector (GeneCopoeia) containing human FcγRIIa or the Q27W mutated FcγRIIa. Two days after transfection, the IIA1.6 transfectants were washed and cultured under selection of 2 µg/mL Puromycin (Sigma) until further use.

*Intracellular calcium flux*

For calcium assays 5 x $10^5$ IIA1.6 cells were incubated for 35 min with 4 µmol/L Fluo-3 and 10 µmol/L Fura Red (Invitrogen, Carlsbad, Calif) and anti-FcγRIIa-APC (AT10 clone, Santa Cruz) at 37C. Cells were washed twice and resuspended in Hank's balanced salt solution (HBSS). Cytosolic calcium levels were measured on Facs Canto II; after 45 sec of baseline measurement a goat anti-mouse IgG (Thermo) antibody was added and calcium flux was measured for 4½ minutes. Subsequently 2 µg/ml ionomycin (Calbiochem, San Diego, CA) was added. Area under the curve was calculated using FACS Diva and GraphPad Prism software.

*pMAPK western blot*

Microtiter plates were coated with 10 µg/mL OVA overnight, washed with PBS and incubated with 25 µg/mL of rabbit anti-OVA for 1 hour. Wild type or Q27W transfected 2A1.6 cells were plated on the OVA-IC coated plates for 2, 5, 10 and 30 minutes at 37°C and lysed in subsequently 20 µL of reducing sample buffer. Total lysates from 0,5 × $10^6$ cells were subjected to SDS-PAGE and Western blotting. The following antibodies were used: phosphorylated extracellular signal-regulated kinase (ERK; clone E10; Cell Signaling) and total ERK (clone 3A7; Cell Signaling). pMAPK levels were calculated in relation to their internal loading control MAPK.

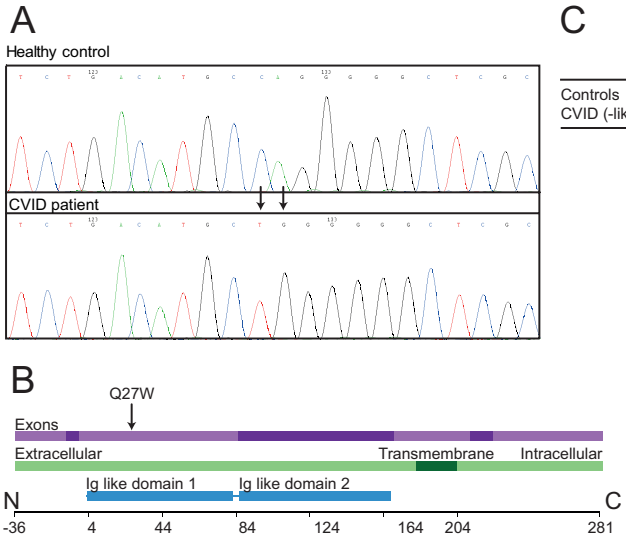
| | **Patient 1** | **Patient 2** |
|---|---|---|
| Diagnosis | Hypogammaglobulinemia | CVID |
| seks | Male | Male |
| Age (y) | 16 | 8 |
| **Immunoglobulin levels (g/l)** | | |
| IgM | 1.5 (0.28-2.4) | 0.34 (0.28-1.9) |
| IgA | 1.2 (0.7-3.6) | <0.003* (0.54-2.5) |
| IgG (total) | 5.12* (5.20-15.6) | 4.66* (5.20-14.3) |
| IgG1 | 2.9* (3.2-8.6) | 3.6* (4.3-10.2) |
| IgG2 | 0.86 (0.64-4.95) | 0.52* (0.72-4.3) |
| IgG3 | 0.08* (0.23-1.96) | 0.27 (0.13-0.85) |
| IgG4 | 0.07 * (0.11-1.57) | 0.01* (0.02-0.93) |
| **Pneumococcal polysaccharide serotype responses (IgG mg/ml)** | | |
| PnPS 1 | 1.0 | 0.07 |
| PnPS 3 | 6.6 | 0.31 |
| PnPS 4 | 2.1 | 0.59 |
| PnPS 5 | 3.9 | 0.11 |
| PnPS 6B | 0.21 | 0.13 |
| PnPS 7F | 2.9 | 0.14 |
| PnPS 9V | 2.7 | 0.62 |
| PnPS 14 | 2.0 | 1.3 |
| PnPS 18C | 1.7 | 0.24 |
| PnPS 19F | 10 | 6.3 |
| PnPS 23F | 18 | 2.8 |
| HiB | 6.76 | > 100 |
| Infections | Upper respiratory tract (otitis) | Respiratory tract infections (mostly upper) |
| Treatment | IVIg | Antibiotic prophylaxis |

**Table 1: Patient Characteristics**

Patient characteristics of two antibodydeficient patients with FcγIIA variation. Pneumococcal polysaccharide responses are measured after diagnostic vaccination with Pneumovax®. At least 8 out of the11 serotypes should reach a post vaccination IgG titer of ≥1 mg/ml for a sufficient response. PnPS: Pneumococal polysaccharide. IVIg: intravenous immunoglobulines. HiB: Haemophilus Influenzae B. *: value below reference, #: value borderline of reference.

## Results

*Detection of an immunodeficiency-associated FcγRIIa variant using next generation sequencing*
The IgG receptor FcγRIIa contributes to dendritic cell-mediated $CD8^+$ T cell activation[31] and is associated with anaphylaxis in common variable immunodeficiency, or CVID.[12] We therefore considered the FcγRIIa gene to be a candidate gene for which receptor variants predispose to CVID development. We analyzed the FcγRIIa gene (NM 001136219.1) in an existing cohort of CVID patients. Two unrelated patients were identified with an identical homozygous variant in the FcγRIIa gene at position 27 (relative to 131H, according to current nomenclature positioned at 63), where a double point mutation (CAG à TGG) leads to a change from glutamine (Q, a hydrophilic residue) to tryptophan (W, a hydrophobic residue) (*Figure 1A, patient characteristics in Table 1*). Of note, both patients were 131H/H. The mutated amino acid 27 position is located in the extracellular part of the receptor, in an Ig-like C2-like domain (*Figure 1B*), and was predicted to be damaging in a computational prediction algorithm (Polymorphism Phenotyping v2, score of 0.960; sensitivity: 0.78, specificity: 0.95). Homozygous expression of this FcγRIIa allele variant occurs with a frequency of 0.5% in a healthy Dutch control population. In our tested CVID cohort this was 4%, suggesting the variant might be a functional polymorphism that contributes to the clinical manifestation of CVID (*Figure 1C*).




C

| | Total | Heterozygous | Homozygous |
|---|---|---|---|
| Controls | 192 | 13.0% (25) | 0.5% (1) |
| CVID (-like) | 48 | 12.5% (6) | 4.2% (2) |

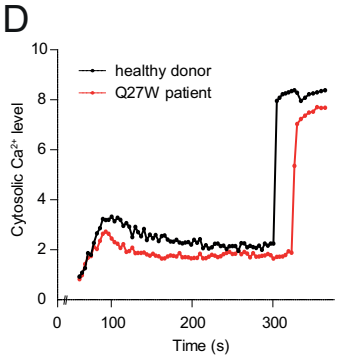



**Figure 1. Detection of the Q27W FcγRIIa polymorphism using next generation sequencing**
(A) Sequence of the FcγRIIa reveals a double point mutation (indicated by arrows) in two patients with CVID. (B) Graphic representation of the FcγRIIa protein with the different exons and introns (purple), the intra-, trans- and extracellular domain (green) and Ig like domains (blue). The position of the Q27W polymorphism is indicated by the arrow. (C) Frequencies of hetero- and homozygous Q27W variant in our CVID cohort and controls. (D) Calcium mobilization kinetics in B lymphocytes from Q27W patients with CVID (red line) and healthy donor (representative, black line).

CVID is a heterogeneous disease that can be partially explained by defective B-cell function. Although B cells predominantly express the inhibitory FcγRIIb, they do express low but functional levels of FcγRIIa.[32] We therefore assayed the Q27W FcγRIIa patients for B cell activation, using a B cell calcium flux assay triggered by B cell receptor (BCR) crosslinking.[7] Fresh patient- and healthy donor (WT) control peripheral blood mononuclear cells (PBMC) were isolated, stained for CD19 and loaded with calcium dyes, and calcium levels were measured using flow cytometry. BCR crosslinking of IgM$^+$ B cells from two separate Q27W patients exhibited calcium mobilization that was near comparable to healthy donors, as measured based on areas under the curve of the calcium tracer fluorescence over time (*Figure 1D*, αIgM $F(ab')_2$-triggering; AUC patient 1: 229, patient 2: 187, while reference healthy control children have AUC values of 209, placing the patients within 1SD of normal AUC).[7] In summary, two antibody deficient patients with an FcγRIIa Q27W polymorphism were identified, but clinical phenotype was not accompanied by defective early B cell activation, as measured by BCR-triggering induced calcium mobilization.

*FcγRIIA mRNA and protein surface expression levels are not affected by the Q27W substitution*
The FcγRIIa receptor is expressed on a variety of immune cells, amongst which are neutrophils, B-lymphocytes, thrombocytes and DCs. We therefore measured mRNA expression levels on PBMCs and neutrophils from a Q27W FcγRIIa patient and healthy control (WT) individuals. We found comparable mRNA expression levels of FcγRIIa in neutrophils of both FcγRIIa genotypes (*Figure 2A*). Next, we FACS-sorted remaining immune cell types based on a set of characteristic surface markers: monocytes (CD14$^+$, CD19$^+$), B cells (CD19$^+$, CD14$^-$), and DCs (CD14$^-$, CD19$^-$, HLA-DR$^+$, CD11c$^+$). DCs were further subdivided in BDCA-1$^+$ DCs (CD1c$^+$, CD141$^-$) and BDCA-3$^+$ DCs (CD141$^{high}$, CD1c$^-$). In all of the sorted immune cells, FcγRII mRNA expression levels were comparable between cells expressing the Q27W FcγRIIa and the common FcγRIIa variant: moderate expression on monocytes and DCs and low, but detectable expression in B cells (*Figure 2A*).

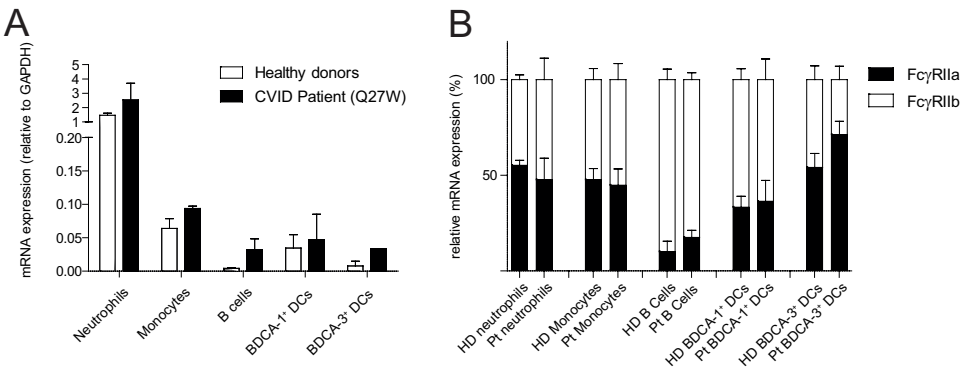



**Figure 2. FcγRII mRNA levels are unaffected by the Q27W mutation**
mRNA expression of FcγRIIa relative to GAPDH. (A) mRNA expression of FcγRIIa relative to GAPDH in different cell types of healthy donors (white bars, mean + SEM, N = 5) and a Q27W CVID patient (black bars, mean + SEM, N = 2 [same patient, 2 time points]). (B) relative FcγRIIa (black bars) and FcγRIIb (white bars) mRNA expression in healthy donor (HD, mean + SEM, N = 5) and Q27W CVID patient (Pt, mean + SEM, N = 2 [same patient, 2 time points]) immune cells.

The signaling cascade incited by FcγRIIa triggering is counteracted by through the inhibitory isoform FcγRIIb that harbors an immunoreceptor tyrosine-based inhibition motif (ITIM). We proposed that a variant in FcγRIIa might impact the relative balance of FcγRIIa signaling and FcγRIIb in cellular subsets, and thereby modulate cell function. We however found unchanged and equal contributions in neutrophils, monocytes and BDCA-3⁺ DCs, with an FcγRIIb relative dominance over FcγRIIa of 75% in BDCA-1⁺ DCs and around 90% in B cells (*Figure 2B*). Taken together these data show no difference in FcγRIIa mRNA expression between the Q27W patient and healthy (WT) controls.

We next asked if the Q27W substitution affects cell surface expression of FcγRIIa. To this end we performed whole blood stainings using surface marker expression, supplemented with neutrophil analysis based on forward and sideward scatter profile and CD14 and CD19

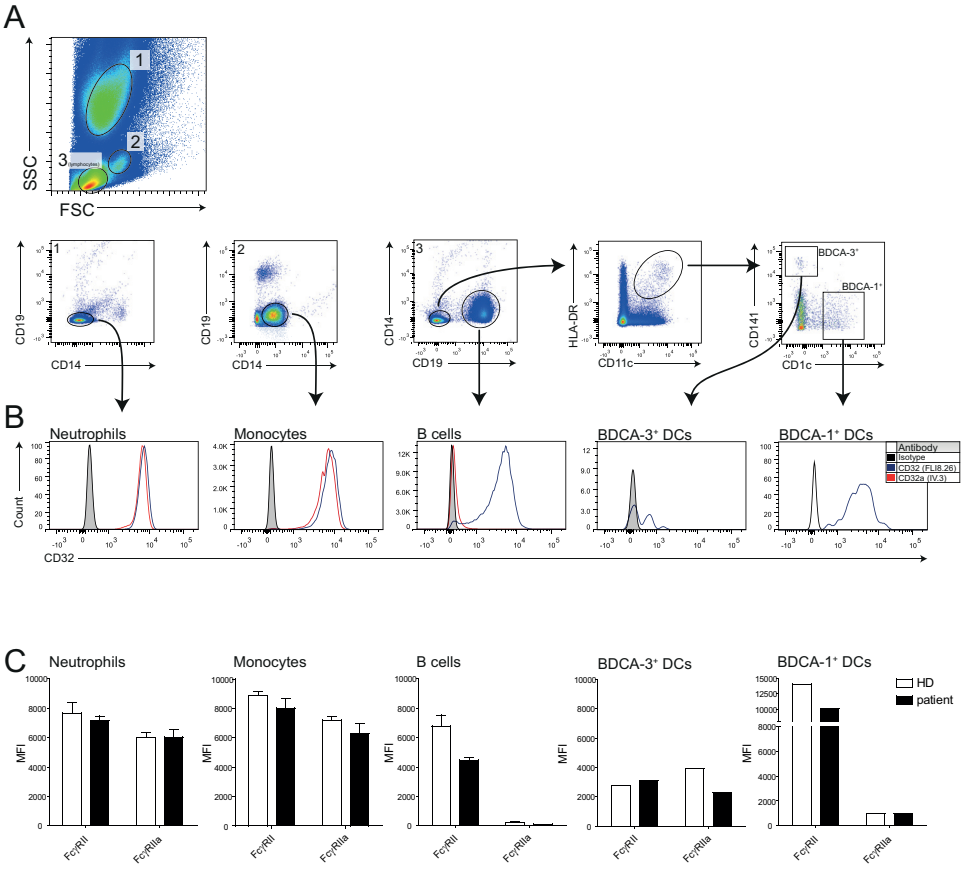



**Figure 3. FcγRII protein surface expression is unaffected by the Q27W mutation**
Gating strategy (A) for FcγRII surface expression in (1) neutrophils, (2) monocytes, (3) B cells, BDCA-3⁺ and BDCA-1⁺ DCs. (B) FcγRII (FL18.26) and FcγRIIa (IV.3) surface expression compared to isotype control (grey) on different immune cells extracted from Q27W patient blood. (C) FcγRII (left 2 bars) and FcγRIIa (right 2 bars) surface expression in healthy donor (HD, white bars, mean + SEM, N = 3) and a Q27W CVID patient (black bars, mean + SEM, N = 2 [same patient, 2 time points]) immune cells extracted from blood (Insufficient events for DC FcγRII expression on patients DC in one experiment expression in right 2 panels of B and C).

negativity (*Figure 3A*). We determined FcγRIIa expression levels using two sets of antibodies: FL18.26 clone that stains both FcγRII types (IIa and IIb), and IV.3 clone with specificity for the FcγRIIa and not IIb (*Figure 3B, representative histograms*). We confirm earlier work stating high surface expression of total FcγRII and specifically FcγRIIa in neutrophils and monocytes (*Figure 3C, left two panels*).[14] Lower relative expression levels were seen in BDCA-3$^+$ DCs, where the contribution of FcγRIIa (IV.3) was relatively high when compared to BDCA-1$^+$ DCs (*Figure 3C*, 4$^{th}$ and 5$^{th}$ panel). In both B cells and BDCA-1$^+$ DCs, the combined FcγRII expression levels were high, while the contribution of FcγRIIa (IV.3) was relatively low (*Figure 3C)*, 3$^{rd}$ and 5$^{th}$ panel, confirming earlier work.[15,31] In conclusion, the expression of FcγRIIa was similar on either cell type from either genotype (*Figure 3C*).

*Neutrophil killing capacity is not affected by the Q27W substitution*

To assess whether the Q27W variant has functional consequences to FcγRIIa functioning, we analyzed neutrophil killing capacity, also considering that neutrophils have the highest expression of the FcγRIIa. If we would find defective function in neutrophil killing capacity, such data might explain the more frequent infections observed in Q27W FcγRIIa patients (*Table 1*). We therefore performed antibody-dependent cellular cytotoxicity (ADCC) assays using $^{51}$Cr release from A431 cells using mAbs targeting the epidermal growth factor receptor (EGFR).[33] We used cetuximab (IgG1 EGFR) and panitumumab (IgG2 EGFR), which trigger ADCC in an FcγR dependent manner. IgA2-EGFR (kind gift from T. Valerius, Kiel Germany) was used as positive control, since it triggers cytotoxicity via FcγRI, and is therefore not impacted by variations in FcγRIIa. Cetuximab induced moderate killing by neutrophils (*Figure 4A*), which was not different between Q27W patient and healthy control neutrophils. Cytotoxicity induced by panitumumab (IgG2 EGFR) was also comparable to healthy controls (*data not shown*).

IgG1-mediated cytotoxicity by neutrophils is inhibited by FcγRIIIb.[34] In line with this, we observed enhanced cytotoxicity when ADCC was repeated in the presence of FcγRIIIb blocking

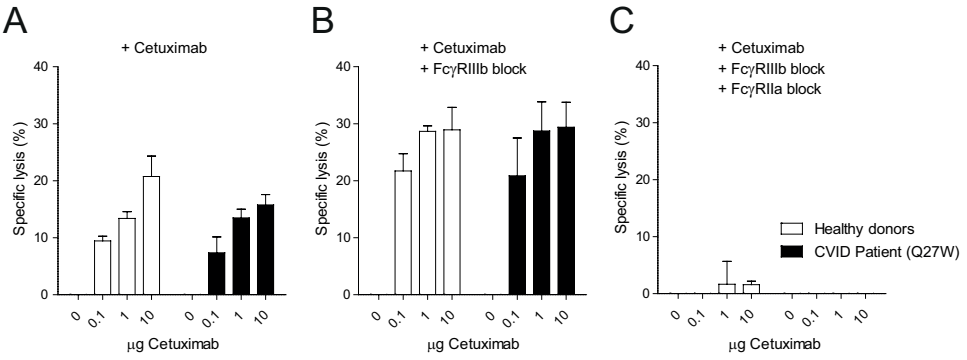



**Figure 4. No effect of FcγRIIa Q27W on PMN-mediated ADCC or BCR mediated B cell calcium mobilization**
(A) Specific lysis of A431 cells by isolated human PMNs (E:T = 80:1) in the presence of the anti-EGFR mAb cetuximab in a 4 hour $^{51}$Cr-release assay. PMNs were pre-incubated with F(ab')2 fragments of the anti-FcγRIII mAb 3G8, which led to increased killing. Bars represent no mAb, 0.1, 1 and 10 μg/ml cetuximab in healthy controls (white bars) and a Q27W patient (black bars). (B and C) Cetuximab induced ADCC in the presence of FcγRIIIb blocking (B) or both FcγRIIIb and FcγRIIa blocking (C). Data of 2 experiments in triplo are presented as mean percentage of specific lysis ± SD.

(*Figure 4B*). Cytotoxicity was fully dependent on FcγRIIa, as blocking FcγRIIa abrogated lysis (*Figure 4C*). No difference was observed between the Q27W patient (black bars) and healthy donors (white bars) in this fully FcγRIIa-dependent model system. Of note, the duration of the assay was 4 hours, possibly obscuring early FcγRIIa signaling defects that occur within the first minutes of receptor crosslinking. In conclusion, the neutrophil killing capacity is normal in patients expressing the Q27W polymorphism, at least under these experimental conditions.

*Decreased intracellular $Ca^{2+}$ mobilization and MAPK phosphorylation upon FcγRIIa cross-linking in IIA1.6 cells transfected with a Q27W variant FcγRIIa*

The antibody-dependent cellular cytotoxicity (ADCC) assays using $^{51}Cr$ release by neutrophils in combination with FcγRIIa blocking antibody is a robust assay to test FcγRIIa signaling outcomes that occur after several hours rather than minutes. To test for early consequences of the Q27W mutation in FcγRIIa, we over-expressed the wild type and Q27W variant FcγRIIa (131H) in IIA1.6 mouse pre-B cells that are normally FcγR deficient, and used these to test for early signaling events. The Q27W FcγRIIa variant was made by site-directed mutagenesis of the common FcγRIIa variant template (*Figure S1A and B*). Receptor surface expression was comparable between wild type and Q27W FcγRIIa vector transfected cells (*Figure 5A*). Next, we tested if the Q27W FcγRIIa variant is defective at cell stimulation when triggered using crosslinking antibodies. We first labelled the FcγRIIa using a mouse-anti-human APC-labelled antibody. After establishing a baseline calcium level, we added a goat-anti-mouse antibody to cross-link the surface-expressed FcγRIIa and measured intracellular calcium mobilization. We noticed that crosslinking of both Q27W FcγRIIa and control FcγRIIa on IIA1.6 cells elicited intracellular calcium flux, although Q27W FcγRIIa cells induced a significantly decreased calcium flux (*Figure 5B and 5C, 6 independent experiments*). Of note, ionomycin stimulation resulted in similar amounts of calcium mobilization in both wild type and Q27W FcγRIIa, indicating that both transfectants could elicit comparable maximal responses when triggered appropriately, and that cells were equally labelled with the calcium-sensitive dye.

We next investigated the temporal regulation of downstream FcγRIIa signaling. MAPK phosphorylation occurs upon FcγRIIa cross-linking and precedes the regulation of several transcription factors.[35] We therefore analyzed phosphorylation levels of mitogen-activated protein kinase (MAPK, originally called extracellular signal-regulated kinase, ERK).[36] We again transfected wild-type or Q27W FcγRIIa into IIA1.6 cells, rested the cells for 2 days followed by overnight starvation to reduce MAPK phosphorylation levels. Subsequently, cells were plated onto OVA (10 μg/ml, O/N) – anti-OVA IgG (25 μg/ml, 1 hour) coated plates for 2, 5, 10, 15, 20 and 30 minutes before resuspension of transfected IIA1.6 cells in lysis buffer. We then performed western blot using MAPK (clone 3A7) and pMAPK (clone E10) antibodies and quantified protein expression. pMAPK levels were calculated in relation to their internal loading control MAPK. Wildtype FcγRIIa induces MAPK phosphorylation initiated immediately and reached maximum values between 15 and 20 minutes (*Figure 5D and E black line, representative of 3 independent experiments*). Q27W FcγRIIa also induces MAPK phosphorylation, but at a reduced rate that reaches maximal phosphorylation between 20 and 30 minutes (*Figure 5D and E red line, representative of 3 independent experiments*). Moreover, at timepoints 5 and 10 minutes, we observed a marked difference in MAPK phosphorylation (*Figure 5F, 3 independent experiments, t=2 in Figure S1*). This data confirms that the Q27W polymorphism results in delayed signaling.

In conclusion, we here show that the Q27W mutation in FcγRIIa causes both decreased intracellular calcium mobilization and delayed MAPK phosphorylation upon receptor triggering, at least in these model B cells. Recent work using B cells of X-linked immunodeficient mice showed a defective MAPK signaling phenotype that linked to CVID-associated defects in generation of T cell independent type 2 antibody responses such as to bacterial polysaccharides. In analogy, delayed signaling of the Q27W FcγRIIa variant in B cells we propose suppresses the elicitation of downstream signaling circuits, which may contribute to a CVID phenotype.

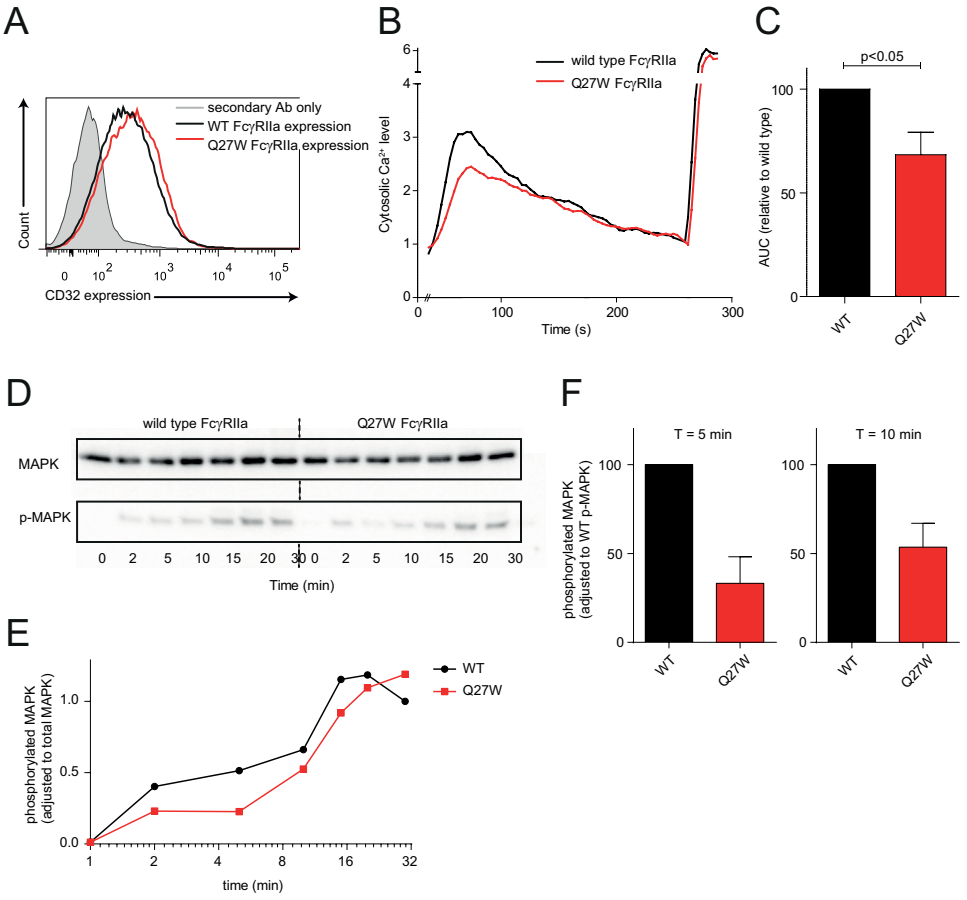



**Figure 5. Decreased early signaling upon FcγRIIa triggering in Q27W transfected IIA1.6 cells**
(A) IIA1.6 cells were transfected with wildtype (black line) or Q27W (red line) FcγRIIa. Transfection efficiencies were comparable (detection using FACS, antibody: AT10-APC). (B and C) Transfected wildtype (black line) or Q27W (red line) were loaded with calcium dyes and FcγRIIa dependent calcium flux was induced using an anti CD32 antibody (AT10), cross-linked with a goat-anti-mouse antibody. (B) Intracellular calcium mobilization was less in Q27W transfected cells (representative). (C) Summary of 6 independent experiments (mean and SEM) comparing Q27W AUC (red bar) to wildtype AUC (black bar). (D-F) Western blot for MAPK phosphorylation. (D) Representative western blot. (E) Kinetics of MAPK phosphorylation over time, relative to total MAPK. (F) Amount of phosphorylated MAPK in the Q27W mutant relative to WT at T=5 and T=10 (3 independent experiments, mean and SEM).

## Discussion

The FcγRIIa is expressed on multiple immune cells and elicits a range of effector functions. Allelic variations within this receptor have been described, and are associated with differences in susceptibility to immune-mediated disease.[12, 23-27] Most of the work done on FcγRIIa gene variation focuses on the 131H/R polymorphism. This commonly found receptor variant relates to changes in IgG subclass binding, and is therefore associated with susceptibility to certain infections as well as auto-immune diseases.[37,38] We here describe a new FcγRIIa variant in which an amino acid exchange is found outside of the IgG ligand binding domain, but in a predicted FcγRIIa dimerization domain. The variant was found in two separate patients that were both of the 131H phenotype. We also used the 131H variant for the assays done on FcγRIIa transfected cells.

Recently, a related but distinct FcγRIIa gene variant was described, also linking this receptor to CVID.[12] Here, not FcgRIIa protein function was changed but rather an FcγRIIa RNA splice variant was formed, that associated with increased propensity to develop anaphylaxis upon intravenous Ig (IVIg) administration. This finding underscores the importance of FcγRIIa function in health and disease. We here describe a novel FcγRIIa variation that we found in CVID patients, although we only succeeded in indirectly linking its functional effects to clinical manifestations of CVID. We found the FcγRIIa variant causes a delay in MAPK signal propagation that manifests immediately upon ligand binding, supporting that a variety of cells that express FcγRIIa may be affected and contribute to disease. A recent paper showed that impaired activation of the MAPK pathway upon BCR triggering relates to defects in antibody production upon vaccination with T cell-independent type 2 antigens, a deficiency that is also seen in CVID patients.[39] In our own experiments, we found that BCR triggering resulted in a small reduction in calcium flux generation (*Figure 1D*). Significant defects we only observed when we analysed MAPK and calcium signal transduction elicited by triggering of the FcγRIIa itself. Our data supports that BCR signalling is probably relatively normal. Instead, our work supports that FcγRIIa serves as a risk factor for the development of CVID due to its role in elicitation of MAPK signaling upon binding of immune complexes, for normal B cell activation and differentiation into antibody producing plasma cells.

CVID is a heterogeneous disease, which develops as a consequence of absence or malfunction of proteins involved with immunoglobulin production by plasma and memory B-cells. Although several genetic predispositions were found in recent years, a specific cause can only be found in 10-15% of patients with CVID.[5] The FcgRIIa Q27W variation was found homozygous in 4% of CVID patients, compared to 0.5% in the healthy Dutch population. The two patients we found were both homozygous for the Q27W variation, and received antibody therapy (IVIg) or antibiotic prophylaxis for their immunodeficiency. We, however, do not argue that the FcγRIIa Q27W mutation is the sole cause of CVID (or hypogammaglobulinemia) in these patients. Instead, we propose that the Q27W substitution in FcγRIIa probably contributes to CVID, and is not solely accountable for the phenotype in these patients.

We and others investigated the role of FcγR in antigen cross-presentation by DCs,[31, 40-44] and it is not unlikely that this mechanism is impaired in cells expressing the Q27W FcγRIIa variant. A decrease in cross-presentation relates to impaired anti-viral $CD8^+$ T-cell priming or activation, and may thereby explain the observed frequent upper airway tract infections

in Q27W FcγRIIa expressing CVID patients. Limited amounts of DCs from these pediatric patients prevented further investigation into the effect of the Q27W FcγRIIa on these cells. However, in transfected IIA1.6 cells, the Q27W FcγRIIa variant cells show a delay in signaling. This causes a delay in elicitation of downstream signalling circuits, including those governed by MAPK pathways. We therefore confirm FcγRIIa a candidate gene that predisposes to the development of CVID, not only through RNA splicing but probably also signaling via the ITAM motif embedded within the FcγRIIa chains.[12]

## Acknowledgements

We thank the LTI and Boes laboratory for helpful discussions. We thank Thomas Valerius for providing us with the IgA2-EFGR antibody.

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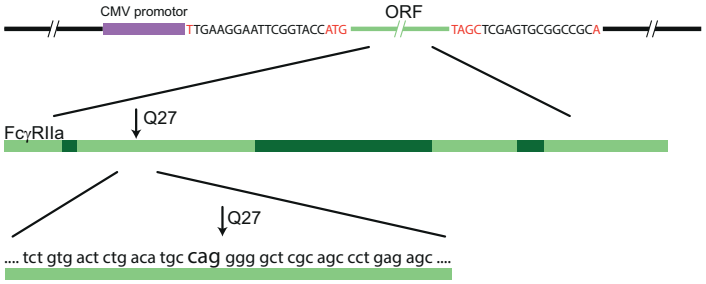
## Supplemental Figures

A

| | | sequence | | |
|---|---|---|---|---|
| Wildype | forward | GTGACTCTGACATGC | CAG | GGGGCTCGCAGC |
| Mutant | forward | GTGACTCTGACATGC | TGG | GGGGCTCGCAGC |
| Mutant | reverse | GCTGCGAGCCCC | CCA | GCATGTCAGAGTCAC |

B




C

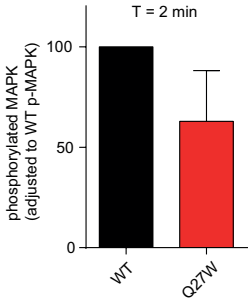
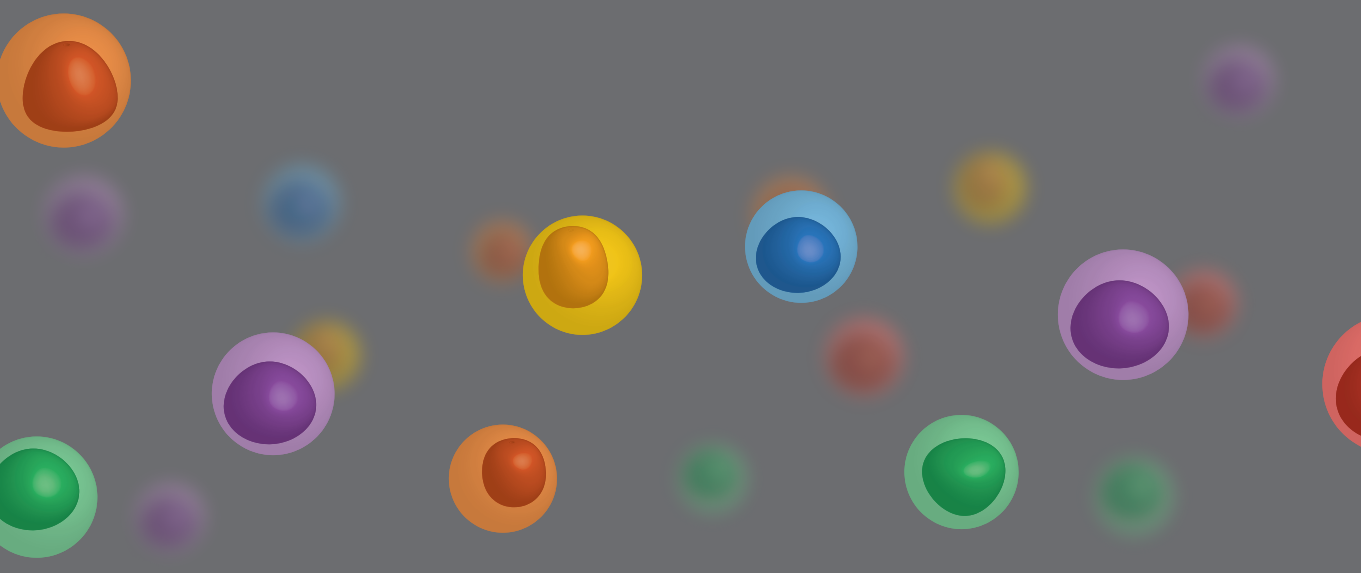



**Figure S1. Site-directed mutagenesis of the FcγRIIa gene**
(A) Primers used for the PCR reaction. (B) Schematic representation of the vector (EX-T2966-M68) expressing the FcγRIIa gene. Shown in detail is the open reading frame with the Q27W position. (C) Kinetics of MAPK phosphorylation over time, relative to total MAPK. Shown are amounts of phosphorylated MAPK in the Q27W mutant relative to WT at T=2 (3 independent experiments, mean and SEM).

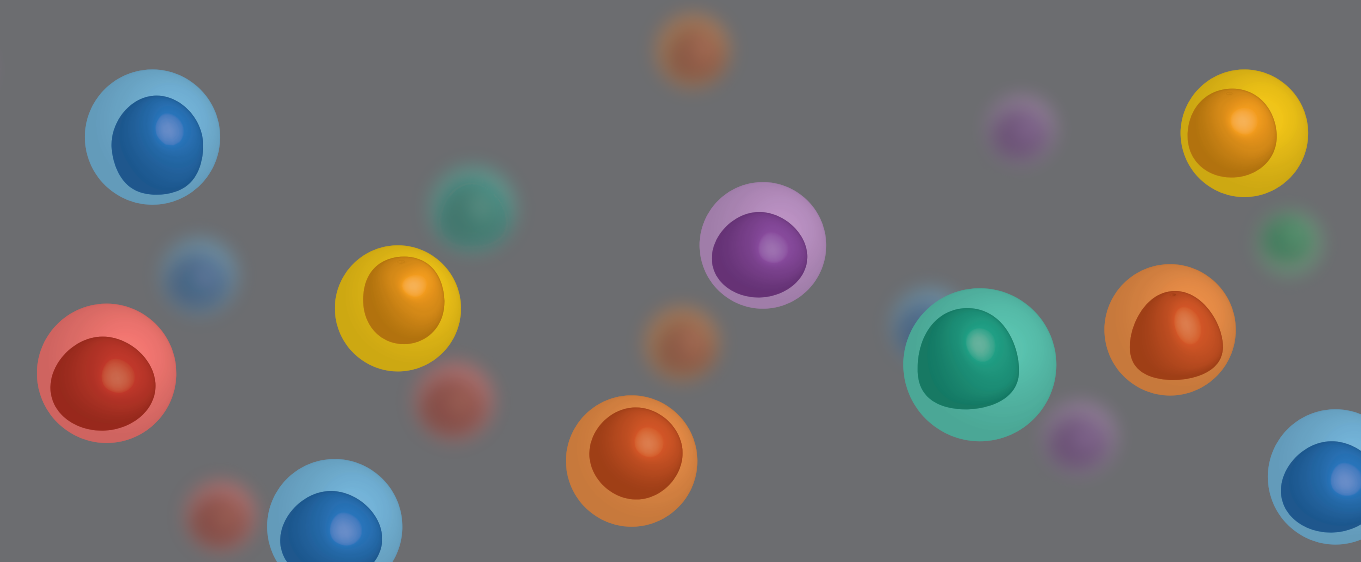
# 5



# Cognate CD4 T-cell licensing of dendritic cells heralds CD8 T-cell immunity after allogeneic umbilical cord blood transplantation

Thijs W.H. Flinsenberg, Manon Jansen, Dan Koning, Colin de Haar, Maud Plantinga, Rianne Scholman, M. van Loenen, Stefan Nierkens, Louis Boon, Debbie van Baarle, Mirjam Heemskerk, Jaap-Jan Boelens, Marianne L. Boes

Submitted



## Abstract

Reactivation of human cytomegalovirus (CMV) is hazardous to patients undergoing allogeneic cord-blood transplantation (CBT), lowering survival rates by approximately 25%. While antiviral treatment ameliorates viremia, complete viral control requires $CD8^+$ T-cell-driven immunity. Mouse studies suggest that cognate antigen-specific $CD4^+$ T-cell licensing of dendritic cells is required to generate effective $CD8^+$ T-cell responses. For humans this was not fully understood. We here show that $CD4^+$ T-cells are essential for licensing of human DCs to generate effector and memory $CD8^+$ T-cell immunity against CMV in CBT patients. First, we show in CBT recipients that clonal expansion of CMV-pp65 specific $CD4^+$ T-cells precedes the rise in CMV-pp65 specific $CD8^+$ T-cells. Second, the elicitation of CMV-pp65 specific $CD8^+$ T-cells from rare naïve precursors in cord blood requires DC licensing by cognate CMV-pp65-specific $CD4^+$ T-cells. Finally, also $CD8^+$ T-cell memory responses require $CD4^+$ T-cell-mediated licensing of DCs in our system, by secretion of IFN-γ by pp65-specific $CD4^+$ T-cells. Together these data show that human DCs require the licensing by cognate antigen-specific $CD4^+$ T-cells to elicit effective $CD8^+$ T-cell-mediated immunity and fight off viral reactivation in CBT patients.

## Introduction

CMV seropositive patients who are immunocompromised are at increased risk for developing potentially life-threatening CMV reactivation. Especially after allogeneic cord-blood (CB) transplantation (CBT), the first weeks of immune reconstitution are hazardous for developing cytomegalovirus (CMV) reactivation, which is associated with decreased survival rates.[1,2] Antiviral treatment can reduce CMV viremia, but effective viral control requires induction of CMV-directed immunity by T-lymphocytes. In particular $CD8^+$ cytotoxic T-lymphocytes (CTLs) fulfill a predominant role in protection against CMV disease.[2-4] Therefore, strategies that increase early CMV-specific adaptive immune responses after transplantation are currently explored, which could ultimately help to establish full clearance of, and long-term immunological memory against CMV. In the human setting, cell–based therapy is being explored, geared towards dendritic cell (DC)-mediated activation of CTLs.[5,6] The elicitation of antigen-specific CTL immunity was in mouse models shown to require cognate $CD4^+$ T-cell licensing.[7-10] Furthermore, priming of naïve $CD8^+$ T-cells requires for both the $CD4^+$ T-helper cells and $CD8^+$ T-cells to recognize antigen on the same antigen presenting cell.[5,11,12] Such $CD4^+$ T-cell help can involve CD40-ligand binding to CD40 on DCs.[9,13,14] For humans, a requirement for $CD4^+$ T-cell help in DC licensing for formation of effector and memory CTLs was not yet demonstrated. It is also not yet clear which are the signaling pathways through which $CD4^+$ T-cells might execute their licensing.



