Cell-biological mechanisms regulating antigen presentation.

Signalling towards adaptive immunity

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Cell-biological mechanisms regulating antigen presentation.
Signalling towards adaptive immunity

Celbiologische mechanismen die antigeen presentatie reguleren.
Signaleren naar de adaptieve afweerreactie

(met een samenvatting in het Nederlands)

Proefschrift

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Contents

Chapter 1.

General introduction.

As humans, we are exposed daily to millions of potential pathogens, through contact, inhalation, or ingestion. Our ability to avoid infection depends on our immune system, which consists of two distinct, yet interrelated and interacting subsystems: the innate and adaptive immune system. The adaptive immune system remembers previous encounters with specific pathogens and destroys them when they attack again. Elicitation of an adaptive response depends on special white blood cells from the innate immune system. This thesis focuses on the central role that the intracellular endosomal transport network plays in elicitation of an adaptive immune response by certain specialized white blood cells, respectively B cells and Dendritic Cells (DCs). It investigates novel mechanisms underlying activation-induced endosomal trafficking in DCs, including endosomal tubulation and receptor-mediated uptake. Moreover, it investigates putative role of dysregulated endosomal processes that leads to diminished class II MHC antigen presentation in disease pathology of the most prevalent type of inherited primary immunodeficiency disease: Common Variable Immunodeficiency (CVID).
Overall, the work in this thesis is aimed to clarify endosomal processes that are pivotal to antigen-specific lymphocyte activation. This will aid to improve (DC-based) vaccination strategies and contribute to elucidation of dysfunctional mechanisms underlying primary immunodeficiencies. The first chapter provides a short overview of key players in the adaptive immune response, the central role of the endosomal compartment in antigen presentation and an overview of primary immunodeficiencies with (partially) defective antigen presentation machinery.

Adaptive immune response.

The immune system is divided into two distinct, yet interrelated, subsystems: the innate and adaptive immune system. Key differences between the innate and adaptive immune response are summarized in **Table 1**.
The innate immune system is composed of cells and mechanisms that rapidly recognize pathogens in order to defend the host from infection. Innate immune cells, such as DCs, are capable of this generic pathogen recognition by expressing proteins that recognize pathogen-associated or cellular damage-associated molecular patterns. These proteins are named pathogen recognition receptors, e.g. PRRs. The best defined family of PRRs are toll-like receptors (TLRs), which consist of 11 members that are together capable of recognizing extra- and intracellular pathogen-associated molecules patterns. As first responders after infection, these innate immune cells relay instructive information to determine the type and/or initiation of adaptive immune response. Unlike the innate immune response, the adaptive immune response confers long-lasting immunity.
The adaptive immune response is organized around two white blood cell types; B and T cells. Activated B cells divide rapidly and their progeny differentiates into plasma cells and long-lived memory B cells. The plasma cells produce large amounts of antigen-specific antibodies into the extracellular milieu. In the extracellular milieu, antigen-

specific antibodies coat antigens that results in their neutralization or facilitates their elimination. There are some antigens that fully activate B cells directly, but for most antigens, mature B cells require help from $CD4^+$ T cells to differentiate into antigen-specific plasma cells. In both cases, the antibody response is stronger when T cell help is available. T cell help consists of both soluble and membrane-bound proteins expressed by antigen-specific T helper cells, such as cytokines and CD40L respectively. Inability of B cells to recruit help from T cells may result in severe immunodeficiency.

In addition to helping B cells, a distinct subset of T cells can directly eliminate infected or altered-self (tumor) cells upon recognition. Thesre are the $CD8^+$ cytotoxic T cells.

For recognition of pathogenic elements, B and T cells are equipped with receptors that have varying specificities, named T- and B cell antigen receptors (e.g. TCR and BCR). In contrast to germline-encoded PRRs, TCRs and BCRs are created in a complex process of gene segment rearrangement within the cells and therefore distribute clonally. This means that cells of the same type have receptors with different specificities, but the progeny of each specific cell (clones) possesses receptors with identical specificity. After the first encounter with an antigen, antigen-specific clones will expand so that, if the same type of antigen invades the body a second time, there will be more antigen-specific clones ready to deal with the pathogen. Clonal expansion, deletion, or anergy, enables the immune system to adapt and to keep pace with rapidly evolving pathogens. Altogether, this allows the adaptive immune system to confer an antigen-specific long-lasting immunological recall responses, coined immunological memory, forming the fundamental basis for vaccination strategies.

BCRs and TCRs recognize their ligands using different mechanisms. BCRs recognize native antigen whereas TCRs recognize only a small portion (peptide) of the antigen in the appropriate context. Activation of T cells relies on antigen processing and presentation of antigenic peptides by innate immune cells in the context of Major Histocompatibility Complexes (MHC). This process is named antigen presentation[1].

Table 1: Key differences between innate and adaptive immune system.

Property	Innate immune system	Adaptive immune system
Cells	DC, NK, Mø.	B cell, T cell.
Receptors	germline-encoded. gene rearrangement not necessary. non-clonal distribution.	encoded in gene segments. Somatic gene rearrangement necessary. clonal distribution.
Recognition	conserved molecular patterns. selected over evolutionary time.	details of molecular structure (antigens). selected over lifetime of individual.
Response	cytokines, chemokines.	clonal expansion, cytokines.
Action time	immediate effector function.	delayed effector function.
Evolution	vertebrates and invertebrates.	vertebrates.

DC is abbreviation for dendritic cells, NK is Natural Killer cells, Mø is macrophages.

Class I and II MHC dichotomy

Two types of MHC molecules have evolved, class I and II respectively. $CD8^+$ T cells can recognize peptide-class I MHC complexes, whereas $CD4^+$ T cells respond solely to

peptide-class II MHC complexes. In general, class I MHC present peptides that are derived from proteins degraded in the cytosol, which contains predominantly self-synthesized (endogenous) proteins. As cytosolic peptides are continuously loaded onto class I MHC, these molecules display a 'peptide fingerprint' of intracellular protein content, which is recognized by cytotoxic $CD8^+$ T cells for immune surveillance of virus infections and tumors.

Class II MHC acquires peptides that are generated by proteolytic degradation in endosomal compartments. These peptides may originate from exogenous components that are internalized from the extracellular environment, as well as endogenous proteins that are present in endosomes such as plasma membrane proteins. These antigens must first be effectively internalized and targeted to the correct processing compartment, therefore presentation of exogenous antigens is more complicated than presentation of endogenous antigens. This is reflected by expression of both MHC classes. Class I MHC is abundantly expressed by nearly all nucleated cells. In contrast, expression of class II MHC is under steady state conditions restricted to a specific group of cells, named antigen presenting cells (APCs). These specialized cells acquire, process, and present antigens and includes cells from the monocyte-macrophage lineage, DCs, and B cells[1].

Thus far, it is clear that class II MHC and endosomal proteases process exogenous antigen, while proteasome-class I MHC process endogenous antigens to confer immunity (**Figure 1**). However, this poses the threat that we cannot resolve viral infections by $CD8^+$ T cells when viruses do not target DCs.

In 1976, a third mechanism was reported that we are now beginning to understand. In this observation, the capture of exogenous class I MHC-restricted antigens by DCs resulted in $CD8^+$ T cell responses[2]. This process was termed antigen cross-priming and was shown to be relevant for numerous cell-associated antigens in various settings, including viral, self and tumor-associated antigens[3]. Antigen cross-priming refers to the presentation of class I MHC-restricted antigen to prime specifically naïve $CD8^+$ T cells. The general term for class I MHC presentation of exogenously acquired antigen is now named antigen cross-presentation.

APC-T cell interaction.

Activation of $CD4^+$ and $CD8^+$ T cells requires presentation of peptide-MHC complexes of appropriate specificity and parallel binding of CD4 to class II MHC or CD8 to class I MHC molecules. This is referred to as signal 1. In general, the stimulation and clonal expansion of $CD8^+$ T cells by antigen presenting DCs requires sequential interaction of an estimated 200 TCR molecules with antigen-specific peptide-class I MHC complexes[4]. Similarly, $CD4^+$ T cells were activated by APC that expressed as few as 210-340 specific peptide/class II complexes[5]. However, T cell activation is not only based on quantity of peptide-MHC complexes. T cell activation requires a second signal through co-stimulatory molecules, which are mainly upregulated during infectious conditions by PRRs[6]. This safety

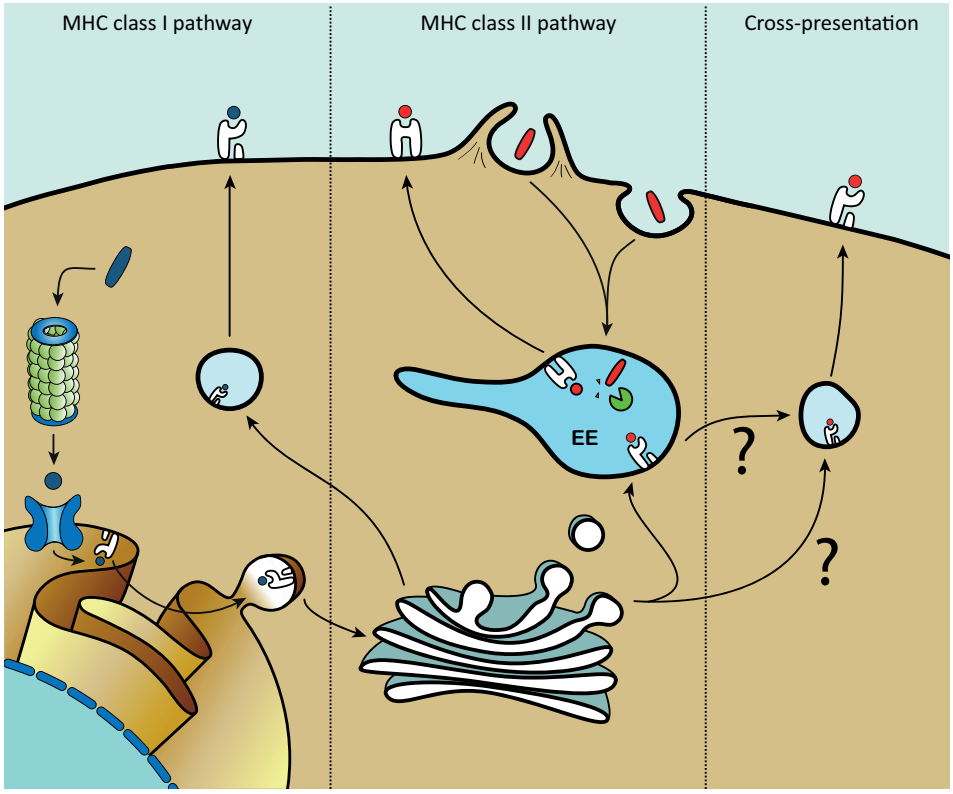

Figure 1. Canonical MHC class I and II antigen presentation pathways and cross-presentation.
MHC class I molecules (left panel) present peptides that are derived from proteins degraded mainly in the cytosol (blue proteins and peptides). MHC class II molecules acquire peptide cargo that is generated by proteolytic degradation of exogenous proteins in endosomal compartments (red proteins and peptides). In addition, various cells including DCs are able to present peptides of exogenous proteins in MHC class I molecules during inflammatory conditions (cross-presentation). Mechanisms involved in cross-presentation are still not completely understood.