CMV-specific $CD4^+$ T-cell clones are present in the healthy population, suggesting a role for antigen-specific $CD4^+$ T-cells in immunity against CMV as 50-80% of adults experiences CMV infection in their lifetime.[6,15] Moreover, effective control of CMV infection was attained in patients when CMV specific T-cells of which 77% were $CD4^+$ T-cells, were infused.[16] Further, human DCs loaded with both HLA class I and II/peptide complexes were more effective at generating antigen-specific CTL responses compared to those loaded with solely MHC class I/peptide complexes.[17] In a different setting, not only the absence of antigen-specific CTLs, but also the absence of specific $CD4^+$ T-helper cells resulted in higher CMV loads.[18] Thus, $CD4^+$ T-helper cells are likely to participate in CMV control.

We set out to clarify the role of human $CD4^+$ T-helper cells in DC-licensing for CTL-mediated immunity for both CTL priming and memory CTL activation. First, we show in CBT recipients that clonal expansion of CMV-pp65 specific $CD4^+$ T-helper cells precedes the expansion of primary CMV-pp65 specific CTLs. We clarified that DC licensing is cognate, as expansion of primary CMV-pp65 specific CTLs from naïve CB precursors requires presence of pp65-specific $CD4^+$ T-helper cells, in co-cultures. Finally, also DC licensing is required for CTL memory, as DCs licensed by $CD4^+$ T-cells, which they do through secretion of IFN-γ, stimulate much more efficient CMV-pp65 specific CTL memory responses. Together these data imply that in humans $CD4^+$ T-helper cells are pivotal DC licensing to elicit $CD8^+$ T-cell-immunity during CMV reactivation in CBT patients.

## Materials and Methods

*Patient inclusion and human samples*
Approval for this study was obtained by the ethics committees of the University Medical Centre Utrecht (METC-05-143, METC-11-063 and METC-13-437). Written informed consent was obtained from all participating patients or their legal representatives prior to CBT. In this consent it was stated that their medical data may be used for research purposes. According to the hospital's standard operating procedures, regular blood samples were taken for viral load detection by qPCR and T-cell number measurements (see next paragraph). All children below the age of 18 who received an allogeneic CBT between 2010 and 2013 at the HSCT unit of the Wilhelmina Childrens Hospital were evaluated. All patients received a Fludarabine and Busulfan containing regimen with early-given (day -9) anti-thymoglobulin.[19] 8 patients suffered from CMV reactivation post-CBT. These patients were all CMV positive prior to transplantation. 2 were excluded because maximum CMV loads did not reach 1000 copies/ml. From the 6 other patients, CMV loads, $CD4^+$ and $CD8^+$ count were evaluated and plotted over time. We also included 8 control patients who did not have a events that are likely to impact the T-cell counts (T-cell impacting events) such as viral reactivations> 1000 cp/mL (HHV6, CMV, EBV or Adenovirus), GvHD >= grade 2 or graft rejection. $CD4^+$ T-cell counts within the first 3 months after CBT were evaluated using the AUC trapezoidal method: $\sum(\text{Cell number}_{\text{timey}} + \text{cell number}_{\text{timex}})/2)*(\text{time}_y - \text{time}_x)$ (ref. 20). Trends in $CD4^+$ T-cell count were evaluated using a Pearson-correlation coefficient.

*Immune phenotyping*
Immune phenotyping was performed on whole-blood samples every other week once leukocyte count was $>0.4 \times 10^9$/L. Absolute numbers of T-cells ($CD3^+$), helper T-cells ($CD3^+CD4^+$), cytotoxic T-cells ($CD3^+CD8^+$) were determined using TruCOUNT technology (BD Biosciences). A volume of 20 µL of CD3-FITC, CD45-PerCP, CD19-APC or CD3-FITC, CD8-PE, CD45-PerCP, CD4-APC reagent (MultiTEST; BD Biosciences) was added to a TruCOUNT tube containing a known quantity of beads, followed by 100 µL of EDTA-treated whole blood and incubated for 15 minutes at room temperature. RBCs were subsequently lysed for 15 minutes with 450 µL of FACS Lysing Solution (BD Biosciences). Samples were acquired using a FACSCalibur and analyzed with Multiset software (BD Biosciences). Qualitative and subset analysis of T-cell compartments were performed as described previously.[21]

*Cord blood dendritic cell culture*
$CD34^+$ cells were isolated according to manufacturers instructions (Miltenyi Biotec) and expanded using 20 ng/ml IL-3 (Invitrogen), 20 ng/ml IL-6 (BD Biosciences, 50 ng/ml SCF and 50 ng/ml FLT3-L (both from Peprotech). For DC culture, $3*10^6$ $CD34^+$ cells were cultured in a T25 flask (Thermo) in X-vivo medium (Lonza) containing 2mM l-glutamine, 100 U/mL penicillin-streptomycin and 5% human serum in the presence of 20 ng/ml GM-CSF, 20 ng/ml IL-4 (all from invitrogen), 20 ng/ml SCF and 100 ng/ml FLT3-L at 37°C and 5% $CO_2$ for 7 days.

*$CD8^+$ T-cell priming assay*
CB $CD34^+$ derived DCs were loaded with 10 µg/ml pp65 (Miltenyi Biotec, purity > 95%, low endotoxin; < 10 EU/mL), medium or 10 µg/ml BSA (Roche, 10.000 DCs in 100 µL X-vivo 5% + human serum per well, 96 wells plate [Thermo]).Then 50.000 donor matched naive $CD8^+$

T-cells were added (separated from $CD34^-$ fraction using Miltenyi Biotec MACS beads according to manufacturers instructions) together with medium, 50.000 $CD4^+$ T-cells (separated using MACS beads) or 10 µg/ml CD40 agonist clone 7 (Bioceros). All was co-cultured for 3 weeks at 37°C and 5% $CO_2$ for 7 days. On day 8 and 15, $CD34^+$ derived DCs were loaded with $1.10^{-6.5}$M NLV peptide and irradiated with 30 Gy. 10.000 DCs were plated per well and the T-cells were added for re-stimulation. Every week at day 2 and 5, IL-7 and IL-15 (Immunotools) were added, both at a final concentration of 5 ng/ml. After 3 weeks, cells were stained with an HLA-A2 $pp65_{495\text{-}503}$ pentamer (ProImmune) and positive cells were single-cell sorted and stimulated for several weeks as described under $CD8^+$ T-cell cloning. Prior to crypopreservation, a small aliquot of T-cells (1-5 x $10^5$) was harvested for TCR sequencing.

*TCRβ chain sequencing*
TCRβ chains were sequenced as previously described.[22] Briefly, a one-sided anchored RT-PCR was performed in order to amplify TCRβ mRNA. Amplified products were purified from agarose gel and ligated into a pGEM-T Easy Vector (Promega), followed by transformation into chemically competent E. coli DH5α bacteria. 32 bacterial colonies were screened for the presence of a TCR construct and subsequently sequenced via capillary electrophoresis. Sequences were analysed using web-based software (www.imgt.org)[23] and TCRs were identified using the official ImMunoGeneTics nomenclature.[24]

*Cross-presentation assay*
Cross-presentation essays with MoDCs and NLV-specific $CD8^+$ T-cell clones were performed as described in (ref. 25), and can also be found in the supplementary methods.

*$CD4^+$ T-cell cloning*
HCMV pp65-specific $CD4^+$ T-cells were isolated from HLA-DRB1*0101$^+$ PBMCs using the IFN-γ secretion assay (Miltenyi Biotec, Bergisch Gladbach, Germany). Briefly, PBMC were stimulated with 2 µg/ml pp65-KYQEFFWDANDIYRI peptide (HLA-DRB1*0101 binding pp65 peptide), and after 4 hours of stimulation, IFN-γ secreting $CD4^+$ T cells were isolated using FACS. CMV-pp65-specific $CD4^+$ T-cell line was three weekly stimulated with irradiated (30 Gy) allogeneic PBMCs (1 x $10^6$ cells/ml), and 800 ng/ml PHA (Murex Biotec Limited, Dartford, UK).

*DC maturation assay*
Day 4½ monocyte-derived DCs (MoDCs) were incubated O/N in the presence of medium, pp65 (3 µg/mL), pp65 and CD40 antibody clone 7 (10 µg/mL, Bioceros), pp65 and CD40L antibody clone 5c8 (10 µg/mL, Bioceros), pp65 and 200.000 $pp65_{509\text{-}523}$ specific $CD4^+$ T-cells or Poly(I:C) 30 µg/mL (Sigma-Aldrich) and LPS (100 ng/mL (Sigma-Aldrich)). Cells were subsequently harvested and analyzed for co-stimulatory marker expression using flow cytometry.

*Detection of cytokines in culture supernatant*
Cytokine concentrations were measured by the MultiPlex Core Facility of the LTI using Luminex technology with in house developed bead-sets and Bio-Plex Manager Version 6.1 software (Bio-Rad Laboratories) as previously described.[26]

*Detection of CMV-specific $CD4^+$ and $CD8^+$ T-cells in patient samples*
NLV specific $CD8^+$ T-cells were detected using HLA-A2 $pp65_{495\text{-}503}$ pentamer (ProImmune) or

HLA-B7 $pp65_{417-426}$ tetramer (produced in house). Antigen specific $CD4^+$ and $CD8^+$ T-cells were detected with intracellular IFN-γ staining after stimulation with a pp65 and IE-1 15-mer overlapping peptide mix (JPT peptide technologies), as described in [27].

## Results

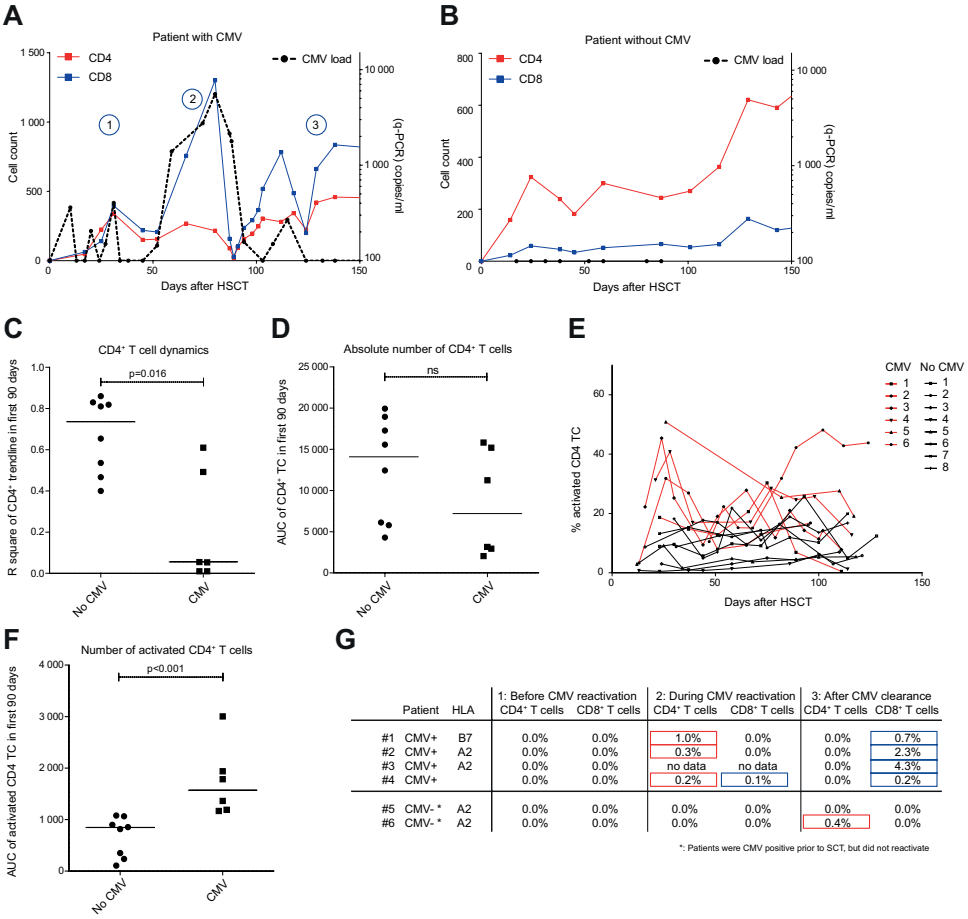
*$CD4^+$ and $CD8^+$ T-cell dynamics after cord blood transplantation in patients with and without CMV reactivation*

We studied early T-cell reconstitution in pediatric patients undergoing complete immune reconstitution through allogeneic cord blood transplantation (CBT), in relation to CMV reactivation. Six CBT recipients experienced CMV reactivation (>1000 virus copies/ml), and eight control patients were included (without infectious complications). We analyzed the reconstitution of $CD4^+$ and $CD8^+$ T-cells, and CMV loads (*Figure 1*). We observed expansion and contraction of the $CD8^+$ T-cell population as CMV viral load increased and regressed (*Figure 1A, Supplementary 1A-C*). The control patients instead experienced a consistent and gradual increase in $CD8^+$ T-cell numbers during reconstitution (*Figure 1B, Supplementary 1D-F*). The $CD4^+$ T-helper cell numbers also fluctuated more in patients with CMV reactivation than in control patients (*Figure 1C*). Such an expansion and contraction pattern for $CD4^+$ T-cells was previously observed in reconstitution under viral pressure.[28] Additional support for $CD4^+$ T-cells in CMV immunity comes from CBT patients, showing that recovery of $CD4^+CD45RA^+$ T-cells is required to clear CMV viremia.[29] Of note, while the total number of $CD4^+$ T-cells was comparable during the first 90 days after CBT (*Figure 1D*), in CMV-reactivating patients the percentage of $CD4^+$ T-cells that was activated, as expressing an $HLA\text{-}DR^+/CD38^+$ phenotype, was increased (*Figure 1E-F*).

*CMV-specific $CD4^+$ T-helper cells precede primary CMV-specific CTL expansion after CBT*

We hypothesized that $CD4^+$ T-cells, through DC-licensing, may support CMV-specific CTL responses, as was shown in mouse-based research.[30-32] Cognate interaction between $CD4^+$ T-helper cells and DCs would thereby enable DC to stimulate more effective CTL responses. To first investigate expansion of the primary CMV-specific CTL population in relation to CMV viremia, we analyzed PBMCs from 4 available CBT-recipients who exhibited CMV reactivation (*Figure 1G, #1-4*). We measured a sample prior to and during CMV reactivation, and after CMV control (*timepoints indicated in Figure 1A*). As control samples, we included samples from two patients, numbers 5 and 6, who carried CMV prior to CBT, yet did not reactivate (*Figure 1G*). In all CBT patients who cleared CMV reactivation we observed expansion of the primary CMV-specific CTL population (*Figure 1G, right column, patients 1-4; mean: 127 days post-SCT*). These cells were of CB origin, as confirmed by chimerism analyses (*data not shown*). The two control patients 5 and 6 did not elicit CMV-specific $CD8^+$ T-cells at 120 and 180 days post-SCT. Early on during CMV reactivation, CMV-specific CTLs did not yet expand, except for in patient number 4 (*Figure 1G*). Considering the CMV-specific $CD4^+$ T-helper cell population, we next measured IFN-γ production in $CD4^+$ T-cells after stimulation with a CMV pp65-overlapping peptide-mix as described (*Supplementary figure 2*).[3,18,33] We observed expansion of the primary CMV-specific $CD4^+$ T-helper cell population in all analyzed samples of reactivating patients, early on during CMV reactivation (*Figure 1G, middle column*). Finally, in control patient 6, we detected CMV-specific $CD4^+$ T-cells at day 180, indicating that CMV-positive patients (IgG-positivity prior to SCT) may eventually develop anti-CMV T-cells, but later than patients that re-

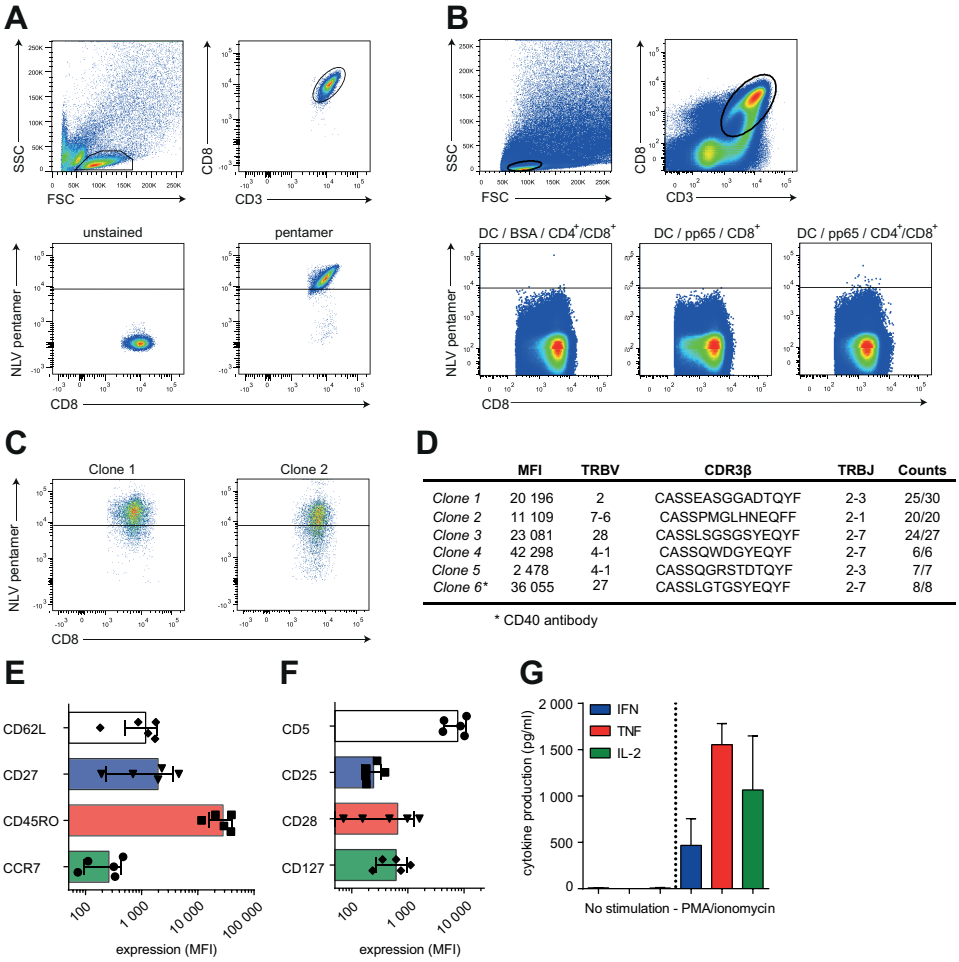
activate. Taken together, recovery of the CMV-pp65 specific CD4⁺ T-cell population precedes expansion of primary CMV-pp65 specific CTLs, supporting a role of CD4⁺ T-cells in CD8⁺ T-cell priming.




| Patient | HLA | 1: Before CMV reactivation CD4⁺ T cells | 1: Before CMV reactivation CD8⁺ T cells | 2: During CMV reactivation CD4⁺ T cells | 2: During CMV reactivation CD8⁺ T cells | 3: After CMV clearance CD4⁺ T cells | 3: After CMV clearance CD8⁺ T cells |
|---|---|---|---|---|---|---|---|
| #1 CMV+ | B7 | 0.0% | 0.0% | 1.0% | 0.0% | 0.0% | 0.7% |
| #2 CMV+ | A2 | 0.0% | 0.0% | 0.3% | 0.0% | 0.0% | 2.3% |
| #3 CMV+ | A2 | 0.0% | 0.0% | no data | no data | 0.0% | 4.3% |
| #4 CMV+ | | 0.0% | 0.0% | 0.2% | 0.1% | 0.0% | 0.2% |
| #5 CMV- * | A2 | 0.0% | 0.0% | 0.0% | 0.0% | 0.0% | 0.0% |
| #6 CMV- * | A2 | 0.0% | 0.0% | 0.0% | 0.0% | 0.4% | 0.0% |

*: Patients were CMV positive prior to SCT, but did not reactivate

**Figure 1: T-cell dynamics in CBT recipients**
(A,B) T-cell development over time in CBT recipients with (A) or without (B) CMV reactivation (load >1000 copies/ml). Red and blue line represents CD4⁺ and CD8⁺ T-cell numbers respectively (absolute counts). The black dashed line represents CMV loads (copies/ml). The numbers 1,2 and 3 (in blue circles) correspond with referred time points in figure 1G. (C) CD4⁺ T cell dynamics (R square of CD4⁺ trend-line) and median in the first 90 days after transplantation in CBT recipients without (dots) or with (squares) CMV reactivation. (D) Total CD4⁺ T-cell numbers (AUC) and median in the first 90 days after transplantation in CBT recipients without (dots) or with (squares) CMV reactivation. (E,F) The percentage of activated CD4⁺ T-cells in CBT recipients with (red lines) or without (black lines) CMV reactivation, AUCs and median in the first 90 days in (F). (G) Percentages of pp65-specific CD4⁺ and CD8⁺ T-cells (tetramer and/or IFN-γ release upon pp65-peptide-mix stimulation) before CMV reactivation (1), during CMV reactivation (2) and after CMV clearance to below detection limits (3, see figure 1A) in 4 CBT recipients with reactivation (1-4) and 2 CBT recipients without (5 and 6) reactivation. No samples were available during CMV reactivation for patient #3. Significance in C, D and F was determined using a non-parametric Mann-Whitney test.




| | MFI | TRBV | CDR3β | TRBJ | Counts |
|---|---|---|---|---|---|
| *Clone 1* | 20 196 | 2 | CASSEASGGADTQYF | 2-3 | 25/30 |
| *Clone 2* | 11 109 | 7-6 | CASSPMGLHNEQFF | 2-1 | 20/20 |
| *Clone 3* | 23 081 | 28 | CASSLSGSGSYEQYF | 2-7 | 24/27 |
| *Clone 4* | 42 298 | 4-1 | CASSQWDGYEQYF | 2-7 | 6/6 |
| *Clone 5* | 2 478 | 4-1 | CASSQGRSTDTQYF | 2-3 | 7/7 |
| *Clone 6** | 36 055 | 27 | CASSLGTGSYEQYF | 2-7 | 8/8 |

* CD40 antibody

**Figure 2: CD4⁺ T-cell licensing of DCs is necessary to prime naïve CD8⁺ T-cell in vitro**
(A) Gating strategy and pentamer staining of CMV-pp65 specific CD8⁺ T-cells. (B) Gating strategy and pentamer staining of primed CB-derived CD8⁺ T-cells. DCs were loaded with 10 µg/ml BSA (left graph and figure S3A) or µg/ml pp65 (middle and right graph) and co-cultured with donor matched naïve CD8⁺ T-cells in the absence (middle graph) or presence (left and right graph) of CD4⁺ T-cells. Pentamer-high events (>log 4 intensity) were single-cell sorted and clonally expanded for 4-6 weeks (representative of 6 independent experiments). (C) Representative pentamer staining of two individually-derived CD8⁺ T-cell clones. (D) Characteristics of 6 clones from 3 independent experiments. Shown are MFI values of pentamer stainings and TCR sequences. Clone 6 was produced using a CD40 agonist antibody. (E,F) Phenotype analysis of pp65-specific CD8⁺ T-cell clones. (G) Cytokine production (IFN-γ, TNF and IL-2, pg/ml) of CD8⁺ T-cells after PMA/ionomycin stimulation.

*CD4⁺ T-cell licensing of DCs is necessary to prime naïve CD8⁺ T-cell in vitro*

To address whether cognate CD4⁺ T-cells facilitate DC licensing for CD8⁺ T-cell priming, we performed co-cultures of CB-derived naïve CD8⁺ T-cells with donor-matched CD34⁺ derived DCs, in the presence or absence of polyclonal donor-matched CD4⁺ T-cells. DCs had been pre-loaded with pp65 protein or BSA, and co-cultures were allowed to proceed for 3 weeks

duration. Using HLA-A2 pentamers loaded with the CMV-derived peptide NLVPMVATV (NLV/A2 in short) we identified $pp65_{495\text{-}503}$ –specific CTLs (*Figure 2A*). Only when $CD8^+$ T-cell priming had been performed in the presence of pp65 protein and $CD4^+$ T-cells, did we observe $CD8^+$ T-cells that bound NLV/A2 pentamers (bright fluorescence >log 4 intensity) (*Figure 2B and Supplementary 3A*). To confirm that NLV/A2-reactivity represents $pp65_{495\text{-}503}$ –specific $CD8^+$ T-cells, we performed single cell-sorting of events over log 4 intensity and derived clones. As control, we sorted several cells from the cultures with BSA or pp65 without $CD4^+$ T-cells (>log 3.5 intensity, as not much NLV/A2-reactivity was present). We succeeded in derivation of 5 independent CMV-specific CTL clones, but only from DC/CTL cultures supplemented with both pp65 and $CD4^+$ T-cells (*Figure 2C, Supplementary 3B*). Of note, we derived one CMV-specific CTL clone from DC/CTL cultures supplemented with both pp65 protein antigen and anti-CD40 antibody but no $CD4^+$ T-cells. Together, these data show that $CD4^+$ T-cells can license DCs to expand a primary CTL population, and that such DC licensing can involve CD40-CD40L interaction, as previously observed in mice (*Figure 2D, clone 6*).[7,9,13,34,35]

We wished to confirm CMV-reactivity of the expanded CTL clones using a second method, by DNA sequencing of the recombined CDR3β TCR regions (*Figure 2C-D*). We found that our derived CDR3β sequences are frequently shared in CMV-specific $CD8^+$ T-cells, supporting CMV specificity (*Figure 2D*).[36] CDR3β sequence variants were unique and not yet described.[36]

We next investigated the cellular characteristics of CTL clones. All clones had an effector memory phenotype ($CD45RO^{high}/CCR7^-/CD62L^{int}/CD27^{int}$ *Figure 2E*).[37] CD28 expression varied between different clones (*Figure 2F*) and none of the clones expressed PD-1 or CTLA-4 in resting state (*data not shown*). Functionally, we could not detect cytokine production, possibly since clonal expansion ensued for nearly three months inducing T-cell exhaustion. Using PMA/ionomycin, we circumvented this state, now yielding high levels of cytokine production (*Figure 2G*), indicating that these clones were able to respond appropriately. In conclusion, we used human CB-derived co-cultures of antigen-loaded DCs and naïve lymphocytes, that $CD4^+$ T-cell licensing of DCs is necessary for antigen-specific $CD8^+$ T-cell priming.

*Cognate $CD4^+$ T-cells induce licensing of DCs for enhanced memory CTL responses*

We next asked whether human cognate $CD4^+$ T-cells are necessary to licensing of DCs for $CD8^+$ T-cell memory.[10] To this end, we derived human HLA-A2*01$^+$/HLA-DRB1*01$^+$ monocyte-derived (Mo)DCs and loaded these with pp65 protein antigen for presentation via HLA-DRB1 and HLA-A2. Next, we induced licensing of the DCs by administration of CMV-$pp65_{509\text{-}523}$-specific $CD4^+$ T-cells recognizing HLA-DRB1*01/$_{\text{KYQEFFWDANDIYRI}}$ complexes presented by the DCs (50,000 DCs and increasing numbers of $CD4^+$ T-cells).[38] Medium was refreshed to avoid the possibility that $CD4^+$ T-cell derived cytokines directly stimulate CTL activation. We added 50,000 memory CMV-$pp65_{495\text{-}503}$-specific CTLs to the licensed DCs and determined memory CTL activation by intracellular cytokine staining after 4-hours of co-culture clones [25] in the presence of golgi-stop. NLVPMVATV peptide ($1x10^{-6}$ M) was added to DCs as positive control for CTL activation (*Figure 3B*). We found that upon licensing of DCs, $CD8^+$ T-cell activation was enhanced, as determined by percentages of IFNγ and TNF producing cells and surface expressing LAMP-1 (*Figure 3A-B*) or amounts of IFNγ and TNF produced (*Figure 3C, 3D and data not shown*). Next, is cognate $CD4^+$ T-cell licensing required for DCs mediated CTL memory responses? We repeated the DC licensing experiment using polyclonal DRB1*01$^+$-restricted $CD4^+$ T-cells, and found that $CD4^+$ T cells needed to be antigen specific to induce DC licensing, as no induction of CTL activation was seen after addition of polyclonal $CD4^+$ T-cells (*Figure 3E*). Finally, $CD4^+$

T-cells enhanced CTL stimulation via DC licensing and not by direct stimulation of the memory CTLs, as co-culture of CD4⁺ T-cells, pp65 and CD8⁺ T-cells in the absence of DCs did not yield cytokine production by the memory CTLs (*Figure 3B*).

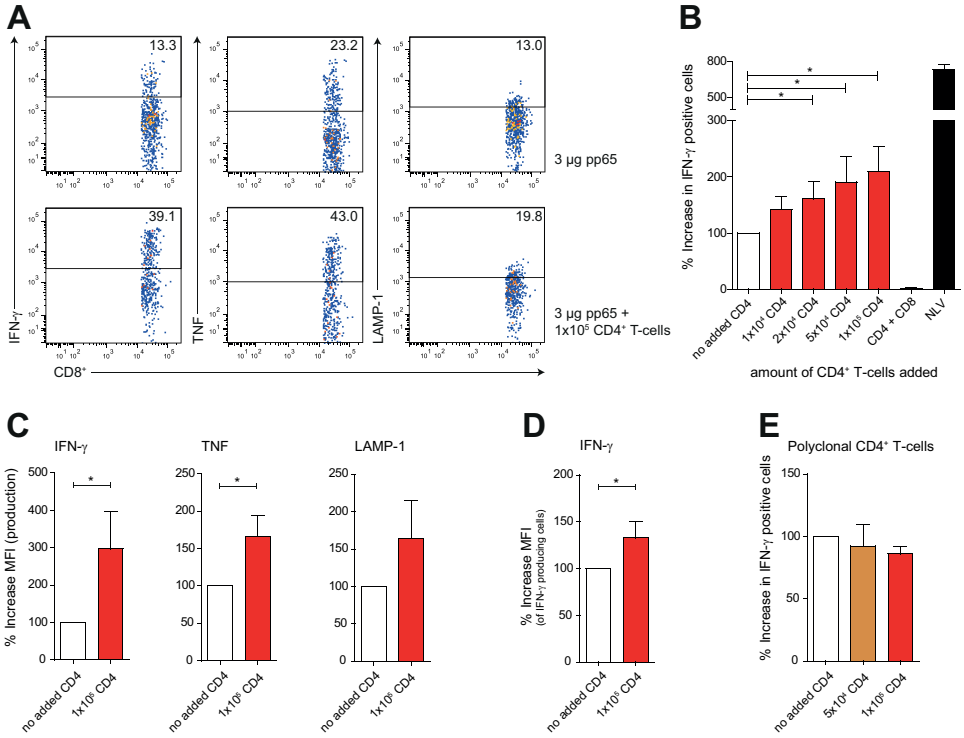



**Figure 3: Cognate CD4⁺ T-cells induce licensing of DCs for enhanced memory CTL responses**
(A-D) Summary and representative plots of CD8⁺ T-cell activation. (A) MoDCs were loaded with HCMV derived pp65 and co-cultured with 50.000 A2/NLVPMVATV specific CD8⁺ T-cells in the absence (upper graphs) or presence (lower graphs) of HLA-DRB1*01/KYQEFFWDANDIYRI specific CD4⁺ T-cells. Freshly thawn T-cells were gated based on CD3 en CD8 expression and analyzed for activation-induced production of IFN-γ (left) and TNF (middle) and LAMP-1 surface expression (right). (B) Summary (mean + SEM) of HCMV $pp65_{495-503}$ cross-presentation. Bars represent production of IFN-γ after co-culture with MoDCs loaded with 3 µg pp65 in the presence of antigen specific CD4⁺ T-cells (mean: 120.9%, SEM: 15.8%, n=6). The black bar shows a maximum response after stimulation with NLV-peptide loaded DCs. (C) MFI of CD8⁺ T-cell cytokine production (IFN-γ and TNF) and LAMP-1 surface expression after co-culture with pp65-loaded DCs with (red bars) or without (white bars) 100.000 antigen specific CD4⁺ T-cells (mean + SEM, n=4). (D) MFI of IFN-γ, gated on IFN-γ producing CD8⁺ T-cell (mean + SEM, n=4). (E) MoDCs were loaded with HCMV derived pp65 and co-cultured with 50.000 A2/NLVPMVATV specific CD8⁺ T-cells in the presence of donor-matched polyclonal CD4⁺ T-cells (mean + SEM, n=4). Significance in all panels was determined using a non-parametric Mann-Whitney test.

*CD40-CD40L and CD80/86-CD28 are not involved in CD4⁺ T-cell mediated memory CD8⁺ T-cell activation*

We next considered that CD4⁺ T-cells may facilitate licensing of DCs via CD40L and CD28 molecules, as was suggested in mouse studies[7,9,13,34,35] and human CD8⁺ T-cell priming (*Figure 2D*). We therefore exchanged cognate CD4⁺ T-cells with a stimulating CD40 antibody in our MoDC/CTL co-cultures described in *figure 3*. As negative control that cannot induce DC licens-

ing, we included an agonist CD40L antibody. We observed that although antibody-mediated CD40 cross-linking does induce DC maturation (*Figure 4D*), its effect on CD8⁺ T-cell activation was modest (*Figure 4A*). The CD40L antibody did not influence CD8⁺ T-cell activation. We confirmed these data by performing the CD4⁺ T-cell co-incubation experiments in the presence of CD40L blocking antibodies (*Figure 4B*). We similarly tested CD80/CD86 (B7-1/B7-2) – CD28 signaling, considering their importance in DC/T-cell interaction[39] and that CD80/86 blockade using abatercept is used in several auto-immune disorders.[40,41] Abatercept treatment did not inhibit the CD4⁺ T-cells induced activation of memory CD8⁺ T-cells (*Figure 4C*). Taken together, we conclude that CD4⁺ T-cells induced licensing of DCs is not attributable to CD40L or CD28-mediated interaction.

Finally, is enhanced stimulation of memory CTLs by licensed DCs a mere consequence of cognate CD4⁺ T-cell-mediated upregulation of DCs surface molecules (*Figure 4D-E*)? This does not seem to be the case, as incubation of pp65-loaded DCs with antigen specific CD4⁺ T-cells or CD40L did not cause an overt increase of HLA-ABC, co-stimulatory markers CD40, CD80, or CD86 or HLA-DR (*Figure 4D, E*). Instead, cognate CD4⁺ T-cell licensing of DCs may involve the enhance stimulation of memory CTLs via increased antigen presentation of HLA-A2/NLVPMVATV complexes.



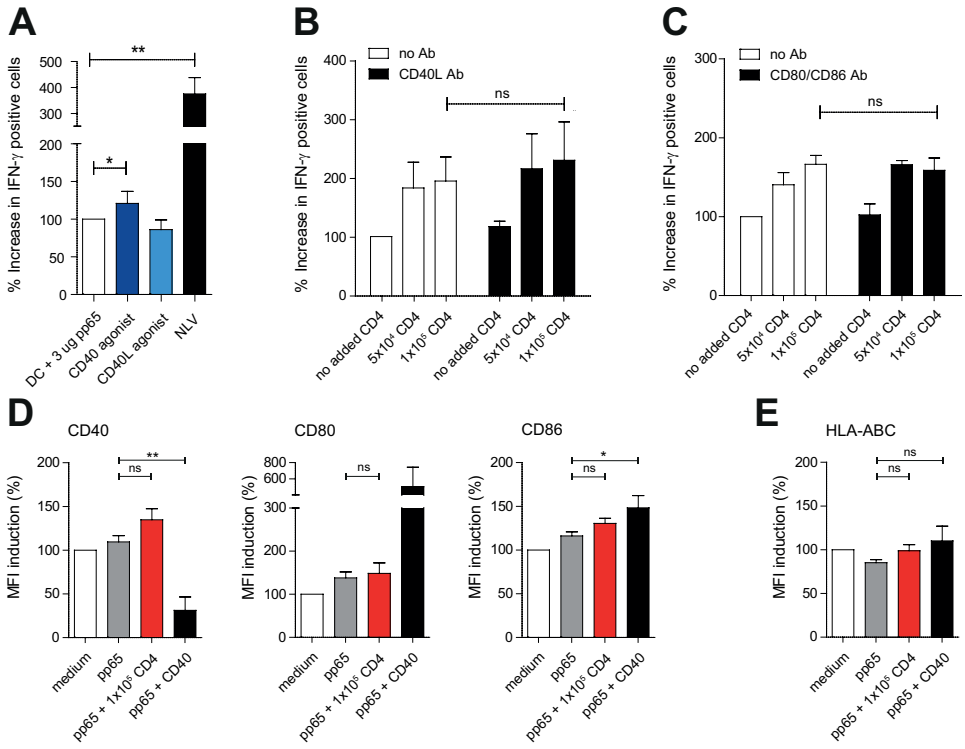



**Figure 4: CD40-CD40L and CD80/86-CD28 are not involved in CD4⁺ T-cell mediated memory CD8⁺ T-cell activation**

(A) IFN-γ production of CD8⁺ T-cells after co-culture with pp65-loaded DCs in the presence of a CD40 (dark blue) or CD40L (light blue) agonist. (B,C) IFN-γ production of CD8⁺ T-cells after co-culture with pp65-loaded DCs and antigen-specific CD4⁺ T-cells in the presence of CD40-CD40L blocking (B) or CD80/CD86 blocking (C) (mean + SEM, n=4). Significance in all panels was determined using a non-parametric Mann-Whitney test.

*Identification of candidate soluble CD4⁺ T-cell-secreted mediators for licensing of DCs*
Cognate CD4⁺ T-cells may mediate DC licensing for enhanced CTL memory responses via secretion of soluble mediators. To this end, we analyzed culture supernatants of co-cultures of pp65-loaded HLA-DRB1*01 MoDCs with cognate CMV-pp65$_{509-523}$-specific CD4⁺ T-cells or polyclonal HLA-DRB1*01-restricted CD4⁺ T-cells (37°C, O/N), by cytokine multiplex-array. The supernatants of cognate DC/T-cell co-cultures but not DC/polyclonal T-cell co-cultures contained increased amounts of IFN-γ, TNF and IL-6 cytokines and CCL3 and CCL4 chemokines (*Figure 5A-B*). Intracellular cytokine staining confirmed that both IFN-γ and TNF are produced by cognate CD4⁺ T-cells when co-cultured with (*Figure 6A and Supplementary figure 4A*). While IL-12 was proposed as a cytokine produced by human CD1c⁺ DCs involved in CD8⁺ T-cell priming[42], we did not detect IL-12 (*Figure 5C*), nor IL-10 or IL-15 (*Supplementary figure 4B*) in our human DC/T-cell co-cultures.

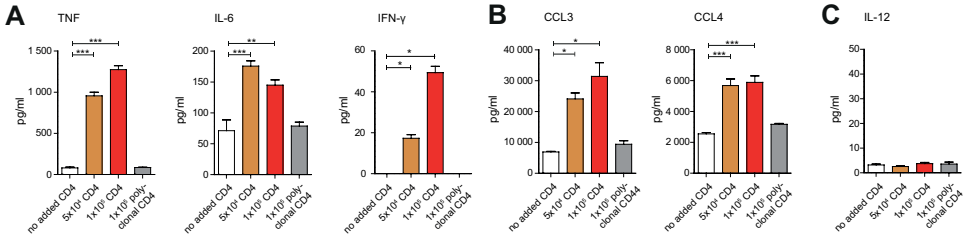



**Figure 5: Identification of candidate soluble CD4⁺ T-cell-secreted mediators for licensing of DCs**
(A-C) Cytokine and chemokine production. MoDCs were loaded with pp65 and co-cultured for 12-16 hours without (white bars) or with antigen-specific (orange and red bars) or polyclonal (grey bars) CD4⁺ T-cells. Shown are amounts (pg/ml) of TNF (80 to 1270 pg/ml), IL-6 (71 to 176 pg/ml) and IFN-γ (0 to 50 pg/ml), CCL3 (6.9 to 31 ng/ml) and CCL4 (2,6 to 5.9 ng/ml, B), IL-12 (C) measured with multiplex (mean + SEM, n=4). Significance in all panels was determined using a non-parametric Mann-Whitney test.

IFN-γ produced by cognate CD4⁺ T-cells enhances memory CTL stimulation by licensed DCs
The multiplex-array revealed IFN-γ as a candidate cytokine produced by cognate CD4⁺ T-cells that enhances DC licensing and consequential memory CTL stimulation (*Figure 5A*). We confirmed these data by intracellular IFN-γ staining of the stimulated CD4⁺ T-cells (*Figure 6A*). To address the possibility that IFN-γ contributes to DC licensing, we added recombinant IFN-γ (ranging from 0.15-150 ng/ml) to 50,000 pp65-loaded HLA-A2*01⁺ MoDCs in the absence of CMV-pp65$_{509-523}$-specific CD4⁺ T-cells. After 12-16 hours, we added 50,000 memory CMV-pp65$_{495-503}$-specific CTLs and analyzed for CTL stimulation by intracellular cytokine staining after 4-hours of co-culture in the presence of Golgi-stop. NLVPMVATV peptide ($1x10^{-6}$ M) was included as positive control for CTL activation (*Figure 6B*). We found increased CD8⁺ T-cell stimulation in an IFN-γ dose-dependent manner, as determined by percentages of IFNγ and TNF producing cells and surface expressing LAMP-1 (*Figure 6B-C*) or amounts of IFN-γ and TNF produced. Thus, the co-culture of CMV-pp65 antigen loaded DCs with cognate CD4⁺ T-cells provokes IFN-γ production by these CD4⁺ T-cells, that consequently facilitates the display of CMV-pp65 peptide/A2 complexes to CD8⁺ T-cells. In conclusion, cognate CD4⁺ T-cells enhanced DC licensing for memory CD8⁺ immunity, in a manner that requires MHC class II-TCR interaction and subsequent release of IFN-γ by CD4⁺ T-cells. Primary antigen-specific CTL expansion also requires cognate CD4⁺ T-cells, but here DC-licensing appears to work via the CD40-CD40L axis

as in mice.[7-9]. Taken together, these data provide mechanistic support how early reconstitution of CD4+ T-cells in CBT recipients helps early antigen-driven CD8+ T-cell-mediated immune protection against viral reactivation.

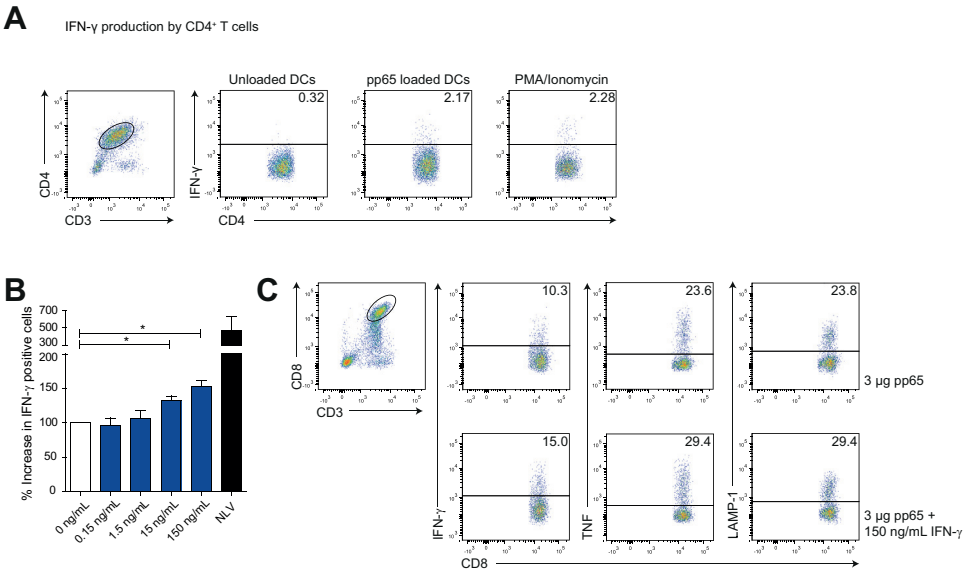



**Figure 6: IFN-γ produced by cognate CD4+ T-cells enhances memory CTL stimulation by licensed DCs**
(A) intracellular staining for IFN-γ gated on CD3 and CD4 positive cells after 12-16 hours co-culture of antigen specific CD4+ T-cells with medium (left graph), pp65-loaded MoDCs (middle graph) or PMA/ionomycin (right graph). (B) representative plots of (C): MoDCs were loaded with HCMV derived pp65 and co-cultured with 50.000 A2/NLVPMVATV specific CD8+ T-cells in the absence (upper graphs) or presence (lower graphs) of recombinant IFN-γ. Freshly thawn T-cells were gated based on CD3 en CD8 expression and analyzed for activation-induced production of IFN-γ (left) and TNF (middle) and LAMP-1 surface expression (right). (C) Summary (mean + SEM) of HCMV $pp65_{495-503}$ cross-presentation. Bars represent production of IFN-γ after co-culture with MoDCs loaded with 3 µg pp65 in the presence of recombinant IFN-γ (0.15 to 150 ng/ml, mean + SEM, n=4). Significance in all panels was determined using a non-parametric Mann-Whitney test.

## Discussion

Infection-related mortality and GvHD are major causes of death after CBT in both adults and pediatric patients.[43,44] Patients are particularly vulnerable to viral reactivation, including reactivation with CMV [1], Epstein-Barr virus (EBV) [45], human herpes virus-6 (HHV6) [46] and varicella zoster virus (VZV) [47]. As the immune system is rebuilt from stem cell precursors, immune protective CD8+ T-cells are formed, that exhibit antigen-specific receptors that recognize epitopes from viruses including CMV. It had not been fully understood, whether and how virus-specific CD4+ T-cells participate in CD8+ T-cell mediated protection against viral reactivation. From mouse-based research, a role of CD4+ T-cells in CD8+ T-cell priming was deducted. For example, effective CTL induction was only seen when CD4+ T-cells were present.[48-51] At the same time, from SCT transplantation studies, there had been rationale that CD4+ T-cells may somehow bolster CD8+ T-cell mediated viral immune protection.[52] For example, studies show that not only CMV specific CD8+ T-cells, but also CMV specific CD4+ T-cell numbers can be used to

predict the risk for reactivation in patients after allo-SCT.[33] From mouse-based research, it was learned that the induction of virus antigen-specific CD8+ T cells requires the prior licensing of DCs by interaction with cognate, antigen-specific CD4+ T-cells.[53] It was our aim to show the possible applicability of such studies to viral reactivation SCT in human patients. We here show that antigen-specific CD4+ T-cells precede the rise of antigen specific CD8+ T-cells after CBT, that are necessary to control CMV reactivation. Using a CB-based culture system, we further show that CD4+ T-cells are required to prime antigen-specific CD8+ T-cells. These results are in line with conclusions based on mouse work.

The role of CD4+ T-cells in providing help in elicitation of CTL-mediated viral control may be different when using bone marrow or mobilized peripheral stem cells, although in these settings, CD4+ T-cell reconstitution is also correlated with long term survival.[54,55] When using adult bone marrow or mobilized peripheral stem cells, CMV specific CD4+ and CD8+ T-cells can be detected independent of CMV viremia, but levels of CMV specific CD4+ [54] or CD8+ T-cells [3,56] are protective in these settings. A major difference is the fact that these patients receive antigen specific CD8+ T-cells from their donor that can clonally expand, circumventing the required priming in the CB setting. Therefore, expansion of antigen specific CD8+ T-cells could be seen as early as 21 days after SCT.[56] The mechanism by which CD4+ T-cells contribute to survival in the bone marrow or mobilized peripheral stem cell transplantation setting is not fully known, although it has been shown that CD4+ T-cell help is important to maintain CTL effector function in chronic viral infections in mice. Our data presented here on how cognate antigen-specific CD4+ T-cells provide DC licensing for effective memory CD8+ T-cell responses by secreting IFN-γ, provide experimental support for the described observations.[57]

As stated in the introduction, CMV reactivation after CBT correlates with decreased survival rates.[58-61] Besides CMV-induced pneumonitis, CMV reactivation is associated with increased risk of GvHD, while GvHD is also a risk factor for viral reactivations.[59,62-65] CMV is the most frequent reactivation, but other viruses also hamper survival. Reactivation of EBV [45], HHV6 [46] and VZV [47] play a major role after CBT. We here describe that CMV control coincides with the presence of CMV-specific CD8+ T-cell expansion, that is preceded by the appearance of a CMV-specific CD4+ T-cell population. Using CB-based co-cultures, we show the requirement for cognate CD4+ T-cells in DC licensing for the expansion of antigen-specific CD8+ T-cells. We believe this is a general mechanism that can be applied broadly to anti-viral and possibly even anti-tumor immune responses. Especially considering the important role of CD8+ T-cells in relapse control, this work supports the importance of monitoring the CD4+ T-cell reconstitution early after CBT, and paving the road to CD4+ T-cell-based intervention strategies.

## Acknowledgements

The authors like to thank members of the Boes laboratory for helpful discussions. We thank Esther Quakkelaar for help with the TCR sequencing.

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## Supplemental Figures

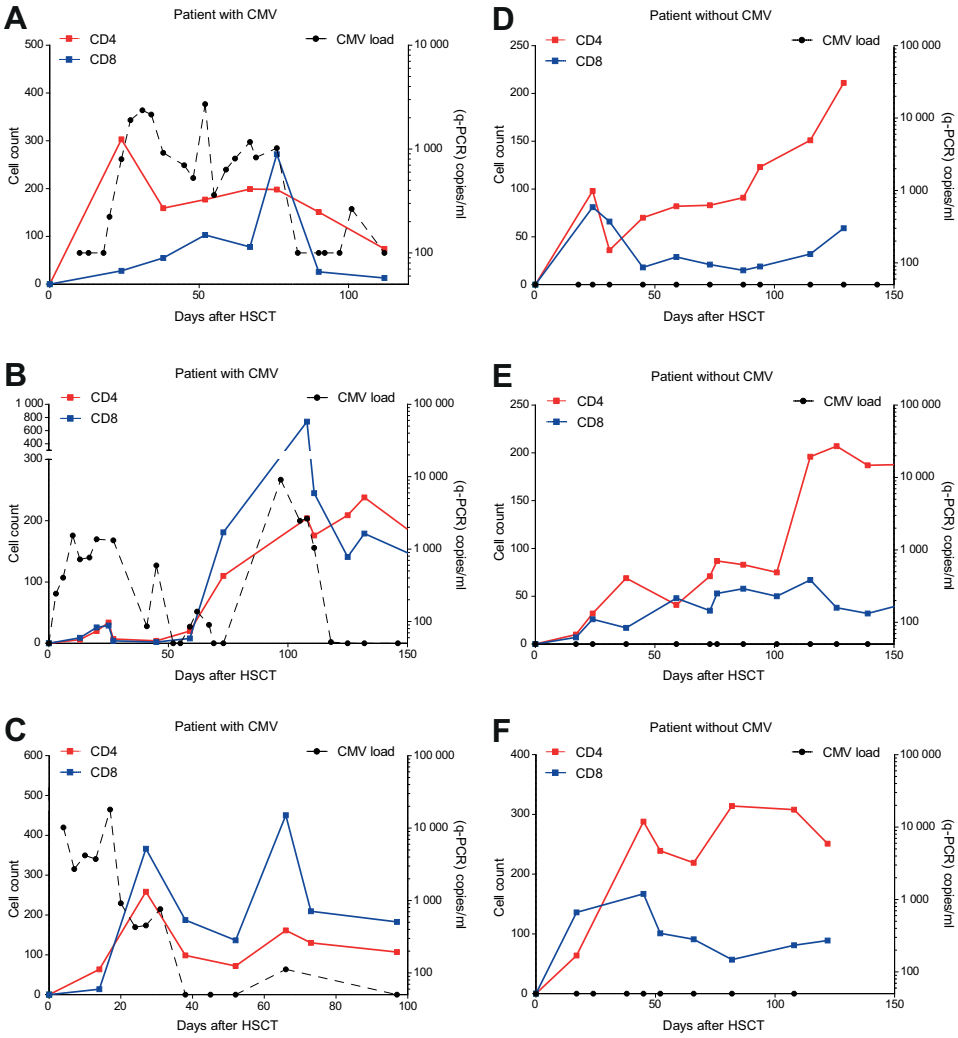



**Figure S1: T-cell dynamics in CBT recipients**
(A,F) T-cell dynamics over time in CBT recipients with (A-C) or without (D-F) CMV reactivation (load >1000 copies/ml). Red and blue line represents $CD4^+$ and $CD8^+$ T-cell numbers respectively (absolute cell count). The black dashed line represents CMV loads (copies/ml).

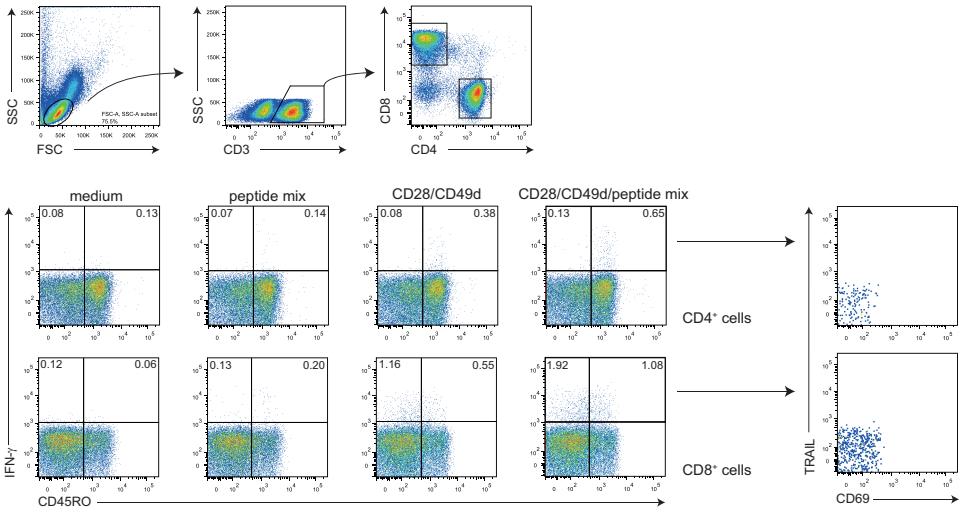



**Figure S2: Detection of antigen specific T-cell in CBT recipients**
Representative gating of PBMCs after stimulated with medium (left panels), pp65 overlapping peptide-mix (2nd panels), CD28 and CD49d (3rd panels) or pp65-overlapping peptide-mix and CD28 and CD49d (4th panels). Live cells were gated on CD3 and CD4 or CD8 expression. Further gating was performed according to CD45RO and IFN-γ expression.

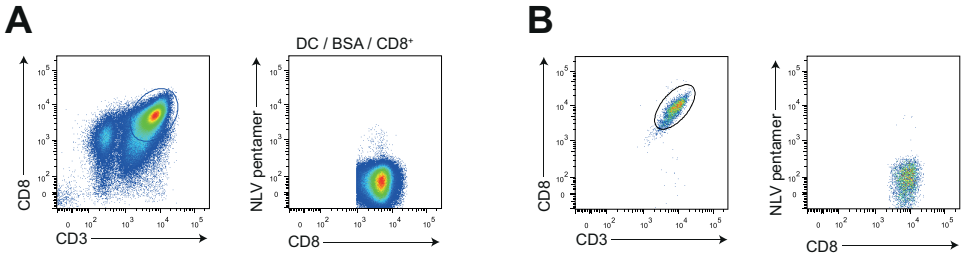



**Figure S3: CD4+ T-cells are required for naïve CD8+ T-cell priming**
(A) Gating strategy and pentamer staining of primed CB-derived CD8+ T-cells. DCs were loaded with BSA and co-cultured with donor matched naïve CD8+ T-cells. (B) Clonal expansion of control-sorted cells stained with NLV-pentamer.

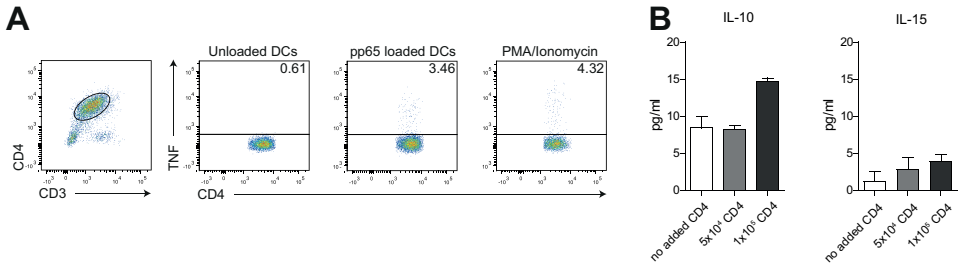
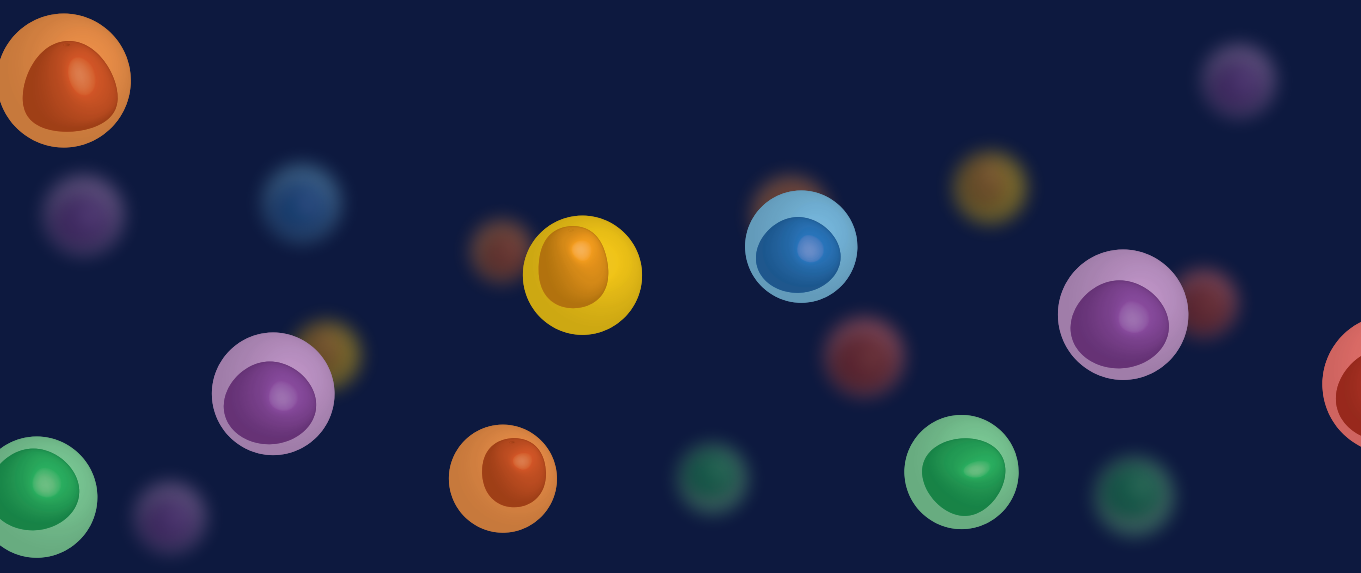
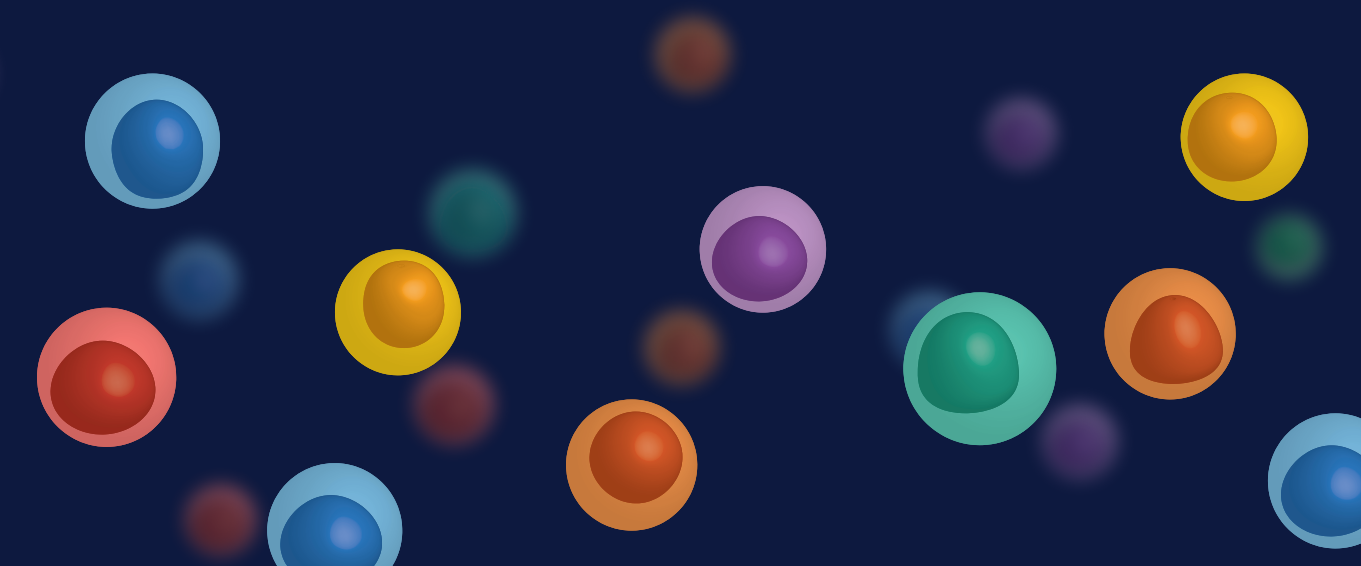



**Figure S4: cytokine production by CD4⁺ T-cells**
(A) Intracellular staining for TNF gated on CD3 and CD4 positive cells after 12-16 hours coculture of antigen specific CD4⁺ T-cells with medium (left graph), pp65-loaded MoDCs (middle graph) or PMA/ionomycin (right graph). (B) MoDCs were loaded with pp65 and cocultured for 12-16 hours without (white bars) or with antigen specific (grey and black bars) CD4⁺ T-cells. Shown are produced amounts (pg/ml) of IL-10 and IL-15, measured with multiplex (mean + SEM, n=4).

# Part B



# DC intrinsic mechanisms involved in cross-presentation



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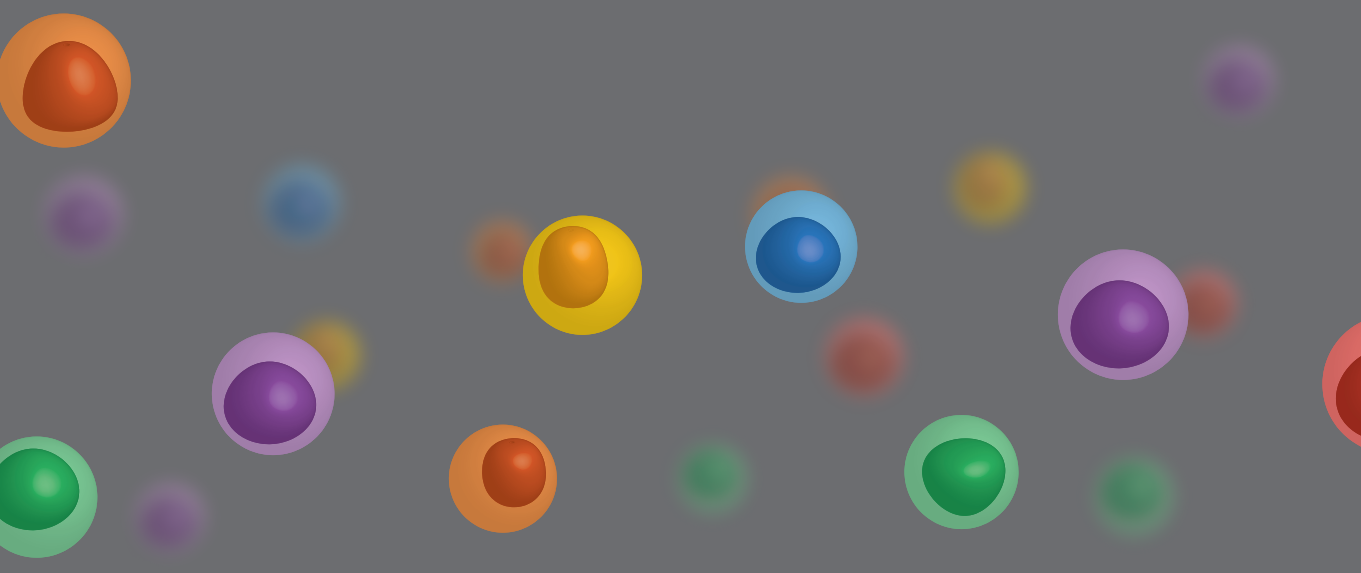

# Antigen processing and remodeling of the endosomal pathway: Requirements for antigen cross-presentation

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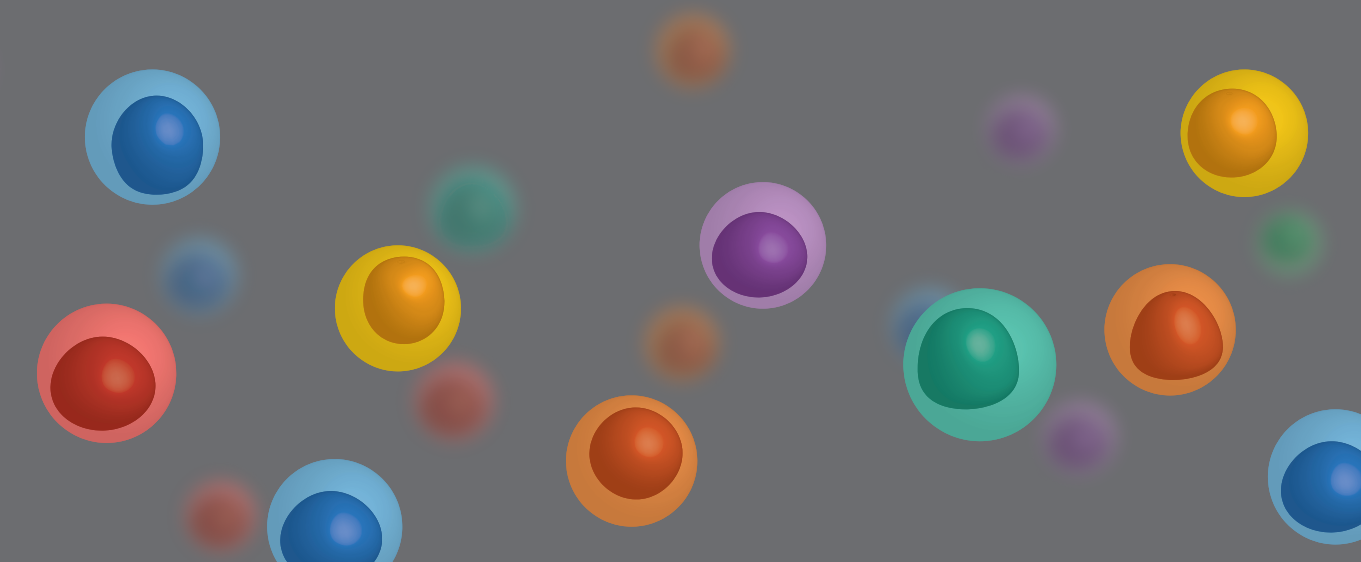
Frontiers in Immunology, 2012 Mar 7;3:37



## Abstract

Cross-presentation of endocytosed antigen as peptide/class I major histocompatibility complex complexes plays a central role in the elicitation of $CD8^+$ T cell clones that mediate anti-viral and anti-tumor immune responses. While it has been clear that there are specific subsets of professional antigen presenting cells capable of antigen cross-presentation, identification of mechanisms involved is still ongoing. Especially amongst dendritic cells (DC), there are specialized subsets that are highly proficient at antigen cross-presentation. We here present a focused survey on the cell biological processes in the endosomal pathway that support antigen cross-presentation. This review highlights DC-intrinsic mechanisms that facilitate the cross-presentation of endocytosed antigen, including receptor-mediated uptake, maturation-induced endosomal sorting of membrane proteins, dynamic remodeling of endosomal structures and cell surface-directed endosomal trafficking. We will conclude with the description of pathogen-induced deviation of endosomal processing, and discuss how immune evasion strategies pertaining endosomal trafficking may preclude antigen cross-presentation.

## Introduction

MHC restriction of T lymphocytes was first reported by Rolf Zinkernagel and Peter Doherty [1]. They provided experimental proof that T cells can only respond to peptide antigens when they are presented in complex with host-derived major histocompatibility complex (MHC) molecules, existing in a class I and class II variant. $CD8^+$ T cells can recognize peptide/class I MHC complexes, whereas $CD4^+$ T cells can respond to peptide/class II MHC complexes. Soon thereafter, Michael Bevan showed that the functional dichotomy of endogenous antigen presentation on class I MHC and exogenous antigen on class II MHC is not absolute[2]. He demonstrated that minor histocompatibility antigens from transplanted cells (e.g. exogenous antigen) could prime cytotoxic $CD8^+$ T cells in a host class I MHC restricted manner and named this process cross-priming. More recent work showed that injected naïve antigen-specific $CD8^+$ T cells accumulate in the lymph nodes that drain tissues expressing a membrane-bound self antigen in a class I MHC-dependent manner. $CD8^+$ T cells can thus survey processed self-antigen delivered from non-lymphoid tissues, without leaving lymphoid organs[3]. In following years, the capability to present exogenous antigens via class I MHC was shown to be relevant for numerous cell-associated antigens in various settings, including viral, self, and tumor-associated antigens[4]. Antigen presentation that results in $CD8^+$ T cell activation is now named cross-priming[5], whereas T cell deletion or induction of anergy is called cross-tolerance[6;7]. Collectively the presentation of exogenous cell-associated antigens via class I MHC molecules to $CD8^+$ T cells is called cross-presentation.

Under homeostatic conditions, cross-presentation of self-antigens harbors the risk of autoreactivity and is therefore strictly controlled. Under these circumstances, antigen cross-presentation is mostly confined to a specific subset of dendritic cells (DC), notably $CD8\alpha^+$ DC in mice[8] and $CD141^+$ DC in human[9-12]. $CD8\alpha^+$ $DEC205^+$ mouse DC not only excel in antigen cross-presentation, but are also specialized in the uptake of dying cells[13]. Receptor-based antigen capture and cross-presentation was shown using a DEC-205 antibody to which protein antigen was chemically coupled[14;15]. Antigen internalized via DEC205 targeting results in the continuous, steady state capture and processing of antigen into peptide/class I MHC complexes in a manner that results in tolerogenic $CD8^+$ T cell responses[16]. Besides DC, macrophages[17]) and liver sinusoidal endothelial cells[18] can also cross-present antigens in the steady state. In contrast, more cell types can cross-present antigen during inflammation. Mouse DC[19], macrophages[20], neutrophils[21] and under specific conditions even B cells[22], have been demonstrated to cross-present antigen in vivo. Additionally, cell types that can cross-present antigen in vitro include basophils[23], γδ T cells[24], mast cells[25] and endothelial cells[26].

Although multiple cells can be involved in cross-priming in vivo, DC are especially important for this process, as shown by abrogated $CD8^+$ T cell priming after depletion of $CD11c^+$ cells (predominantly DC) in mice[27]. Hence, this review focuses mainly on DC, for most cell-intrinsic mechanisms involved in cross-presentation of endocytosed antigen are described in this cell type.

In the last 15 years, at least 50 DC vaccination-based trials, aimed in part at harnessing effective $CD8^+$ T cell responses were performed with overall minimal success (reviewed in[28]). This review aims at providing insight in molecular mechanisms that are pivotal to cross-presentation.

## Antigen processing compartment for cross-presentation

Antigen recognition can trigger receptor-mediated endocytosis and can bring forth the ability of cells to cross-present the receptor-bound antigen[29;30;31]. In contrast to complement-opsonized antigen, immunoglobulin (Ig)-opsonized antigens are delivered in an endosomal compartment that favors cross-presentation by murine DC[32]. Because Ig-opsonized antigen is predominantly endocytosed via Fc receptor and complement-opsonized antigen via complement receptor, this study indicates that antigen recognition dictates antigen delivery in distinct endosomal compartments. Ultimately, this may favor either the class I or class II MHC presentation pathway (reviewed in[33]).

Aside from antigen recognition, antigen size plays a role in its handling by phagocytes: particulate antigens that are larger than roughly 0.5µm are internalized by phagocytosis, whereas smaller antigens are brought in by pinocytosis[34;35]. One major route of antigen internalization that yields cross-presentation seems to be phagocytosis, as particulate antigens are often more efficiently targeted for cross-presentation in comparison to their soluble counterparts[36]. Thus, DC internalise antigens via distinct routes that are dictated by the structure of the antigen (i.e., particulate or soluble, size) and possible involvement of a recognizing endocytic receptor.

Receptor-mediated endocytosis is considered to be a highly efficient process that permits the selective retrieval of macromolecules present in the extracellular fluid[37]. Such uptake depends on structural proteins that mediate the formation of lipid vacuoles, e.g. clathrin or caveolin[38]. Within minutes of internalization from the plasma membrane into the endosomal pathway, antigen is located in vesicular compartments named early endosomes/phagosomes, characterized by a near neutral pH (pH 7.5)[39] and presence of the small GTPase Rab5[40]. Endosomal maturation causes the fusion of early endosomes (EE) with late compartments, accompanied by transition of Rab5 expression to Rab7-positivity. The endosomal compartment is now renamed to late endosomes (LE) with their hallmark of a lowered pH of the endosomal lumen (pH 5.5). Further maturation of the LE leads to fusion with lysosomes in which the acidic environment (pH 4.7) and lysosomal proteases and hydrolases with low pH optima can mediate full degradation of luminal content (i.e., internalized antigen, but also cellular components for turnover). Especially DC harbor cellular mechanisms that prevent the rapid maturation-induced acidification of endosomal compartments, thereby allowing protein antigen fragments to remain intact for a prolonged time (as proteolytic activity by pH-sensitive proteases is restrained). Thereby, DC can cross-present antigen-derived peptides more efficiently than other phagocytes[41;42]. Micro-organisms exploit these mechanisms to prevent their display as peptide/class I MHC complexes as immune evasion strategies (i.e., Mycobacteria and Salmonella[43;44]), as will be discussed in paragraph 6.

Peptides of 8-10 amino acids in length fit within the antigen-binding groove of class I MHC molecules, leading to a stable formation of peptide/class I MHC complexes[45;46]. The proteasome is the foremost contributor to cleaved peptides for the classical class I MHC presentation pathway. Therefore its role in cross-presentation was assessed. The use of proteasome-selective inhibitors clarified the existence of both proteasome-dependent[47] and independent antigen processing[48;161] in distinct cross-presentation model systems. To date, two main routes leading to cross-presentation have broad experimental support: the cytosolic and vacuolar pathway. The cytosolic pathway proposes that endocytosed antigen is transported into the cytosol for proteasome/cytosolic peptidase-mediated degradation, whereas the vacuolar pathway relies on proteases for antigen processing within endosomes.

The cytosolic pathway model is supported by phagosome-to-cytosol translocation of OVA-beads[49], OVA-IgG and HRP-IgG immune complexes in murine cells[50]. Lin et al. demonstrated that cross-presentation competent CD8α$^+$ and not the incapable CD8α$^-$ DC were sensitive for exogenously added cytochrome-c (cytochrome-c induces apoptosis when cytosolic concentrations are elevated)[51]. Antigen translocation from phagosome to cytosol involves processes that are antigen-specific, have antigen size-restrictions, may involve the reduction and unfolding of protein antigen and are Sec61 complex mediated[52-54]. In addition, cytosolic transfer of apoptotic peptides by neighboring- and dendritic cells can occur via gap-junctions into the cross-presenting cell[55].

The processing of antigen that is translocated into the cytosol involves the proteasome, as well as amino- and carboxy-terminal peptidases[56;57]. The Transporter associated with Antigen Processing (TAP) translocates the peptides into the endoplasmic reticulum (ER) which thereby enter the conventional class I MHC pathway[58], or back into the phagosomal pathway in an MyD88-dependent manner[59-61]. All necessary components to enable peptide trimming, loading, and translocation appear present and functional in early phagosomes[62]; Houde et al., 2003). It was proposed that phagosome-ER fusion occurs to deliver the necessary components to the phagosome[63]. It now appears that rather than complete phagosome-ER fusion, which was disputed[64], only selective ER-derived components are delivered to phagosomes[65;66]. The SNARE Sec22b is shown to recruit ER-resident proteins to phagosomes that are necessary for phagosome-to-cytosol translocation[67].

Several groups demonstrated that peptide generation for cross-presentation may occur independent of the proteasome[68], while requiring endosomal acidification[69;70]. The proposed vacuolar pathway does not require phagosome-to-cytosol translocation, but relies on endosomal proteases for generation of antigenic peptides[71;72]. Shen et al. showed that cell-associated OVA can be degraded by both cathepsin S in the endosomal pathway or the cytosolic proteasome within one population of DC. This indicates that the proteasome-independent vacuolar pathway may co-exist with the cytosolic pathways. This possibility is supported by reports demonstrating that plasmacytoid DC cross-present in both proteasome-dependent and independent pathways[73;74]. In summary, antigen processing for cross-presentation depends on distinct protealytic enzymes and may occur in the endosomal compartment as well as the cytosol.

These studies strengthen the concept that both antigen recognition and its physical characteristics affect antigen sorting into the given processing pathways, thereby influencing antigen presentation. Immunization studies showed that appropriate endosomal sorting is essential for efficient cross-presentation. Immunization with bead-coupled OVA caused CD8$^+$ T cell responses and proliferation in an Fcg receptor and DAP12-dependent manner. Cross-presentation of soluble OVA was independent of Fcg receptors and DAP12[75].

## Class I MHC in the endosomal compartment

Early studies showed that TAP-dependent cross-presentation is sensitive to Brefeldin A through its ability to block ER-to-Golgi transport. These data fuelled the initial proposal that peptide loading occurs in the ER[76;77]. However, the identification of Brefeldin A-independent antigen cross-presentation[78;79] and the discovery that components for peptide loading are present in phagosomes[80;81] suggest that peptide loading onto class I MHC may occur also outside of the ER. Class I MHC molecules are distributed in endosomal compartments, as shown in human melanoma epithelial cells (Mel JuSo cells) and lymphoblastoid cells (B-LCLs)[82;83]. In

contrast to endosomal class II MHC molecules, that can directly transit from the Golgi system to the endosomal pathway via association with the invariant chain chaperone, a major route for endosomal localization of class I MHC involves internalization from the plasma membrane. Peptide-class I MHC interactions are destabilized in late endosomal compartments (pH around 5.0), thereby facilitating peptide loading[84]. Further support came from TAP inhibition studies in which TAP function in the early and recycling endosomal (transferrin-positive) compartment was selectively disrupted[85]. Endosomal peptide loading would contribute to rapid cross-presentation of a selective set of endocytosed antigen-derived peptides, while decreasing the risk for competition with endogenous peptides that are assembled into peptide/class I MHC complexes in the ER.

For efficient endosomal peptide/class I MHC loading, class I MHC molecules must be delivered into the peptide-loading compartment. Class I MHC molecules are constitutively internalized[86]. Mutational analysis of the cytoplasmic domain of class I MHC molecules identified several key residues that are essential for internalization[87;88]. An evolutionary-conserved tyrosine residue mediates the delivery into lysosomes[89]. This tyrosine residue is part of a known targeting motif YXXØ (Y= tyrosine, X= any amino acid, Ø= bulky hydrophobic amino acid) that has been shown to bind directly to adaptor protein (AP)-1, 2, or 3[90]. AP-tyrosine motif interaction results in selective incorporation of motif-containing cargo, such as the transferrin receptor, in clathrin-coated vesicles for uptake[91]. Besides endosomal targeting mediated by the tyrosine-based motif, the cytoplasmic domain of class I MHC molecules contains two or three conserved lysine residues[92]. Lysines are targets for ubiquitination that can also induce clathrin-mediated endocytosis. Studies on immune evasion strategies employed by Kaposi sarcoma associated herpesvirus (KSHV) identified two viral proteins, K3 and K5, that can downregulate cell-surface bound class I MHC molecules via poly-ubiquitination[93]. Two human homologous proteins of K3 and K5, the membrane-associated RING-CH family MARCH IV and IX, are key regulators in class II MHC surface expression in B cells and DC[94]. Moreover, MARCH IV and IX ubiquitinate class I MHC molecules and induce its internalization in an overexpression system[95]. Possibly these proteins can facilitate class I MHC endocytosis under physiological conditions, but this remains to be established. Thus, class I MHC molecules are taken up into the endosomal pathway of DC in a clathrin-dependent manner, enabling for sufficient amounts of endosomal class I MHC molecules to assemble into antigenic peptide/class I MHC complexes. As T cell activation requires presentation of multiple antigen-specific peptide/class I MHC complexes, the efficient transport of peptide/class I MHC complexes from peptide loading compartment to the cell-surface is a further cross-presentation requirement that needs to be attained.

## Recycling of endosomal class I MHC

The intracellular location where peptide/class I MHC complexes are assembled dictates the trafficking route that is taken. Peptide loading within the ER probably results in transport via the biosynthetic pathway to the cell surface. In contrast, endosomal peptide/class I MHC assembly suggests an alternative route of transport.

The endosomal pathway contains both vesicular and tubular structures[96;97]. During endocytosis, cell surface-derived membrane proteins and lipids are concomitantly taken up with antigen into endosomal vesicles[98]. To ensure steady surface display, most of the proteins and lipids are rapidly returned to the plasma membrane via the endosomal recycling pathway that consists of two main routes. Within minutes, retrograde recycling of membrane proteins from the EE to plasma membrane may occur, whereas a slower recycling route exists via juxtanu-

clear endosomal recycling compartments (ERC)[99]. It was estimated that cells internalize the equivalent of their cell surface one to five times per hour[100], demonstrating the importance of endosomal recycling to normal cellular function.