: MHC I/endogenous peptide, : MHC I/exogenous peptide, : MHC II/exogenous peptide, : exogenous protein, : endogenous protein, : exogenous peptide, : endogenous peptide, : Proteasome, : TAP : Proteases, EE: Early endosome

mechanism enhances T cell activation towards non-self antigens and limits responses to self-antigens. Best known examples of co-stimulatory molecules are the B7 family members CD80 and CD86 that bind to CD28[1]. However, many more co-stimulatory molecules exist. For example, several SLAM family members are known to co-stimulate for T cell activation[7]. The signalling through co-stimulatory molecules is also referred to as signal 2. Upon interaction between APCs and T cells, APCs secrete various cytokines and chemokines that can further direct T cell development into various subsets. The release of these mediators, is referred to as signal 3 and also depends on PRR signalling[6].

To ensure effective T cell activation, T cells are recruited to APCs by APC-secreted chemokines. This process occurs prior to APC-T cell interaction and is therefore often referred to as signal 0. Upon recruitment, T cells are scanning APCs in an antigen-independent manner by APC expressed ICAM-1 and T cell expressed LFA-1. Upon the encounter of peptide-MHC complex of appropriate specificity, TCR signalling enforces ICAM-1/LFA-1 interaction by putting LFA-1 in a higher affinity state. In parallel, ICAM-1 and/or MHC clustering recruits ICAM-1 to the APC surface. This results in tight interaction between APC and T cell. This cognate interaction induces a relatively long-lived interface, referred to as the Immunological Synapse (IS) between an APC and a T cell. The IS remodels into a bull's eye configuration with a central part named the central supramolecular activation complex (cSMAC). This concentrates the TCRs and peptide-MHC complexes, with co-stimulatory molecules, just on the edge of the bull's eye. The ring-shaped area that surrounds the cSMAC is referred to as the peripheral central supramolecular activation complex (pSMAC) and contains LFA-1 and ICAM-1. A region outside of the pSMAC, the distal SMAC or dSMAC, contains CD45 and other tyrosine phospatases. The secluded space between APC and T cells at the IS is used for the containment of locally secreted soluble molecules, such as aforementioned cytokines, thereby only affecting interacting cell and not neighbouring cells. Peptide-MHC complexes, co-stimulatory molecules, and cytokines, are all transported to the IS in a polarized fashion by the transport system inside the cell, referred to as the endosomal compartment.

Endosomal compartment.

The endosomal pathway is a highly dynamic membrane system composed of vesicular and tubular compartments, which are involved in intracellular transport and thereby crucial for the aforementioned T cell activation. Endosomal compartments are best known for incorporating membrane molecules derived from the cell surface and sorting these molecules for either degradation or recycling back to the cell surface. This cell surface-directed recycling is crucial for many processes, including antigen presentation. Endosomal recycling is tightly controlled by a variety of proteins that include microtubules, motor proteins, SNARE proteins, and various small GTPases.

Despite the high plasticity of endosomes, a rough classification of endosomes can be made based on morphology, luminal pH, relative abundance of certain proteins or lipids, and intracellular localization, as illustrated in **Figure 2**. Within seconds after the uptake of cell-surface or extracellular molecules, the cargo reaches Early Endosomes (EEs). EEs are peripherally localized, transient, have a vesicular-tubular morphology, enriched in sterols and specific phosphoinositides (PtdsIns(3)P), are characterized by a near neutral pH (pH 7.5)[8] and the presence of the small GTPase Rab5[9].

Cargo that is destined for degradation is targeted and concentrated in membrane subdomains that are remodelled to form intraluminal vesicles (ILVs). Incorporation in

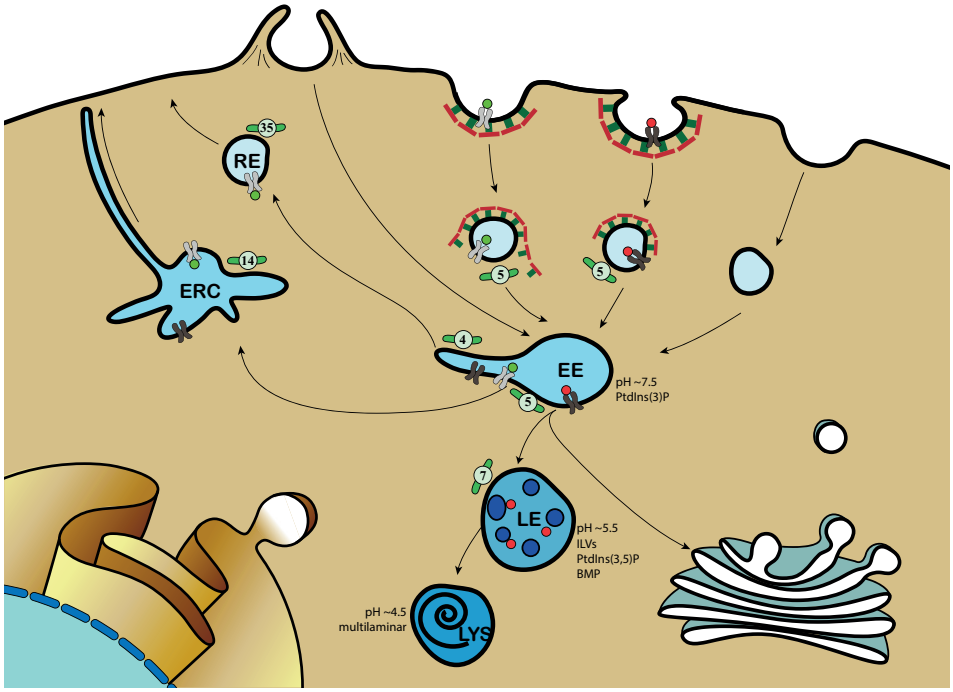

Figure 2. Characteristics of distinct endosomes in the endosomal network.

Endosomes alter their composition as they undergo a transition from early into late compartments, by continued influx and efflux of various proteins. Characteristics of Early Endosomes (EE) are a neutral pH, presence of Rab4 and/or Rab 5 and predominantly transferrin. In parallel, Late Endosomes (LE) have an acidic pH of 5.5, Rab7 and Low-density-lipoprotein positivity. Finally, endosomal cargo is degraded in lysosomal compartments (LYS). Recycling of cargo back to cell surface occurs mainly from EE. <10 min recycling occurs via Rab35 positive recycling endosomes (RE), whereas slower recycling occurs via the Rab14 positive juxtanuclear endosomal recycling compartment (ERC).

transferrin LDL Rab GTPase.

ILVs terminates ligand-induced signalling of many plasma membrane receptors[10]. As time passes by, ILVs accumulate in this compartment, accompanied by transition of Rab5 to Rab7 expression, and dramatic increase in bis(monoacyl-glycero)phosphate (BMP)[11] and PtdsIns(3,5)P. Over time, this results in maturation of EE into Late Endosomes (LEs). LEs are localized further away from the cell surface and in addition to Rab7-positivity characterized by a lowered pH of the endosomal lumen (pH 5.5). An example of cargo that is destined for LE is Low-Density-Lipoprotein (LDL). Therefore, LDL can be used as marker for the identification of LEs[12].

Eventually, LE can fuse with lysosomes upon which the luminal content is fully degraded by lysosomal proteases. Characteristics of lysosomes are accumulated lipid sheets and the descent of luminal pH to 4.5-5.

In parallel, cargo may be rescued from this degradation pathway by sorting cargo towards tubular transport intermediates that direct cargo to the trans-Golgi network, or recycles

these back to the cell surface either directly or via the perinuclear endocytic recycling compartment (ERC)[13]. Direct recycling is very fast and completed approximately within 10 minutes after internalization mainly via Rab35-positive vesicles[14]. Indirect recycling takes more time and occurs via the ERC. The ERC is an endosomal compartment co-localizing with the juxtanuclear-located microtubule-organizing centre (MTOC). It has a mild pH, similar to EE, and endosomal tubules can emanate from this organelle. Transferrin (Tfn) is often internalized and recycled via the ERC. Therefore, Tfn is used as marker for this organelle[15].

Assembly of peptide/class II Major Histocompatibility Complexes occurs in late endosomes.

DCs constitutively express MHC class II molecules and costimulatory molecules CD40L and CD83[16]. In contrast, expression of class II MHC and costimulatory molecules are potently induced upon activation in macrophages (most notably by IFNγ exposure), while B cells constitutively express class II MHC but require stimulation to express costimulatory molecules.

Next to professional APCs, other cell types can express class II MHC but only upon efficient stimulation. These cells are called non-professional antigen presenting cells, to emphasize that these cells are not specialized to present and process acquired antigens. Non-professional antigen presenting cells includes fibroblasts, glial cells, thymic epithelial cells, and pancreatic B cells[51].

Human MHC is encoded by polymorphic genes clustered on the short arm of chromosome 6, named “human leukocyte antigen”, or HLA. Considering HLA Class II, there are -DR, -DP, -DQ, and two non-classical class II MHC genes, HLA-DM and -DO[17]. In mice two major groups of class II MHC genes exist, I-A and I-E, as well as non-classical H-2M and H-2O. To date, more than 870 MHC II allotypes have been identified in the human population from which approximately 600 proteins were expressed. This high polymorphism produces a great diversity of peptide receptors and enables us to survey a variety of peptide sequences derived from the multitude of pathogenic organisms[18].

Class II MHC is composed of two non-covalently linked type I membrane polypeptides, subunit α (35kDa) and β (28kDa). After synthesis and translocation into the endoplasmic reticulum (ER), both Class II MHC subunits are assembled into heterodimeric complexes guided by their transmembrane domain[19] and an associating invariant chain (Ii) that functions as a chaperone[20]. The invariant chain and MHC II subunits associate into a nonameric complex. Binding of Ii to class II MHC molecules prevent binding of endogenous peptides that reside in the ER[21]. In addition, Ii matches isotypic α and β-chains. This is necessary since α- and β- subunits are not examined by antigenic peptides in the ER to form functional peptide receptors, because class II MHC heterodimers do not acquire antigenic peptides in the ER[18]. After proper assembly in the ER, class II MHC-Ii

complexes are transported via the Golgi network[22] either directly or indirectly via the plasma membrane to late endosomes (LE)[23]. The trafficking is dictated by sorting signals present in the cytoplasmic tail of Ii[22] and depending on associated Ii-isoform of which 4 are present in humans[24], but always delivering class II MHC complexes to endosomal compartment. Electron microscopic studies showed that 60% of total class II MHC resides in LE of immature DCs[25].

Class II MHC-peptide loading is confined to late endosomes.

The peptide-binding cleft of class II MHC is comprised of an eight-stranded β-sheet topped by two long α-helical segments, forming an elongated binding groove for a diverse range of peptides. The size of the groove favours peptides of 12 to 20 amino acids in length, which protrude from both ends of the groove. The peptide-binding groove protects the core peptide from further proteolysis. Antigenic proteolysis by endosomal proteases generates peptides of an appropriate length for this class II MHC groove. Cathepsin S, L, and F has been shown to play a role in the formation of class II MHC-peptide complexes[26-29]. In addition, the asparagine endopeptidase AEP was shown to initiate processing of tetanus toxoid in human B cells[30]. These proteases are pH-sensitive with their optimum at a mildly acidic pH, limiting their catalytic activity to late endosomal/lysosomal compartments[26-29]. In the endosomal compartment, similar endosomal proteases progressively degrade Ii, removing the c-tail sorting signals in Ii and leaving only the class II-associated invariant chain peptide (CLIP) bound to the peptide-binding groove on the surface of the αβ dimers[31]. CLIP is eventually exchanged by antigenic peptides in a process that is catalyzed by HLA-DM and hampered by HLA-DO[32]. Initially, HLA-DM releases CLIP from the class II groove[33], stabilizes empty class II molecules[34], and finally shapes the range of peptides that are loaded by biasing the peptide pool that binds class II MHC[35]. The peptide loading activity of HLA-DM has an acidic pH optimum at pH 4.5-5.5 and limited enzymatic activity at pH 7 and moreover involves physical interaction with class II MHC, thus the association and related function of HLA-DM to class II MHC molecules is localized to LE in which the pH is acidified.
Binding of HLA-DO to HLA-DM complexes decreases its peptide loading efficiency[32]. Both α and β subunits of HLA-DO weakly dimerize and upon HLA-DM binding the complexes can exit the ER to localize in LEs containing class II MHC[36]. In contrast to class II MHC and HLA-DM, which are constitutively expressed by B cells, DCs, and macrophages, HLA-DO expression is restricted to B cells, thymic epithelial cells and some subsets of DCs, but not on macrophages[37, 38]. HLA-DO inhibition on HLA-DM function was more prominent at pH 5.5, whereas HLA-DO/DM complexes regain ability to catalyze peptide loading at pH less than 5.5[38, 39]. This indicates that HLA-DO preferentially inhibits HLA-DM activity in EE (pH > 5.5), while allowing efficient peptide loading in LE and lysosomal compartments (pH < 5.5). Furthermore, expression of HLA-DO is downregulated upon activation of DCs and B cells. This probably enhances HLA-DM catalyzed peptide loading under inflammatory

conditions, while HLA-DO limits self-antigen presentation under homeostatic conditions.

Peptide loading onto class I Major Histocompatibility Complexes occurs in the endoplasmatic reticulum and endosomal compartment.

In contrast to class II MHC, class I MHC is expressed on most nucleated cells in steady-state conditions although the level of expression differs amongst different cell types. The class I MHC heavy chain is composed of a 45 kDa α-chain associated with a 12 kDa β2-microglobulin molecule. In humans, class I MHC heavy chains are encoded from HLA-A, -B, and -C. In mice, class I MHC heavy chains are encoded from genes designated H-2K,-2D, and -2L. The β2-microglobulin molecule is encoded on another chromosome from a highly conserved gene fragment.
As previously mentioned, in general class I MHC is loaded with endogenous peptides that are generated by proteasomal protein degradation although, exogenous antigens can also be loaded in class I MHC in a process named cross-presentation. As antigen cross-presentation is discussed in further detail in Chapters 2 and 3, this introduction focuses only on classical class I MHC peptide loading in the endoplasmatic reticulum (ER).

Proteasomal degradation.

In contrast to 12-20 residue size peptides for class II MHC, class I MHC binds peptides of 8-10 residues with equal affinity[40], which matches with the majority of trimmed protein fragments after proteasomal degradation. The proteasome continuously generates peptides, thereby class I MHC molecules can display a 'peptide fingerprint' of intracellular protein content to cytotoxic $CD8^+$ T cells for immune surveillance of viruses and tumors. To determine proteins for proteasomal degradation, poly-ubiquitin is conjugated to the substrate protein. At first, the evolutionarily highly conserved polypeptide ubiquitin is activated by ubiquitin activating enzyme E1. Secondly, E2 ubiquitin-carrier or ubiquitin-conjugating proteins (UBCs) catalyze the transfer of the activated ubiquitin from E1 to the substrate that is specifically bound to a member of the ubiquitin-protein ligase E3 family. Thirdly, repeatedly adding activated ubiquitin moieties to internal lysine residues (Lys-21, or -22) on previously conjugated ubiquitin generates the poly-ubiquitin chain.
The main eukaryotic proteasome is composed of a proteolytic active 20S core subunit and two 19S ATPase subunits that are responsible for recognition, unfolding and translocation of poly-ubiquitinated proteins[41]. The 20S core particle is composed of 28 subunits; four rings containing seven members that are stacked on top of each other[42]. Fourteen β subunits form the inner cavity and contain the proteolytically active sites, whilst 7 α subunits are situated at each end of the rod-like structure to regulate proteasome specificity. Importantly, the β subunits can be substituted in response to exposure to pro-inflammatory signals such as cytokines as TNFα and IFNβ, but in particular IFNγ. The proteasome composed of these alternative subunits is known as the

immunoproteasome. Analysis of peptide fragments generated *in vitro* showed that the presence of immunosubunits altered the cleavage site preference of proteasomes. This altered the relative abundance of certain peptides within the generated peptide pool.

Peptide loading onto class I MHC in Endoplasmatic Reticulum.

After proteasomal degradation in the cytosol, the transporter associated with antigen processing (TAP) recognizes and translocates peptides into the ER lumen. TAP is an ATP-binding cassette (ABC) transporter that is composed of two half-transporters, TAP1 and TAP2[43]. Both subunits are essential for ATP-dependent peptide translocation into the ER lumen and recruitment of the chaperon tapasin that stabilizes the complex[44]. After translocation into the ER lumen, the protein fragments are further trimmed by ER lumen residing proteases for loading onto class I MHC because many peptides are longer than the optimal 8-10 residues. Tripeptidyl peptidase II (TPPII) plays a role in trimming of long peptides to enable class I MHC binding[45]. The ER-residing aminopeptidase ERAP1 and ERAP2 are also pivotal in trimming of class I MHC ligands[46]. ERAP1 favors peptides of 9-16 residues and cleaves peptides with a hydrophobic N-terminus. In contrast, ERAP2 cleaves peptides with basic residues[47]. The trimmed peptides of 8-10 amino acids in length are rapidly recruited into the peptide loading complex (PLC). The assembly of the PLC occurs in a tightly regulated sequential process. At first, the heavy chain of the class I MHC molecule is synthesized. The unfolded heavy chain will associate with the transmembrane-bound chaperone calnexin[48], which folds the heavy chain and recruits the ER residing protein ERp57. ERp57 is able to form several direct conjugates with the heavy chain[49]. When the class I MHC molecule is finally properly folded, β2-microglobulin associate with the heavy chain. Upon binding of β2-microglobulin, calnexin is replaced by calreticulin. This complex binds via tapasin to the TAP complex and together form the PLC[44]. In the PLC, tapasin acts as a bridge between TAP and the remaining PLC components, thereby stabilizing empty class I MHC and promoting binding of high affinity peptides in the ER[50]. PLC components are also observed in the endosomal network, enabling endosomal class I MHC peptide loading[15].

Dysfunctional antigen presentation can underlie Primary Immunodeficiency.

Primary immunodeficiencies (PIDs) are inherited defects of the immune system, presenting with diverse clinical characteristics but overall increased susceptibility to various types of infections. Despite their low prevalence, PIDs are very useful to acquire mechanistic knowledge as they are 'experiments by nature'. Currently, over 170 PIDs have been described and categorized by their most prominent effect including T and B cell dysfunction. As antigen presentation is crucial for elicitation of proper T and B cell function, it is highly likely that defects in antigen presentation can underlie PIDs. **Table 2** provides an overview of PIDs with T-B cell dysfunction and known associated cell-biological defects that derail antigen presentation.

Table 2. Primary immunodeficiencies with contribution of dysregulated antigen presentation.