There is experimental support that the recycling pathway may play a considerable role in antigen cross-presentation. Pharmacological inhibition of the recycling pathway by inclusion of primaquine in murine DC cultures abrogates cross-presentation of exogenous soluble antigen, without affecting class I MHC-mediated presentation of endogenously expressed antigen[101]. Similarly, Di Pucchio et al. report cross-presentation of a viral antigen by plasmacytoid DC in a brefeldin A-resistant, but primaquine-sensitive manner[102]. Furthermore, silencing of the small GTPases Rab3b and 3c, that colocalize with class I MHC molecules in recycling endosomes (RE) of DC2.4 cells, inhibits cross-presentation[103]. Finally, mouse DC lacking class I MHC in recycling compartments due to expression of class I MHC with an aberrant tyrosine-based motif, are defective in cross-presentation[104]. Together, these reports demonstrate that interfering with the recycling pathway of class I MHC can abrogate cross-presentation, but the exact DC-intrinsic mechanisms for class I MHC molecule recycling that are involved in cross-presentation remain elusive.

The endosomal targeting of internalized antigen involves the selective recruitment of signaling molecules (i.e. EHD1[105] and Rab effector molecules[106]). One factor that regulate selective recruitment of signaling molecules is the small GTPase Arf6[107]. GDP/GTP cycling affects Arf6 function in membrane lipid and protein recycling. Active GTP-bound Arf6 localizes to the cytosolic side of the plasma membrane for clathrin-independent endocytosis, whereas GDP-bound Arf6 localized to tubular-like endosomal structures[108].

The Rab family of small GTPases are considered key regulators of endocytic trafficking[109] (figure 1). Rab22a colocalizes with class I MHC in Arf6-associated tubules[110], and the expression of dominant active or inactive versions of the Rab22a protein, or depletion of Rab22a, impairs class I MHC recycling to the cell surface[111]. Also other members of the Rab family are pivotal to class I MHC recycling. Both Rab35[112] and Rab11[113] are implicated in recycling from the ERC to the cell surface. Rab11 in complex with its effector Rab11-FIP2 interacts with one of four known mammalian C-terminal Eps15 homology (EH) domain containing proteins (EHD1)[114] that all play a role in endosomal trafficking[115]. EHD1 is essential for recycling of both clathrin-dependent and independent endocytosed molecules, including but not restricted to class I MHC[116] and class II MHC[117].

Next to the recycling pathway via the juxtanuclear ERC, class I MHC molecules may also be directed towards the TGN for entering the biosynthetic pathway. Retrograde transport from the endosomal compartment to the TGN involves a hetero-pentameric complex called the retromer (reviewed in[118]). Thus far, however, interaction between class I MHC molecules and retromer complexes is not reported. However, depletion of the retromer-distinct sorting nexin SNX4 results in disruption of the ERC, and miss-sorting of the transferrin receptor to lysosomes. Therefore, SNX4 appears important for shuttling selective cargo between EE and the ERC[119]. In conclusion, the endosomal recycling compartment is a highly dynamic compartment composed of vesicular and tubular membrane structures, in which proper interplay between molecules including GTPases and Rab proteins contributes to antigen cross-presentation.

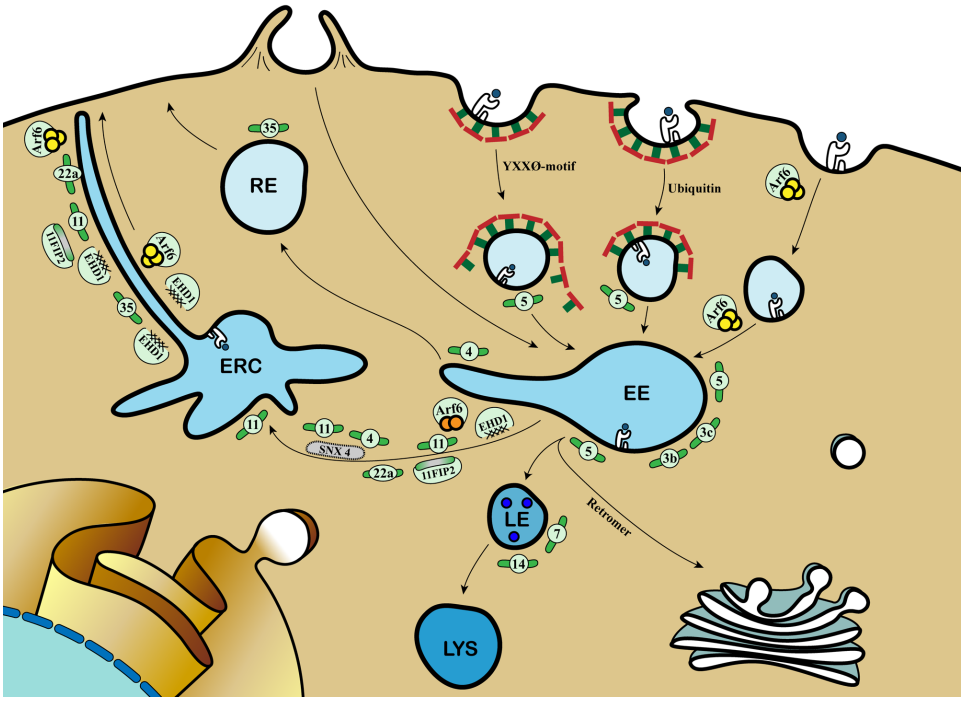



**Figure 1: Molecular mechanisms coordinating cargo recycling in the endosomal compartment**
Antigens can be internalized by phagocytosis and (receptor-mediated) endocytosis and converge into early endosomes (EE). Class I MHC molecules are taken up by either clathrin-mediated endocytosis dependent on tyrosine-based internalization motif or poly-ubiquitination, or clathrin-independent but GTP-bound Arf6-dependent mechanism. The small GTPase Rab proteins dictate the selection of different effectors and binding partners, thereby directing cargo to distinct endosomal compartments, involving late endosomes (LE), lysosomes (lys), recycling endosomes (RE), and the endosomal recycling compartment (ERC). Rab3b and 3c are involved in rapid recycling of transferrin and are involved in cross-presentation. Rab4 together with Rab11 and Sorting Nexin 4 (SNX4) sort cargo into the ERC. Rab22a regulates class I MHC recycling via Arf6-positive tubules. Rab35 mediates recruitment of EHD1 for class I MHC recycling from early endosomes. EHD1 also colocalizes with Rab11 and its Rab11-FIP2, Arf6, and the Rab4 and Rab5 effector Rabenosyn-5. During Rab5-to-Rab7 transition, the retromer complex directs cargo to the *trans*-Golgi Network (TGN). The increased blue coloration illustrates the drop in endosomal pH. Further information and references are mentioned in the main text.

= Class I MHC; = peptide; = Clathrin; = Rab, = GTP-bound Arf6, = GDP-bound Arf6

## Directed migration enabled by association of endosomal compartments with cytoskeletal elements

Membrane-associated cargo, including class I MHC molecules, is selectively transported to distinct endosomal compartments. But what regulates the structural support necessary for endosomal trafficking?

All eukaryotic cells have a filamentous network of cellular proteins, collectively termed the cytoskeleton. It mainly comprises three distinct classes of fibers: microfilaments, microtubules, and intermediate filaments. The cytoskeleton has multiple tasks. It gives the cell its

rigidity and strength that helps maintaining cell shape. Moreover, it provides tracks that allow directed movement of organelles and their transport intermediates during intracellular trafficking processes. Microtubules are major components of the cytoskeleton, and are composed of α- and β-tubulin heterodimers. Disruption of these microtubules perturbs Arf6-associated recycling tubules[120], and thereby may abrogate endosomal transport of peptide/class I MHC complexes.

The continuous assembly and disassembly of microtubules creates a temporal and spatial dynamic network that allows for long-range endosomal transport[121]. This network allows directional movements of motor proteins that associate with these microtubule tracks. Kinesins and dyneins are two families of such motor proteins. Most kinesins migrate over the microtubules in plus-end direction towards the cell periphery, whereas dyneins are directed to the minus-end, towards the microtubule organizing center (MTOC)[122]. Various cargo-selecting molecules or complexes from the endosomal compartment are linked to the motor proteins, allowing separation and movement of endosomal vacuoles over these microtubule tracks. For instance, several Rab proteins are associated directly to motor proteins, such as Rab14 with kinesin[123] and Rab4 with dynein[124]. Similar to SNX4, most Rab proteins are indirectly linked to motor proteins via adaptor proteins, allowing separate trafficking processes in distinct responses. For example, Rab6 can interact with Bicaudal-related protein 1 (BICDR-1) or Bicaudal D-2 to associate with kinesin-3 or 1 respectively[125;126]. Thus, endosomal small GTPase activity of Rab proteins can affect motor-microtubule interaction, thereby altering the segregation or guidance of cargo transport.

Directed assembly of microtubules may also allow for polarized trafficking and delivery of membrane proteins or (soluble mediator) cargo in high concentration to one specific spot. Upon cognate interaction between an antigen presenting cell and a T cell, the cytoskeleton forms a highly organized structure called the immunological synapse (IS). The IS is a region of spatially and temporally organized, highly concentrated motifs of membrane proteins and cytosolic molecules, formed at the T cell interaction site. The formation of the immunological synapse in DC is critical for subsequent T cell activation and depends on cytoskeletal rearrangement[127]. Perturbation of the cytoskeleton abrogates IS formation and subsequent T cell activation[128]. Endosomal compartments that transport class II MHC molecules converge at the IS upon cognate DC-T cell interaction[129-131]. In addition, a recent study demonstrates that ICAM-1, an adhesion molecule involved in strengthening the DC-T cell interaction, is targeted to the IS. This occurs either via the cell surface by cytoskeleton-dependent active transport, or via RE, where it colocalizes with class II MHC molecules. The latter pathway depends on continuous endocytosis and recycling of ICAM-1. Polarization of the recycling ICAM-1 to the DC-T cell interaction site in its turn depends on the high-affinity state of the ICAM-1 binding partner LFA-1 on T cells[132]. This was not unexpected, as it was described earlier that blocking LFA-1 with an antibody on antigen-specific $CD4^+$ T cells hampers remodeling of the endosomal class II MHC-containing compartment in murine DC[133]. Taken together, these data demonstrate that T cell-directed, cytoskeleton-supported recycling of antigen cargo is crucial for cellular immune responses.

In all cell types mentioned, the small GTPase CDC42 of the Rho family was shown to be responsible for MTOC polarization[134-136]. Specifically, Pulecio et al. show that CDC42-mediated polarization mediates both MTOC polarization and directed transport of the cytokine IL-12 to the DC-T cell interaction site, which was crucial for antigen-specific $CD8^+$ T cell proliferation and IFNγ production[137]. Yuseff et al. demonstrated that atypical PKC is a downstream target

of CDC42, required for MTOC polarization[138]. CDC42 may be responsible for MTOC relocation by a mechanism that recruits the PAR6-atypical PKC complex to the plasma membrane in an Arf6-dependent manner, as was demonstrated to facilitate the establishment of polarity in migrating astrocytes[139;140]. Moreover, PAR6 overexpression reduced MTOC reorientation in murine macrophages[141]. Taken these data together, CDC42-based polarity machinery plays an instrumental role in the polarization of the microtubule network and influences the direction of RE and other microtubule-associated trafficking.

### Implications to human infectious diseases

Abrogated transport or recycling of class I and class II MHC complexes leads to immune-related disorders, as might be expected considering their importance in the initiation of immune- or tolerogenic responses. Indeed, an inefficient MHC transport leads to severe combined immunodeficiency as in patients with Bare Lymphocyte Syndrome type I. This disease can be caused by mutation in the TAP1, TAP2, or TAPBP genes, all leading to inefficient peptide/class I MHC transport and ultimately decreased cell surface expression[142]. Hampering the transport of peptide/MHC complexes also plays a major role in viral infections. Herpes and Pox viruses can evade immune responses. They do this by several mechanisms including preventing the presentation of newly synthesized class I MHC molecules at the cell surface by blocking peptide translocation via TAP, block of peptide loading, retention of MHC/peptide complexes in the ER or their retrograde translocation into the cytosol for degradation, as illustrated in figure 2[143-145]. Increased MHC internalization also limits plasmamembrane displayed peptide/class I MHC complexes and subsequent T cell activation[146]. Thus far no inborn mutations are known that correlate with mechanisms of MHC recycling or degradation. However, pathogens developed immune evasion strategies that interfere with endosomal transport of MHC or its recycling from the plasma membrane, with possible implications to antigen cross-presentation.

For example, EBV-derived BILF-1 and the previously mentioned K3 and K5 of KSHV decrease class I MHC surface expression by increasing its internalization, aiding the virus in escaping the immune system[147-151]. HIV uses multiple strategies to evade the immune system[152;153]. HIV-1 expresses the Nef protein that targets newly synthesized class I MHC from the TGN to the destructive lysosomal compartments, thereby preventing cell surface expression of peptide/class I MHC. Moreover, Nef increases the turnover of MHC surface molecules by targeting MHC to lysosomes via the clathrin-dependent retromer-mediated pathway[154]. Additionally, Nef and host-derived sorting protein PACS1 interfere with MHC recycling by targeting and retaining MHC from the plasma membrane in the TGN via the previously described clathrin-independent ARF6 endocytic pathway[155]. Recycling of MHC is also abrogated by Murine Cytomegalovirus, which induces an arrest of MHC in EE[156]. The retromer is also targeted for immune evasion as was reported for Herpesvirus Saimiri (HVS) infection. HVS-derived tyrosine kinase-interacting protein binds and redistributes the retromer subunit Vps35 from the EE to lysosomes, thereby inhibiting retromer activity. This is physiologically linked to $CD4^+$ T cell downregulation and immortalization[157], but possible retromer-targeted effects by HVS on cross-presentation remain to be shown.
The enormous number of viruses targeting peptide/MHC expression and endosomal trafficking illustrates its crucial role in anti-viral responses. Bacteria can also use these mechanisms to create an environment in which they can thrive[158]. Intracellular pathogens can replicate in vacuoles that retain an elevated pH, show limited hydrolytic activity, and intersect poorly

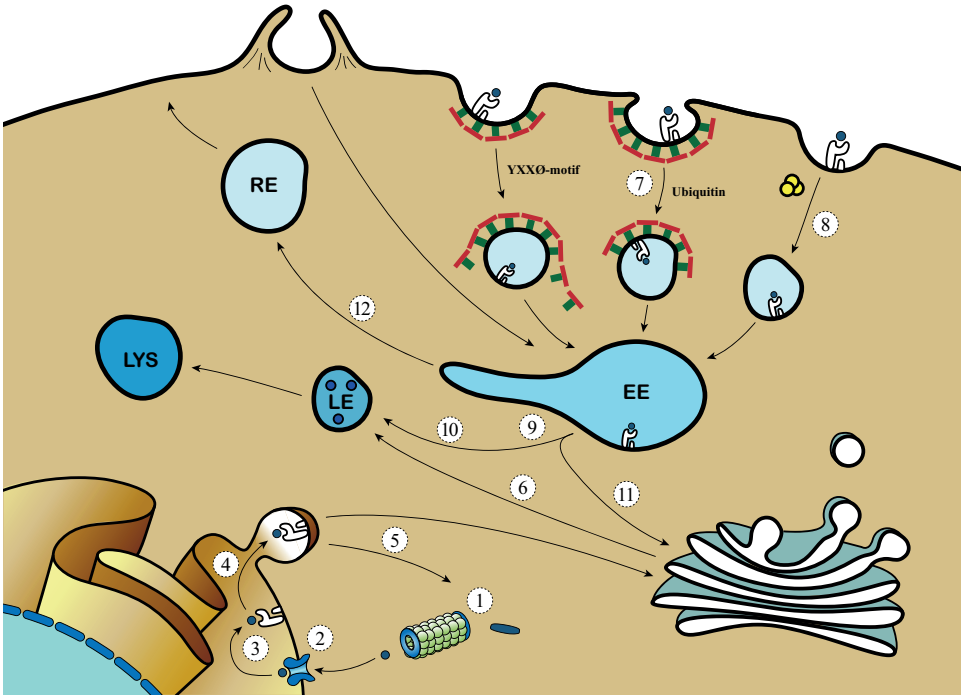



**Figure 2: Viral evasion strategies aim at different pathways of MHC transport**
1: Inhibition of proteasomal processing (EBV); 2: Inhibition of TAP (EBV, HSV, CMV); 3: Inhibition of peptide transport (EBV, CMV, adeno); 4: Retaining MHC molecules in the ER (adeno, coxpox); 5: Target MHC for proteasomal degradation (CMV); 6: Target MHC from TGN to endo/lysosomal compartments (HIV); 7: Clathrin dependent MHC internalisation (EBV, KSHV, HIV, CMV); 8: Clathrin independent MHC internalization (HIV); 9: Arresting phagosomal maturation (mycobacteria, salmonella, chlamydia, and leishmania); 10: Targeting MHC to the LE/lysosome (HSV); 11: Inhibition of retromer activity (HVS); 12: Inhibition of progression to the RE (CMV, HSV).

= protein; = peptide; = TAP; = Clathrin; = proteasome; = GTP-bound ARF6

with antigen presentation pathways. To achieve this, pathogens such as Mycobacteria hamper phagosome-lysosome fusion and Salmonella delays vacuolar acidification, thereby inducing arrest of phagosomal maturation[159;160]. Bacteria can also use endosomal remodeling and recycling for their own benefit. For example, Rab14 and syntaxin 6, which are together with IRAP involved in cross-presentation[161;162], are recruited to Chlamydial inclusion vacuoles[163;164]. Also, a recent investigation demonstrates that Salmonella induces kinesin activity by the expression of Arl8B, an Arf family member[165]. Concomitant with increased kinesin activity, endosomal remodeling into tubular-like structures is promoted by Arl8B[166], thereby creating an opportunity for Salmonella to transfer from cell to cell.
In summary, peptide/MHC surface expression is pivotal in initiating T cell responses and is therefore an important target in pathogen evasion strategies. Pathogens interfere with endosomal transport of MHC molecules to the plasma membrane, internalization of MHC and subsequent recycling or degradation. Knowledge of these processes is important in therapeu-

tic interventions aiming at clearance of infections via appropriately activated MHC-restricted T cell responses. Drugs specifically targeting viral evasion molecules could re-establish proper peptide/MHC presentation, thereby allowing the immune system to clear the virus. Secondly, it is important to clarify evasion strategies employed by prevalent pathogens in future cellular vaccination developments, e.g. DC-based vaccine strategies, since such evasion could impair vaccine effectiveness.

## Concluding remarks

Efficient cross-presentation of $CD8^+$ T cells that initiates balanced anti-viral and anti-tumor immune responses depends on DC-intrinsic mechanisms that enable the sequential interaction of specific TCR molecules with peptide/MHC complexes in the context of activating or inhibiting (tolerogenic) signals. The molecular mechanisms described in this review all aid to ensure the quantity and quality of this DC-derived signal towards the $CD8^+$ T cells.

Antigen recognition by specific receptors permits the selective and rapid retrieval of antigens present in the extracellular fluid, focussing the antigen pool that is directed towards cross-presentation. Additionally, targeting antigen to specific receptors allow it to target towards superior cross-presenting DC subsets[167], or to overcome prior incapability of antigen cross-presentation[168]. Not surprisingly, targeting antigen increases antigen cross-presentation in vivo[169] and is currently used in first phase clinical trials (140, DCVax-001). It now appears that next to efficient antigen uptake, receptor selection is instrumental for antigen delivery to cross-presentation competent compartments. Antigen introduction, as well as co-presence of 'danger signals' appears to optimize, at least in some circumstances, the capability of selective endosomal compartments to support antigen cross-presentation, by recruitment of necessary components for cross-presentation[170-173].

DC maturation is accompanied by dramatic changes in cell shape. Since the cytoskeleton is responsible for cell shape, danger signaling is likely involved in cytoskeletal reorganization. Indeed, Toll-like receptor 4 (TLR4) signaling induces actin cytoskeleton remodeling in a MyD88-dependent manner[174]. In addition, MTOC reorientation in DC by binding of antigen-specific T cells required TLR signaling[175]. However, this could be an indirect effect due to the fact that mature DC form more stable synapses than immature DC[176]. Moreover, innate signals via MyD88 are demonstrated in murine DC to remodel the late endosomal compartment in which class II MHC peptide loading occurs[177]. All together, these data demonstrate a beneficial role for innate signaling in presentation of antigens. However, exact molecular mechanisms that link innate signaling with directed cargo transportation remain elusive.

A large amount of viral immune evasion strategies generated by the evolutionary pressure of the endosomal recycling pathway on anti-viral responses suggests that efficient recycling of class I MHC molecules is essential for an effective $CD8^+$ T cell response. An exon7-deleted variant of class I MHC clearly demonstrates that only a small delay in class I MHC recycling greatly affects $CD8^+$ T cell responses[178]: antigen cross-presentation by exon7-deleted class I MHC molecules-expressing cells results in more robust $CD8^+$ T cell responses.

The quality of the MHC/TCR interaction (e.g. signal 1) affects DC-$CD8^+$ T cell interaction strength, thereby affecting $CD8^+$ T effector function[179], memory differentiation[180] and survival[181]. Hence, primary immune deficiencies with defective quality of signal 1, such as Wiskott-Aldrich syndrome and DOCK8 immunodeficiency patients, share clinical characteristics (e.g. eczema, elevated IgE levels, cutaneous M. contagiosum or Papilloma and Herpes viral infections, and increased tumor incidence[182;183]. Both Dock8 and WAS protein are important

for T cell synapse formation[184;185], and crucial for interactions between naive $CD8^+$ T cells and DC[186;187]. Thus, the endosomal recycling pathway may prove of importance for antigen cross-presentation and prevention of correlated diseases via distinct mechanisms, some of which are outlined above, and likely with more to be uncovered in the years to come.

## Acknowledgements

We acknowledge financial support from the Wilhelmina Research Fund and an NWO/NGI Horizon grant (to M.B.). We thank Michiel de Jong (www.TomatoesandPotatoes.com) for his aid to design the figures. We additionally thank Henk Schipper and Robert van der Burgh for critical reading of the manuscript, the members of the Boes laboratory, and members of the Center for Molecular and Cellular Intervention (CMCI) for helpful discussions.

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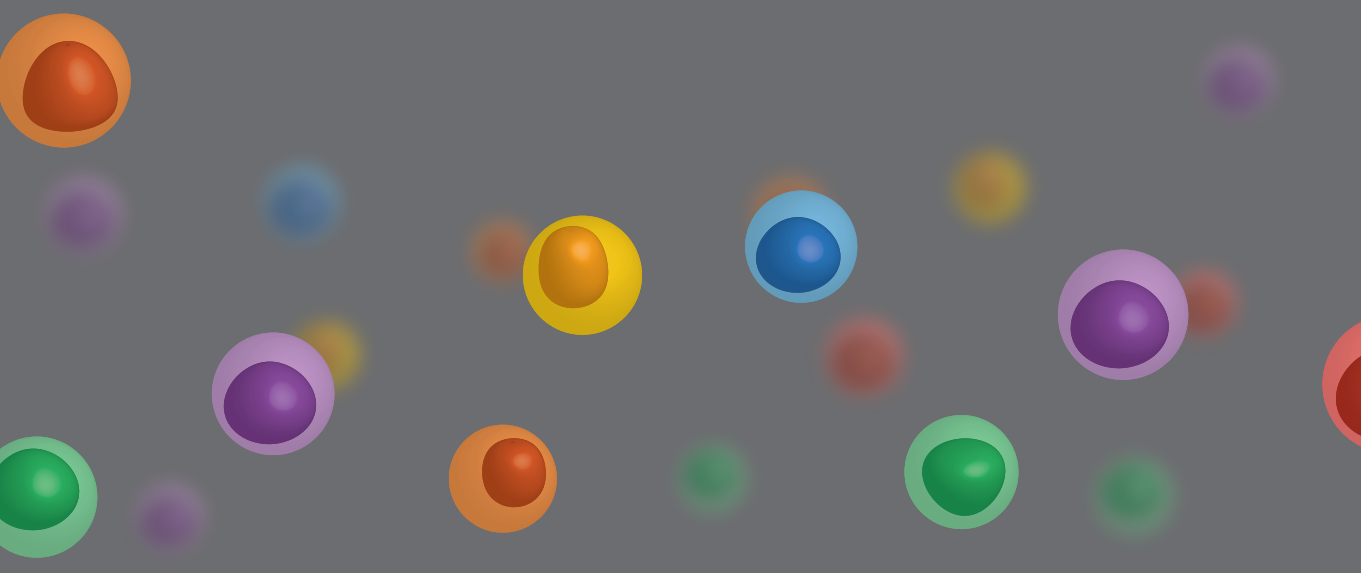

# Tubulation of endosomal structures in human DCs by TLR ligation and lymphocyte contact accompanies antigen cross-presentation

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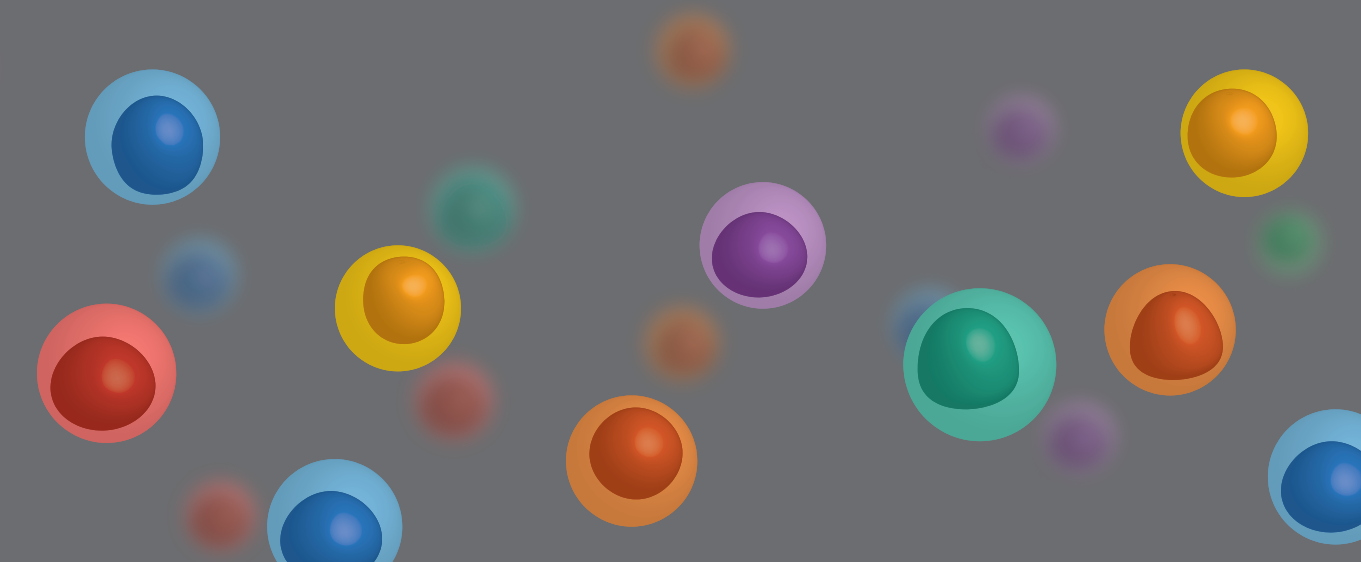
Journal of Biological Chemistry 2014 Jan 3;289(1)520-8



## Abstract

Mouse dendritic cells (DCs) can rapidly extend their Class II MHC-positive late endosomal compartments into tubular structures, induced by Toll-like receptor (TLR) triggering. Within antigen-presenting DCs, tubular endosomes polarize towards antigen-specific $CD4^+$ T cells, which are considered beneficial for their activation. We here describe that also in human DCs, TLR triggering induces tubular late endosomes, labeled by fluorescent LDL. TLR triggering was insufficient for induced tubulation of transferrin (Tfn)-positive endosomal recycling compartments (ERCs) in human monocyte-derived DCs. We studied endosomal remodeling in human DCs in co-cultures of DCs with $CD8^+$ T cells. Tubulation of ERCs within human DCs requires antigen-specific $CD8^+$ T cell interaction. Tubular remodeling of endosomes occurs within 30 minutes of T cell contact and involves ligation of HLA-A2 and ICAM-1 by T cell-expressed T cell receptor and LFA-1, respectively. Disintegration of microtubules or inhibition of endosomal recycling abolished tubular ERCs, which coincided with reduced antigen-dependent $CD8^+$ T cell activation. Based on these data, we propose that remodeling of Tfn-positive ERCs in human DCs involves both innate and T-cell-derived signals.

## Introduction

Endocytosis and recycling of lipids and receptor-bound proteins in the plasma membrane is a highly dynamic and organized process [1]. Engulfed material enters the endosomal pathway, first localizing to early endosomes (EE). From here, most cargo is recycled back to the cell surface via two main recycling pathways that each consists of vesicular and tubular structures. The fast recycling route recycles cargo directly from EE to the plasma membrane, whereas slow recycling occurs via a juxtanuclear-positioned endocytic recycling compartment (ERC) [2]. Non-recycled cargo in EEs transits into late endosomes (LE) that eventually fuse with lysosomes where remaining cargo is degraded [3].

Activation of $CD4^+$ and $CD8^+$ T cells requires presentation of peptide/MHC complexes of the appropriate specificity. DCs are the most adept antigen presenting cells. Presentation of extracellular antigens requires antigens to be internalized into endosomes, their processing into peptides, assembly of antigenic peptide/MHC complexes, and transport of these complexes to the cell surface. While loading of exogenous antigen-derived peptides onto Class II MHC molecules occurs in LE, the loading of Class I MHC molecules occurs in the endoplasmic reticulum or, as recent work supports, in endosomal compartments [4-6].

Encounter of antigen-specific $CD4^+$ T cells induces the formation of late endosomal Class II MHC-positive tubules in murine DCs. These intracellular tubules extend approximately to 5-15µm in length [7-11]. The LE tubules transform in a TLR-dependent manner, and rearrange transport of Class II MHC molecules between LEs and the DC surface, presumed to facilitate the ensuing $CD4^+$ T cell response [12-15]. In early studies, next to LE tubules also elongated tubular recycling endosomes (REs) are observed. In HeLa cervical cancer cells, infection by *S. typhimurium* or overexpression of Eps 15 homology domain (EHD) 1 induces formation of these long endosomal tubules [16;17]. These RE tubules mediate efficient Class I MHC recycling towards the cell surface [18]. For human DCs, morphology of the endosomal pathway and signals that induce rearrangement of endosomal structures during immune activation are not understood. We used live cell confocal microscopy to investigate endosomal remodeling in human DCs stimulated with TLR-ligands and upon cognate interaction with $CD8^+$ T cells. We demonstrate 3 modes of inducing endosomal tubulation, triggered by distinct signals for tubular remodeling of late and recycling endosomes. Live cell confocal microscopy experiments reveal an unexpected role for ICAM-1 and class I MHC molecules in remodeling of the endosomal recycling compartment.



## Experimental Procedure

*In vitro generation of human monocyte-derived DC*

Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors after informed consent by centrifugation (2300 rpm, 20 min, RT) on ficoll-paque (GE Healthcare) . Monocytes were isolated from PBMCs by centrifugation (2900 rpm, 45 min, RT) on 3 layer isotonic percoll density gradient (from top-to-bottom: 34%, 47,5% and 60%; Sigma Aldrich). Monocytes are stored in freeze medium (10% DMSO (Sigma Aldrich) in heat inactivated-FCS) at -80°C for maximally 8 weeks. Monocytes were cultured in 8 wells Nunc® Lab-Tek® II chambered coverglass (Thermo Scientific). These were pre-coated with 1% w/v Alcian Blue 8GX (Klinipath) in PBS for 30 min at 37°C. Cells were maintained in a humidified incubator at 37°C with 5% $CO_2$ for 5 days in differentiation medium: RPMI 1640 with 1% v/v PenStrep (Invitrogen), and 1% v/v

Gibco® GlutaMAX (Invitrogen), and 10% v/v human AB+ serum (Sanquin) + 500 U/ml GM-CSF and 100 U/ml IL4 (Immunotools).

*Monocyte-derived DC maturation*
MoDC maturation was induced by addition of 200 ng/ml Lipopolysaccharide Ultrapure from E. coli strain 0111:B4 (LPS-EB ultrapure, Invitrogen), 5 μg/ml pI:C (Sigma Aldrich) 4 hours prior to microscope analysis. This occurred in presence or absence of viral antigens; 3 μg/ml HCMV-derived pp65 antigen (Miltenyi Biotec) or dialysed recombinant EB2 protein.

*Confocal microscopy and imaging analysis*
Mature moDCs were washed with RPMI 1640 without phenol red, supplemented with 0.2% v/v Bovine Serum Albumin (BSA) (Roche) and 10 mM HEPES, and subsequently incubated for 30 min at 37°C with 20 μg/ml DiI-conjugated LDL (Biomedical Technologies) and 5 μg/ml Alexa Fluor 647-conjugated transferrin (Invitrogen Molecular Probes). Hereafter, cells were washed twice with RPMI 1640 without phenol red, supplemented with 0.2% BSA and 10 mM HEPES, and used for live cell imaging. Prior to any stimulation, at least ten positions are chosen and locked to be able to track the exact same cells over time. Scoring was done for presence of motile LDL or Tfn-positive tubular structures emanating from the center of moDCs, by two independent observers in a double-blinded manner. The percentage of $LDL^+$ tubular moDCs is relative to LDL-loaded moDCs, whereas $Tfn^+$ tubular moDCs is determined relative to $LDL^+$ tubular moDCs. Live cell imaging was performed on a Zeiss LSM710 confocal microscope equipped with live-cell chamber device to maintain 37°C and 5% $CO_2$ condition during experiments. Images are obtained with 1.3x optical zoom using "Plan-Apochromat" 63x 1.40 oil DIC M27 objective (Zeiss) and processed using Zen 2009 software (Zeiss Enhanced Navigation).

*T cell clone antibody-blocking experiments*
HLA-A*0201–restricted, HCMV pp65–specific $CD8^+$ Tcell clones were prepared as published [19]. HLA A2/NLVPMVATV-restricted $CD8^+$ T cell clones were freshly thawed and incubated in ice cold PBS at 4°C for 1 hour in presence or absence of 1 μg/ml mouse-anti-human CD11a antibody (anti-LFA-1, HI111, Biolegend) or mouse-anti-human CD127 antibody (purified in house from R34-34 hybridoma). T-cells were washed with ice cold RPMI 1640 without phenol red, supplemented with 0.2% BSA (Roche) and 10 mM HEPES and kept on ice; 5 min prior to incubation with moDCs, T cells were warmed up to 37°C and used for live cell confocal microscopy.

*Beads-antibody coating and bead binding assays*
Dynabeads® M-450 Epoxy beads (Dynal) were coated with mouse-anti-human CD19-biotin antibody (HIB19, BD Pharmingen), or mouse-anti-human CD54-biotin (ICAM-1) antibody (HA58, eBioscience), or mouse anti-human HLA-A2 (Thermo Scientific Pierce), or combination of both anti-ICAM-1 and anti-HLA-A2 antibodies, according to the manufacturers instructions. For imaging experiments, beads were warmed up to 37°C prior to administration.

*Pharmacological inhibition of endosomal remodeling*
Human moDCs (day 5) are pulsed for 4 hours with 3 μg pp65 in presence of 200 ng/ml LPS (LPS-EB ultrapure, Invitrogen) and 5 μg/ml pI:C (Sigma Aldrich). Upon staining of ERCs (30 min, 37c) with 5 μg/ml Alexa Fluor 647-conjugated transferrin (Invitrogen Molecular Probes),

vesicle-to-tubule transformation is stimulated by co-culture of antigen-specific (NLV) CD8+ T cells for 1 hour. MoDC with tubular ERCs are imaged prior to any stimulation, and 20 to 40 minutes after administration of either 50 μM primaquine biphosphate (Sigma Aldrich), 10 μM nocodazole (Sigma Aldrich), PBS, or DMSO (Sigma Aldrich).

*Pharmacological inhibition of antigen cross-presentation*
Human moDCs (day 5) are loaded with 3 μg/ml CMV pp65 antigen (Miltenyi Biotec) or NLVP-MVATV-peptide (Pepscan) overnight in presence of 200 ng/ml LPS (LPS-EB ultrapure, Invitrogen) and 5 μg/ml pI:C (Sigma Aldrich). Subsequently, moDCs are exposed for 30 minutes to either primaquine biphosphate (50 μM), or nocodazole (10 μM), or carrier controls PBS, and DMSO, respectively (all Sigma Aldrich). Hereafter, DC cultures are thoroughly washed to remove inhibitors. HLA-A2/NLVPMVATV-restricted CD8+ T cells are added, and DC/CD8+ T cells are co-cultured for a further 5 hours at 37°C. Activation of CD8+ T cells is measured by induced antigen-driven production of IFNγ and TNF, and surface-expressed LAMP1 by flow cytometry. DC viability was determined by Annexin V and 7-AAD staining (Biosciences). Surface expression was determined by staining DC with fluorchrome conjugated anti-HLA-A2, CD80 (both Biosciences), HLA-DR and ICAM-1 (both Biolegend).

*Statistics*
Flow cytometry data were collected on FacsCanto II and analyzed with BD FACSDiva v6.1.3 and Flowjo 7.6 software (Treestar). All data were statistically analyzed and plotted with GraphPad Prism® 5 software. $P<0.05$ was considered statistically significant.

## Results

*TLR stimulation of human dendritic cells triggers remodeling of late endosomes into tubular structures*
LPS stimulation of murine DCs induces elongated tubular structures emanating from LEs in a time- and dose-dependent manner [20-24]. To examine the effect of TLR triggering in LE remodeling of human DCs, we cultured monocyte-derived DCs (moDCs, 5 day culture in presence of IL-4 and GM-CSF[25]), and performed live cell confocal microscopy. To allow visualization of LE, DCs were pulsed with fluorescently labeled DiI-Low Density Lipoprotein (LDL, 30 minutes, 37°C [26]), followed by washes to remove unbound LDL.
We visualized DiI-LDL-pulsed moDCs after addition of TLR4 ligand LPS, or PBS as control in a time-lapse manner (500,000 DCs/coverslip well, LPS 200ng/ml; 0, 20, 60 and 240 minutes, schematically depicted in *Figure 1A*. Scoring was done for presence of motile LDL-positive tubular structures emanating from the center of moDCs, by two independent observers in a double-blinded manner. LPS treatment induced a rapid and steady increase in long tubular endosomes in the majority of moDCs (55% of DCs at 1 hour; 80% of DCs at 4 hours, *Figure 1B and C*). PBS-treated DCs never showed tubular endosomes in more than 25% of DCs, a background level that may relate to spontaneous DC maturation (*our unpublished data*).
Polyinosinic:polycytidylic acid (pI:C)/LPS combined treatment induced a similar level of tubular endosomes as LPS treatment alone, measured after 4 hours. Of note, moDC-expressed tubular endosomes are stable, yet dynamic structures that last at least 6 hours, or the duration of experiments (*Figure 1D*, individual tubules time-lapse captures, and *Figure S1*, movie).

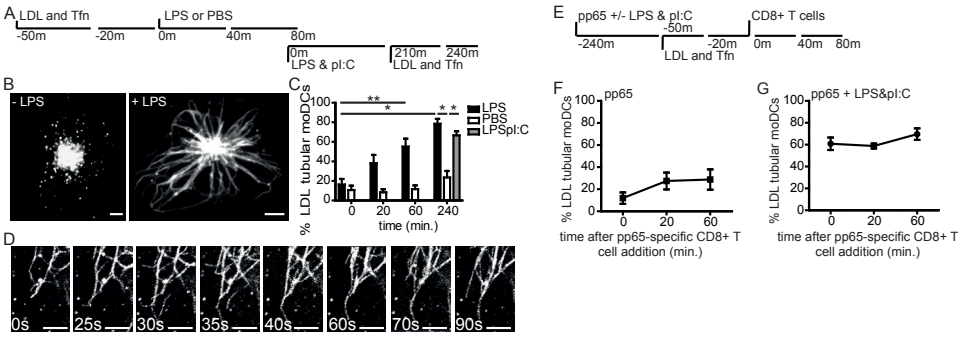



**FIGURE 1. TLR stimulation of human dendritic cells triggers remodeling of Late Endosomes into tubular structures**

(A) Schematic outline of live cell confocal microscopy experiment in B and C. Fluorescent cargo LDL-DiI and Transferrin-Alexa647 are staining after 30 minutes incubation the late and recycling endosomes, respectively. Just before or after stimulation with LPS or PBS. (B) Confocal image of moDCs with vesicular (left) or tubular (right) LDL+ endosomes (60 minutes 37˚C, 200 ng/ml LPS). (C) Percentage of moDCs expressing tubular LDL+ endosomes. Time points indicate a 20-minute time window immediately before treatment and around indicated time points (20, 60 minutes); PBS (white bars), 200 ng/ml LPS (black bars), or mix of 200 ng/ml LPS and 5 µg/ml pI:C (grey bars). Data represent mean ± SEM of 4 independent experiments. (D) Time-lapse captures of tubular LDL+ endosomes in LPS-treated moDCs (time points indicate seconds). (E) Schematic outline of live cell confocal microscopy experiment in F and G. (F and G) Percentage of moDCs expressing tubular LDL+ endosomes after culture in the presence of pp65 (3 µg antigen, 4 hour time point) and pp65-specific CD8+ T cells (1:1 ratio) in absence (F) or presence of LPS and pI:C combined (G). Data represent mean ± SEM of at least 3 independent experiments. Two-tailed Mann-Whitney U test. *P<0.05, **P<0.01. bar 5µm.