Disease	Affected cells	Associated features	presumed pathogenesis
APC-T cell interaction:			
Class I MHC deficiency	Decreased $CD8^+$ T cell numbers	Vasculitis	Mutations in TAP1, TAP2, or tapasin.[52-55]
Class II MHC deficiency	Decreased $CD4^+$ T cell numbers	Diarrhea, respiratory tract infections	Mutation in transcription factors for class II MHC; CIITA, RFX5,RFXAP, RFXANK.
DOCK8 deficiency	Decreased T and B cell numbers	Low NK cells, hypereosinophilia, recurrent infections, severe atopy, extensive cutaneous viral and bacterial infections, susceptibility to cancer.	Defects in DOCK8, defective IS formation.[56]
Wiskott-Aldrich syndrome	Progressive decrease in T cells, abnormal lymphocyte response to anti-CD3. Decreased IgM to polysaccharides.	Excema, lymphoma, autoimmune disease, IgA nephropathy,bacterial and viral infections. Thrombocytopenia with small platelets.	Mutation in WAS; cytoskeletal and immunological synapse defect.[57,58]
CD40 ligand deficiency	IgG^+ IgA^+ IgE^+ B cells lost.	Neutropenia, thrombocytopenia; haemolytic anemia, biliary tract, and liver disease, opportunistic infections.	Defective T-B interaction and impaired DC signalling.
CD40 deficiency	IgG^+ IgA^+ IgE^+ B cells lost.	Neutropenia, gastrointestinal, and liver/biliary tract disease, opportunistic infections.	Defective T-B interaction and impaired DC signalling.
ICOS deficiency	Low IgG and IgA and/or IgM		Mutation in ICOS.
XLP	Decreased effector CD8 T cells.	Very vulnerable for EBV infection, fulminant infectious mononucleosis, hypogammaglobulinemia, and malignant lymphoma	Mutation in SAP. Specifically inhibiting Germinal Center T-B interaction, not DC-T cell.[59,67]
LAD type	Decreased T and B responses.	delayed umbilcal cord separation, had frequent skin infections with little or no leukocyte infiltrate in the severely deficient patients	Mutation in CD18.[60,61]
Hermansky-Pudlak syndrome type 2 (HPS-2),	Reduction of circulating NKT cells and of $CD56^{bright}CD16^-$ Natural Killer (NK) cells subset	oculo-cutaneous albinism, bleeding disorders,recurrent bacterial infections, neutropenia	TLR recruitment to endosomes. CD1b failed to efficiently gain access to lysosomes, resulting in a profound defect in antigen presentation.[62-64]
B cell-receptor dependent antigen pt			
CD19 deficiency	Low IgG and IgA and/or IgM to T-cell dependent antigens.	Hypogammaglobulinemia, glomerulonephritis.	Diminished T-cell dependent antigen responses by defective T-B interaction.[65]
BTK deficiency	Lack of B cells, low antibody levels	otitis media, pneumonia, and septicemia.	Mutation in Btk.[61,66]

Common Variable Immunodeficiency.

With a prevalence of 1 in 25.000-50.000 Caucasians, Common Variable Immunodeficiency (CVID) is the most common PID. It is a very heterogeneous disease, characterized by recurrent infections of predominantly the respiratory tract. The diagnosis of CVID is based on several criteria; the patient should have a marked decrease (at least 2 SD below the mean for age) in serum IgG and IgA; onset of immunodeficiency at greater than 2 years of age; absent isohemagglutinins and/or poor response to Pneumococcal Polysaccharide (PnPS) vaccine. In addition, prior to CVID diagnosis other direct or indirect causes of hypogammaglobulinemia need to be excluded. Some genetic mutations are associated with CVID and aid in establishing CVID diagnosis. However, thus far only 10%

of CVID patients can be genotypically diagnosed. Mutations are found in T cell-expressed ICOS-L, CD27, CD81, and in B cell-expressed CD19, CD20, CD21, and BLK. Concomitantly, phenotypic and functional B and T cell defects are observed. This illustrates that both defective B cell- and T cell-intrinsic mechanisms may underlie CVID pathology.

Scope and outline of this thesis

In conclusion, tight control of endosomal processes is crucial for efficient antigen presentation, for elicitation of adequate adaptive immune responses. Therefore, this thesis is aimed to clarify the endosomal processes that are pivotal to antigen-specific lymphocyte activation (both B and T lymphocytes). Improved understanding will aid to improve (DC-based) vaccination strategies and contribute to elucidatation of dysfunctional mechanisms underlying primary immunodeficiencies.

The research questions of this PhD thesis are the following:

1.) Is remodelling of the endosomal network in human dendritic cells involved in antigen cross-presentation?
2.) What is the mechanism involved in activation-induced remodelling of the endosomal network in human dendritic cells?
3.) Could we exploit Fc receptor (FcR)-mediated uptake to improve antigen cross-presentation?
    And are there differences between subsets of DCs?
4). Are early B cell receptor signalling perturbations involved in (pediatric) Common Variable Immunodeficiency patients? And may perturbations in early B cell receptor signalling underlie patients disease pathology?

Part I: Regulating antigen cross-presentation in human dendritic cells.

In chapter 2, recent insights in distinct antigen cross-presentation pathways and the necessity to apply novel insights in DC vaccination development are reviewed. Both chapters 2 and 3 propose that elucidation of molecular and cell-biological mechanisms underlying antigen cross-presentation is required to obtain effective DC vaccination strategies. Chapter 2 focuses on antigen-receptor targeting whereas chapter 3 focuses on tightly controlled cell-surface directed transport from antigen processing compartments for possibilities to improve antigen cross-presentation. Hence, chapter 3 illustrates the importance of cell-surface directed transport by summarizing immune evasion strategies that pathogens have developed to interfere with class I MHC recycling and antigen cross-presentation. In addition, chapter 3 summarizes recent data acquired on the regulation of endosomal transport relevant to cross-presentation, including endosomal remodelling into elongated tubules.
Both chapters 4 and 5 discuss and investigate putative mechanisms underlying this

endosomal tubulation. Chapter 4 demonstrates that human DCs are capable of remodelling both EE and LE compartments into elongated tubules. Collapse of these tubules coincides with reduced antigen cross-presentation mediated $CD8^+$ T cell activation. Chapter 5 demonstrates that MICAL-L1 expression is required for early endosomal tubulation in human DCs.
Finally, in chapter 6, we potentiate antigen cross-presentation by antigen receptor targeting as proposed in chapter 2, by targeting antigen to Fcγ-receptors in human DCs.

PART II: Common Variable Immunodeficiency: dysregulated B cell activation and antigen presentation.

The second part of this thesis describes pathogenic mechanisms that contribute to CVID-related disease, including defective antigen presentation. Chapter 7 summarizes recent insights in B cell intrinsic defects that underlie CVID disease and defective B cell activation. Chapter 8 demonstrates that a novel identified mutation in two CVID patients render the protein BLK dysfunctional and diminishes BCR signalling in their B cells. Expression of the pathogenic BLK mutant diminishes antigen presentation required for T cell help recruitment. Therefore, this mutation is likely to contribute to CVID disease in these patients. This suggests that defective BCR signalling and interdependent antigen presentation are underlying factors of CVID disease. Additionally, chapter 9 demonstrates that a substantial number of pediatric CVID disorder patients exhibit significant deficits in early BCR signalling, potentially harbouring interdependent antigen presentation defects. Moreover, chapter 9 demonstrates that defective BCR-signalling dependent calcium cytosolic influx relates to defective CD20-BCR dissociation and may predispose to the spectrum of CVID disorders.

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PART I

Regulating antigen cross-presentation in human dendritic cells.

Chapter 2.

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

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Abstract

The initiation of adaptive immune responses requires antigen presentation to lymphocytes. In particular, dendritic cells (DCs) are equipped with specialized machinery that promote effective display of peptide/major histocompatibility complexes (MHC), rendering them the most potent stimulators of naive T lymphocytes. Antigen cross-presentation to $CD8^+$ T cells is an important mechanism for the development of specific cytotoxic T lymphocyte (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 been under investigation in recent years, 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/major histocompatibility complexes (MHC) 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-haematopoietic cells were virally infected showed a requirement for cross-presentation by haematopoietic 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 receptor interacting protein-membrane-bound ovalbumin (RIP-mOVA) mice that express a membrane-bound form of ovalbumin 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 autoimmune 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 already shown elegantly in 1996, the injection of OVA peptide-specific naive 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 a 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 to cross-prime foreign peptides and elicit CTL responses. Thus far, B cells[17,18], neutrophils[19,20], basophils[21], mast cells[22] and endothelial cells[23] were also 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 particularly 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 especially investigated in the last decade, mainly 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 represented uniformly in all DC subsets. Some DC 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 naive T cells that reside within lymph nodes. The role of the different subsets of DCs in antigen cross-presentation has been studied extensively in mice. DCs are characterized in the 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 Toll-like receptor-3 (TLR-3)[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 compared directly 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 haematopoietic precursors is possible, the low frequency of naturally occurring $CD141^+$ DCs (1 in 104 peripheral blood mononuclear cells (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[45,46]. In addition, the nature of the 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-sized fragment. Further restriction to the formation of peptide/class I MHC complexes involves the amino and carboxyl ends of the peptide to harbour charged anchor residues that complement those of the peptide-binding groove of the class I MHC molecule. Because the proteasome is demonstrated to be the main source of peptides in the classical MHC class 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 (Fig. 1).

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 internalization, antigens

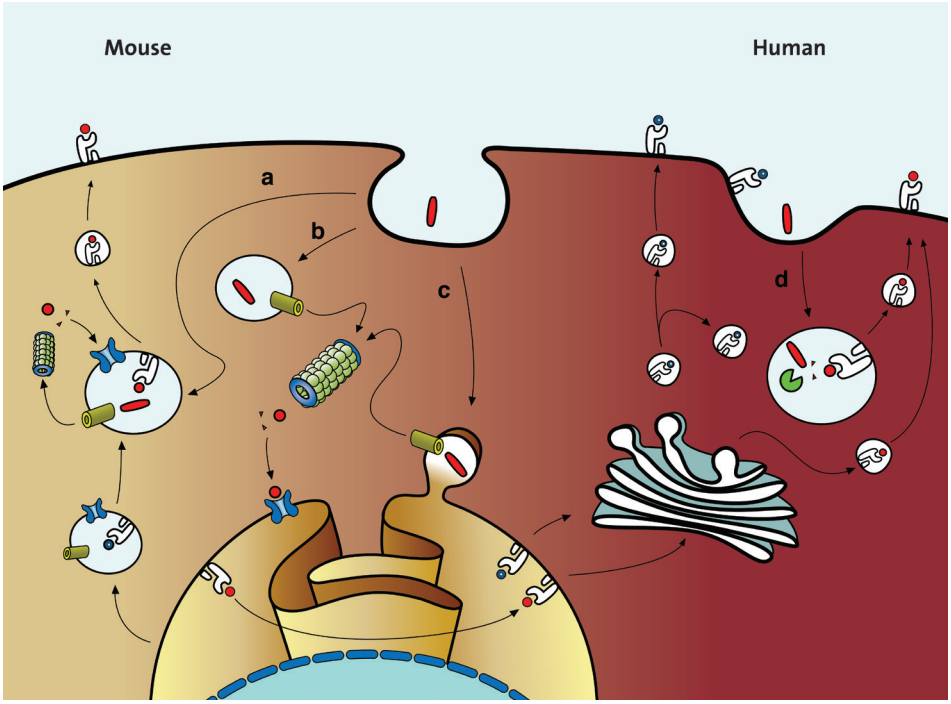

Figure 1. Proposed pathways of antigen cross-presentation.

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 endoplasmic reticulum (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 transporter associated with antigen processing (TAP) where loading on class I major histocompatibility complex (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.

are located initially in phagosomes. These phagosomes fuse with early endosomes (characterized by a near-neutral, slightly acidic pH) and later with late endosomes (pH approximately 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 as to 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−/− interferon (IFN)-IR−/− mouse model, where macrophages were unable to degrade mammalian DNA and started the production of tumour necrosis factor (TNF), activation of synovial cells was observed resulting in chronic polyarthritis symptoms[53].

It can be deduced 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 min after phagocytosis[55]. Acidification of the phagosome, thereby increasing the lysosomal protease activity, has been shown to counteract cross-presentation in mice in a nicotinamide adenine dinucleotide phosphate (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 recruited continuously 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 adenosine triphosphate (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 $CD8\alpha^+$ 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, as TAP knock-out mice are not capable of cross-presenting exogenously acquired viral peptides[7]. Moreover, efficient cross-presentation required TLR-4- and signalling 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 endoplasmic reticulum (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, as 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]. As 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 have already suggested that peptide-receptive class I MHC molecules can be generated under late endosomal/lysosomal pH conditions[72]. Multiple pathways can co-exist in the same cell type, indicating that these pathways are compartmentalized and require sorting and specific antigen targeting to specialized endosomal compartments[49].

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 DCs 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 be used thereafter 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, IIA and III 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. Secondly, small proteolytic complement protein fragments (i.e. C3a, C4a, C5a) act as chemoattractants to recruit and activate new phagocytes. CRs and FcRs allow for internalization 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 human[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 anti-viral or anti-tumour CTL responses[46]. In both mice and humans, the presence of many different uptake receptors has been shown (Table 1).

Receptors involved in targeting antigen to the class I or class II major histocompatibility complex (MHC) antigen presentation pathway in mouse and human dendritic cells

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 humans, but opposing effects of some receptors 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 humanized mouse model[95]. However, in contrast to the results in mice, MR-mediated antigen uptake induced $CD4^+$ T cell responses by

Table 1. Receptors involved in targeting antigen to the Class I or Class II MHC antigen presentation pathway in mouse and human dendritic cells [23;34;35;74-76]

	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]

n.d., not determined; n.a., not applicable

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 be translated immediately to the human system. Because enhancing cross-presentation can be an effective means to improve CTL responses in diverse DC vaccination programmes, more research about receptor targeting in the human system is needed.

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 of 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 such as influenza A[91], Epstein–Barr virus[100] and canarypox virus[101] or bacterially infected dying cells from Salmonella 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 approximately 1 million cells turn over each second, which does not generally 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 such attempts are exemplified in experiments on heat shock protein (HSP)-associated antigen uptake. HSPs are intracellular chaperone molecules that associate readily 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 knowledge is being increasingly 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 be extrapolated to the human system without risk.

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 paediatric 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' tumour 1 (WT1) protein induced complete remission in three 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 loaded exogenously with peptides derived from the human epidermal growth factor 2 (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, targeted mainly 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 five of 24 SCT patients at risk for HCMV after allogeneic SCT[119].

As described, the results from anti-tumour and anti-viral clinical trials show a modest immunological response, which may not yet result in an increase in patient survival. As

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 relatively high residual tumour load or already with virus-associated disease). Patients with a substantially lower tumour load and absent viral disease may be more likely to show benefit from induced specific anti-tumour or anti-viral activity, as also suggested by recent results[118]. Conversely, the potency of immunological 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 non-obese diabetic-like receptors (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 a 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 the development of next-generation DC-based anti-tumour and anti-viral intervention strategies.

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Chapter 3.

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

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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],[63] 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 $CD8\alpha^+$ and not the incapable $CD8\alpha^-$ 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[61,62]. 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 Fcγ receptor and DAP12-dependent manner. Cross-presentation of soluble OVA was independent of Fcγ 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 juxtanuclear 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.

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

Membrane-associated cargo, including class I MHC molecules, is selectively transported

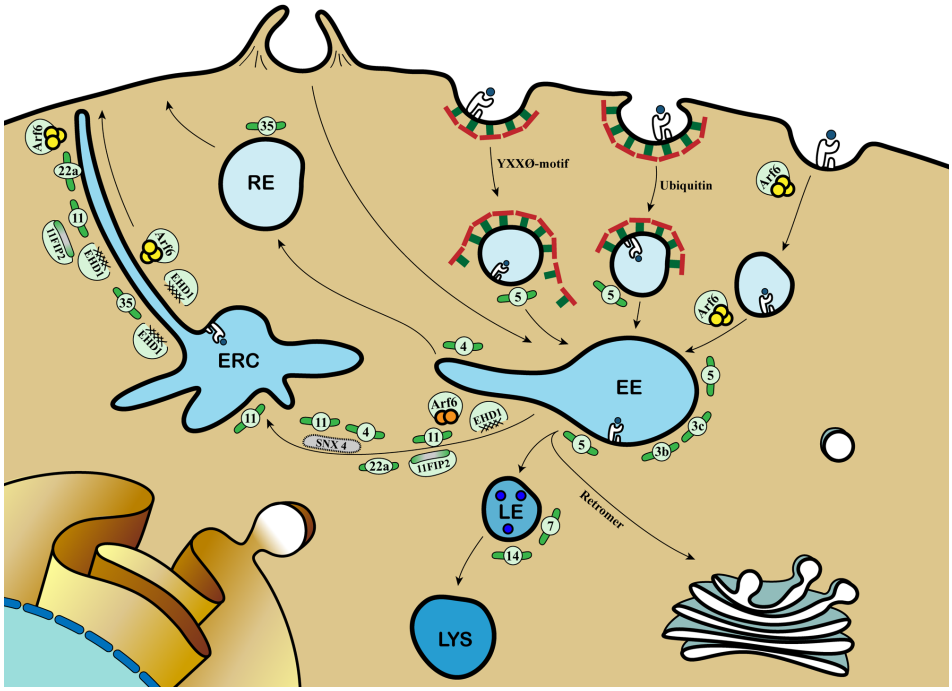

Figure 1: Molecular mechanisms coordinating cargo recycling in the endosomal compartment. Antigens can be internalized by phagocytosis and (receptor-mediated) endocytosis and converge into early endosomes (EE). Class I MHC molecules are taken up by either clathrin-mediated endocytosis dependent on tyrosine-based 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.

Figures legend: = Class I MHC molecules; = peptide; = Clathrin; = Rab, = GTP-bound Arf6, = GDP-bound Arf6

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

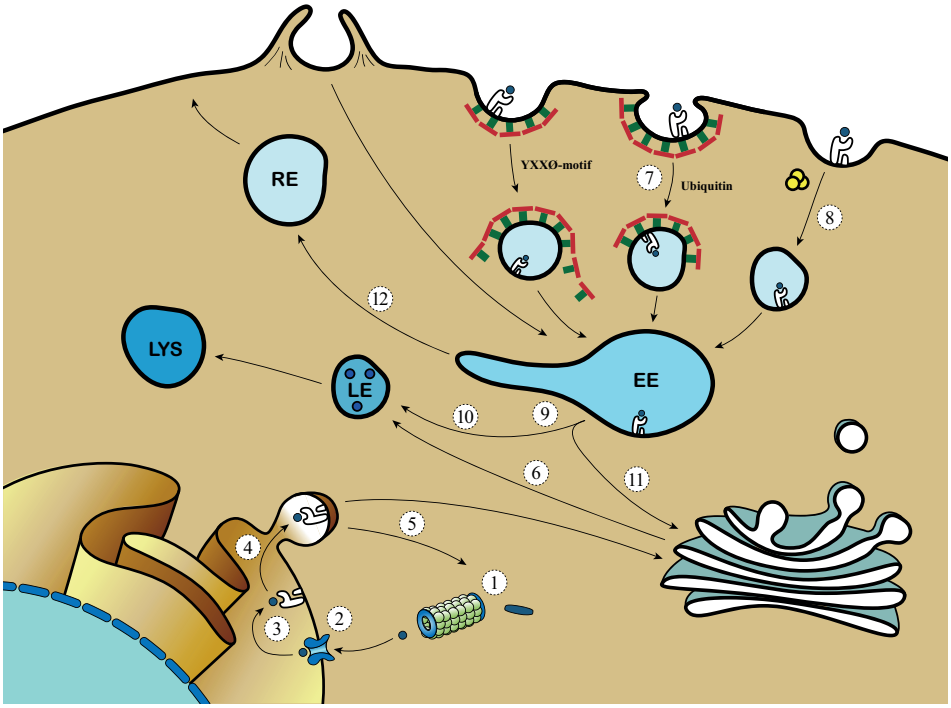

Figure 2: Viral evasion strategies aim at different pathways of MHC transport.

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).

Figures legend: = protein; = peptide; = TAP; = Clathrin; = Class I MHC molecules; = proteasome; = GTP-bound ARF6.

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 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 therapeutic 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.

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Chapter 4.

Tubulation of endosomal structures in human dendritic cells by Toll-like receptor ligation and lymphocyte contact accompanies antigen cross-presentation.

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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 toward antigen-specific $CD4^+$ T cells, which are considered beneficial for their activation. Here we 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-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 min 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 transferrin-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 DCs. 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 $CD8^+$ T cell 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 NLVPMVATV-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).