*Late endosome tubular remodeling in human dendritic cells occurs independently of cognate T cell interaction*

Cognate DC-T cell interaction induces T cell-polarized tubular endosomes in murine DCs, in a TLR-dependent manner [27;28]. We now asked whether in human moDCs, cognate T cell interaction in itself causes tubulation of LE or that TLR triggering is required. Therefore we pulsed 5-day moDCs with human CMV protein pp65 that is cross-presented to HLA-A2/NLVP-MVATV-specific CD8⁺ T cell clones [29]. We then added PBS or TLR ligands LPS and pI:C (200 ng/ml and 5µg/ml, respectively; 4 hours). Next, DCs were pulsed with DiI-LDL for 30 minutes (according to the scheme in *Figure 1E*), to allow for visualization of LE compartments. DCs were washed, T cells were added (1:1 DC-T cell ratio) and DCs were assayed for development of tubular endosomes by time-lapse confocal microscopy (0, 20, 60 minutes after T cell addition). Cognate DC-CD8⁺ T cell interaction in the absence of LPS/pI:C could not significantly induce LE tubular remodeling (*Figure 1F*), indicating the crucial role of TLR ligation in LE tubulation. In contrast, we observed tubular LDL⁺ compartments in 60-70% of antigen-laden LPS/pI:C-treated DCs. The fraction of antigen-laden DCs expressing tubular LDL⁺ endosomes did not significantly increase further upon addition of antigen-specific CD8⁺ T cells (*Figure 1G*). In conclusion, TLR-induced formation of tubular endosomes is conserved between murine and human DCs. We found no effect of additional antigen-specific CD8⁺ T cell contact on LE remodeling as in contrast to addition of antigen-specific CD4⁺ T cells in murine DCs [30].

*Efficient tubular remodeling of recycling endosome in human dendritic cells requires cognate T cell interaction*

Does antigen-specific T cell contact perhaps trigger tubular remodeling of other endosomal compartments in human DCs? To address this question, we visualized within moDCs the juxtanuclear located ERC, which is characterized by presence of Tfn receptors [31]. Endosomal recycling can occur via tubular recycling endosomes extending intracellular, as shown in EHD 1 over-expressing system within HeLa cells. These elongated recycling endosomes facilitate efficient recycling of both Tfn and Class I MHC molecules [32].

We visualized ERC in moDCs by incorporation of fluorescent Tfn (according to scheme shown in *Figure 1A*). Prior to stimulation, most moDCs have a vesicular Tfn$^+$ ERC (*Figures 2A*). In contrast to LE compartments, the addition of LPS or a combination of LPS and pI:C in absence (*Figure 2B*) or presence of viral antigens pp65 (*Figure 2D*) or EB2 (*Figure 2E*) to moDCs did not induce significant vesicular-to-tubular transformation of the ERC. When pp65-specific CD8$^+$ T cells were added to pp65-laden LPS/pI:C-treated moDCs, tubular transformation of Tfn$^+$ compartments ensued in 60% of LDL-tubular moDCs (*Figure 2C and 2D*). Similar results are obtained by using EB2-specific CD8$^+$ T cell clone and EB2-laden LPS/pI:C treated moDCs (*Figure 2E*). Incubating EB2-laden LPS/pI:C treated moDCs with pp65-specific CD8$^+$ T cells, avoiding cognate DC-T cell interaction, showed significantly reduced induction of tubular Tfn$^+$ endosomes compared to Tfn$^+$ tubulating pp65-laden DCs (*Figure 2C and 2D*, 60 minutes after

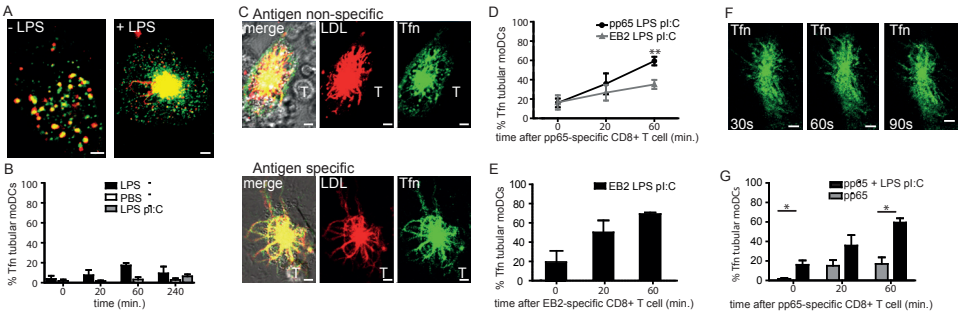



**FIGURE 2. Efficient Recycling Endosome tubular remodeling in human dendritic cells requires cognate T-cell interaction**

(A) Representative images of moDCs with LDL$^+$ late endosomes (red), and Tfn$^+$ recycling endosomes (green) in absence or presence of LPS stimulation (60 minutes, 200 ng/ml). (B) Percentage of LDL$^+$ tubular moDCs expressing tubular Tfn$^+$ endosomes prior to stimulation (t=0) or around indicated time points. 200 ng/ml LPS (black bars), or mix of 200ng/ml LPS and 5 µg/ml pI:C (grey bars). Data represent mean ± SEM of at least 3 independent experiments. (C). Representative images of moDCs upon CD8$^+$ T cell contact in antigen-independent (upper images) or antigen-dependent manner (lower images); LDL (red), Tfn (green), colocalization LDL and Tfn (yellow), pp65-specific CD8$^+$ T cell ("T"). (D) Percentage of LDL$^+$ tubular moDCs expressing tubular Tfn+ endosomes after 4 hour culture in the presence of 3 µg HCMV-derived pp65/LPS/pI:C (black circles) or EBV-derived EB2/LPS/pI:C (grey triangles), at indicated time points. Two-tailed, Mann-Whitney U test P<0.01. Data represent mean ± SEM of at least 4 independent experiments. (E) Percentage of LDL$^+$ tubular moDCs expressing tubular Tfn$^+$ endosomes after 4 hour culture in the presence of 3 µg EBV-derived EB2 with LPS and pI:C (black circles) at indicated time points. Data represent mean ± SEM of at least 3 independent experiments. (F) Time-lapse captures of Tfn$^+$ tubular endosomes in moDCs (time points indicate seconds). bar 5 µm. (G) Percentage of LDL$^+$ tubular moDCs expressing tubular Tfn$^+$ endosomes upon culture in the presence of 3 µg HCMV antigen pp65 (4 hours) in absence (grey bars) or presence of LPS/pI:C (black bars). Data represent mean ± SEM of at least 3 independent experiments; *P<0.05.

addition of pp65-specific CD8⁺ T cells).
In conclusion, TLR signaling alone is not sufficient to drive remodeling of Tfn⁺ ERC. Instead, functional cognate DC-CD8⁺ T cell interaction induces remodeling into elongated Tfn⁺ tubular structures. This remodeling requires TLR stimulation, as tubular transformation did not occur when pp65 antigen-specific CD8⁺ T cells were cultured with pp65-laden moDCs in absence of TLR stimulation (*Figure 2G*). The TLR-dependent remodeling of Tfn⁺ compartments in moDCs upon cognate interaction of antigen-specific CD8⁺ T cells is rapid and dynamic (*Figure 2F and Figure S2*: single cell images and movie of Tfn⁺ compartments).

*ICAM-1 clustering provokes tubulation of Tfn⁺ endosomal recycling compartments in human dendritic cells*
Efficiency of tubular remodeling of Tfn⁺ ERC is higher upon cognate moDC/CD8⁺ T cell interaction compared to antigen independent DC-CD8⁺ T cell interaction. We hypothesized that interaction of DC-expressed intercellular adhesion molecule-1 (ICAM-1) with lymphocyte function-associated antigen 1 (LFA-1) on interacting T cells facilitates tubulation of Tfn⁺ ERC in human moDCs for the following reasons. First, TLR4 ligation stimulates surface expression of ICAM-1 within a few hours (*our unpublished data*). Second, when antigen-bearing DCs enter the lymph node and are scanned by T cells, the initial DC-T cell interaction is antigen-in-

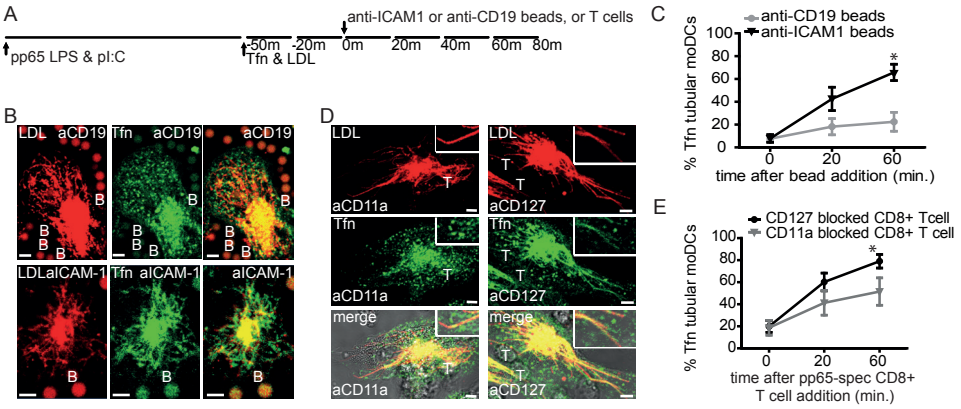



**FIGURE 3. ICAM-1 clustering provokes tubulation of Tfn⁺ endosomal recycling compartments in human dendritic cells**
(A) Schematic outline of live cell confocal microscopy experiment in B and D. (B) Representative images of moDCs upon anti-CD19 (upper images) or anti-ICAM-1 (lower images) mAb-coated beads contact. (C) Percentage of LDL⁺ tubular moDCs expressing tubular Tfn⁺ endosomes after 4 hour culture in the presence of 3 µg HCMV-derived pp65/LPS/pI:C. Prior to stimulation (t=0) or around indicated time points upon addition of anti-CD19 (grey circles), anti-ICAM-1 (black triangles) mAb coated beads (1:4 DC/bead ratio). One-tailed, Mann-Whitney U test; *P<0.05. Data represent mean ± SEM of 4 independent experiments. (D) Representative images of moDCs upon CD11a (LFA-1, left 3 images) or CD127 (IL7R, right 3 images) blocked pp65-specific CD8⁺ T cell contact. (E) Percentage of LDL+ tubular moDCs expressing tubular Tfn⁺ endosomes after 4 hour culture in the presence of 3µg HCMV-derived pp65/LPS/pI:C. Prior to stimulation (t=0) or around indicated time points upon addition of CD11a (LFA-1, grey triangles) or CD127 (IL7R, black circles) blocked pp65-specific CD8⁺ T cells (1:1 DC/T cell ratio). One-tailed, Mann-Whitney U test; *P<0.05. Data represent mean ± SEM of 6 independent experiments. Boxes are zoomed part of images. LDL (red), Tfn (green), colocalization LDL and Tfn (yellow), CD11a or CD127-blocked pp65-specific CD8⁺ T cell ("T"), mAb coated bead (illustrated by "B"). bar 5 µm.

dependent and involves association of LFA-1 with ICAM-1 [33]. And third, upon recognition of peptide MHC complexes by antigen specific T cell receptors (TCRs), TCR signaling drives LFA-1 in a state that binds with increased affinity to ICAM-1 [34]. Of additional consideration was that ICAM-1 clustering facilitates antigen presentation by recruiting HLA-A2 to the T cell contact zone [35] and that blocking of LFA-1 on antigen-specific CD4$^+$ T cells hampers tubular remodeling of LE in murine DCs [36].

To productively engage ICAM-1, we coated beads with stimulating antibodies (Ab) against ICAM-1.[37] As negative control we used beads coated with isotype-identical Ab specific for CD19, which is not expressed on moDCs. We performed the live-cell confocal imaging experiments using Ab-coated beads similar as earlier imaging experiments (*Figure 3A*). Addition of anti-ICAM-1 mAb coated beads to these moDCs induced tubular transformation of Tfn$^+$ juxta-nuclear positioned endosomes within 30 minutes, reaching 60-70% of moDCs showing tubular recycling endosomes at 60 minutes. Addition of anti-CD19 mAb coated beads did not induce remodeling of Tfn$^+$ compartments (*Figure 3B and 3C*). Similar data was obtained using anti-CD45 beads (*our unpublished data*).

To confirm whether absence of ICAM-1/LFA-1 interaction counteracts CD8$^+$ T cell-induced tubular remodeling of ERCs, we pre-incubated CD8$^+$ T cells with anti-LFA-1 (anti-CD11a) blocking Ab and added these to antigen-laden moDCs. CD127 (IL-7 receptor alpha) molecules are not involved with DC-T cell interaction, and were therefore blocked on CD8$^+$ T cells as a control. We found that CD8$^+$ T cell pre-treatment with anti-LFA1 mAb counteracted the induction of tubular Tfn$^+$ ERCs in interacting moDCs, whereas pre-treatment with anti-CD127 did not (*Figure 3D and 3E*). Thus, ICAM-1/LFA-1 interaction between moDCs and interacting CD8$^+$ T cells instigates ERC remodeling into elongated tubular structures in moDCs.

*Class I MHC clustering, or simultaneous ICAM-1/Class I MHC clustering, provokes tubulation of Tfn$^+$ endosomal recycling compartments in human dendritic cells*

ICAM-1/LFA-1 binding induces moderate levels of tubular transformation of Tfn$^+$ ERC in moDCs in comparison to cognate DC-CD8$^+$ T cell. During cognate DC-CD8$^+$ T cell interaction, both peptide/Class I MHC binding to the antigen-specific TCR and binding of ICAM-1 to LFA-1 occurs in parallel. We next asked whether ligation of MHC complexes, or co-ligation together with ICAM-1, is responsible for more tubular endosomal transformations in moDCs. To address this question, we exposed moDCs to anti-Class I MHC (anti-HLA-A2) mAb coated beads or beads coated with both anti-HLA-A2 and anti-ICAM-1 (*Figure 4A*). After 60 minutes of bead binding, up to 60-70% of LE remodeled human DCs showed tubular recycling endosomes (*Figure 4B and C*). Thus, both anti-ICAM-1 mAb-coated and double-coated (anti-A2/anti-ICAM-1 mAb) beads efficiently induced tubular remodeling of Tfn$^+$ ERC.

In conclusion, sufficient cross-linking of HLA-A2 and/or ICAM-1 molecules on the DC surface by mAb-coated beads or antigen-specific T cells drives Tfn$^+$ endosomal tubular remodeling.

*Elongated recycling endosomal tubules require an intact microtubule cytoskeleton and unperturbed endosomal recycling in human dendritic cells*

In murine DCs, tubulation of LE compartments requires the support of an intact microtubule-driven cytoskeleton [38]. The cellular requirements for ERC remodeling are unknown. Therefore, we tested whether recycling from the endosomal pathway to the DC surface is necessary, and whether an intact microtubule cytoskeleton is required. We made use of the reversible inhibitors primaquine (50 μM) or nocodazole (10 μM) (*Figure 5A-C*) [39;40]. We used

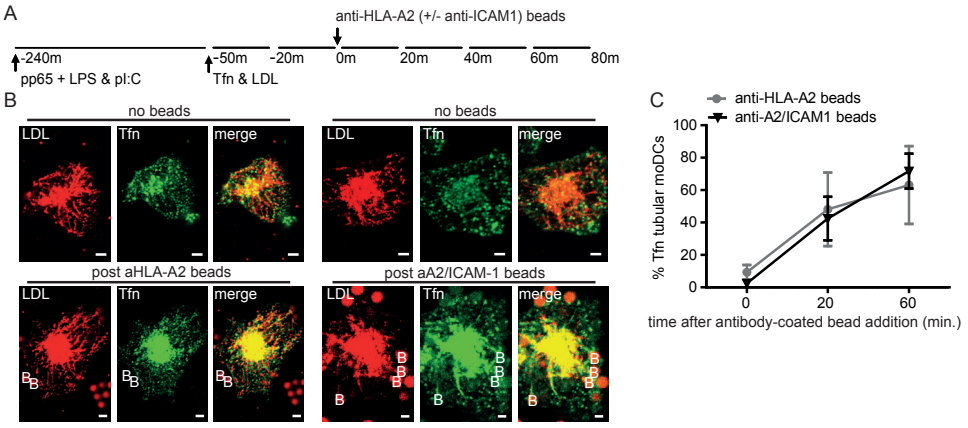



**FIGURE 4. Class I MHC clustering, or simultaneous ICAM-1/Class I MHC clustering, provokes tubulation of Tfn⁺ endosomal recycling compartments in human dendritic cells**

(A) Schematic outline of live cell confocal microscopy experiment in B. (B) Representative images of moDCs upon anti-HLA-A2 mAb (left 6 images) or anti-ICAM-1/anti-HLA-A2 (right 6 images). LDL (red), Tfn (green), colocalization LDL and Tfn (yellow), mAb-coated beads (illustrated by "B"), Bar 5 µm. (C) Percentage of LDL⁺ tubular moDCs expressing tubular Tfn⁺ endosomes after 4 hour culture in the presence of 3 µg HCMV-derived pp65/LPS/pI:C. Prior to stimulation (t=0) or around indicated time points upon addition of anti-ICAM-1/anti-HLA-A2 (black triangles) or anti-HLA-A2 mAb (grey circles) coated beads (1:4 DC/bead ratio). One-tailed, Mann-Whitney U test; *P<0.05. Data represent mean ± SEM of 3 independent experiments.

these inhibitors as published in DCs [41;42]. We induced tubulation of Tfn⁺ ERCs by 1-hour culture of antigen/LPS/pI:C-stimulated moDCs with antigen-specific CD8⁺ T cells (*Figure 5, A*: schematic outline of the experiment; *B*:, confocal image). Thirty minutes of primaquine or nocodazole treatment results in a significant reduction in moDCs with tubular Tfn⁺ endosomes to 8 and 17%, respectively (*Figure 5B and C*). Thus, tubular Tfn⁺ recycling endosomes require intact microtubules and continuous endosomal recycling.

*Abolishment of tubular ERC compartments in human dendritic cells associates with reduced ability to activate CD8⁺ T cells*

Does tubular transformation of ERCs have a functional consequence to antigen presentation by DCs? Molecules that selectively support tubular endosomes are not yet described, precluding a knock-down based approach. We therefore used pharmacological reagents primaquine and nocodazole to address this question, as in *Figure 5A, B and C*. We recently established a human DC-based cross-presentation model [43], which we adapted to compare antigen-specific CD8⁺ T cell activation by moDCs that are either able or temporarily unable to express tubular ERCs, by thirty minute-treatment of DCs with primaquine (50 µM) or nocodazole (10 µM) just prior to administration of T cells (schematically depicted in *Figure 5D*). Using this approach, advert effects of the drugs on moDC-mediated antigen uptake or processing, as well as direct effects on CD8⁺ T cells, were prevented [44;45]. Antigen-specific CD8⁺ T cells were next added to untreated, primaquine or nocodazole-treated moDCs (5 hour culture, 37°C). Both reversible primaquine and nocodazole thirty minute-treatment significantly reduced antigen-specific CD8⁺ T cell activation, as measured by decreased IFNγ production (*Figure 5F*, 27% and 22% re-

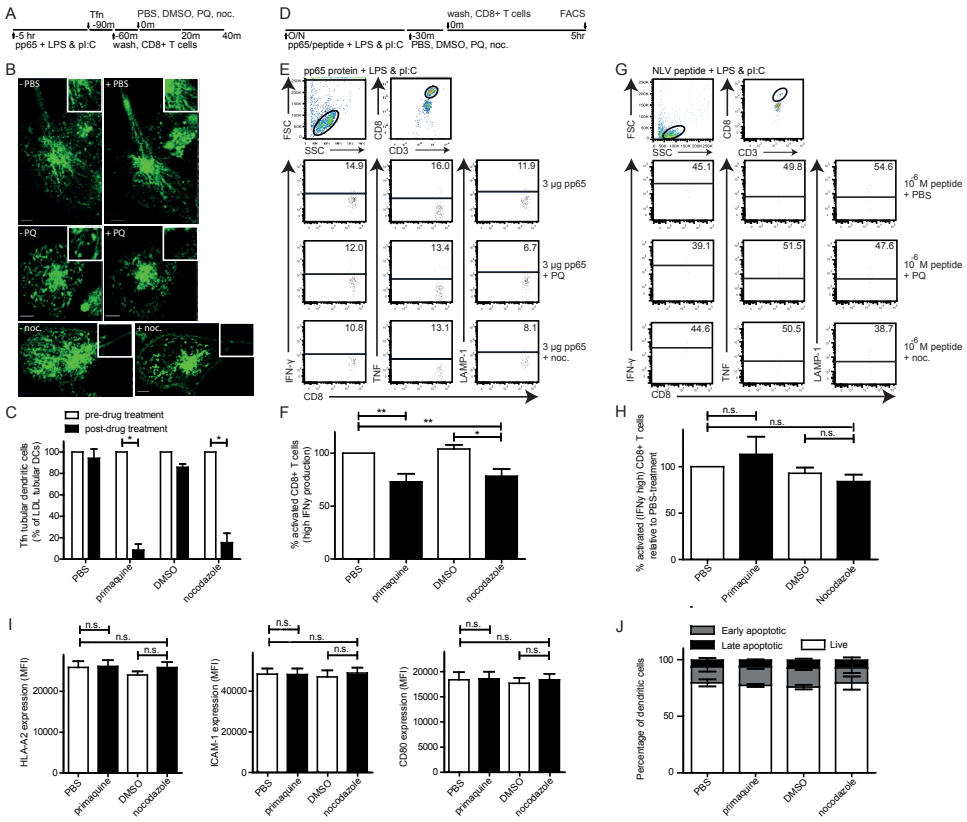



**FIGURE 5. Disintegration of tubular Tfn⁺ endosomal recycling compartments in human dendritic cells associates with reduced ability of dendritic cells to activate antigen-specific CD8⁺ T cells.**

(A) Schematic outline of live cell confocal microscopy experiment in 5B and C. Representative images (B) and the percentage (C) of selected 3 µg HCMV-derived pp65/LPS/pI:C-laden moDCs after 1 hours of co-culture with pp65-specific CD8⁺ T cells and pre-treatment (left 3 images in B, white bars in C) or after 30 minutes of indicated drug treatment (right 3 images in B, black bars in C). "PQ" (primaquine, 50 µM), "noc" (nocodazole, 10 µM), Transferrin (green), Bar 5 µm. Data represents mean ± SEM of at least 4 independent experiments. (D) Schematic outline of experimental setup for 5E, F, G and H. (E) Human moDCs were loaded with 0 or 3 µg HCMV-derived pp65 in presence of 200 ng/ml LPS and 5µg/ml pI:C. MoDCs were treated with either 50 µM primaquine biphosphate, 1 µg/ml nocodazole, or carrier controls PBS, and DMSO, followed by extensive washing. Next, pp65-specific CD8⁺ T cells were added for co-culture with the treated moDCs (1:1). DC mediated activation of antigen-specific CD8⁺ T cells was measured by analysis of induced production of IFNγ, TNF, or surface expressed LAMP1 high. (F) Percentage of antigen-specific activated CD8⁺ T cells, determined relative to matched PBS treated-moDCs. (G) Human moDCs were loaded with pp65-derived NLV-peptide in presence of 200 ng/ml LPS and 5µg/ml pI:C. MoDCs were treated with either 50 µM primaquine biphosphate, 1 µg/ml nocodazole, or carrier controls PBS, and DMSO, followed by extensive washing. Next, pp65-specific CD8⁺ T cells were added for co-culture with the treated moDCs (1:1) and antigen-specific CD8⁺ T cell activation was determined. (H) Percentage of antigen-specific activated CD8⁺ T cells, determined relative to matched PBS treated-moDCs. Human moDCs were loaded with 3 µg HCMV-derived pp65 in presence of 200 ng/ml LPS and 5µg/ml pI:C. MoDCs were treated with either 50 µM primaquine biphosphate, 1 µg/ml nocodazole, or carrier controls PBS, and DMSO, followed by extensive washing. Hereafter, HLA-A2, ICAM-1, and CD80 expression on (I) or viability (J) of moDCs is determined by flow cytometry analysis. Data represents mean ± SEM of at least 3 independent experiments. Two-tailed, Mann-Whitney U test; *P<0.05, **P<0.01.

duction, respectively). Concomitantly, TNF production and surface expressed LAMP1 on CD8+ T cells were reduced as well (*Figure 5E and 5F*). Both reversible primaquine and nocodazole thirty minute-treatment did not significantly affect presentation of pre-processed pp65-derived NLV-peptide (*Figure 5G and 5H*), DC surface expression of HLA-A2, ICAM-1, and CD80 (*Figure 5I*), and DC viability (*Figure 5J*). Because both inhibitors were added after antigen uptake and overnight antigen processing, possible effects on antigen uptake and processing are excluded. All together, this shows that primaquine and nocodazole affect endosomal tubulation, but does not interfere with other processes that are pivotal to antigen-dependent CD8+ T cell activation. Taken together, abolishment of the tubular structure of ERC in human DCs associates with reduced ability of DCs to activate antigen-specific CD8+ T cells.

## Discussion

Various environmental cues, including TLR ligands, induce DC maturation. During maturation the DCs rapidly transform from endocytic cells that survey their immediate surroundings, into cells dedicated to antigen presentation. As the processing of antigen and assembly of peptide-loaded MHC complexes occurs at intracellular locations, transport of peptide/MHC complexes to the DC surface is critical for display to T cells. We here show that TLR triggering-induced maturation rapidly drives vesicle-to-tubule transformation of late endosomal compartments in human DCs. This corroborates earlier studies performed on murine DCs [46-48]. TLR signaling does not suffice to drive Tfn+ recycling endosomal tubulation, which suggests that induction of endosomal tubulation does not necessarily direct DCs towards maturation. However, as antigen-specific CD8+ T cells only induced tubulation of recycling endosomes in presence of LPS and polyI:C, we believe that DC maturation is a prerequisite for endosomal tubulation. Whether there is selection of endosomal tubulation in response to distinct TLR stimuli, as was proposed for phagosome maturation, is yet unknown [49].

It is reported that in absence of TLR stimuli, T cells cannot stimulate late endosomal tubulation in both human and mice [50]. In contrast to murine DCs, addition of T cells in presence of TLR ligand does not further stimulate late endosomal tubulation. Whether this is due to usage of human CD8+ T cells instead murine CD4+ T cells is not known.

We confirmed the necessity of ICAM-1/LFA-1 and HLA-A2/TCR interactions in remodeling of Early/Recycling endosomal compartments in DCs that bind T cells, by use of antibody-coated beads as surrogate T cells as well as with blocking experiments. Of note, in our bead experiments, we found equal efficiency at inducing tubular remodeling of Tfn+ endosomal compartments using HLA-A2 mAb-, ICAM-1 mAb-, or double mAb-coated beads (*Figure 3C, 4B and 4C*). Whether this finding is relevant to DCs that interact with T cells or only true to those that interact with mAb-coated beads, we could not fully address. However, since DC-T cell contact induces the rearrangement of HLA-A2 and ICAM-1 into immune synapse-like structures on the DC surface [51], we consider it unlikely that singular HLA-A2 clustering drives ERC tubular remodeling. In our bead assays, supra-physiological cross-linking of either HLA-A2 or ICAM-1 molecules may already facilitate immune synapse-like structures. Indeed, in live cells, HLA-A2 and ICAM-1 have increased association with each other upon cross-linking of either ICAM-1 or HLA-A2 [52].

We are not the first to relate Tfn+ compartments in DCs to Class I MHC-mediated stimulation of antigen-specific CD8+ T cells. It had been known that peptide-receptive Class I MHC molecules are present in endosomes [53]. Moreover, Class I MHC molecules are present in primaquine sensitive or Tfn+ compartments [54;55]. In murine DCs, soluble antigen derived peptide loading

onto Class I MHC molecules occurs within Tfn$^+$ endosomes in an LPS-dependent manner [56]. Murine DCs that lack Class I MHC molecules in recycling endosomes due to an aberrant tyrosine-based internalization motif, were shown to be defective in cross-presentation [57]. Finally, tubular recycling endosomes can mediate efficient Class I MHC recycling in HeLa cells, and HLA-A and ICAM-1 signaling is essential in viral antigen presentation to CD8$^+$ T cells [58;59]. All together, data by us and others provide experimental support that in human DCs tubular transformation of Tfn$^+$ ERCs modulates the recycling peptide/Class I MHC complexes, and their display to antigen-specific CD8$^+$ T cells.

Recently, it was shown that infection of HeLa or RAW cells by *S. typhimurium* promote LE tubulation in these cells to increase cell-to-cell transfer of Salmonella [60]. These data show that endosomal tubular transformation is not restricted to DCs. In addition, it raises the possibility that pathogens may exploit interference of endosomal tubulation to inhibit surface-directed transport of peptide/MHC complexes. Discovery of pathogen-derived molecules that selectively inhibit endosomal tubulation would be beneficial to determine molecular mechanisms involved in endosomal remodeling.

The stimulation and clonal expansion of CD8$^+$ T cells by antigen presenting DCs requires the sequential interaction of an estimated 200 TCR molecules with antigen-specific peptide/Class I MHC complexes [61]. As DC maturation does not drastically increase surface expression of Class I MHC molecules [62-64] selective recruitment of specific peptide/MHC complexes towards the DC-T cell contact zone must occur. We believe such recruitment is supported by endosomal tubules that polarize towards the cell surface. We show that the induced transformation of tubular ERC structures occurs efficient only when (a) sufficient clustering of HLA-A2 and/or ICAM-1 occurs at the DC surface and (b) TLR stimulation is provided. The requirement for innate stimulation through for example TLRs restricts remodeling to “dangerous” antigens and not endogenous self-peptides. The requirement of TLR triggering prior to CD8$^+$ T cell activation also ensures that DCs are optimally primed for antigen presentation. Our findings collectively support a two signal-model in which the DC through tubular endosome transformation facilitates selective clonal CD8$^+$ T cell expansion. Only antigen-specific CD8$^+$ T cells induce sufficient ICAM-1 and HLA-A2 clustering that allow for the tubular transformation of Tfn$^+$ ERC in DCs. Accordingly, only a sufficiently high qualitative signal, triggering of the high affinity TCR would rally the quantitative response (peptide/MHC I complexes) that is required for full CD8$^+$ T cell activation.



## Acknowledgements

We thank members of the Laboratory Translational Immunology, especially the Boes lab members for their expertise. We are grateful to Wim Burmeister for providing us with EB2 plasmid. This study was supported by the Wilhelmina Research Fund (to Marianne Boes).

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## Supplemental material

*LPS-induced tubular Late Endosomes in human dendritic cells are dynamic (.avi, 3.1 MB)*
Movie of LPS-treated moDC (200 ng/ml, 60 minutes) expressing tubular LDL+ LE compartments. MoDC imaged at a frame rate of 5 seconds by confocal microscope (63x lens, 2x digital zoom, at 37°C with 5% $CO_2$ in serum-free medium without phenol red, 0.2% BSA, 10 mM HEPES).

*Lymphocyte ligation-induced tubular Tfn⁺ recycling endosomes are motile (.avi, 2.3 MB)*
Movie of LPS-treated moDC (200 ng/ml, 60 minutes) displaying tubular Tfn⁺ recycling endosomes. MoDC imaged at a frame rate indicated in the movie (in seconds) using confocal microscopy (63x lens, 2x digital zoom, at 37°C with 5% $CO_2$ in serum-free 0.2% BSA and 10 mM HEPES-buffered medium without phenol red).

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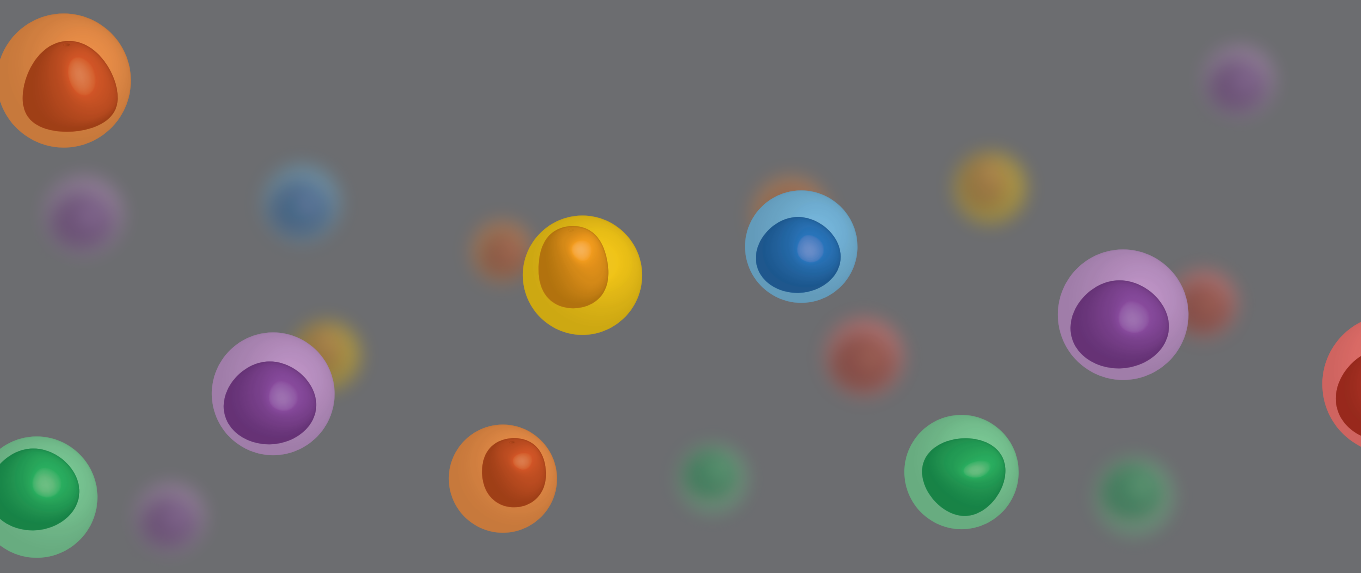
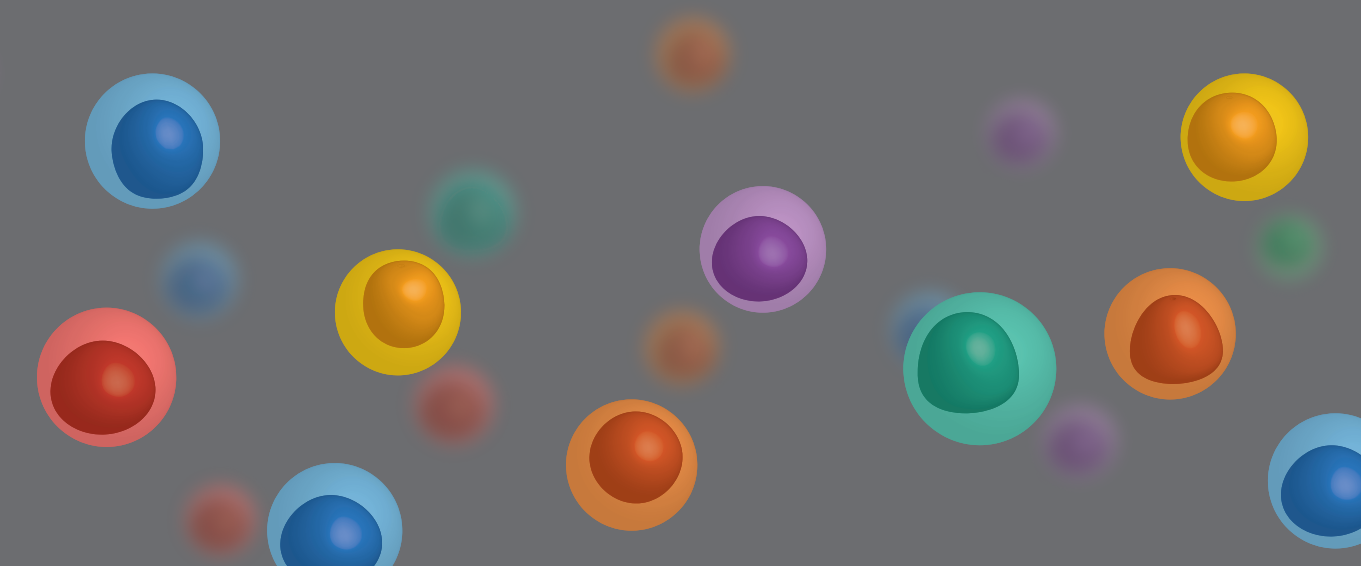


# Application of antigen cross-presentation research in patient care

Thijs W.H. Flinsenberg and Marianne L. Boes

Frontiers in Immunology 2014 Jun 17;5:287



## Application of antigen cross-presentation research into patient care

Dendritic cell (DC) based cellular immunotherapy is being explored as a treatment modality for several malignancies, for viral diseases and auto-immune disorders. More than four decades of preclinical research on DC biology has created a strong foundation for clinical application of DC-based clinical trials, which already have been performed since the 1990s. Although sometimes met with limited success, clinical trials yield better understanding of the requirements for optimal DC-based therapy. Recent advancements in the understanding of human DC biology and subset characteristics now give rise to ample opportunities to explore for a next generation of DC-based immunotherapy. This Research Topic is focussed on articles that can help understanding the biology involved in DC antigen presentation, for future DC-based immunotherapy.

DCs are professional antigen presenting cells (APCs) that are particularly well endowed to elicit adaptive immune responses, via the presentation and cross-presentation of antigen-derived peptide/MHC complexes to T-lymphocytes. These processes decide how the host interacts with its environment, and therefore can be a target for pathogen interruption. Van Montfoort et al. (1) provide an overview of cross-presentation features and describe how the study of various viral pathogens can elucidate anti-viral immune strategies. They further describe how DC maturation is crucial in immunity against viruses and how viruses may dampen this response to their own advantage. Understanding the presentation pathways is pivotal for optimal DC-based immunotherapy development.

DC-based immunotherapy comes in two flavors. Either DCs are cultured and manipulated ex-vivo before infusion, or endogenous DCs can be targeted in-vivo. Concerning the latter approach, local administration of long peptides has proved effective in several diseases giving opportunity for further exploration. Both Delamarre and Cohn (2) and Rosendahl Huber et al. (3) discuss the requirements for improved antigen presentation, providing considerations on $CD4^+$ and $CD8^+$ activation, choice of antigen and desired adjuvants. Delivering antigen to DCs is a next hurdle to take.

Regarding antigen delivery, it matters to engage specific receptors, for these receptors can decide the intracellular pathway of antigen routing and enzymatic processing. Antigens are thereby directed towards assembly into peptide/MHC class I (cross-presentation) or peptide/MHC class II complexes, and induction of immunity or tolerance. Fehres et al. (4) describe the biology of receptor-mediated uptake in the context of antigen presentation, with special emphasis on C-type Lectin receptors. They further discuss the possibilities to formulate antigen in order to provide directed antigen delivery. Another import route of uptake involves the family of Fc Receptors, which is discussed by Platzer et al. (5) Here, the role of this receptor family is highlighted in antigen presentation with emphasis on the opposing roles of activating and inhibiting Fc Receptor isoforms. Furthermore, they underscore that mechanisms of antigen-presentation in mice are not always identical to the human pathways. Thus, the need for more research on human DC biology is warranted, for DC-vaccination strategies are still heavily based on mouse-biology.

When designing a DC-based immunotherapy, it is relevant to consider the subtype of DCs that one aims for. Boltjes and van Wijk (6) present an overview of all phagocyte subsets that are present throughout the human body in steady-state and under inflammatory conditions. They also emphasize the differences between mouse and human cells, and review cell types that should be considered for immunotherapy. Until recently, monocyte-derived DCs (MoDC) were used mostly in DC-therapy, for their relative ease to culture in large quantities ex-vivo. But while MoDCs can be found in human tissue under inflamed conditions, other DC subsets are more prevalent overall and may be more specifically endowed at stimulation of particular T-cell subsets, to be explored in immunotherapy. One subset that was suggested to be superior in $CD8^+$ T-cell priming is the recently identified BDCA-$3^+$ ($CD141^+$) DC, characterized by CLEC9A and XCR1 expression. Tullett et al. (7) highlight recent findings explaining why these cells are effective at $CD8^+$ T-cell priming and discuss in-vivo antigen targeting towards these DCs. Wimmers et al. (8) also describe the use of naturally circulating mDCs and pDCs for DC-based immunotherapy. They discuss the division of labor between pDCs and mDCs and the clinical trials that are being performed using these subsets. Interestingly, they highlight that mDCs and pDCs work in synergy, supporting each other to enhance the effector phase of the adaptive immune response. Based on this observation, a next step in DC-based vaccination should include a cocktail of mDCs and pDCs, or in-vivo antigen targeting to both subtypes.

DCs are often called 'master regulators' of the immune response. Besides firing up immune reactions, DCs play an equally important role in the maintenance of tolerance, either by dampening specific T-cell responses or by inducing regulatory T-cell subsets. Loss of tolerance

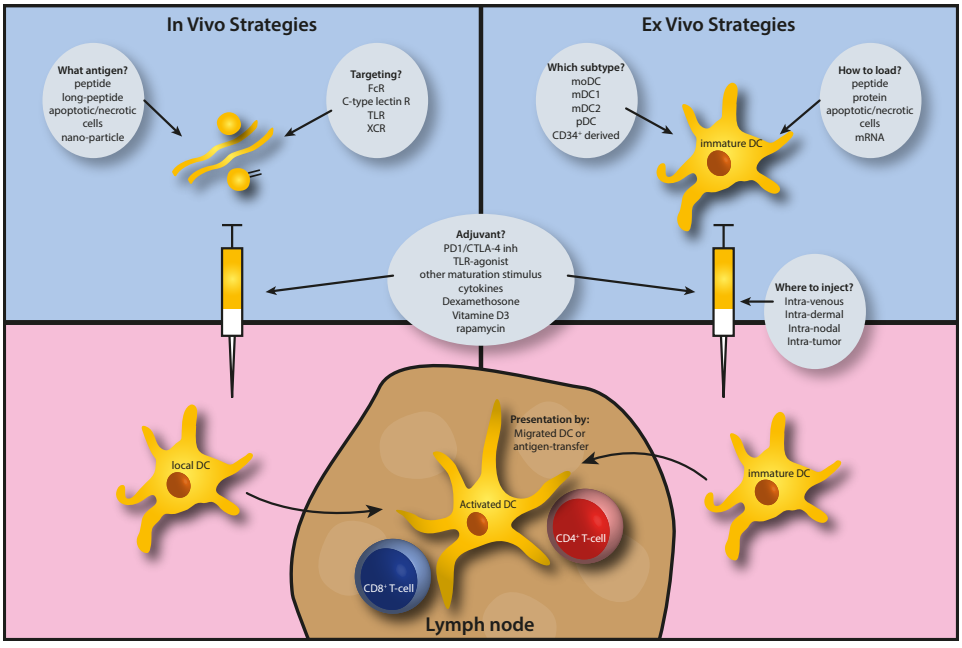



**Figure 1: Schematic outline of the considerations to apply antigen cross-presentation research to the clinic, most readily by dendritic cell-based immunotherapy.**

is of pivotal importance in auto-immune diseases as described by Hopp et al. (9) Their review concerns the presentation of self-antigen, which they discuss in the context of mechanisms in tolerance induction, DC maturation status, DC uptake and processing mechanisms and tolerance-associated intracellular signaling pathways. The regulation of DC function is also controlled by metabolic pathways, as described by Pearce et al. (10) Recent advancements concerning regulation of DC metabolism include the identification of key-proteins like PI3K, Akt and mTOR in DC function. The awareness that manipulaton of DC metabolic pathways changes DC function should be explored for designing DC-based cellular therapy, especially since it may give opportunity to steer towards more immunogenic or tolerogenic consequences. This could be of upmost importance in the setting of auto-immune diseases, anti-cancer or graft-versus-host therapy.

Plantinga et al. (11) finally discuss recent developments in DC therapy in the setting of allogeneic-hematopoietic cell transplantations (HCT). Such transplantations are considered a last-resort treatment for several malignancies of hematological origin. DCs grown from the same donor background as the HCT are now being explored for their potency to prevent cancer relapses early after allogeneic HCT. The various considerations for such DC vaccinations are discussed, such as the stem cell source, type of tumor antigen and vaccination strategy.
The breath and quality of the work discussed in this research topic underscores the strong translational push of DC research towards clinical settings. Immunotherapy is now being incorporated into standard cancer care, with antibody-based treatments currently being at more advanced stages than cellular therapies. The abundance of currently ongoing DC-based cellular immunotherapy trials should benefit patient care in the near future, considering that the roots for translational success lies in pre-clinical laboratory research.

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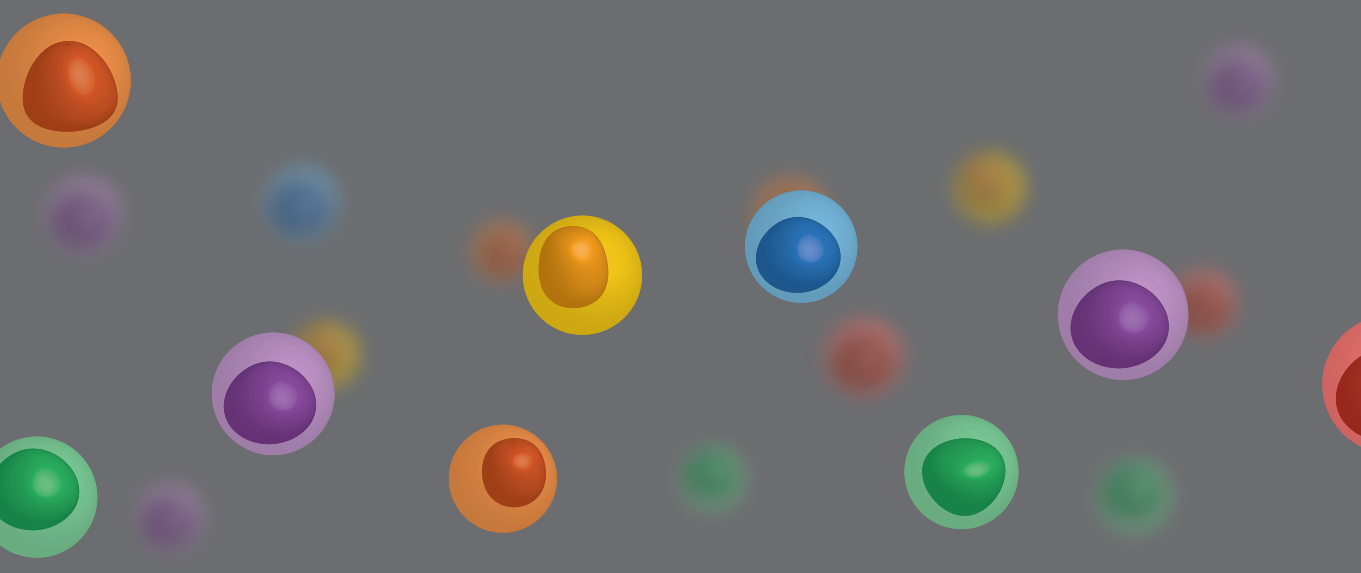
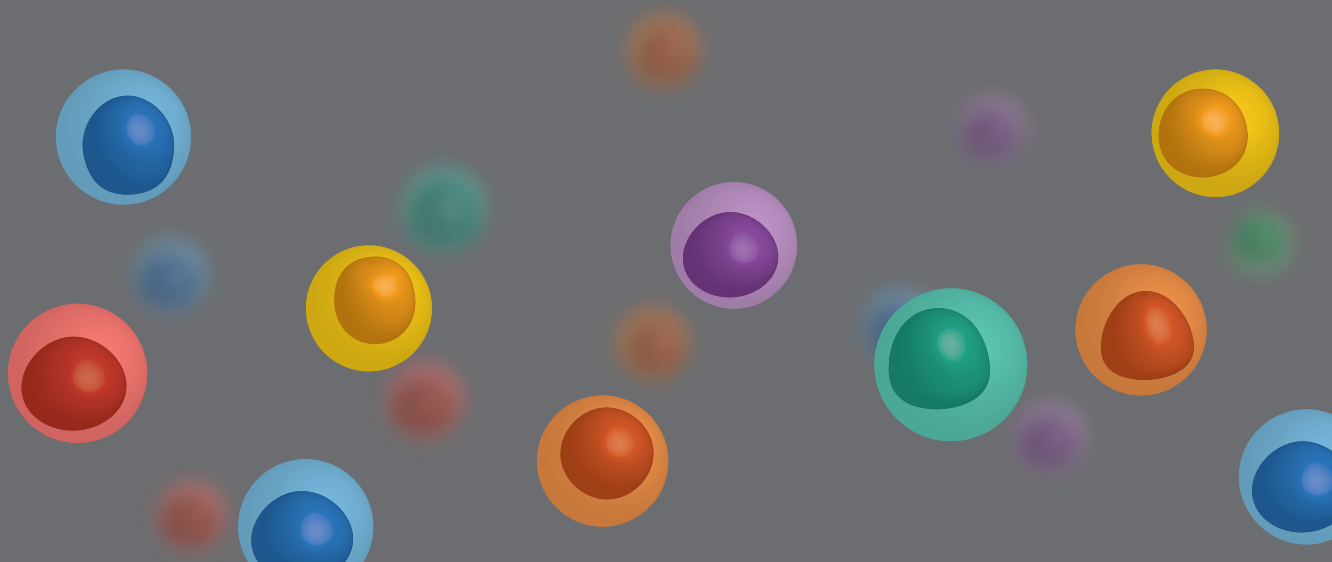


# General discussion



## Discussion

Dendritic cells are the most important cells of our immune system [1], for these cells orchestrate the various immune cells towards tolerance or immune-stimulatory reactions. Especially the carefully balanced and bidirectional interaction with lymphocytes is essential in maintaining health in a continuous battle with both external and internal threats. They protect us against a microbial invasion, while preventing outgrowth of malignancies.[2-4] The downside of such a complex regulation is its susceptibility to dysbalance, thereby contributing to a spectrum of auto-immune diseases.[5] Considering the communication with T-lymphocytes, we differentiate between the presentation of endogenous and exogenous proteins via MHC class I and II towards $CD8^+$ and $CD4^+$ T-cell respectively (described in more detail in chapter 1 and 2)[6]. The presentation of exogenous protein via MHC class I, termed cross-presentation, is the main focus of this thesis.

Dendritic cell cross-presentation is a fascinating topic for academic research, and attempts to clarify its processes, underlying biology and function gave rise to over 5000 papers in the last decade (*ISI web of knowledge*). In this thesis, we aimed to contribute to the understanding of cross-presentation biology. This research was done within a clinical environment and part of the research focused towards the development of a DC-based vaccine, for which the first steps are described in the supplemental chapter.

### The rise of new DC subtypes

The human DC cross-presentation field was boosted in 2010, when three independent groups identified a human counterpart of the mouse $CD8^+$ cross-presenting DC, termed BDCA-$3^+$ or $CD141^+$ DC.[7-9] This DC characterized by the expression of CLEC9A and XCR1 was shown to be superior in antigen cross-presentation when compared to other DC subtypes (pDC, MoDC or BDCA-$1^+$ DC). In the years to follow, other groups confirmed the existence of these DC subtypes, but disputed its superior role in antigen cross-presentation.[10-15] Looking back to the initial papers, we can point out several clues to this discrepancy.

*BDCA-$3^+$ DCs are superior?*

The initial papers by Bachem *et al.* and Poulin *et al.* showed superiority of BDCA-$3^+$ cells in antigen cross-presentation, mostly independent of TLR-ligation.[7;9] We confirmed these findings, although differences were not striking in our experimental conditions.[16] Jongbloed *et al.* and later Mittag *et al.* also compared cross-presentation without TLR ligation, however, did not find a difference between the subsets.[8;11] Whether BDCA-$3^+$ DCs are really better cross-presenters, or that this finding is dependent on assay conditions is yet unclear. BDCA-$3^+$ DCs were indisputably superior in cross-presenting antigen from necrotic cells, which is also explained by high expression of CLEC9A and XCR1.[7-9]

*Interplay of cross-presentation capacity with TLR-ligation*

Jongbloed *et al.* did show superior antigen cross-presentation by BDCA-$3^+$ DCs compared to BDCA-$1^+$ DCs after stimulation with antigen in the presence of poly I:C. However, the expression of TLR3 was 10-fold higher in BDCA-$3^+$ positive DCs, possibly explaining differences in cross-presentation.[8] Nizzoli *et al.* reported in 2013 that BDCA-$1^+$ DCs are the most relevant cross-presenters, at least in the priming situation.[12] They attribute this effect to the produc-

tion of IL-12, which is secreted at high abundancy by BDCA-1$^+$ DCs. This was also shown by Mittag *et al.*[11] Furthermore, they show that either BDCA-1$^+$ or BDCA-3$^+$ can be superior at antigen cross-presentation, depending on the TLR-trigger that was additionally added. Interestingly as stated before, no cross-presentation was seen in the absence of TLR stimulation.

*Cross-presentation capacity relates to the tissue background of DCs*
In 2012, Segura *et al.* compared lymph node (LN) and blood DCs and described higher T-cell polarization after contact with LN DCs, consistent with the notion that blood DCs are immature versions of LN DCs.[17] They however found no difference in CD4$^+$ T-cell polarization between BDCA-1$^+$ and BDCA-3$^+$ DCs, which appears to contradict the conclusion by Jongbloed *et al.*[8;17] Indeed, Jongbloed *et al.* noted superior Th-1 responses by BDCA-3$^+$ DCs. Moreover, Segura *et al.* show that only LN derived DCs (not blood DC) can cross-present long-peptide and claim that this is due to the immature status of blood DCs. This is in contrast to our data and the data by Bachem *et al.*, which shows cross-presentation by blood BDCA-3$^+$ DCs.[7;16;17] In 2013, Segura *et al.* again showed similar cross-presentation capacity of soluble antigen between different DC subtypes, including pDCs.[13] One of the differences in experimental setup that may explain the inconsistencies in research findings is that Segura *et al.* used MelanA long peptide, where the other papers use full pp65 protein. Mittag *et al.* also compared DC subtypes in spleen and blood.[11] They show that cross-presentation capacity is not confined to BDCA-3$^+$ DCs, but is shared with BDCA-1$^+$ and CD16$^+$ DCs. Furthermore, DC function was comparable between spleen and blood derived DCs.

*What about other DC-subtypes?*
In 2013, Tel *et al.* showed that not mDCs, but pDCs are superior in antigen cross-presentation. They state that pDCs are able to preserve antigen cargo for prolonged periods of time and are able to present antigen-derived peptides efficiently even after 100 hours.[15] Furthermore they state that the high frequency of pDCs in blood combined with their antigen presenting capacity puts pDCs on the frontline of anti-tumor defense. Interestingly, this is in sharp contrast to the data published by Bachem *et al.* where pDCs were of all subsets the only one that could not cross-present soluble pp65 protein.[7] Also Nizzoli *et al.* showed only very modest cross-presentation capacities by pDCs.[12]

Recently, research on cross-presentation by human DCs became even more interesting, when Gehring *et al.* showed that hepatitis B virus (HBV) in infected humans was predominantly taken up, processed and cross-presented by monocytes, that subsequently differentiated into MoDCs.[10] Since this work is actually done with real patients and an actual disease, and considering the fact that DCs are rather rare, while monocytes are very frequent, this work gives rise to even more food for discussion on the relevance of cross-presentation research on selective DC subtypes.

*In conclusion*
It appears that the initially proposition that DC subtypes exhibit differential functions is not clear-cut or black and white. Depending on the location of DC retrieval, antigen used and DC stimulation, all DC subtypes and monocytes are able to cross-present to more or lesser extent. Superiority of one subtype over the other with respect to cross-presentation capacity even so depends on these factors. Based however on all currently available literature, it seems unlikely

to me that one subset is generally superior over another in antigen cross-presentation. Probably, we need all subsets to optimally trigger CD8+ T-cell responses in vivo. Therefore, concerning treatment strategies, we should in my opinion not focus on a single DC subtype but rather keep an open minded view and try different subtypes, if possible combinations, to optimally serve the need for cellular treatment.

## DC licensing, how does it work?

DC licensing by CD4+ T-cells has been extensively studied in mice. More than 2 decades ago, it was already shown that the induction of virus antigen-specific CD8+ T cells requires the prior licensing of DCs by interaction with cognate, antigen-specific CD4+ T-cells.[18-22] This help could in some cases be replaced by CD40 agonist antibodies.[23-25] It has until now been assumed that these findings could be translated to the human situation. In chapter 5, we show that this indeed is the case, but mechanistically, details still need to be worked out.[26]

*CD40-CD40L interaction*

The initial model of DC licensing via CD40 that was proposed was based on CD40 upregulation by DCs, that interacted with CD8+ T-cell expressed CD40L, thereby together with the peptide/MHC, giving the essential signals necessary for T-cell differentiation to acquire full cytolytic effector function.[25;27;28] Later however, it was shown that the CD40 signal was derived from activated CD8+ T-cells that transiently expressed CD40. In this model, the DC functions only as a facilitator bringing the cognate CD4+ and CD8+ T-cell in close proximity.[29] However, sorted DCs could only prime CD8+ T-cells after they had been in contact with cognate CD4+ T-cells, further establishing the DC-licensing model.[30] We showed in *chapter 5* a role for CD40 in DC licensing, but based on these experiments, it is not yet proven that CD4+ T-cell based DC-licensing is CD40 dependent. Performing priming in the presence of CD40-40L blocking antibodies should provide a useful answer here. How CD4+ T-cells contribute to eventually memory development yet has to be cleared. We are until now not able to show memory T-cell activation after in vitro priming. Once this can be measured, the effects of CD4+ T-cells and CD40-40L interaction can be further investigated, with respect to memory function.

*Licensing cytokines*

CD4+ T-cells are known to produce various cytokines, which have in mice been implicated to be essential for CTL priming.

Firstly, IL-2 seems to be important in CTL priming/survival. In a cross-priming model in the absence of CD4+ T-cells, CTL induction could be induced by exogenous IL-2 injection.[31] Moreover, when making CTL clones for in vitro studies, adding IL-2 is essential for growth and survival.[16]

Secondly, IL-12 has been implicated important in priming. Although it has been shown that IL-12 can overcome the requirement for CD40 activation [32], IL-12$^{-/-}$ mice were perfectly capable of inducing effective CTL priming.[33] In human DCs, IL-12 was again termed important for CTL priming by BDCA-1+ DCs.[12] In this paper however, polyclonal CD8+ T-cell expansion in combination with PMA/ionomycin stimulation after 7 days was used as a read-out for priming, which is in sharp contrast to the low levels of CTL expansion seen after multiple weeks of restimulation in other papers, making it very unlikely that this paper actually describes priming instead of polyclonal T-cell expansion.

Finally, we showed a role for IFN-γ in CD4+ T-cell mediated DC licensing, at least in the memory

situation.[26] IFN-γ has already been investigated in the priming situation in mice, where it was shown to be crucial in CTL induction, where IL-12 was not.[34] Further experiments with IFN-γ blockade and addition need to be done to further characterize its role in human CTL priming.

*Enabling CD4⁺ T-cell mediated presentation through acquisition of DC-derived exosomes*
Another model proposes that CD4⁺ T cells can acquire APC-like properties by recruiting peptide/MHC-I (and co-stimulatory factors) from DCs in response to cognate pMHC-II-TCR interactions.[35] This is proposed to happen through DC-exosomes that are transferred within the immunological synapse. Although this is a possible scenario that deserves further investigation, this possibility appears to contrast existing data. Smith et al. showed that sorted, already licenced DCs, could effectively prime naïve CD8⁺ T-cells in culture, proving that it is the DCs, rather than the CD4⁺ T-cells that prime the CTLs.[30] The latter paper supports the sequential two-cell interaction model, which is also in contrast with papers describing the requirement for both the CD4⁺ and CD8⁺ T-cell to interact with the same APC.[36-38]

*In conclusion*
It is still unclear how DC licensing exactly works. As already stressed by Xiang et al, there are three current models (*Figure 1*). For all models, there are experiments in support and against. It could still be that one model is solely responsible for priming, but the more likely scenario in vivo would be a combination of models dependent on the pathogen and location.

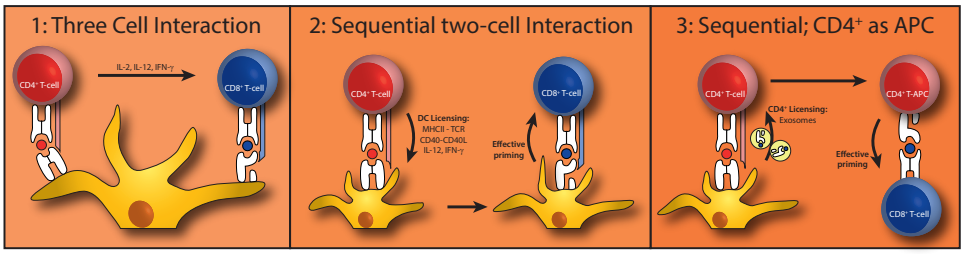



**Figure 1: Three suggested mechanisms for CD4⁺ T-cell mediated CTL priming (Adapted from Xiang et al [39]).**
1: The three cell interaction, where an antigen specific CD4⁺ T-cell and CD8⁺ T-cell are in close proximity, interacting through the same DC. The CD4⁺ T-cell directly influences CTL priming by cytokine release.[36-38] 2: Sequential two-cell interaction, where the DC is licensed by an antigen specific CD4⁺ T-cell. This licensing happens by a combination of signal 1,2 and 3.[25-28;30] 3: Sequential; CD4⁺ as APC, where the CD4⁺ T-cell acquires APC capacities by receiving peptide-MHC complexes. One proposed mechanism is via exosomes.[39]

## The future of DC vaccinations, how can we optimize this cellular therapy?

In the era of PD-1 and CTLA-4 checkpoint blockade[40], is there a future place for DC vaccinations as an immunotherapy? I think there is.

Anti-tumor therapies focus on the 'escaped cancer-cells' that circumvented removal by the immune system or anti-cancer medical treatment but are never truly specific, giving rise to major side effects. For many anti-cancer treatments, it's even these massive side-effects that hamper further treatment. This is one of the main reasons why the future of anti-cancer ther-

apy will include the use of our own defense system, whether in the form of antibodies like the PD-1 blockade, or cellular therapy.

Cellular therapies could potentially be a major arm in anti-cancer treatment. The problem so far is that we do not actually understand well enough our immune system to fully control a therapy based on its actions. While our knowledge expands, cellular therapies based on different immune cells are being tested with mixed results. Especially DCs, the orchestrators of all adaptive immune responses, still holds promise in anti-cancer therapy. Potentially, DC-vaccinations could provide a useful tool, for it could be used in different cancer- and other immune settings. Moreover, once better understood, it could be used in anti-viral therapy post SCT or even in chronic viral infections such as HIV or hepatitis B virus. However, the results so far are disappointing to a degree that many people are giving up on this method. Still, new developments happen, and clinical trials pursue.[41] The question remains, how can we optimize DC-vaccination?

*The optimal DC subtype*
With all new insights in DC biology, it is up to groups and trials to bring this knowledge into practice. One of the recent relevant questions is: which DC-subtype should we use? If we look upon the literature of the past few years, the discussion on DC subtypes has been lively, and until now, it is not exactly known how each DC-subtype is involved in immune responses (see previous paragraph). This however creates a lot of new opportunities to the DC-vaccination field. In the past, DC-vaccinations were predominantly performed using MoDCs. However, now it is generally accepted that primary DCs are more set to the priming task, we need to re-evaluate DC trials. Also trials done with $CD34^+$ DCs need to be seen in a different perspective, since it is not clear what type of DCs are generated in this process, and how they compare to primary DCs. Within the U-DANCE (Utrecht DCs Against CancEr) group, a CB-derived DC vaccine is being developed with extra attention to the exact DCs that are cultured using different protocols.[42] We should understand the DC that is infused in the patient as good as possible, so we are able to make adjustments according to the clinical outcome, specifically based on DC function. Therefore it is of extreme importance to evaluate generated DC function prior to infusion as well as during a clinical trial in a laboratory setting.

*What is the correct antigen loading strategy?*
Loading ex-vivo generated DCs with tumor lysates has been tried in the vaccination setting in over 80 trials.[6] Though it is safe, well tolerated and even immune responses can be detected, the clinical response was generally low[43;44]. Another way to load DC-vaccines is by mRNA-pulsing, which lead to amazing clinical results in AML[45]. Also this work is being pursued by several groups, and gave rise to many clinical trials.[41] Hopefully within the next few years, this technique will be used for DC-vaccines in children with AML (M. Plantinga and C. de Haar, work in progress).
In-vivo DC targeting, where coupling protein to antibodies that target in vivo DC-receptors (CLEC-9A, DEC-205, DCIR) is a promising therapy, but has not been tested in the clinical setting[46]. Injection of long-peptides resulted, at least in HPV induces vulvar carcinoma, in complete remission in several patients.[47] Since this cancer is locally distributed, this technique could well be useful in many skin-cancers.

*What antigen configuration should be loaded?*
As already mentioned, a DC vaccine could be loaded with different substrates like; lysates, protein, long peptides or mRNA. The effect of different loading strategies or preparation mechanisms (especially in case of lysates) could influence the outcome and should therefore be investigated. With respect to the effect of lysate preparation and its effect on T-cell priming, progress is being made, hopefully leading to new insights in the near future (L. Spel, work in progress). The perfect antigen furthermore would be very cancer specific, leading to minimal side effects. Loading DCs with such tumor-associated antigens (TAA) is another strategy that has been fairly successful in many studies. It has also lead to the first DC-based FDA approved medication[48], although its efficacy is debated.[49] Nonetheless, the idea of TAA pulsed DCs gave rise to over 40 clinical trials that are being pursued. Especially when a specific, yet in a tumor generally expressed TAA is found, this could be a suitable option. Finding new and good TAA's is also an important task that could improve DC vaccinations in the future.

*The optimal T-cell priming*
An optimal DC-vaccine should also focus on $CD4^+$ and $CD8^+$ priming. Although it is generally accepted that a successful anti-tumor response will involve $CD8^+$ T-cell mediated killing [50-52], we showed in chapter 5 the importance of $CD4^+$ T-cells in $CD8^+$ priming.[26] This is supported by a recent trial in which DCs pulsed with both MHC class I and class II peptides induced better anti-tumor responses than DCs pulsed with only MHC class I.[53] We also propose that (antigen-specific) $CD4^+$ reconstitution should be taken along as an outcome for DC-vaccination trials. Furthermore, the outcome of trials using cord-blood derived DCs will be of interest, since this source mainly contains naïve T-cells.

*Vaccine administration*
With respect to the injection site there is a lot of knowledge to gain. DC vaccines are generally infused in the periphery and it is thought that these DCs will migrate to lymph nodes. However, this has never been carefully evaluated. We do know, that locally targeted DC vaccines can be really effective for specific tumors[47], but failed to induce responses in different settings.[54] Recently, Aarntzen *et al.* showed that pre-treatment of the skin did not induce DC-migration, where the amount of injected DCs did matter.[55] Moreover, intranodal injection or intradermal injection could be explored.[56;57] If it is decided that DCs should be infused in the periphery, we should evaluate migration, and optimize DCs to migrate better. To do so, the effect of maturation on chemokine receptors could be investigated, and perhaps manipulated, to pursue specific migration towards desired tissue.

*In summary*
An effective anti-tumor therapy will eventually rely on combination of strategies. However promising the next generation DC vaccination will be, at this moment, checkpoint blockade is proven more valuable. The problem is that even this medication does not work in all patients, and has major side-effects. Checkpoint blockade is very useful once a patient has tumor-infiltrating lymphocytes (TILs) that are inhibited. However, in instances when there are no TILs, or T-cells yet have to be primed (in the case of SCT), a checkpoint blockade will have little use. This is where DC vaccination can come into good use. In the latter scenario, DCs will prime T-cells that can migrate to the tumor area. Once at the site, checkpoint blockade can assist the newly acquired T-cells to overcome checks and effectively kill tumor cells.

## In conclusion

In this thesis, I present new insights in DC cross-presentation biology aimed at future DC vaccinations. Hopefully, we can implement this knowledge in the development of future DC vaccinations against various kinds of cancer. However, the studies here presented are of cell-biological nature, making our conclusions more broadly applicable.

For instance, knowledge of $CD4^+$ T-cell involvement in DC licensing and CTL priming may prove relevant for antiviral drug development in a more general sense. Considering for example the case of HIV, where $CD4^+$ T-cells are key players in the disease, better understanding of these mechanisms is important. DC vaccination in HIV has already been tried with limited success.[58] Knowing however that the $CD4^+$ T-cells are crucial in CTL induction, the levels of $CD4^+$ T-cells are important parameters to be considered in such trials. As recently demonstrated by Garcia et al. DC-vaccinations against HIV can be effective after appropriate $CD4^+$ T-cell numbers were measured (>450 cells/$mm^3$, after combination antiretroviral therapy).[59]

To conclude, and I hope this applies to DCs as orchestrators of the immune system: "Education is the most powerful weapon which you can use to change the (biomedical) world" (paraphrased from Nelson Mandela).

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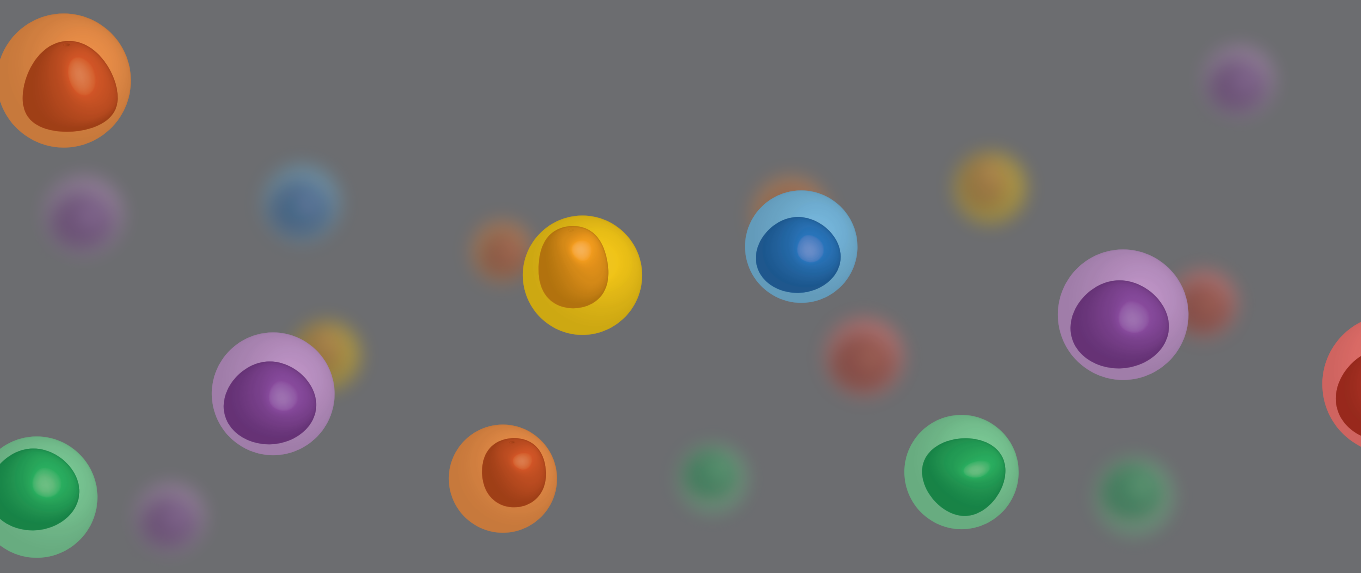
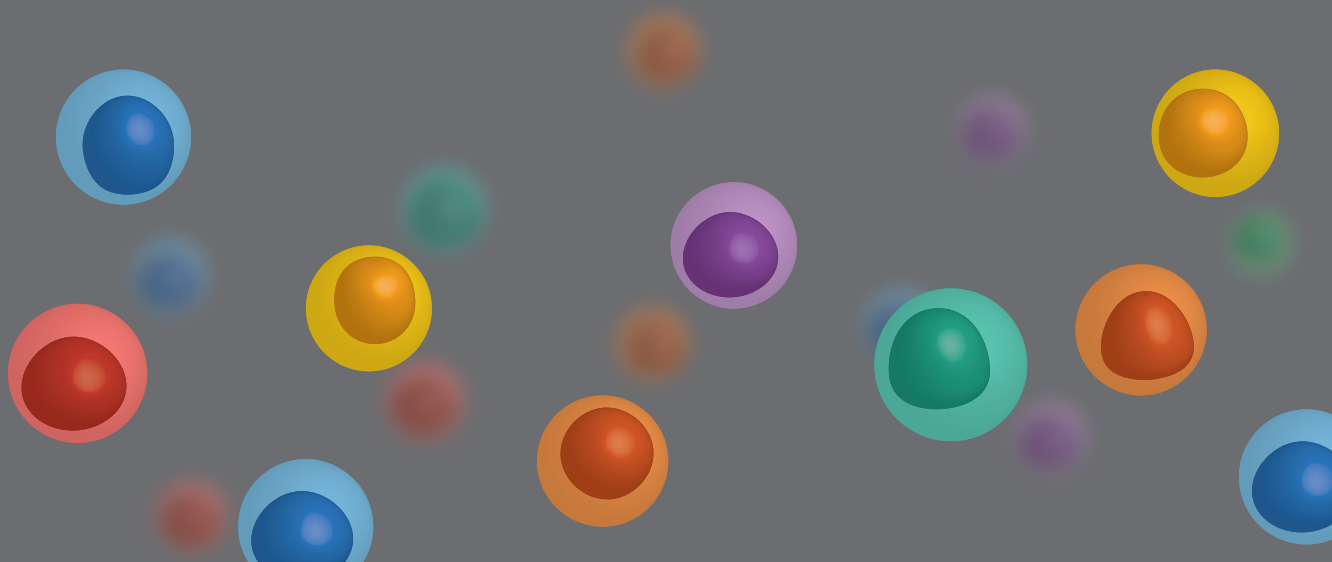


# Summary & Samenvatting



## Summary

Cord blood (CB) stem cell transplantation (SCT) is a last resort treatment for several malignancies, immune- and metabolic disorders. Although the safety of this procedure has improved over the past decades, room for improvement remains. Two of the major causes contributing to post-SCT mortality are disease relapse (in case of malignancies) and infectious complications. To be able to increase chances of survival, better therapies are warranted. As an approach for improved therapy, we focus on the development of a dendritic cell (DC)-vaccine aimed at inducing a rapid anti-viral or anti-tumor immune response. The effectively of such a response is strongly correlated to specific anti-viral or anti-tumor $CD8^+$ T cell activity. In this thesis, we investigate the induction of antigen-specific $CD8^+$ T cells by DCs, and explore DC extrinsic and intrinsic factors to boost this process.

### Cross-presentation and cross-priming by dendritic cells

DCs are key players in initiation and control of adaptive immune responses due to their exquisite ability to present antigenic fragments in the form of peptide/major histocompatibility complexes (MHC) to T cells. This involves the classical antigen presentation routes where endogenously produces antigen is presented on class I MHC, while exogenously produces antigen is presented on class II MHC. A combination of these pathways also occurs. This process called cross-presentation, is most efficiently carried out by DCs and involves presentation of exogenously produces antigen on class I MHC molecules. Cross-presentation is an important mechanism by which DCs initiate $CD8^+$ T cell immunity against viruses and cancer.

In *Chapter 2*, we review current literature regarding cross-presentation and describe how this process is involved in DC vaccinations. We elaborate on different DC subsets in mice and men, and their known capacity for cross-presentation. Although most DC subsets can cross-present under certain conditions, it appears to be especially the recently identified subset of $BDCA3^+$ ($CD141^+$) DCs that is specialized in this process. Mechanistically, the biology involved in cross-presentation is still a subject of intense research. Depending on the model system used, two major pathways of cross-presentation are proposed. In the first model called the cytosolic pathway, antigen is taken up and requires transportation from the endosome into the cytosol for degradation by the proteasome. The second model called the vacuolar pathway involves proteasome- and TAP (transporter associated with antigen-presentation) independent processing that occurs within the endosome. This mechanism relies on recycling class I MHC molecules. Receptor mediated uptake can direct antigen towards the MHC class I or class II antigen presentation route. One of these receptor families, the Fc gamma Receptors (FcγR) was shown to skew toward cross-presentation in mice and was the main initiator for the research that led to chapter 3.

We investigated cross-presentation after FcγR mediated uptake of antigen in *chapter 3*. First, we investigated the expression of several FcγRs on the surface of DCs and showed that the FcγRII was the main receptor expressed. We then continued to investigate cross-presentation after uptake by this receptor. To do so, we first set up a model using monocyte derived DCs (MoDCs), which we loaded with a viral protein (pp65). When DCs cross-presented peptides from this protein on class I MHC molecules, $CD8^+$ T cells specific to one of the pp65-derived

peptides reacted by cytokine production. We measured this initiation of cytokine production by $CD8^+$ T cells as a read out for the amount of cross-presentation. We found that antigen targeting to the FcγR (using IgG-opsonised antigen) resulted in increased cross-presentation. Using several blocking agent, we found that both the cytosolic and vacuolar pathway appeared to be involved. Finally, we repeated these experiments in human primary $BDCA1^+$ and $BDCA3^+$ DCs and showed that FcγR mediated uptake also led to increased cross-presentation in these DCs. Interestingly, particularly the $BDCA3^+$ subset of DCs revealed increased cross-presentation upon antigen delivery to the FcγR. We found that these DCs expressed relatively high levels of the stimulatory isoform FcγRIIa. These observations lead to the next chapter of this thesis.

*Chapter 4* describes two unrelated common variable immunodeficiency (CVID) patients expressing the same polymorphism in the FcγRIIa gene. We used next generation sequencing and found a double point mutation leading to an amino-acid change at position 27 (Q>W). Bioinformatic analysis suggested this polymorphism to be pathogenic, which is why we investigated the biological consequences of this mutation. FcγRIIa mRNA levels and protein surface expression was normal in patients compared to healthy donors. We screened several immune cells expressing FcγRIIa for receptor dependent defects and found that the aminoacid substitution delayed signaling in patient cells from the cell surface inward. We confirmed this observation using an overexpression model system in cells that normally do not express FcγRs.

We examined the role of $CD4^+$ T cells in antigen cross-priming of naive $CD8^+$ T cells and cross-presentation of effector/memory $CD8^+$ T cells in *Chapter 5*. In multiple situations, $CD4^+$ T cells were shown to contribute somehow to $CD8^+$ T cell priming in mice. Moreover, also in mice, priming of $CD8^+$ T cells not always requires $CD4^+$ T cell help. Although these findings are often extrapolated to humans, there was no functional proof that $CD4^+$ T cells are required for priming in the human system. We therefore developed a human system to investigate a possible role for $CD4^+$ T cells in cross-priming of $CD8^+$ T cells, using cord-blood derived DCs and donor-matched naïve T cells. Culturing antigen loaded DCs with naïve $CD8^+$ T cells only lead to effective outgrowth of $CD8^+$ T cell clones, identified using pp65-peptide/HLA-A2 tetramer staining, when $CD4^+$ T cells were present. We confirmed that the elicited $CD8^+$ T cells were antigen-specific using sequencing of the TCR-Vß regions and evaluation of functional capacity by cytokine release upon stimulation. We next looked into the role of $CD4^+$ T cells on memory T cell activation using the cross-presentation model we developed (as described in chapter 3). We found that antigen specific $CD4^+$ T cells contribute to DC mediated $CD8^+$ T cell stimulation (cross-presentation) in an antigen and MHC restricted manner. Co-stimulation was not essential. Furthermore, we found that interaction between $CD4^+$ T cells and loaded DCs lead to cytokine and chemokine production, involving the secretion of IFN-γ, contributing again to antigen cross-presentation by these DCs. Finally, we correlated these findings to patients T cell reconstitution after CB-SCT. We found that patients with active CMV reactivations had more $CD4^+$ T cell activation that preceded specific $CD8^+$ T cell outgrowth, providing experimental support that also in vivo, $CD4^+$ T cells are involved in effective cross-priming.

### Intracellular mechanisms contributing to cross-presentation

Chapter 2-5 describe beneficial effects of FcγR targeting and $CD4^+$ TC involvement on cross-presentation. These findings all involve DC-extrinsic factors contributing to cross-presentation. The DC intrinsic mechanisms (pathways) involved in cross-presentation are also

focus of intense research, mainly focusing on distinctive compartments and proteins involved. Chapter 6 and 7 discuss a particular mechanism called endosomal tubulation, associated with antigen presentation and cross-presentation.

*In chapter 6*, we review the trafficking of internalized antigen within the DC, and the requirements for cross-presentation. As also mentioned in chapter 2, there are two pathways involved in cross-presentation; the (endoplasmic reticulum and TAP peptide transporter-dependent) cytosolic pathway, and the vacuolar (endosomal) pathway. For cross-presentation to occur via the vacuolar pathway, class I MHC needs to be present in the endosomal compartment. We elaborate on evidence supporting the internalisation of class I MHC molecules in a clathrin-dependent manner, enabling for sufficient amounts of endosomal class I MHC molecules to assemble into antigenic peptide/class I MHC complexes. These complexes can subsequently be recycled to the plasma membrane, for display and cross-presentation to $CD8^+$ T cells. Recycling occurs by two major routes. Fast recycling (direct from the early endosome to the plasma membrane) happens within minutes, whereas a slower recycling route also exists, that involves the juxtanuclear endosomal recycling compartment (ERC). There is compiling evidence for involvement of the recycling endosomes in antigen cross-presentation (including experiments with the recycling inhibitor primaquine in chapter 3). Finally, we discuss the directional and conformational changes of endosomal compartments into tubular structures. These tubules enable fast and T cell contact-site directed transport of class II MHC molecules in mice. Recent evidence also indicated the involvement of ERC in cross-presentation in mice. This lead to the questions investigated in chapter 7.

*Chapter 7* elaborates on the finding that mouse dendritic cells (DCs) rapidly extend their Class II MHC-positive late endosomal compartments into tubular structures when triggered via Toll-like receptors (TLRs). These tubules are directional, which is considered beneficial to the activation and delivery of peptide/MHC complexes towards T cells. In this chapter, we investigated the process of tubulation in human DCs. First, we confirmed the induction of late endosomal tubulations within 30 minutes after TLR triggering, using labeling with fluorescent LDL. However, TLR triggering was insufficient for induction of tubulation of transferrin-positive endosomal recycling compartments (ERCs). As mentioned earlier (as discussed in detail in chapter 6), the ERC is thought to play a role in antigen cross-presentation through the vacuolar (endosomal) pathway. With this in mind, we stimulated antigen-loaded DCs with TLR ligands in the presence of antigen-specific $CD8^+$ T cells. Under these conditions, tubulation of the ERC was induced. This phenomenon was antigen specific and required T cell contact. Furthermore, we showed the requirement of ligation of both HLA molecules and ICAM-1 by T cell-expressed T cell receptor and LFA-1, respectively. Finally, to provide experimental support that ERC tubulation contributes to cross-presentation, we used selective reversible blockers, already introduced in chapter 3. Both disintegration of microtubules using nocodazole or inhibition of endosomal recycling using primaquine abolished tubular ERCs, which coincided with reduced antigen-dependent $CD8^+$ T cell activation.

In *conclusion*, this thesis describes the research of several DC extrinsic and intrinsic mechanisms that contribute to the understanding of antigen cross-presentation to $CD8^+$ T cells. Findings described here should eventually lead to more effective DC vaccination regimens, in the end contributing to patient survival.