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/NLVPMVATV-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

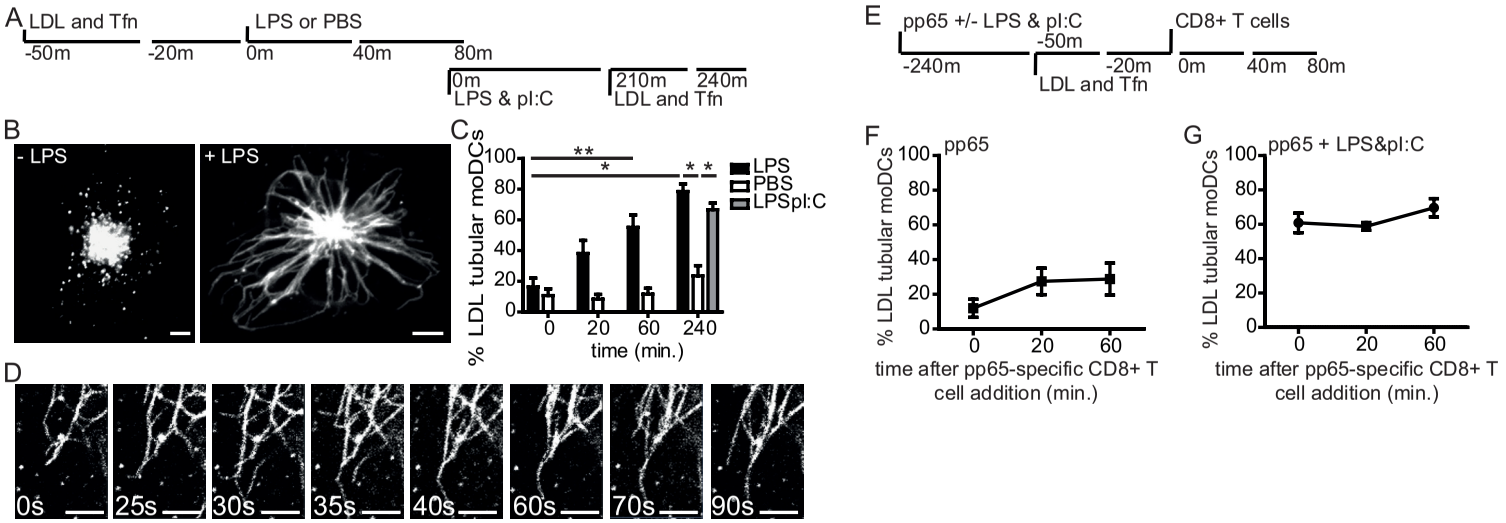


FIGURE 1. TLR stimulation of human dendritic cells triggers remodeling of Late Endosomes into tubular structures.

(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.

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 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

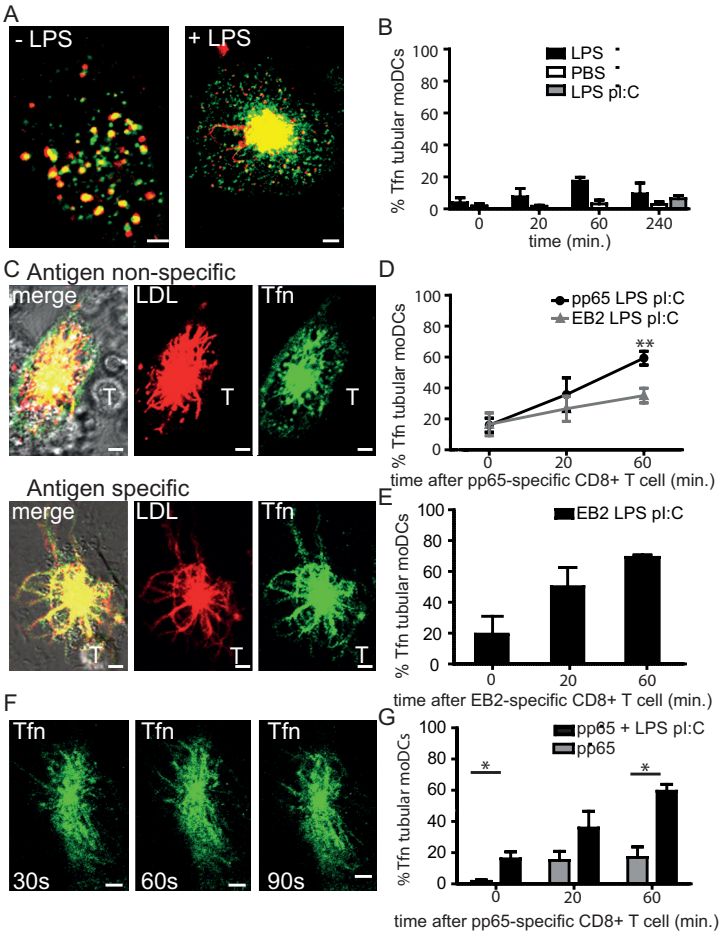


FIGURE 2. Efficient Recycling Endosome tubular remodeling in human dendritic cells requires cognate T cell interaction.

(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.

(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-independent 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 (Fig 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.

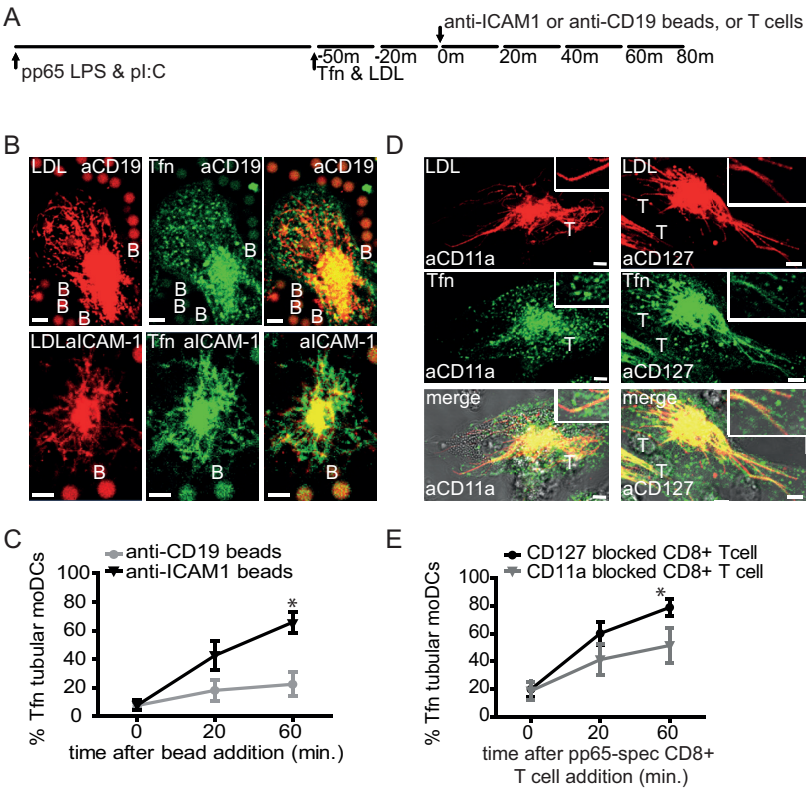


FIGURE 3. ICAM-1 clustering provokes tubulation of Tfn⁺ endosomal recycling compartments in human dendritic cells.

(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.

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 these

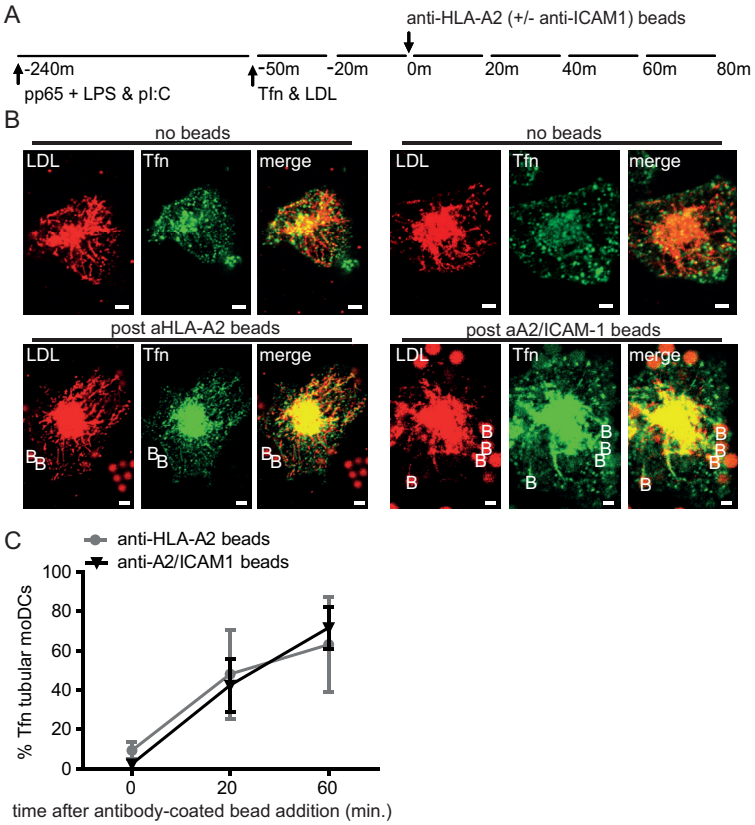


FIGURE 4. Class I MHC clustering, or simultaneous ICAM-1/Class I MHC clustering, provokes tubulation of Tfn⁺ endosomal recycling compartments in human dendritic cells.

(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.