## Samenvatting

Een stamceltransplantatie (SCT) met navelstrengbloed is een relatief nieuwe behandelmethode die een laatste redding kan bieden aan patiënten met diverse soorten kanker, immuun- of metabole stoornissen. Ondanks het feit dat de veiligheid van deze procedure de laatste jaren drastisch is verbeterd, is er nog steeds veel winst te behalen. Twee van de belangrijkste oorzaken voor sterfte na een SCT zijn het terugkomen van de ziekte (een recidief) of infectieuze complicaties. Om deze twee problemen te bestrijden zijn nieuwe therapieën nodig. In dit onderzoek richten wij ons op het ontwikkelen van een vaccinatie strategie die gebruik maakt van dendritische cellen (DCs). Het gebruik van dit vaccin kan hopelijk leiden tot een snel herstel van een belangrijk deel van het immuunsysteem, zodat het lichaam adequaat kan reageren op recidieven of virale infecties. Het succes van deze behandeling is afhankelijk van de activatie van specifieke anti-tumor en/of anti-virale $CD8^+$ T cellen (cellen die tumoren of virussen kunnen doden). In dit proefschrift onderzoeken we het proces waarbij DCs deze $CD8^+$ T cellen aansturen, en kijken we naar manieren in en buiten de DC om dit proces te beïnvloeden.

### Cross-presentatie en cross-priming door dendritische cellen

DCs zijn een van de belangrijkste cel types bij het opstarten en dirigeren van een immuunrespons. Dit komt omdat deze cellen gespecialiseerd zijn in het presenteren van delen van antigen (bv tumoren of virussen) in de vorm van een peptide/MHC complex, aan T cellen. Klassiek wordt deze presentatie onderverdeeld in twee processen. Eiwitten die in de cel zelf worden geproduceerd worden afgebroken en gepresenteerd op MHC klasse I. Eiwitten die worden opgenomen vanuit het externe milieu worden afgebroken in speciale blaasjes in de cel en gepresenteerd op MHC klasse II. Er bestaat ook een route die deze twee combineert. Dit proces heet cross-presentatie. Bij dit proces worden eiwitten vanuit het externe milieu opgenomen, en toch op klasse I gepresenteerd. Meerdere cellen zijn hier in principe toe in staat, maar DCs kunnen het buitengewoon efficiënt. Via cross-presentatie zijn DCs in staat om de ontwikkeling van $CD8^+$ T cellen gericht tegen tumoren of virussen te induceren.

In *Hoofdstuk 2* hebben we de literatuur over cross-presentatie samengevat en de rol van dit proces bij DC-vaccinaties beschreven. We gaan in op de verschillende soorten DCs in muizen en in mensen, en beschrijven wat bekend is over de relatie tussen deze cellen en het in staat zijn tot cross-presentatie. De meeste DC subtypen lijken onder specifieke condities in staat te zijn om antigen via cross-presentatie te presenteren. Er zijn echter aanwijzingen dat de recent ontdekte BDCA-$3^+$ ($CD141^+$) DC gespecialiseerd is in dit proces. Naast de interesse in subsets, wordt er veel onderzoek gedaan naar de mechanismen van cross-presentatie. Er zijn grofweg twee routes te onderscheiden. De eerste route, de cytosolische route genoemd, is afhankelijk van het transport van opgenomen eiwit vanuit het endosomale compartiment naar het cytosol alvorens het kan worden afgebroken door het proteasoom. De tweede route, genaamd de vacuolaire route, is niet afhankelijk van het proteasoom en het TAP-systeem, maar gebeurt in het endosoom. Voor dit mechanisme is de recycling van klasse I MHC van belang. De opname van antigen via specifieke receptoren kan bepalend zijn voor de methode van presentatie. Zo zorgt bijvoorbeeld opname via de familie van Fc gamma receptoren (FcγR) in muismodellen voor meer cross-presentatie. Dit gegeven heeft geleid tot de hypotheses uitgewerkt in hoofdstuk 3.

We hebben het effect van antigen opname via FcγRs op cross-presentatie bekeken in *hoofdstuk 3*. Allereerst hebben we gekeken naar de expressie van de verschillende soorten FcγRs op het celmembraan van DCs en zagen dat vooral FcγRII tot expressie kwam. Vervolgens hebben we gekeken naar het effect op cross-presentatie nadat eiwit werd opgenomen via deze receptor. Om dit te doen hebben we een modelsysteem gemaakt waarbij we gebruik hebben gemaakt van DCs die uit monocyten kunnen ontwikkelen (MoDCs) en een eiwit uit het CMV virus (pp65). Als DCs dit eiwit opnemen, verwerken en presenteren op klasse I MHC, kunnen speciaal hiervoor gemaakte $CD8^+$ T cellen hierop reageren met de uitstoot van cytokines (signaal eiwitten). Deze cytokine uitstoot kunnen we meten en functioneert dus als een indirecte uitleesmethode van de hoeveelheid cross-presentatie. Met deze methode vonden we dat de opname van eiwit via de FcγR (hiervoor maakte we gebruik van IgG-geopsoniseerd eiwit) resulteerde in een toename van cross-presentatie. Met behulp van verschillende remmers vonden we verder dat zowel de cytosolische als de vacuolaire route een rol speelt bij dit proces. Daarna hebben we deze proeven herhaald in DCs die in het lichaam voorkomen. We laten zien dat FcγR gemedieerde opname ook in de primaire $BDCA\text{-}1^+$ en $BDCA\text{-}3^+$ cellen voor een toename in cross-presentatie zorgt. Voornamelijk in de $BDCA\text{-}3^+$ DCs was dit effect aanwezig. Ten slotte vonden we dat deze DCs een relatief hoge expressie van de stimulerende FcγRIIa hadden. Deze observatie was de aanleiding voor het volgende hoofdstuk.

*Hoofdstuk 4* beschrijft twee patiënten met een immuunstoornis (CVID) waarbij een polymorfisme in het FcγRIIa gen werd gevonden. Met behulp van een nieuwe "sequence" methode vonden we dat deze patiënten een dubbele puntmutatie hadden welke leidde tot een aminozuurverandering op positie 27 (Q>W). Na bioinformatische analyse van dit polymorfisme bleek dat deze verandering waarschijnlijk consequenties zou hebben. We hebben daarom gekeken of de cellen met deze receptor van bovengenoemde patiënten anders functioneerden. De mRNA en eiwit expressie was normaal vergeleken met gezonde donoren. Nadat we de functie van verschillende immuuncellen hadden bekeken bleek dat de signalering van deze receptor anders was bij de cellen van de patiënten. We hebben dit vervolgens bevestigd door deze receptor na te maken en in twee modelsystemen te testen.

In *Hoofdstuk 5* hebben we de rol van $CD4^+$ T cellen bekeken op zowel cross-priming van naïeve $CD8^+$ T cellen als cross-presentatie aan effector/memort $CD8^+$ T cellen. Uit diverse studies met muismodellen blijkt dat $CD4^+$ T cellen een rol spelen bij de priming van $CD8^+$ T cellen. Verder is in muismodellen laten zien dat $CD4^+$ T niet altijd noodzakelijk zijn bij de priming van $CD8^+$ T cellen. Alhoewel deze gegevens regelmatig direct naar de mens worden vertaald is nooit goed bewezen dat $CD4^+$ T cellen ook belangrijk zijn bij de priming van humane $CD8^+$ T cellen. We hebben om dit te onderzoeken een modelsysteem opgezet waarbij we gebruik maken van DCs uit navelstrengbloed en de naïeve T cellen van dezelfde donor. We zagen dat het kweken van pp65-beladen DCs samen met naïeve $CD8^+$ T cellen alleen leidde tot $CD8^+$ geheugen T cellen, aangetoond met pp65-peptide/HLA-A2 tetrameer kleuring, als er ook $CD4^+$ T cellen in de kweek aanwezig waren. We hebben vervolgens TCR-Vß sequencing verricht en functionele testen gegaan om te bewijzen dat het ging om antigen-specifieke $CD8^+$ T cellen. Ook hebben we gekeken naar de rol van $CD4^+$ T cellen op de geheugen T cel activatie. Hiervoor hebben we gebruik gemaakt van het cross-presentatie model dat in hoofstuk 3 staat beschreven. Op deze manier vonden we dat antigen specifieke $CD4^+$ T cellen bijdragen aan DC gemedieerde $CD8^+$ T cel stimulatie (cross-presentatie). Co-stimulatie was hiervoor niet nodig. Verder zagen

we dat de interactie tussen $CD4^+$ T cellen en beladen DCs leidde tot cytokine en chemokine (signaalstoffen) productie, met name de productie van IFN-γ, welke bijdragen aan cross-presentatie door deze DCs. Als laatste hebben we gekeken of we deze bevindingen ook aantroffen in patiënten na SCT. We vonden ook hier dat patiënten met actieve CMV reactivatie meer geactiveerde $CD4^+$ T cellen hadden, die voorafgingen aan de ontwikkeling van antigen specifieke $CD8^+$ T cellen. Op deze manier laten we zien dat ook in vivo, $CD4^+$ T cellen betrokken zijn bij cross-priming in de mens.

**Intracellulaire mechanismes die bijdragen aan cross-presentatie**

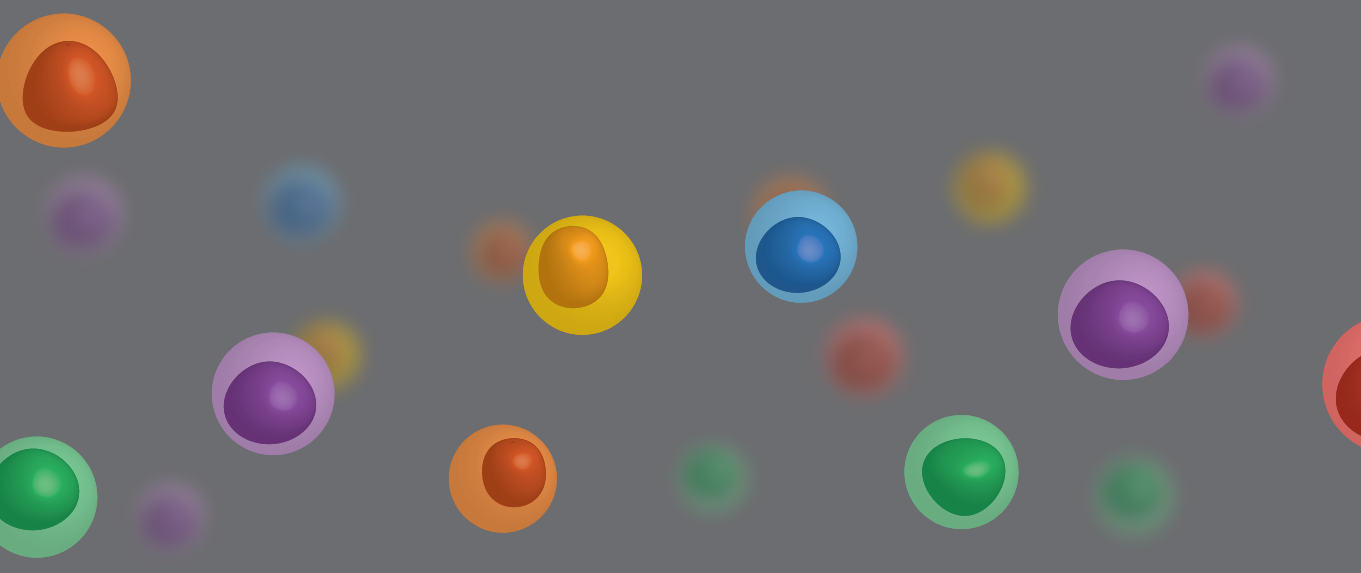
Hoofdstuk 2 t/m 5 gaan over de rol van FcγR gemedieerde opname en de betrokkenheid van $CD4^+$ T cellen bij cross-presentatie. Beide zaken betreffen DC extrinsieke mechanismen die bijdragen aan cross-presentatie. Er wordt de laatste jaren ook veel onderzoek verricht naar DC intrinsieke mechanismen die betrokken zijn bij cross-presentatie. Hierbij gaat het over de verschillende compartimenten en intracellulaire eiwitten die betrokken zijn bij dit proces. Hoofdstuk 6 en 7 beschrijven een van deze mechanismen genaamd endosomale tubulatie. Deze endosomale tubulatie is betrokkene bij antigen presentatie en cross-presentatie.

In *hoofdstuk 6* bespreken we de literatuur over internalisatie van eiwitten door DCs en het vervoer van deze eiwitten in de cel, met name met betrekking tot cross-presentatie. Zoals reeds in hoofdstuk 2 beschreven zijn er 2 routes die betrokken zijn bij cross-presentatie; de cytosolische route (deze betrekt het endoplasmatisch reticulum en TAP eiwitten) en de vacuolaire (endosomale) route. Als cross-presentatie plaats vindt via de vacuolaire route moet MHC klasse I in het endosomale compartiment terecht komen. We laten zien dat er aanwijzingen zijn dat MHC klasse I moleculen geïnternaliseerd worden vanaf het plasma-membraan, waarbij in principe genoeg MHC klasse I moleculen in het endosoom terecht kunnen komen om voor cross-presentatie te kunnen zorgen. Deze moleculen kunnen in het endosoom worden beladen met peptide, en vervolgens recyclen naar de celoppervlakte, om een interactie met $CD8^+$ T cellen aan te gaan. Recyclen van MHC I gebeurt via 2 routes. Snelle recycling (direct van een vroeg endosoom naar het plasma membraan) gebeurt binnen enkele minuten. Er bestaat ook een langzamere recycling route via het juxtanucleaire endosomale recycling compartiment (ERC). Er zijn redelijk wat aanwijzingen dat recycling betrokken is bij cross-presentatie (inclusief experimenten met de recycling remmer "primaquine" beschreven in hoofdstuk 3). Ten slotte bespreken we in dit hoofdstuk de structurele en gerichte verandering van endosomale compartimenten in buisvormige (tubulaire) structuren. Deze tubules zorgen voor snel transport van MHC klasse II naar de plek van DC-T cell interactie in muizen. Recentelijk is in muismodellen ook laten zien dat recycling via het ERC betrokken is bij cross-presentatie. Dit heeft geleid tot het onderzoek beschreven in hoofdstuk 7.

*Hoofdstuk 7* komt voort uit de observatie dat DCs uit muizen in staat zijn om de klasse II MHC laat endosomale compartimenten te veranderen in tubulaire structuren, afhankelijk van Toll-like receptor (TLR) signalering. Deze tubules zijn gericht en zorgen voor een snel transport van klasse II MHC richting de $CD4^+$ T cel. In dit hoofdstuk kijken we naar tubulatie in humane DCs. Allereerst laten we zien dat ook in humane DCs tubulatie kan ontstaan vanuit het laat endosomale compartiment nadat TLR liganden worden toegevoegd. Om dit te laten zien maakten we gebruik van fluorescentie gelabeld LDL. Alleen TLR ligatie was echter onvoldoende om tubulatie te induceren van het transferrine-positieve endosomale recycling

compartiment (ERC). Zoals eerder beschreven (in detail in hoofdstuk 6), zijn er vanuit de literatuur aanwijzingen dat het ERC betrokken is bij antigen cross-presentatie via de vacuolaire route. Gebaseerd op dit gegeven hebben we de proeven herhaald in de aanwezigheid van $CD8^+$ T cellen en pp65. In deze situatie zagen we tubulatie van het ERC. Tubulatie van het ERC was antigen specifiek en T cel contact was noodzakelijk. Verder laten we zien dat de binding van HLA moleculen en ICAM-1 aan respectievelijk de T cel receptor en LFA-1 belangrijk is. Om aan te tonen dat tubulatie van het ERC belangrijk is voor cross-presentatie hebben we gebruik gemaakt van selectieve remmers, zoals ook beschreven in hoofdstuk 3. Zowel de verstoring van microtubules middels nocodazol als inhibitie van endosomale recycling met primaquine zorgde voor verstoring van ERC tubulatie en voor remming van cross-presentatie.

*Samengevat* beschrijft dit proefschrift het onderzoek naar verschillende DC extrinsieke en intrinsieke mechanismen dat bijdraagt aan het begrip van cross-presentatie aan $CD8^+$ T cellen. Deze bevindingen dragen bij aan de ontwikkeling van een effectiever DC vaccin en uiteindelijk hopelijk aan een betere overleving voor patiënten.



# Generation of a cord blood-derived Wilms Tumor 1 dendritic cell vaccine for AML patients treated with allogeneic cord blood transplantation

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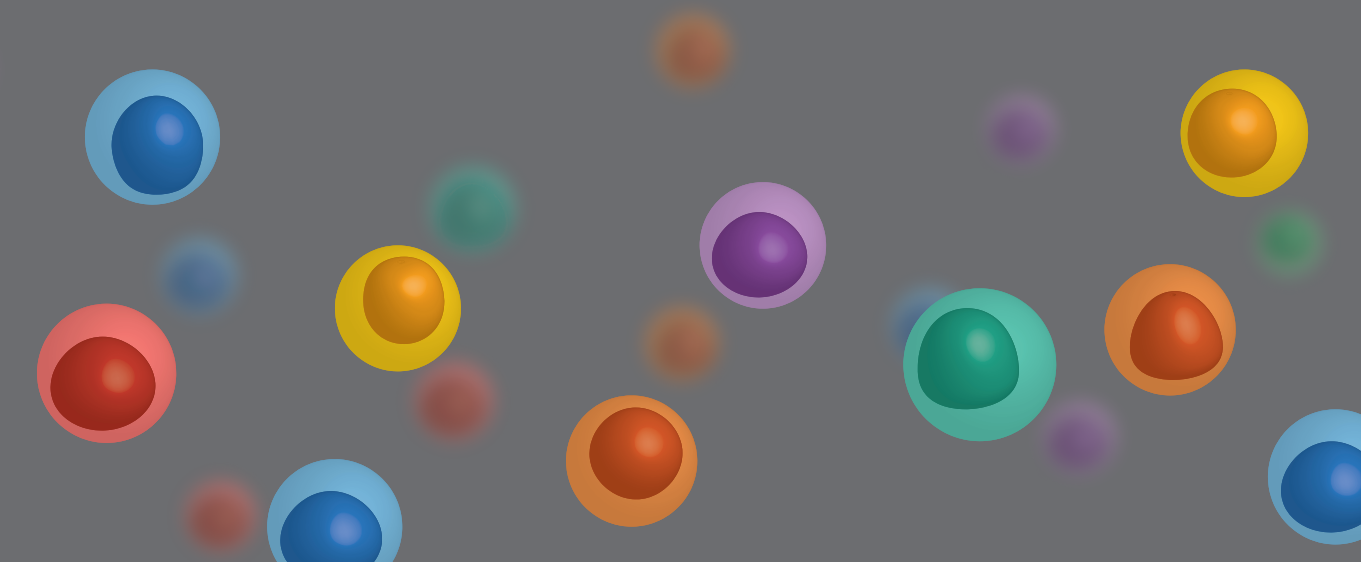
Submitted



## Abstract

The poor survival rates of refractory/relapsed acute myeloid leukemia (AML) patients after hematopoietic cell transplantation (HCT) require the development of additional immune therapeutic strategies. As the elicitation of tumor antigen-specific cytotoxic T lymphocytes (CTLs) is associated with reduced relapses and enhanced survival, enhanced priming of these CTLs using an anti-AML vaccine may result in long-term immunity against AML. Cord blood (CB), as allogeneic HCT source, may provide a unique setting for such post-HCT vaccination, considering its enhanced Graft-versus-Leukemia (GvL) effects and population of highly response naïve T cells. It is our goal to develop a powerful and safe immune therapeutic strategy composed of CB-HCT followed by vaccination with CB $CD34^+$-derived dendritic cells (DCs) presenting the oncoprotein Wilms Tumor-1 (WT1) which is expressed in AML-blasts in the majority of patients. Here, we describe the optimization of a clinically applicable DC culture protocol. This two-step protocol, consisting of an expansion phase followed by the differentiation towards DCs, enables us to generate sufficient CBDCs in the clinical setting. The CBDC exhibit a mature surface phenotype, are able to migrate, express tumor antigen (WT1) after electroporation with mRNA encoding the full-length WT1 protein, and stimulate WT1-specific T cells.

## Introduction

Survival rates of pediatric patients with acute leukemia have improved significantly over the last decades. However, patients suffering from AML still have a poor prognosis with an estimated 5-year probability of overall survival (pOS) rate of only ~50%, even after the last and only potentially curative treatment option, HCT.[1-5] Hence, there is clear medical need for additional therapies for these patients.

As relapse remains the main obstacle even after HCT, novel immunotherapeutic strategies are being developed aimed at preventing relapses after HCT.[6-9] CB is an emerging allogeneic stem cell source with important advantages over conventional stem cell sources (bone marrow or mobilized peripheral blood), including reported enhanced GvL,[4,10] in addition to lower Graft-versus-Host Disease (GvHD). It has been recognized that the fast generation of tumor-specific CTLs early after HCT (i.e., in the period of minimal residual disease) may be crucial for the GvL effects.[11] As such, enhanced priming of these CTLs using an anti-AML vaccine may result in long-term immunity against AML, further reducing the relapse rates and enhancing survival after HCT.

In vivo T cell priming and proliferation requires the presentation of antigens and activation signals provided by a specialized group of antigen presenting cells; in particular dendritic cells (DCs). The inefficiency of host DCs to initiate anti-tumor immunity led to the development of vaccination strategies, where ex vivo-generated DCs are loaded with tumor-specific antigens and given back to the patients to stimulate the in vivo generation anti-tumor CTLs.[12] In either bone marrow or mobilized peripheral blood HCT settings, the donor can provide the source of DCs needed for vaccination, either via primary DCs[13] or $CD14^+$ monocytes.[14] In the CB HCT setting, where donor material is limited, the $CD34^+$ cell population (containing the hematopoietic stem cells) can be used to generate CB-derived DCs (CBDC).[15]

Our goal is to develop a powerful and safe immune therapeutic strategy composed of CB-HCT followed by vaccination with CBDCs presenting the oncoprotein WT1, recently ranked the number one cancer vaccine target antigen.[16] WT1 is overexpressed in the majority of AML (~90%) while expression is very low in normal tissues.[17] Using mRNA electroporation to express full length WT1 protein warrants cytoplasmic expression and optimal class I presentation of WT1 peptides.[18] This approach was used by Van Tendeloo et al, who observed clinical responses as well as WT1-specific CTLs in AML patients.[14] We hypothesize that vaccination with WT1-loaded CBDCs combined with the intrinsic increased proliferative capacity of the T cells in the CB graft will result in fast differentiation and proliferation of WT1-specific CTLs after HCT.[19,20] Here we describe the optimization of a GMP (Good Manufacturing Practice)-applicable culture protocol to generate sufficient CBDCs from a limited amount of $CD34^+$ cells, that are mature, able to migrate and express tumor antigen (WT1) to stimulate specific T cells.

## Materials and Methods

*CB collection and CD34 isolation*
Umbilical cord blood was collected after informed consent was obtained according to the Declaration of Helsinki and approved by the ethics committee of the UMC Utrecht. CB mononuclear cells were isolated from human umbilical CB using Ficoll-Paque (GE Healthcare Bio-Sciences AB) density centrifugation. $CD34^+$ cells were isolated from fresh CB using magnetic bead separation (Miltenyi Biotec) resulting in a 80-95% purity. The $CD34^-$ population was stored at -80°C.

*CBDC culture*
In the single-step protocol $CD34^+$ cells are cultured in X-VIVO 15 supplemented with GM-CSF (100 ng/ml), SCF (25 ng/ml) and TNF (2.5 ng/ml) and 5% human AB serum (Sanquin) for seven days.[21] In the two-step protocol the $CD34^+$ cells are first expanded by culturing $5x10^4$ $CD34^+$ cells/ml in X-VIVO supplemented with Flt3L (50 ng/ml), SCF (50 ng/ml), IL-3 (20 ng/ml) and IL-6 (20 ng/ml) for seven days. Next, these cells are differentiated by culturing $2x10^5$ cells/ml in X-VIVO 15 containing 5% human AB serum and supplemented with Flt3L (100 ng/ml), SCF (20 ng/ml), GM-CSF (20 ng/ml) and IL-4 (20 ng/ml) for another seven days.[22] In indicated experiments the IL-4 was replaced with IL-15 (100 ng/ml) during the differentiation.[23] Recombinant GM-CSF, SCF, Flt3L, IL-1beta, IL-3 and IL-6 were obtained from Miltenyi Biotec and IL-4, IL-15 and TNF from Immunotools. To induce maturation an agonistic anti-CD40 (Bioceros), or CYTOMIX, a combination of IL-1beta, IL-6 and TNF (all 10 ng/ml) and PGE2 (1 mg/ml) from Pfizer, was added to the DCs for 24 hrs.

*Flow cytometry*
Anti-CD1c (L161), anti-CD3 (UCHT1), anti-CD16 (3G8), anti-CD64 (10.1), anti-CD207 (10E2), anti-CD209 (9E9A8), anti-CLEC9A (8F9), anti-HLA-DR (L243) and anti-TNF (Mab11) were purchased from Biolegend. Anti-BDCA-2/CD303 (AC144) and anti-BDCA-3/CD141 (AD5-14H12) were obtained from Miltenyi Biotec. Anti-CD1a (HI149), anti-CD8α (RPA-T8), anti-CD11c (B-ly6), anti-CD14 (M5E2), anti-CD56 (B159), anti-CD80 (L307.4), anti-CD83 (HB15e), anti-CD86 (IT2.2), anti-CD123 (7G3) and anti-IFNg (4SB4) were from BD Bioscience. Anti-CD4 (RPA-T4), anti-CD40 (5C3) and isotype-controls were from eBioscience. Anti-CCR7/CD197 (FR11-11E8) was from R&D.
All flow cytometric data acquisition was performed using a FACS Canto II (BD) and all analyses were performed using FlowJo software (Tree Star, Inc.).

*Sorting and histochemical staining CBDC*
$CD11c^+$ $HLA\text{-}DR^+$ cells within the different CBDC culture were sorted using a FacsARIA II cytometer and checked for purity after the sort (>95%). Cytospins were prepared from $5x10^4$ sorted CBDCs and were fixed in methanol for 5-10 min and May-Grünwald Giemsa staining was used to analyze DC cultures.

*Uptake and processing assay*
Endocytic activity of the CBDC culture was measured by the uptake of FITC-labeled Dextran (m.w. 70.000) from Sigma. Briefly, cells were suspended in X-VIVO 15 (5% AB), and incubated with 10, 100 mg/ml or 1 mg/ml Dextran-FITC for 30 min at 4°C to measure nonspecific binding

or at 37°C to measure specific uptake. Cells were then washed extensively with ice-cold PBS, 0.1% FCS, and 0.05% $NaN_3$ and labeled on ice with appropriate antibodies. The active uptake was determined as the percentage of $FITC^+$ cells at 37°C minus those at 4°C.
For analysis of processing by CBDC cultures we used DQ-Green BSA, a self-quenched dye conjugate of BSA. CBDCs were incubated with 0.5 µg/ml DQ-Green BSA at 4 or 37°C for 10 minutes. After extended washes CBDCs fluorescence signal was assessed at indicated time points.

*Transwell migration assay*
In vitro migration assays were performed using 24 transwell (3 mm pore size) plates (Greiner). In brief, 400.000 CBDCs in 200 ml culture medium (X-VIVO 15 with 5% human AB serum) were plated in the upper compartment. Culture medium, either alone or supplemented with 250 ng/ml CCL19 (R&D systems), was added to the lower compartment. After 2 hrs cells were collected from the lower compartment and analyzed using flow cytometry.

*Mixed leukocyte reaction (MLR)*
The allostimulatory capacity of the CBDC cultures was tested in a MLR. The responder cells were CD3 cells purified from an allogeneic $CD34^-$ population using anti-CD3 microbeads (Miltenyi). Responder cells ($1x10^5$) were then labeled with celltrace violet (5 µM; Invitrogen), and co-cultured with matured CBDCs ($2x10^4$) as stimulator cells in a 96-well round-bottom plate (Corning). Unstimulated celltrace violet-labeled cells served as negative control. After 3 and 5 days, cells were stained and analyzed using a flow cytometry. T-cell proliferation was assessed by quantification of the celltrace violet-dilution signal within both the CD4 and CD8 T cell populations.

*T cell suppression assay*
Part of the $CD34^-$ population was labeled with 2 µM celltrace violet for 7 min at 37°C. These responder cells ($2,5x10^4$) were plated into anti-CD3-coated wells (OKT-3, 1.5 µg/ml). Another part of the $CD34^-$ population is used for sorting regulatory T cells (Tregs) as a positive control for suppression. First, the $CD4^+$ cells are enriched using a CD4 T Lymphocyte Enrichment Set (BD Biosciences). Subsequently, $CD4^+CD25^+CD127^-$ T cells were sorted as Treg by FACS Aria (BD Biosciences) and added in different ratios to the responder cells.
Different populations of the CBDC culture were flow-sorted based on their CD11c and HLA-DR expression, representing $CD11c^-HLA\text{-}DR^+$, $CD11c^+HLA\text{-}DR^+$ (DCs) en $CD11c^-HLA\text{-}DR^-$ (Neg) or the total (unsorted) population was used and added in different ratios to the responder cells. After 4 days the proliferation was measured using flow cytometry.

*In vitro mRNA transcription, mRNA electroporation and protein expression analysis*
In vitro transcribed WT1-mRNA and EGFP-mRNA were produced from linearized pGEM4Z/WT1/A64 and pGEM4Z/EGFP/A64 plasmids using the T7 mMessage mMachine Ultra kit (Ambion, Life Technologies, NY, USA). CBDCs from $HLA\text{-}A^*0201^+$ individuals were loaded with mRNA by means of electroporation as previously described,[24] with minor modifications. Briefly, $5\text{-}10x10^6$ cells in 200 ml Optimem media were transferred to a 4-mm electroporation cuvette (Bio-Rad, Hercules, CA, USA) and electroporated with 10-30 µg RNA by an exponential decay pulse of 300 V for 7 ms using the Gene Pulser Xcell (Bio-Rad). After electroporation, cells were transferred into 6 wells plates containing culture medium for a least two hrs.

To evaluate protein expression, the EGFP expression could directly be assessed using flow cytometry, whereas western blot was used to determine the expression of WT1 protein after mRNA electroporation. WT1 mRNA and sham electroporated CBDCs or the K562 cell line (positive control), were lysed by adding laemmli buffer (2% SDS, 10% glycerol, 62.5 mM Tris pH 6.8) and samples are boiled for 10 min at 95°C. Aliquots of 25 mg of protein were electrophoresed on 10% SDS- polyacrylamide gels, transferred to polyvinylidene difluoride membranes and probed with mouse-anti-WT1 (Dako; 0.5 mg/ml) or goat-anti-actin (Santa Cruz; 1:5000). Horseradish peroxidase-conjugated rabbit-anti-goat or rabbit-anti-mouse (Dako, 1:5000) were used as secondary antibody. The fluorescent intensity was measured on the ChemiDoc system (Bio-Rad).

*WT1 antigen presentation*
WT1 mRNA or sham-electroporated CBDCs ($5x10^4$) were co-cultured with an HLA-A2–restricted WT1-specific T cell clone recognizing the $WT1_{126–134}$ epitope ($5x10^4$) for 4 hrs in the presence of Golgi-stop (1/1500; BD Bioscience). Sham-electroporated CBDCs loaded with/without WT1 peptide (Think Peptides) were used as a positive control. The T cells were subsequently stained for surface markers and, after fixation and permeabilization with the BD fix/perm (BD Biosciences), labeled with anti-IFNg and anti-TNF antibodies (BD Biosciences), followed by flow cytometry–based analysis.

## Results

*Generation of CBDCs from $CD34^+$ stem cells*
The first aim was to develop a culture protocol to generate sufficient CBDCs for multiple vaccinations, requiring a 250-500-fold expansion of $CD34^+$ cells. GMP-grade culture conditions excludes the use of animal derived components, for instance fetal calf serum, as serum-supplement. We tested human AB serum, which has been used before in various clinical trials using DCs, platelet lysate, or TGF-beta to supplement differentiation media. Platelet lysate and TGF-beta resulted in poor CBDC induction compared with AB serum (*data not shown*).
In the one-step protocol $CD34^+$ cells were cultured for one week with GM-CSF, SCF and TNF (GST) in 5% AB serum (*Figure 1A*). The two-step protocol consisted of a one-week culture of $CD34^+$ cells in expansion medium containing Flt3L, SCF, IL-3 and IL-6 (FS36), followed by a one-week culture in differentiation medium containing 5% human AB serum, further supplemented with GM-CSF, Flt3L, SCF, and IL4/L15 (GFS4 or GFS15) or GM-CSF and IL-4 (G4) (*Figure 1A*). The one-step GST protocol induced immediate differentiation of the $CD34^+$ population as depicted by expression of $CD11c^+$, $HLA\text{-}DR^+$ and $CD1a^+$ DCs after the first week (*supplementary Figure 1A*). The expansion potential averaged 6.5 times over one week of culture (*Figure 1B*). As this resulted in insufficient cell numbers for multiple vaccinations, this protocol was excluded in further studies.
The two-step protocols produced an average expansion of 600 fold for FS36/GFS4, 300 fold for FS36/GFS15 and 150 fold for FS36/G4 (*Figure 1B*). The expansion phase could be repeated for at least two more weeks (with an additional 10 x expansion each week) without affecting final DC differentiation (*supplementary Figure 1B*). Flow cytometric analysis of the cells after the expansion phase showed that all cells were of myeloid lineage ($CD33^+$), showed no differentiation into $CD11c^+$ DC, and that some $CD34^+$ CB cells remained (*supplementary Figure*

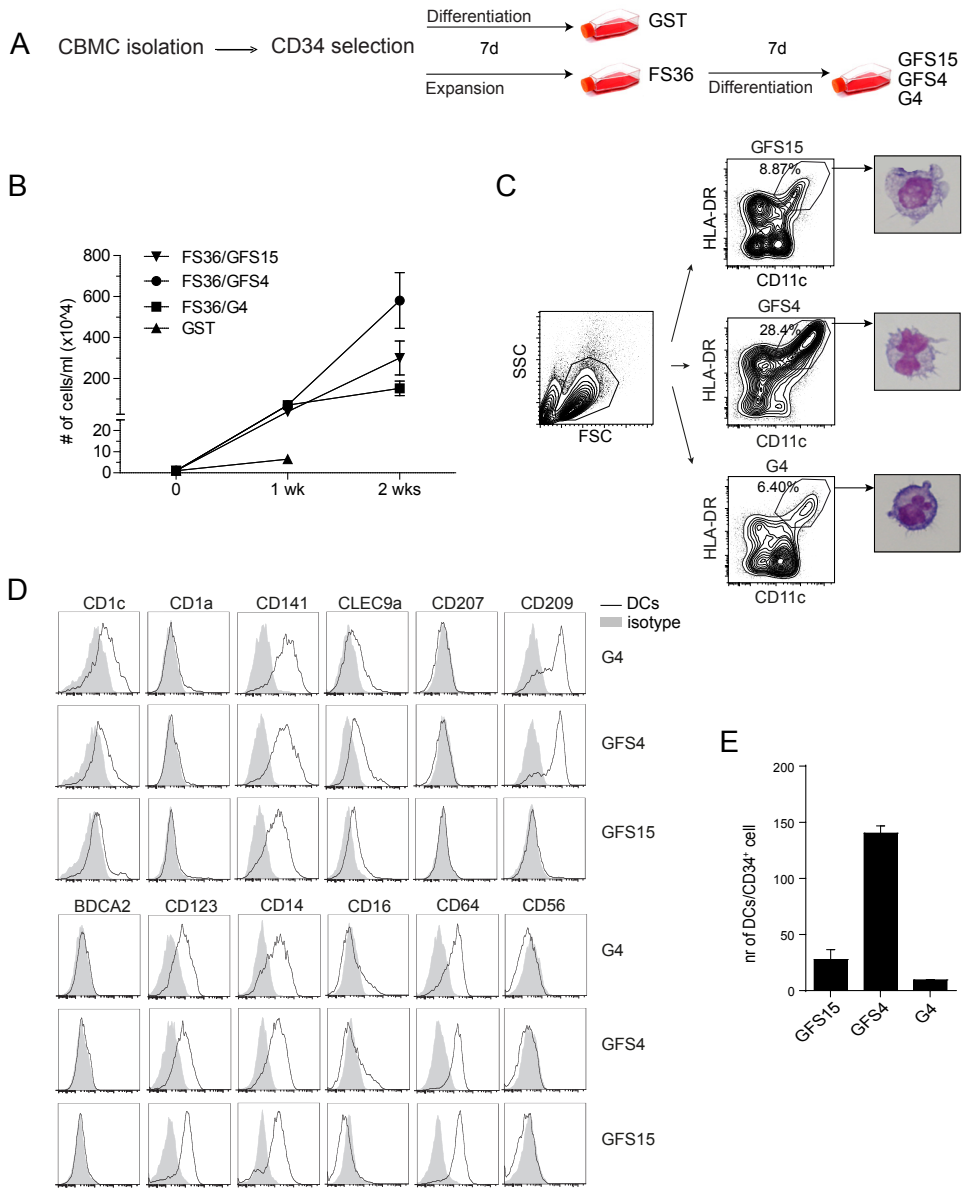



**Figure 1. Generation of CBDCs from CB-derived CD34⁺ stem cells**
(A) Different protocols for generating DCs from CB-derived CD34⁺ cells. (B) Expansion of CD34⁺ cells for the different protocols. (C-E) Phenotype of DCs for the G4, GFS4 and GFS15 protocols. (C) Gating strategy with flow cytometry and cytospins of DCs stained with May-Grünwald-Giemsa. (D) Expression of CD1c, CD1a, CD141, CLEC9A, CD207, CD209, BDCA2, CD123, CD14, CD16, CD64 and CD56 on DCs compared to isotype control. (E) Number of DCs generated from CD34⁺ cells. Data represent at least three independent experiments.

*1C*). In the second week these myeloid precursors were stimulated to differentiate into DCs using the various differentiation media. After differentiation, DCs were identified based on CD11c$^+$ HLA-DR$^+$ expression, and typical dendrites were seen with May-Grünwald staining after flow sorting (*Figure 1C*). Differentiation with GFS4 induced the highest percentage of CD11c$^+$ HLA-DR$^+$ CBDCs. Further analysis of the expression levels of various DC markers showed no clear differences between the different protocols, except for the CD209 expression that was absent after differentiation with GFS15 (*Figure 1D*). Although CD141 (BDCA3) was expressed on the majority of the CBDCs, no CLEC9A expression was detected on the CBDCs. Based on the absence of BDCA2 and CD123 expression on the CD11c$^-$ HLA-DR$^+$ population we concluded that pDC were absent in these CBDC cultures (*supplementary Figure 1D*).
Based on the total cell expansion data (*Figure 1B*) combined with the flow cytometry analyses of the percentage CD11c$^+$ HLA-DR$^+$ CBDCs, the number of CD11c$^+$ HLA-DR$^+$ CBDCs generated per CD34$^+$ CB was calculated (*Figure 1E*). Based on these data the GFS4 protocol was selected for further studies, since this protocol showed the highest potential to generate sufficient numbers of CBDCs.

*Antigen uptake and processing by CBDCs*
Antigen uptake and processing are essential features of DCs. In order to further analyse the functionality of the CBDCs, we first assessed the antigen uptake and processing capacity. Uptake of dextran-FITC (70 kD) increased dose-dependently in the CD11c$^+$ HLA-DR$^+$ population, while there was minimal uptake in the CD11c$^-$ HLA-DR$^+$ CBDCs (*Figure 2A*). Pulse/ chase experiments using DQ-BSA, a protein that becomes fluorescent upon unfolding as a consequence of proteolysis, however showed maximal fluorescence irrespective of the different HLA-DR$^+$ CBDCs. Within one day, all DQ-BSA was completely degraded as seen by a decline in DQ$^+$ signal (*Figure 2B*). The differences within the uptake and processing could be mediated due to different uptake mechanisms. Dextran-FITC is taken up by mannose-receptor mediated endocytosis, while DQ-BSA enters the cell via macropinocytosis.[25] In conclusion, the CD11c$^+$HLA-DR$^+$ population has the capacity of antigen uptake and processing.

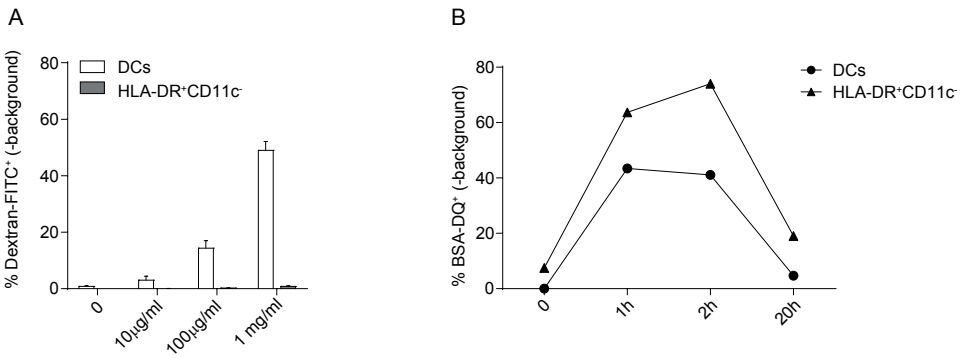



**Figure 2. Antigen uptake and processing by CBDCs**
(A) Uptake of increasing doses of FITC-Dextran by DCs and HLA-DR+CD11c- populations. The % of FITC+ signal taken up at 37°C is shown minus the FITC+ signal detected at 4°C. (B) The processing of BSA-DQ is shown by DCs or HLA-DR+CD11c- population at indicated time points. The DQ-signal seen at 4°C is subtracted from the DQ+ cells at 37°C. Data represent three independent experiments.