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

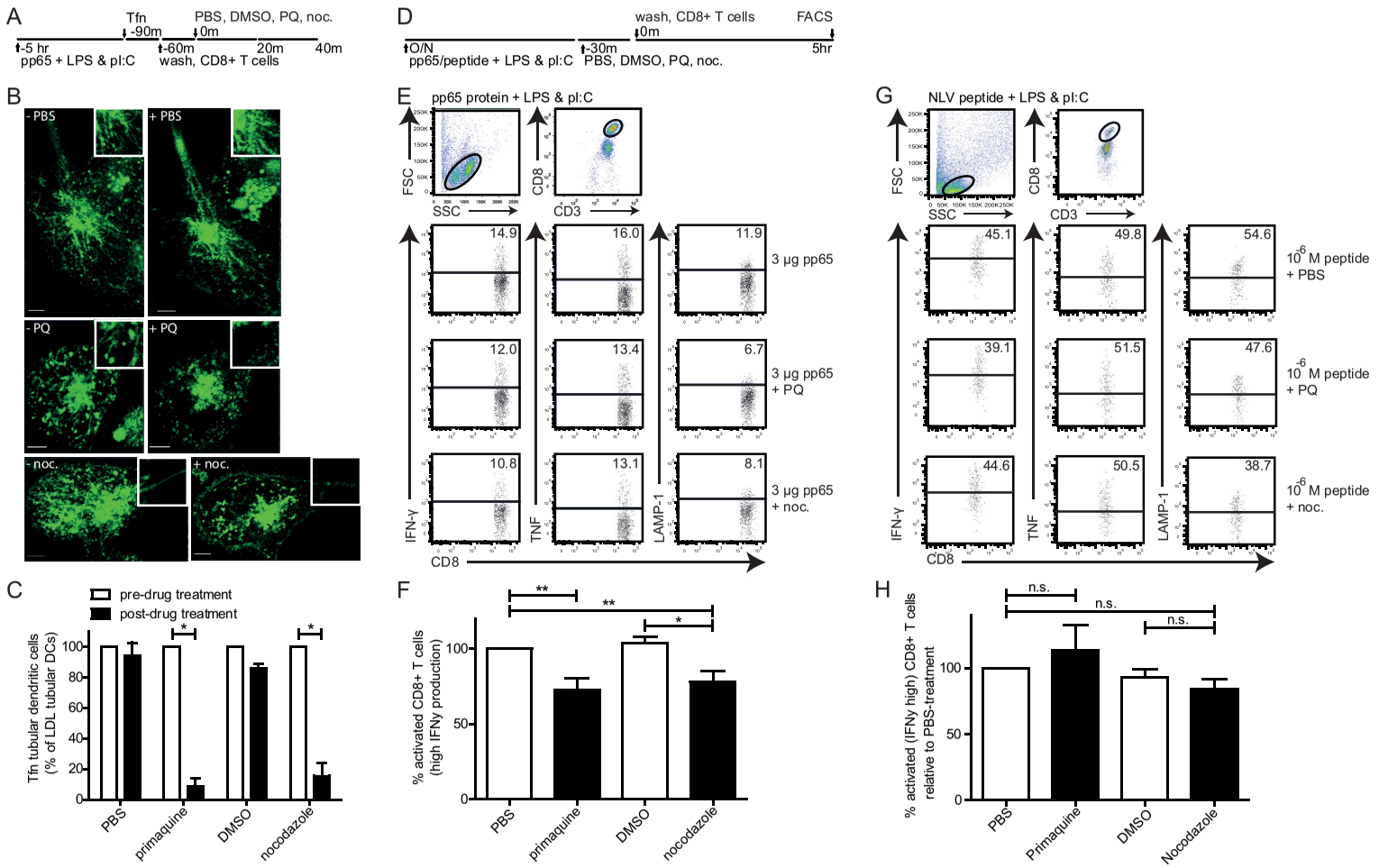
or nocodazole treatment results in a significant reduction in moDCs with tubular Tfn$^+$ endosomes to 8 and 17%, respectively (Figure 5B and C). Thus, tubular Tfn$^+$ recycling endosomes require intact microtubules and continuous endosomal recycling.

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% reduction, 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


A
Tfn
-90m
PBS, DMSO, PQ, noc.
0m
-5 hr
pp65 + LPS & pI:C
-60m
wash, CD8+ T cells
20m
40m
B
- PBS
+ PBS
- PQ
+ PQ
- noc.
+ noc.
C
pre-drug treatment
post-drug treatment
Tfn tubular dendritic cells (% of LDL tubular DCs)
PBS
primaquine
DMSO
nocodazole
D
wash, CD8+ T cells
FACS
0m
O/N
pp65/peptide + LPS & pI:C
-30m
PBS, DMSO, PQ, noc.
5hr
E
pp65 protein + LPS & pI:C
FSC
SSC
CD8
CD3
14.9
16.0
11.9
3 µg pp65
12.0
13.4
6.7
3 µg pp65 + PQ
10.8
13.1
8.1
3 µg pp65 + noc.
IFN-γ
TNF
LAMP-1
CD8
F
% activated CD8+ T cells (high IFNγ production)
PBS
primaquine
DMSO
nocodazole
G
NLV peptide + LPS & pI:C
45.1
49.8
54.6
10-6 M peptide + PBS
39.1
51.5
47.6
10-6 M peptide + PQ
44.6
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% activated (IFNγ high) CD8+ T cells relative to PBS-treatment
PBS
Primaquine
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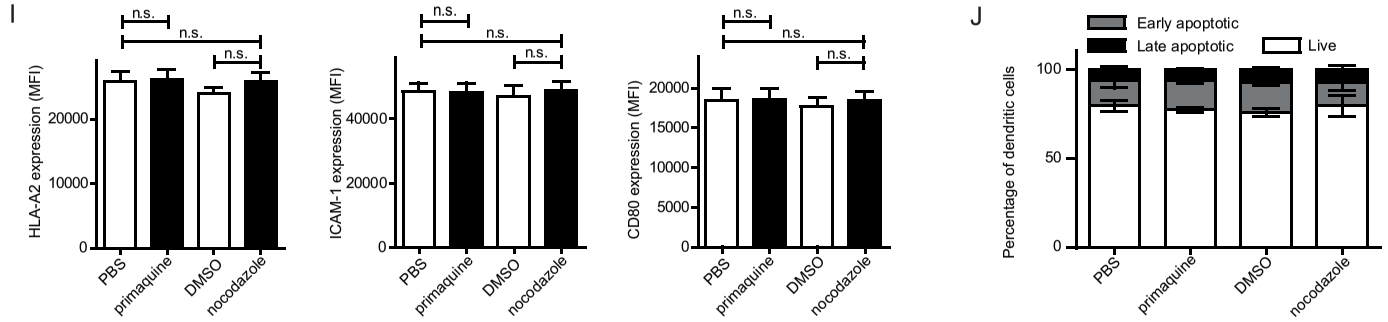

FIGURE 5. Disintegration of tubular Tfn⁺ endosomal recycling compartments in human dendritic cells associates with reduced ability of dendritic cells to activate antigen-specific CD8⁺ T cells.

(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 (O/N, 37°C) with 0 or 3 µg HCMV-derived pp65 in presence of 200 ng/ml LPS and 5µg/ml pI:C. MoDCs were treated (30 min., 37°C) with either 50 µM primaquine biphosphate, 1 µg/ml nocodazole, or carrier controls PBS, and DMSO, followed by extensive washing (3 washes). Next, pp65-specific CD8⁺ T cells were added for co-culture with the treated moDCs (1:1, 5 hours, 37°C). DC mediated activation of antigen-specific CD8⁺ T cells was measured by analysis of induced production of IFNγ, TNF, or surface expressed LAMP1 high (bar in dot plot is background, based on 1% cytokine positive CD8⁺ T cells upon culture with LPS and pI:C-treated moDCs in absence of antigen). (F) Percentage of antigen-specific activated CD8⁺ T cells, determined relative to matched PBS treated-moDCs. (G) Human moDCs were loaded (O/N, 37°C) with pp65-derived NLV-peptide in presence of 200 ng/ml LPS and 5µg/ml pI:C. MoDCs were treated (30 min., 37°C) with either 50 µM primaquine biphosphate, 1 µg/ml nocodazole, or carrier controls PBS, and DMSO, followed by extensive washing (3 washes). Next, pp65-specific CD8⁺ T cells were added for co-culture with the treated moDCs (1:1, 5 hours, 37°C) 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 (O/N, 37°C) with 3 µg HCMV-derived pp65 in presence of 200 ng/ml LPS and 5µg/ml pI:C. MoDCs were treated (30 min., 37°C) with either 50 µM primaquine biphosphate, 1 µg/ml nocodazole, or carrier controls PBS, and DMSO, followed by extensive washing (3 washes). 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.

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.

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Chapter 5.

MICAL–L1–related and unrelated mechanisms underlying elongated tubular endosomal network (ETEN) in human dendritic cells.

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Abstract

The endosomal pathway constitutes a highly dynamic intracellular transport system, which is composed of vesicular and tubular compartments. Endosomal tubules enable geometry-based discrimination between membrane and luminal content. Extended tubular endosomes were suggested to deliver a steady stream of membrane proteins to one location more reliable and effective than vesicular endosomes. Recently, we demonstrated that human dendritic cells (DCs) form a large elongated tubular endosomal network, e.g. ETEN, upon distinct triggers. LPS-stimulation triggered late endosomal tubulation. Additional clustering of class I MHC and ICAM-1 by a cognate interaction between antigen-laden DC and antigen-specific $CD8^+$ T-cells induces formation of transferrin-positive tubules emanating from the endosomal recycling compartment (ERC). We here discuss cell-biological mechanisms that are involved in membrane bending and possibly underlie initiation, elongation, and stabilization of ETEN in human DCs. Using a knock-down approach we demonstrate that MICAL-L1 is necessary for ETEN remodeling originating from ERC in human DCs.

Introduction

The endosomal pathway is a highly dynamic membrane system composed of vesicular and tubular lipid bodies, which is involved in intracellular transport. Endosomal compartments are best known for incorporating membrane molecules derived from the cell surface, and sorting these molecules for either degradation or recycling back to the cell surface. Within seconds after uptake of cell-surface molecules through endocytosis, these molecules are targeted to Early Endosomes (EEs). Components that are destined for degradation are enriched in membrane subdomains that are remodelled to form intra-luminal vesicles. As time passes by, more intra-luminal vesicles accumulate in this compartment, while simultaneously the vesicles mature into a Late Endosomal compartment (LE). Eventually, LE can fuse with lysosomes upon which the luminal content is degraded. In parallel, cargo may be rescued from this degradation pathway by sorting vesicles towards tubular transport intermediates that direct cargo to the trans-Golgi network, or recycles these back to the cell surface either directly or via the perinuclear endocytic recycling compartment (ERC)[1].