*Maturation and Migration of CBDCs*

The next step was the optimization of CBDC activation/maturation. In this regard we tested the effect of a GMP-grade available cytokine mixture (IL-1beta, IL-6, TNF and $PGE_2$) on CBDCs that has been regularly used in clinical DC vaccination trials [26,27]. The effect of this "CYTOMIX" was compared to that of an agonistic anti-CD40 antibody known for its stimulatory effect on Mo-DC maturation. Both CYTOMIX and anti-CD40 induced maturation of CBDCs as shown by

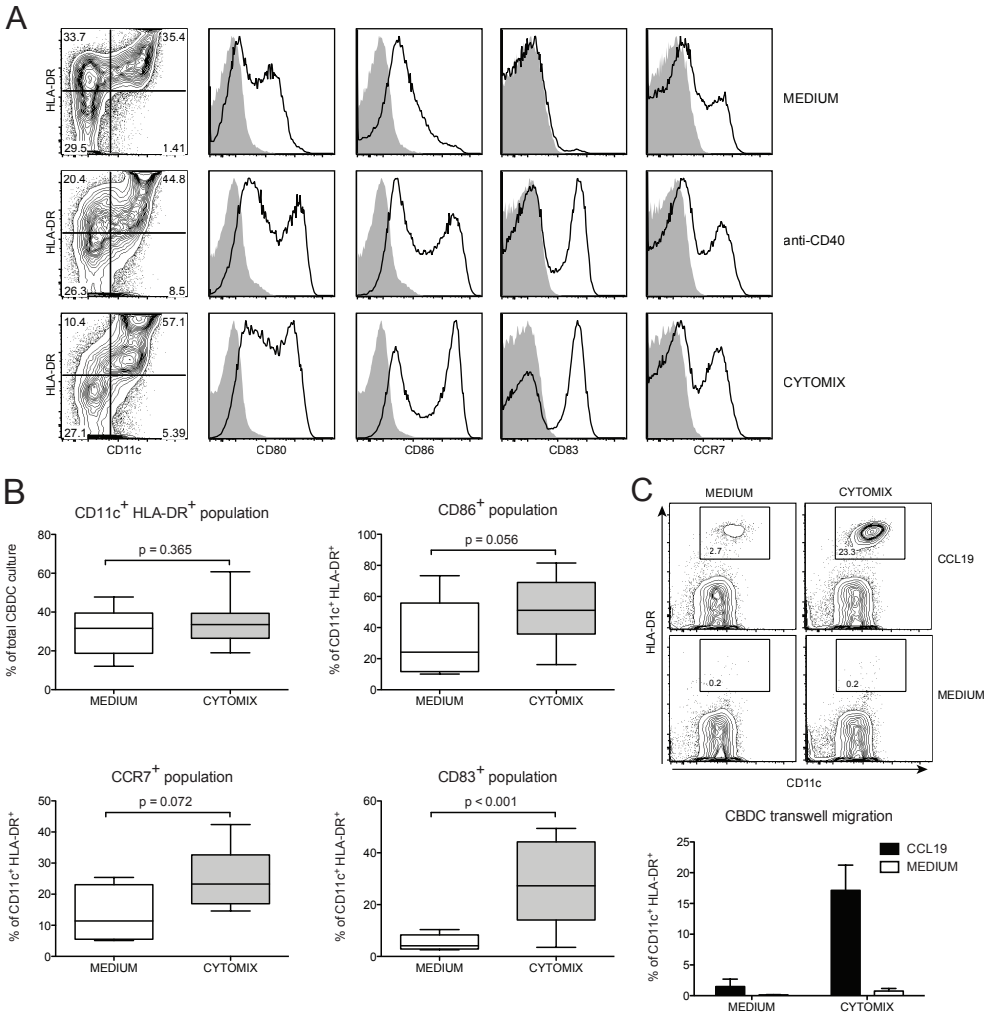



**Figure 3. Maturation and Migration of CBDCs**

(A) CBDCs are matured for 24 hrs with either anti-CD40 or CYTOMIX containing IL-1β, IL-6, TNF and PGE2. Live cells are further gated on HLA-DR and CD11c to analyze CD80, CD86, CD83 and CCR7 expression or isotype as a control (solid grey graph). (B) Plots are shown of whole culture followed by CD86, CCR7 and CD83 expression on DCs after maturation for 24 hrs with CYTOMIX. (C) Migration assay in a transwell system with the whole culture matured for 24 hrs with CYTOMIX or medium as a control in the upper compartment. In the lower compartment CCL19 was added, or medium as a control for 2 hrs. One representative FACS plot is shown and a bar graph of three independent experiments. Data represent three (C) or more (A,B) independent experiments. Error bars represent the SEM. *, $P < 0.05$.

the upregulation of CD80, CD86, CD83 and CCR7 (*Figure 3A*). All mature cells were CD11c$^+$ HLA-DR$^+$ supporting the notion that these cells are differentiated further into primary DC, whereas the CD11c$^-$ fraction seems still in a myelo/monocytic developmental stage. Combined results from 30 individual experiments/donors (*Figure 3B*) showed a highly significant increase in the percentage of CD83-expressing cells. The increase in percentages of CD86 and CCR7 positive cells were smaller and not significant since for both markers the control samples sometimes already expressed these markers. The exact reason for this is not known but may relate to handling of the individual cultures.[28] In these experiments CD83 is therefore the most specific and discriminative marker for CBDC maturation.

The migratory capacity of DCs is an important functional feature to ensure travelling to lymph nodes and subsequent T cell activation. As depicted in *Figure 3C*, maturation of the CBDC culture with CYTOMIX induced a strong increase in CCL19 specific migration of CD11c$^+$ HLA-DR$^+$ CBDCs in an established in vitro assay for CCR7-dependent migration.[18,29]
To address the T cell stimulatory capacity of the matured CBDC culture, we first performed an allo-MLR. CBDCs were able to stimulate allogeneic T cell proliferation (*Figure 4A*) compared to responder cells alone, which did not show any proliferation. Both CD4$^+$ as well as CD8$^+$ T cells are stimulated by CBDCs, although CD8$^+$ T cells to a greater extent (*Figure 4B*). These data confirm that CBDC express the necessary signals to stimulate CD8$^+$ and CD4$^+$ cells in a non-antigen driven manner.

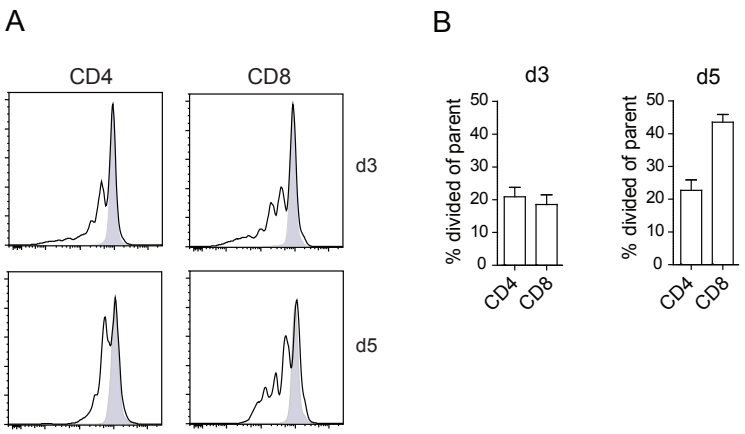



**Figure 4. Allogeneic-Mixed Leukocyte Reaction**
(A-B) Allo-MLR reaction of CBDCs and CD3 lymphocytes. (A) Cell proliferation was studied within the CD4 and CD8 population after 3 and 5 days by celltrace violet dilution. Shaded histogram represents the unstimulated CD3 lymphocytes. (B) Percentages divided within the CD4 and CD8 lymphocyte gate after 3 and 5 days of proliferation. Data represent three independent experiments.

*Characterization of HLA-DR negative population in the CBDC culture*
Using the whole heterogeneous CBDC culture as vaccine, without any need for further purification steps, is attractive for clinical application. This however requires a full characterization of the cells in this culture, especially the population of cells negative for HLA-DR. All cells in the CBDC cultures express CD33 and lacked the expression of CD3, CD19, CD20, and CD56 (*data not shown*) indicating that all cells are of the myeloid lineage. Using the

combined expression of CD11b and CD66b, four different subsets could be detected within the HLA-DR$^-$ population with the double negative and double positive representing the major populations (*Figure 5A and B*). Since similar markers are used to identify myeloid-derived suppressor cells,[30,31] we tested whether HLA-DR$^-$ cells could suppress T cell proliferation using a T cell suppression assay. In summary, none of the populations inhibited the anti-CD3 induced T cell proliferation but rather stimulated the proliferation of T cells in a dose dependent manner (*Figure 5C and D*).

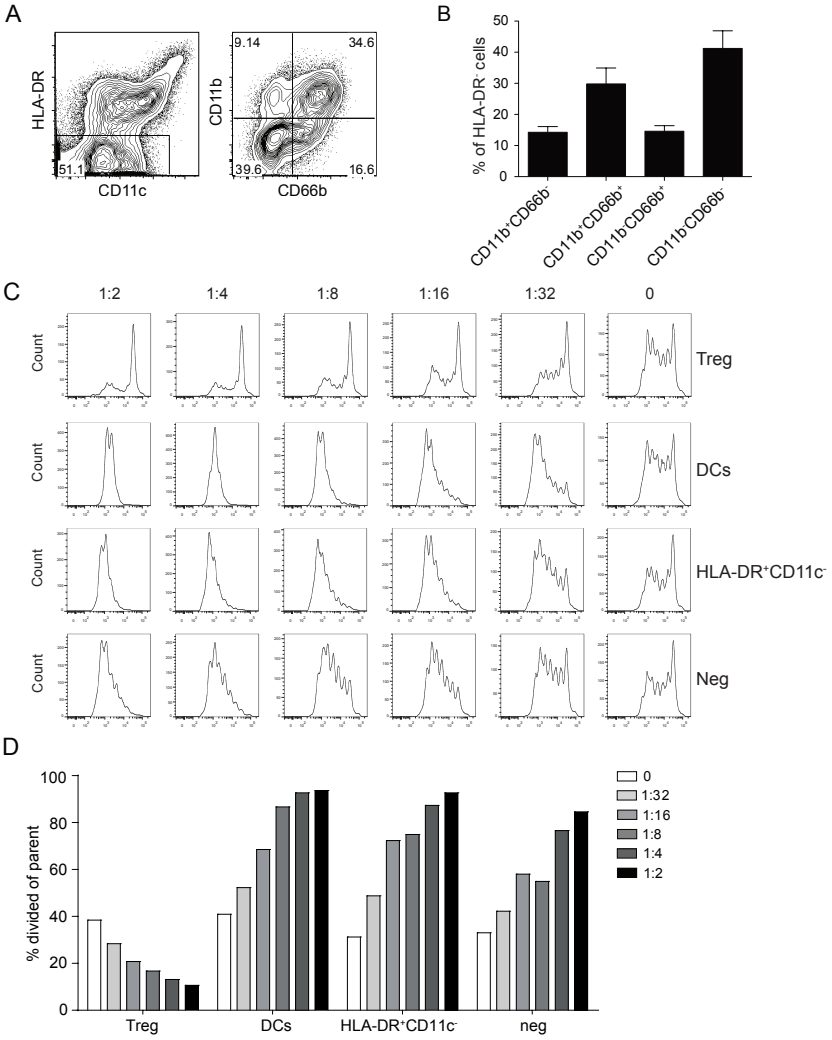



**Figure 5. HLA-DR negative population in the CBDC culture**
(A-D) Characterization of HLA-DR-CD11c- population (A) Gating strategy used for identifying different subsets. (B) % of different subsets. (C) Suppression assay with celltrace violet labeled effector cells, activated by anti-CD3. Different amounts of sorted cell populations are added as indicated, Tregs were sorted as a positive control. After 4 days of co-culture, effector T cell proliferation is assessed by celltrace violet dilution as shown with FACS plots or (D) in a bar graph. Data represent at least three independent experiments.

*CBDCs stimulate WT1-specific T cells*

There are several possibilities to load DCs with tumor antigens, all of which may have specific advantages or disadvantages for the induction $CD4^+$ and $CD8^+$ T cell responses. For WT1, electroporation with the full length WT1 mRNA has been shown to induce intracellular expression, processing and presentation in monocyte-derived DCs (moDCs).[32] In addition, this technique has been used in several clinical trials without severe side effects and thus is considered safe.[14] An optimal concentration of mRNA during electroporation of CBDC was determined using different amounts of EGFP mRNA (*Figure 6A*). Electroporation of 20 mg WT1 mRNA per 200 ml of cells resulted in WT1 protein expression in CBDC cultures from various donors as observed by Western blot (*Figure 6B*).

We next electroporated CYTOMIX-matured CBDCs with WT1 or sham as control and, after an incubation step to allow protein expression, processing and MHC loading, co-cultured the CBDCs with a $WT1_{126–134}$-peptide specific T cell clone. Notwithstanding a large donor-to-donor variation, four out of seven donors showed elevated levels of IFNg (*Figure 6C*) and/or TNF production (Figure 6D) confirming the presentation of $WT1_{126–134}$ peptides in the HLA-A2 molecules after electroporation with full length WT1 mRNA.

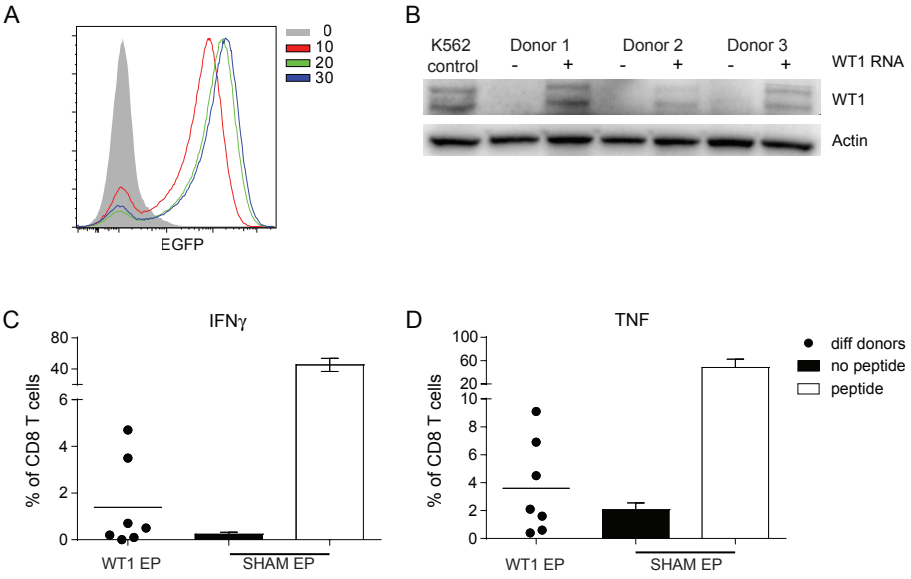



**Figure 6. CBDCs stimulate WT-1-specific T cells. RNA- electroporation of CBDCs**

(A) GFP expression 4 hrs after increasing doses of GFP-electroporation measured by FACS. (B) WT1 Western blot of three different donors 4 hrs after electroporation with WT1 or sham as a control. K562 cell-line was used as a positive control. (C-D) Cytokine production by WT-1 specific T cell clone after 4 hrs co-culture with CBDCs of 7 different donors (dots) after WT1 electroporation. Sham electroporated DCs with (black bar) or without (white bar) WT1 peptide serve as controls. (C) IFNγ and (D) TNF production. Data represent at least three independent experiments.

## Discussion

The poor survival rates of refractory/relapsed AML patients after HCT warrant the development of additional immunotherapeutic treatments strategies.[1-5] As such, we optimized strategies to generate sufficient amounts of CBDCs with the capacity to initiate activation and proliferation of AML antigen-specific CTLs in a pre-clinical setting.

Since only about 20 percent of the CB graft will be available for the preparation of the DC vaccine (80 percent is used for the actual HCT) extensive cell expansion is an essential part of the culture strategy to generate sufficient CBDCs. The present data show that a two-step protocol consisting of a massive expansion of myeloid precursors from the HSC followed by the differentiation of part of these precursors towards DCs induced an average of 600 CBDC culture cells for each initial $CD34^+$ cell. In the worst case scenario the 20% fraction of the CB-unit contains about $5x10^5$ $CD34^+$ cells, resulting in about $2,5x10^5$ cells after $CD34^+$ CliniMACS (personal observations). Taking into account an additional loss of about 25% of the cells during the remainder of the protocol, it will still generate up to $90x10^6$ cells which should be enough for three vaccinations using $1x10^6$ cells/kg in a 30 kg pediatric patient. However, since the CB graft is, in addition to the regular selection criteria, also selected based on a minimal number of $CD34^+$/kg infused per CB graft, the odds are that higher numbers will be generated. Maturation of the CBDC culture using CYTOMIX-induced upregulation of maturation markers and allowed specific migration toward a CCL19 chemokine gradient comparable with previous reports with other DC sources.[18,29] The CYTOMIX-treated CBDC culture stimulated CD4 and CD8 proliferation in an allogeneic MLR setting and electroporation with full-length WT1 mRNA led to expression of the protein and presentation of a HLA-A2-specific WT1 peptide. As such, the proposed protocol fulfills the requirement for application as a DC vaccine for treatment of pediatric AML patients.

Although this optimal culture strategy resembles to some extend the protocol described by Poulin et al,[22] we were unable to generate substantial amounts of $CD141^+CLEC9A^+$ DC. This could be due to the use of human AB serum instead of FCS and/or the use flask instead of 96-well plates since the latter strongly reduced the viability of the CBDC cultures (*data not shown*). The addition of human AB serum is essential for the differentiation of DC in this presented system, since possible alternatives for AB serum like platelet lysate or TGFb induced less DC differentiation (*data not shown*). It has been reported that the use of IL-15 instead of IL-4 may result in the generation of more powerful DC, mostly MoDC.[33,34] In contrast to MoDCs, IL-15 failed to noticeably modify the CD56 expression CBDC (not shown). CD209 (DC-SIGN) expression was absent in the IL-15 differentiated CBDC, which fit with the observations that IL-4 is the inducer of CD209 expression in MoDC cultures.[35]

Cumulative data (n=20) showed that about 35% (+/-10%) of our CBDC culture consisted of $CD11c^+$ $MHCII^+$ expressing DCs after the described protocol. We are currently investigating whether the expression of specific markers or genes at the end of the expansion period could predict the differentiation of a certain subset of cells towards DCs. Although small adaptations like the addition of low levels of TNF to the differentiation medium did increase the percentage of DCs,[36] we ended up with a smaller amount of DCs because of its detrimental effect on viability (*not shown*).

With regard to the antigen uptake, a clear difference was observed between $CD11c^+$ $HLA\text{-}DR^+$ and the $CD11c^-$ $HLA\text{-}DR^+$ population, with the latter being unable to take up 70 kD Dextran molecules probably because of a lack of a specific uptake receptor.[37] Although this difference in uptake may not be important in our current setting using mRNA electroporation for loading the DCs with tumor antigen, we are currently further exploring the expression of different uptake receptors that may be useful for different antigen loading strategies, like antibody-mediated targeting or uptake of apoptotic or necrotic tumor cells.[38] Both CYTOMIX and agonistic anti-CD40, an antibody developed for use in cancer immune therapy, provided strong maturation of CBDC. In addition, newly developed methods for antigen loading and DC maturation, like the use of long peptides linked with TLR ligands or loading this compounds in PLGA particles may in the future provide alternative clinical-grade available maturation and antigen-loading strategies.[39,40]

With regard to the presentation of WT1 peptides after electroporation of the CBDC, using mRNA encoding the full-length protein, we found highly variable induction of generally low levels of IFNg expression by a $WT1_{126–134}$-peptide specific T cell clone. These data are in line with those obtained stimulating a similar $WT1_{126–134}$ T cell clone with MoDCs electroporated with the same WT1 mRNA construct.[41] Considering that the T cell clone used here is only responsive to one of the many possible peptides in the WT1 proteins the low percentage of responding T cells is not surprising.[42] The clinical potential of a WT1 DC vaccine is supported by a clinical trial using a MoDC vaccine electroporated with the same WT1 mRNA construct.[14] In this trial WT1-specific tetramer-positive CD8 cells were enhanced in some HLA-A2 positive patients and this was associated with the induction of clinical and molecular (WT1 mRNA levels) remission.[14] Variation between the donors was not related to the amount of DCs generated in the culture or the success of electroporation (generally between 80-95%). Another attractive explanation may simply be that the variability between donors in protein degradation and processing leads to different WT1 peptides being loaded into MHC molecules.

In summary we describe the pre-clinical development of a WT1 mRNA-electroporated DC vaccine with feasibility to generate sufficient amounts of cells for clinical application from the limited number of $CD34^+$ CB stem cells. In addition, we provided proof-of-principle for the capacity of these cells to present antigen to WT1-specific T cells. The next step in the bench to bedside approach is the translation of our pre-clinical protocol towards generation of this DC-vaccine under GMP conditions, apply for ethical approval and study the above described concept in a phase II clinical study.

## Acknowledgements

This work was supported by the Dutch Children Cancer Free Foundation (KiKa), Amstelveen, The Netherlands.

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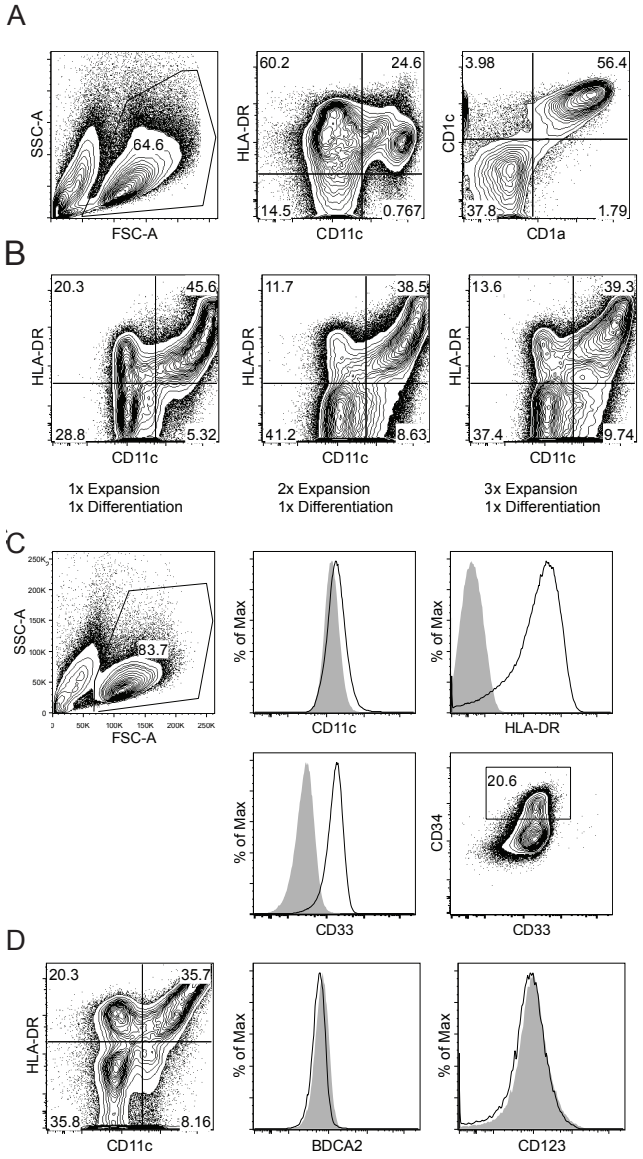
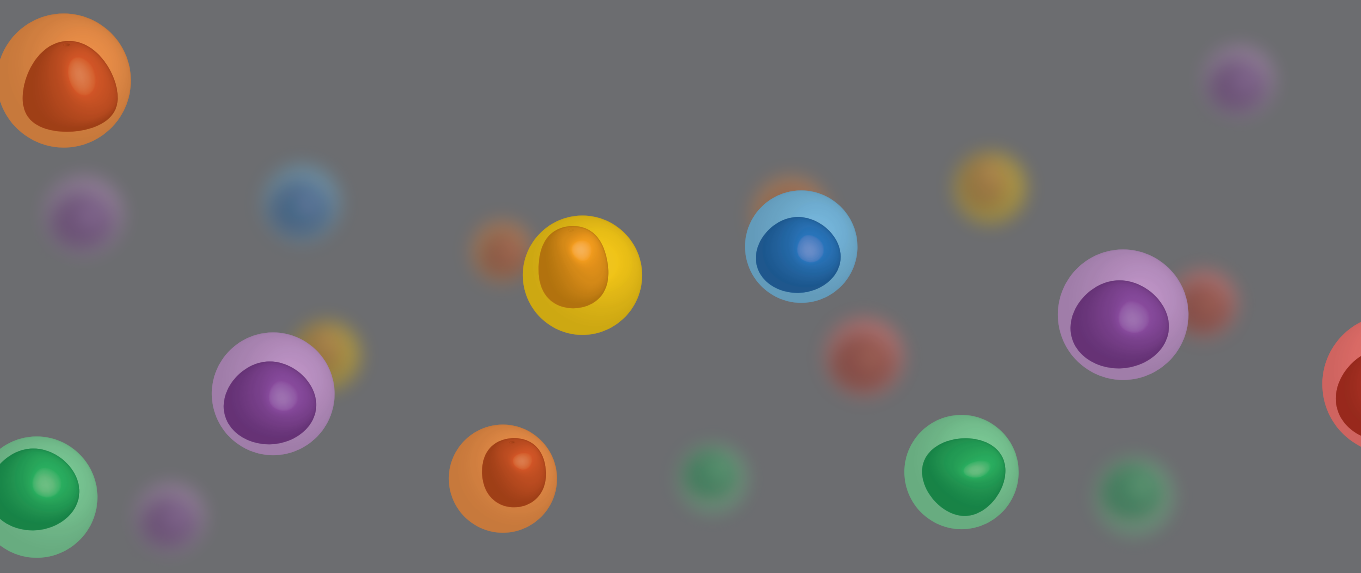
## Supplemental Figure 1





# Dankwoord
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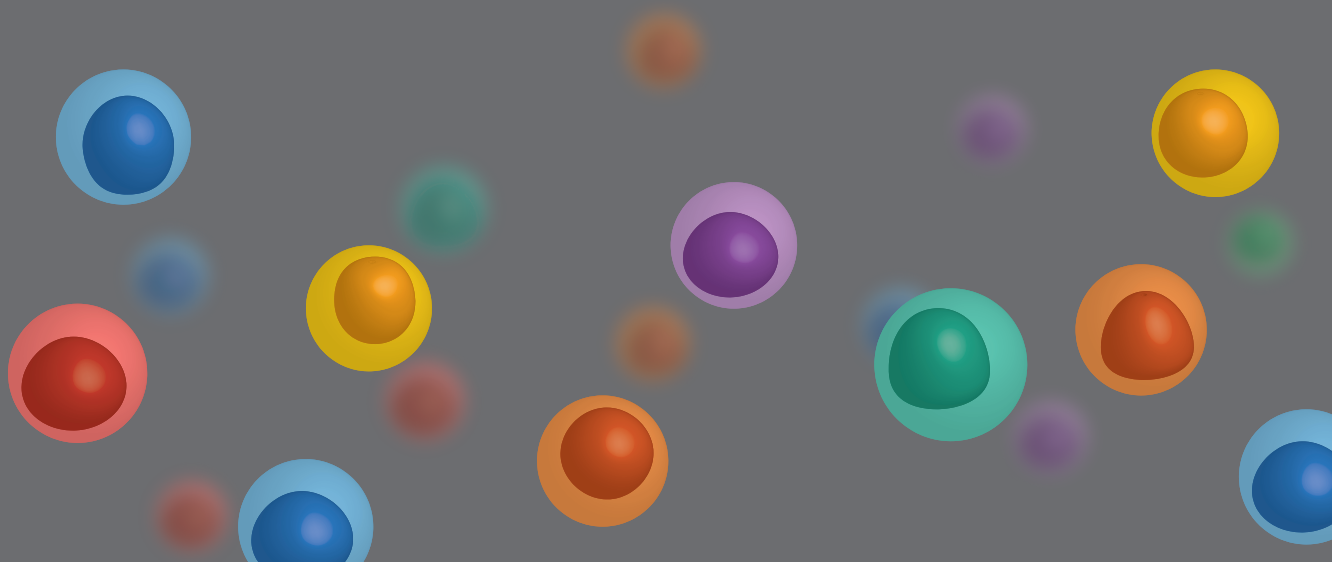

## Dankwoord

Toen ik met dit project begon, was ik nieuwsgierig naar wat een PhD zou brengen. Wie had gedacht dat het zo'n mooie tijd zou worden? Voorheen dacht ik weleens alleen klinisch werk te willen doen, maar als ik nu andere mensen dit hoor zeggen denk ik: Je weet niet wat je mist! Dat dit zo'n mooie tijd is geweest komt vooral door de vele leuke, lieve en enthousiaste mensen met wie ik heb mogen samenwerken (en van wie ik per persoon meer buisjes bloed heb gekregen dan dit proefschrift aan pagina's rijk is). Flinsenberg et al. zou daarom eigenlijk op de kaft moeten staan.*

Lieve Marianne, jij bent de beste supervisor die een AIO zich kan wensen. Ik bewonder jouw vermogen om verschillende mensen met wisselende sterke en zwakke kanten op individuele basis te benaderen, waarmee je ieder op een eigen manier naar de eindstreep helpt. Jouw aanhoudende positiviteit en vermogen om zelfs aan de slechtste data een mooie draai te geven zorgt niet alleen dat je alles durft te bespreken, maar ook dat je altijd met strijdlust jouw kantoor verlaat. De dag dat politiek wijkt voor beoordeling op kwaliteit, ben jij een van de beste professoren van het LTI, maar ik hoop eerder. Ik hoop dat we nog lang (wetenschappelijk) bevriend blijven.

Beste Jaap-Jan, er zouden meer kinderartsen moeten zijn zoals jij. Met jouw visie en verschillende onderzoekslijnen verbeter je de levensverwachting van de meest kwetsbaren. Maar daarnaast ben je ook een goede dokter, bij je patiënten geliefd. Ik heb me regelmatig opgetrokken aan jouw humor en vermogen groot te denken, vooral toen ik twijfelde over welk toekomst-pad te kiezen. Jij maakt een congresbezoek naast leerzaam tot een feestje. Je hebt mij een kans gegeven mezelf te bewijzen en mij mijn gang laten gaan in de keuzes die ik maakte. Dank voor het vertrouwen dat je in mij stelde.

Beste Edward, we hebben elkaar niet vaak gesproken tijdens mijn promotie, maar ik heb er elke keer iets aan gehad. Het is leerzaam te zien hoe jij alles in een breder kader plaatst. Daarnaast waren jouw tips veel waard in de voorbereiding op mijn sollicitatiegesprek. Ik jat nu soms jouw speech over rode, oranje en groene vragen als iemand mij om sollicitatie-advies vraagt. Dank dat je mijn promotie wilde steunen.

Beste Berent, je staat niet op pagina 4 van dit boekje, maar dat zou wel moeten. Als schaduw promotor ben ik jou ontzettend dankbaar. Ik durf te stellen dat ik zonder jouw steun niet tot dit boekje was gekomen. Er zijn veel eigenschappen van jou die ik bewonder. Natuurlijk je people-skills, maar zeker ook je humor. Daarnaast had je altijd even tijd voor me als ik grover geschut nodig had om een probleem op te lossen. Maar wat ik vooral van jou geleerd heb, is dat een lab met een goede sfeer de basis vormt voor goed onderzoek en een geslaagde promotie, en die goede sfeer begint bij de baas.

De Boesmannen+; Ewoud (of Ewout Compaan), Robert, Arie (AJ) en Theo. We hebben volle zalen getrokken met rockband, en met enige regelmaat de wereld gered, maar het meest legendarisch waren toch wel onze 4-dagelijkse werkbesprekingen die zich concentreerden rond het nespresso-apparaat. Ook al gelooft niemand dat het over werk ging, wij weten wel beter. Mannen, een goede sfeer is heel belangrijk, en jullie waren top teamgenoten!

* Geinspireerd door Annick vd Ven, Dankwoord 2011

Speciale dank aan mijn paranimf en dikke vriend Henk Schipper. Ik heb enorm genoten van de licht filosofische en werk overstijgende koffie momenten. Gelukkig zetten we deze trend voort in het Ledig Erf, maar nu met een schuimkraag.

De Boesvrouwen; Lieneke, Lotte, Friederike, Willemijn en Annick. Enig tegenwicht tegen al dat testosteron is minstens zo belangrijk. Bedankt voor de gezelligheid en de goede samenwerking. Annick, voor het kinderlijk uitschrijven van mijn eerste protocol (BLCLs doorzetten). Willemijn, voor de samenwerking in ons Q27W project. Lotte voor de paradiso en tivoli reminders, en voor het oppakken van het priming project. Lieneke voor je technische hulp op diverse vlakken, en voor een wit gezicht tijdens je danoontje overwinningen.

De CBDC gang; Colin, Maud, Stefan, Nina en Esther. Dank voor jullie bijdrage aan de projecten en de goede en kritische input bij de werkbesprekingen. Stefan voor de hulp bij het maken van mijn eerste MoDCs. Maud en Nina, voor de CBDCs. Colin, voor het spotten van Obama.

Dan de sfeermakers van het UMC. De groep die ervoor zorgt dat je ondanks de met enige regelmaat tegenvallende resultaten toch met plezier naar je werk blijft gaan, de rest van het fantastische CMCI lab; Allesandra, Alvin, Annemieke, Annemarie, Arjan, Bas, Caroline, Ellen, Eva, Eveline, Felicitas, Femke, Genoveva, Gerdien, Judith, Kerstin, Lieke, Maja, Marloes, Michal, Nico, Nienke, Sabine, Sanne, Selma, Sylvia, Sytze en Yvonne. Special thanks aan de superondersteuning van sympathiek opperhoofd Mark, sexy bitch Jenny, generaal Scholman, Mariska, Willie Wortel Wilco, Koos, Gerrit en Pien. Ook dank aan Angela en Erica voor alle ondersteuning achter de schermen.

Alle studenten die de afgelopen jaren de sfeer hebben versterkt; Anneta, Manon, Femke, Laura, Mirjam, Annelies, Inge, Joost, Lotte, Kitty, Krijn, Nienke, Popi, Ron, Serge, Suzanne en Verena. Met name mijn co-auteurs Manon, Femke, Mirjam en susanna, dank voor jullie inzet.

Een goede buur is beter dan een verre vriend. Daarom dank aan de Coffers/Beekmanners, in de eerste plaats voor de gezelligheid en niet op de laatste plaats voor de technische hulp bij moleculaire bezigheden; Anita, Anna-rita, Cornelieke, Florijn, Janneke, Jeffrey, Jorg, Koen, Koen, Lodewijk, Lena, Marije, Marit, Pauline, Ruben, Stephin, Pernilla en Veerle. Daarnaast ook dank aan Paul voor de feedback en Caroline voor de richtinggevende gesprekken.

Verder dank aan de vele leuke mensen die het LTI te bieden heeft. Eszter, Jeanette, Joris, Kees, Maaike, Marco, Marielle en Peter voor de samenwerking rondom Fc-Receptoren. Dan en Debbie voor jullie expertise omtrent T-cellen. Alsya, Anouk, Barbara, Bas, Claudia, Emmerick, Evelien, Jonas, Kevin, Marit, Michiel, Michiel, Rutger, Shamir, Stefanie, Steven, Tamar, Tessa, Wouter en Zsolt voor de lol.

De long-dames, die altijd klaar staan om de ondoorgrondelijke wirwar van de statistiek te verhelderen tijdens een gezellig kneuterige kamer-lunch; Anne, Francine, Jacobien en Kim.

PhD-stress kan je het best van je afslaan, of spelen. Daarom dank aan Heren 1.1 (en jullie vrouwen); Jan-Willem, Martijn, Pieter, Thijs en Wouter. Daarnaast dank aan de Morning Due mannen en dame; Bram, Edwin, Maj, Sven en Thomas (en ook aan Bas, Gidde en Maurits).

Michiel, mijn trouwe vriend sinds mijn geboorte! Jij hebt heel wat uren in dit boekje zitten. Dank voor je creatieve en technische inbreng! En Marja voor het onderhoud tijdens de lange computer-sessies.

Bart, Ody, Thom en Lotta; dank voor de gezelligheid op Terschelling en bij familieweekendjes. Echte nerds ondertekenen hun post met een lab-techniek; ondanks dat ik zover nog niet heen ben, zijn jullie een voorbeeld voor me.

Opa en Oma, het is echt supercool om zulke hippe grootouders te hebben. Ik bewonder jullie onbreekbaar rasoptimisme. Ik ben dankbaar voor jullie voorbeeld en jullie steun en ik vind het te gek dat jullie erbij zijn vandaag!

Lieve Maartje en Huib, knappe zus en zwager, eigenlijk zien we elkaar te weinig. Ik vind het altijd heel gezellig met jullie tijdens een mooie wandeling of lekkere barbecue. Dank voor jullie interesse en voor de snelle feedback als ik in een winkel schoenen sta te passen.

Lieve pa en ma. Ik besef heel goed wat een geluk ik heb met zulke ouders. Naast dat jullie er al heel mijn leven voor me zijn, heb ik de afgelopen jaren extra genoten van de regelmatige telefoongesprekken op de fiets en de heerlijke relax-weekendjes in het mooie Limburg. Er zijn maar weinig mensen die hun ouders tot beste vrienden rekenen, maar voor mij geldt dat zeker.

Lieve Lisa, mijn engel. Dat ik dit proefschrift succesvol heb kunnen afronden is mede te danken aan jouw hulp, luisterend oor en geduld. Ook nu ga ik met een gerust hart de volgende professionele fase tegenmoet, wetende dat jij hoe dan ook achter mij staat. Ik ben jou dankbaar voor alles wat je voor me doet, voor je sprankelende energie en voor de fantastische reisgenoot die je bent. Ik hoop dat we samen nog veel mooie avonturen gaan beleven!

## Curriculum Vitae

Thijs Willem Hendrik Flinsenberg was born October 3rd 1984 in Beek, the Netherlands. In 2002, he completed secondary education at the Trevianum in Sittard, with a focus on physics and biology. Probably inspired by his mothers' career as a nurse, he started medical school at the University of Maastricht the same year. Based on his interest in other cultures, during his medical training he did a clinical rotation at the Christian Medical College in Vellore and at the Kalafong Medical Centre in Pretoria, South Africa. As part of his study he wrote a thesis on the effects of sex and estrogen on chicken ductus arteriosus reactivity, which resulted in his first paper published in 2010 in an international peer-reviewed journal.

After graduating in 2008, he started working in the department of paediatrics at the Orbis Medical Centre in Sittard under supervision of drs. Jan Heynens and dr. Adèle Engelberts. In 2009, he continued in pediatrics at the Wilhelmina Childrens Hospital in Utrecht. During a rotation at the stem cell transplantation department under supervision of dr. Jaap-Jan Boelens, his interest for immunology related to stem cell transplantation was born. In 2010 he began his PhD training at the Eijkman Graduate School for Infection and Immunity at the UMC-Utrecht. He worked in the Center for Molecular and Cellular Intervention (CMCI), now part of the Lab Translational Immunology (LTI), under supervision of dr. Marianne Boes and dr. Jaap-Jan Boelens. During this PhD program, he studied the biology of antigen cross-presentation by dendritic cells and the subsequent regulation of T cell responses, aimed to improve dendritic cell-based vaccination as adjuvant therapy for children that receive a stem cell transplantation. The results obtained during this PhD project are presented in this thesis.

In May 2014, he started his residency program in internal medicine at the UMC-Utrecht. Hoping to combine travelling, medicine and research, for the near future he dreams of a post-doc in translational medicine in an academic research setting in Australia, together with his life-partner in crime, Lisa.

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## This thesis

Flinsenberg TW, Compeer EB, Boelens JJ, Boes M. Antigen cross-presentation: extending recent laboratory findings to therapeutic intervention. Clin.Exp.Immunol. 2011;165:8-18.

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Flinsenberg TW, Janssen W, Herczenik E, Boross P, Nederend M, Jongeneel L, Scholman R, Boelens JJ, Maas C, van Gijn M, van Montfrans J, Leusen J, Boes ML. A novel Fcγ-Receptor IIa Q27W gene variant associates with common variable immune deficiency through defective FcγRIIa downstream signaling. Clinical Immunolgy 2014; accepted for publication

Flinsenberg TW, Jansen M, Koning D, de Haar C, Plantinga M, Scholman R, van Loenen M, Nierkens S, Boon L, van Baarle D, Heemskerk M, Boelens JJ, Boes M. Cognate CD4 T-cell licensing of dendritic cells heralds CD8 T-cell immunity after allogeneic umbilical cord blood transplantation. Submitted 2014

Compeer EB, Flinsenberg TW, van der Grein SG, Boes M. Antigen processing and remodeling of the endosomal pathway: requirements for antigen cross-presentation. Front Immunol. 2012;3:37.

Compeer EB, Flinsenberg TW, Boon L, Hoekstra ME, Boes M. Tubulation of endosomal structures in human dendritic cells by Toll-like receptor ligation and lymphocyte contact accompanies antigen cross-presentation. J Biol Chem. 2014 Jan 3;289(1)520-8

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de Haar C, Plantinga M, Blokland NJG, Flinsenberg TW, van Tendeloo VF, Smits EL, Boon L, Spel L, Boes M, Boelens JJ, Nierkens S. Generation of a cord blood-derived Wilms Tumor 1 dendritic cell vaccine for AML patients treated with allogeneic cord blood transplantation. Submitted 2014

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