It was estimated that cells internalize the equivalent of their cell surface one to five times per hour[2], demonstrating the importance of endosomal recycling to normal cellular function. Recycling is under tight structural and motor control by a variety of proteins that include microtubules, motor proteins, SNARE proteins, and various small GTPases. Multiple recycling pathways co-exist in parallel; including the Rab22a, Rab8-EHD1, Rab35-EHD1, and Rab11-Rab4-SNX4 as previously reviewed by us and others[3, 4].

We recently demonstrated that LPS stimulation of human dendritic cells (DCs) drives LE (LDL^+) endosomes remodelling into elongated tubular structures leading to formation of an elongated tubular endosomal network, e.g. ETEN[5]. This corroborates earlier studies in murine DCs[6-8], thereby showing conservation of this mechanism between mice and man. Electron microscopic studies showed that tubules emanating from LEs extend towards the periphery with small transport vesicles protruding towards the plasma membrane[9]. These data suggest that activation-induced LE tubules represent transport intermediates for cell surface-directed transport.

However, the vast majority of endosome-cell surface transport occurs normally via aforementioned ERC, which is known to form short tubular transport intermediate structures[10]. One apparent question was whether ETEN is derived from ERC-derived membranes. Therefore, we investigated whether the characteristic recycling marker transferrin (Tfn) may also be recycled via ETEN[5]. Tfn^+ ETEN in human DCs did not arise upon LPS stimulation as in mouse DCs, but did so upon subsequent cognate interaction with antigen-specific $CD8^+$ T cells. These Tfn^+ elongated endosomal tubules emanate from a juxta-nuclear region where the ERC is located. Further support that ERC contributes to ETEN came from use of the recycling inhibitor primaquine: Tfn^+ tubules were lost when human DCs are briefly treated with a low dose of primaquine at 30 minutes prior to T cell contact. These data suggested that these induced tubular structures are indeed

involved in cell-surface directed transport, which is crucial for DC function as antigen presenting cells. We underscored the importance of this tubular cell-surface directed transport to adaptive immune activation by showing decreased antigen-specific $CD8^+$ T cell activation upon treatment of DCs with either primaquine or nocodazole (disruptor of microtubules)[5]. Below we will discuss possible requirements and underlying mechanisms involved in ETEN formation.

Tubular transport intermediates in endosomal transport

Endosomal tubulation is a phenomenon known for many years, with the vast majority of reported endosomal tubules having a size maximal up to 1.5µm in length. In contrast, tubule length of ETEN can extend up to 15 µm in DCs[5, 6]. The short tubules that are more established form the tubular endosomal network (TEN) or tubular sorting endosomes (TSEs)[1, 10, 11] and account for about two-thirds of the surface area of an endosome, but only one-third of its volume[12]. This high surface-to-volume ratio enables a geometry-based discrimination between membrane and luminal content, by which mainly lipids and membrane-bound cargo, but not luminal content, are exchanged between endosome and receiving organelle. It also suggests that endosomal tubules are designated to transport mainly lipids and membrane proteins. Indeed, many surface receptors are shown to be transported via tubular transport intermediates. Examples include transferrin receptors, class I MHC molecules, β-integrins, and several more[10, 13].
Endosomal tubules may be a more reliable transport intermediate than vesicular endosomes in terms of delivering high amount of cargo to one target membrane location, as a tubule may deliver the same amount of transmembrane cargo in a single package as what would be transported by multiple distinct endosomes. Moreover, efficient endosomal trafficking relies on collaboration of multiple motor proteins. Kinesin and myosin-V were shown to enhance each others processivity *in vitro*[14]. During endosomal transport across the cytoskeletal meshwork, proper function of motor proteins requires their temporal detachment of a particular cytoskeletal element and attachment to another cytoskeletal element. Therefore endosomal tubules may also be a more reliable transport intermediate as they should allow for more interaction with motor proteins connected over an extended distance.
Another important feature of tubules is compartmentalization of endosomal signalling. High membrane curvature in endosomal tubules can induce sorting of lipids and proteins. Lipids may respond to membrane curvature by concentrating into lipid microdomains induced by the curvature, as was demonstrated by lipid segregation into tubules during tubule-pulling experiments[15]. In support, endosomal tubules have a lipid composition significantly different from the compartment from which they originate[16]. In its turn, proteins may be directed to endosomal tubules by recognizing these sorted lipids and/or the high curvature [17].
Recently the importance of endosomal tubules in compartimentalization of endosomal

signaling was demonstrated *in vivo*[18]. Nakamura *et al.*, demonstrated that TLR-stimulated ETEN emanating from LE are a preferred compartment for bacterial sensing and NOD2 signalling *in vivo*[18].

A final consideration regarding endosomal transport and compartment morphology, is that the endosomal membrane is a typical lipid bilayer, in which the tendency to be continuous and avoiding edges outcompetes the resistance to bending by intramolecular interaction between the lipid molecules. The same fundamental rules steer the spherical shapes of vesicular endosomes. Tubules are simply not the energetically favoured conformation of lipid bilayers in soluble surroundings. However, we have shown that both LDL^+ and Tfn^+ ETEN are stable for at least 6 hours in human DCs, thus there should be a concerted action of several factors that model and stabilize these endosomal tubules. Such factors are subject of the next paragraphs.

Membrane remodelling mechanisms.

Initiation of tubules

Changing the energy-favourable spherical shape of endosomes in order to initiate tubular remodelling requires changes of the bilayer structural properties thereby making it asymmetric, or to apply force to the bilayer surface that provides sufficient energy proportional on the extent of deformation[19].

As schematically depicted in Figure 1A, bilayer asymmetry can be achieved by altering the lipid composition and/or different number of lipids per layer in the bilayer[20]. For example, flippases facilitate translocation of phospholipids from one side of a membrane to the other. As a consequence, a transmembrane asymmetrical lipid distribution is established. Every inserted lipid molecule lowers the energy required for bending the lipid bilayer. Hence, insertion of even a small proportion of lyso-PC into a Giant Unilamellar vesicle (GUV) is accompanied by the formation of a single bud[21]. Another approach to asymmetrically displace lipids in the outer leaflet, is the shallow insertion of short hydrophobic or amphipathic protein domains in only the outer lipid monolayer, such as achieved by BAR domain proteins [22].

The lipid composition may be altered by chemical modification of lipids, or by displacement of lipids themselves, by *de novo* generation, retention, selection, or recruitment from elsewhere. As lipids can diffuse fast within membrane planes, local generation would be insufficient unless lipid diffusion can be deterred. Indeed, actin alone or in combination with Ezrin/Radixin/Moesin proteins can limit lipid diffusion[23]. Phosphatidic acid (PA) is an example of such a generated lipid. It has an intrinsic negative curvature and is synthesized by phospholipase D. Adding PA to v-SNARE vesicles increases the rate of fusion with targeted t-SNARE containing vesicles. In contrast, addition of PA to t-SNARE vesicles inhibits SNARE-mediated fusion[24]. As PA also affects other unrelated molecular mechanisms in a similar manner[25], it is believed that PA promotes fusion via a biophysical mechanism, probably with its negative curvature. Thus, lipid-lipid interactions such

as PA present in outer leaflet of an endosome may contribute to (limited) bending of membranes.

Alternatively, presence of PA may act as a signpost for the recruitment of proteins and complexes that can apply mechanical forces required for membrane tubulation[26]. This corroborates with the notion that lipid headgroups commonly serve as attachment sites for peripheral membrane proteins, thereby recruiting proteins necessary for the generation of membrane curvature. Especially, phosphoinositides (PtdIns) seem pivotal for recruitment of curvature generating proteins as their inositol headgroup can easily reversibly phosphorylated and thereby acting as chemical switch.

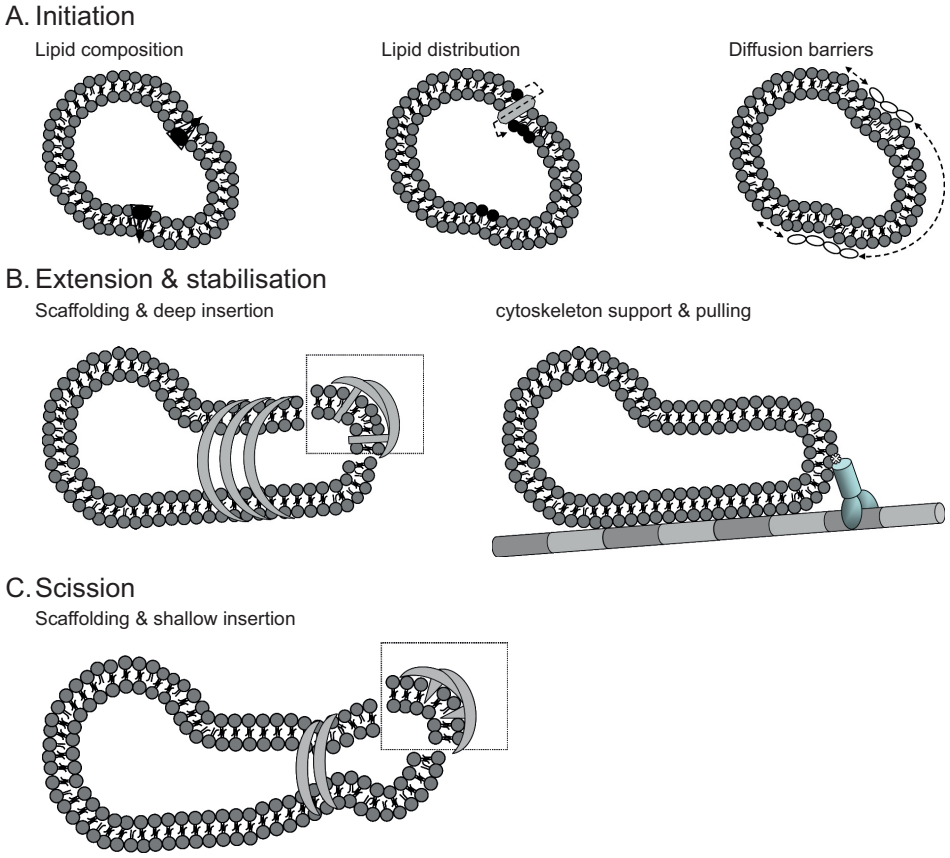

Figure 1. Candidate molecular mechanisms involved with endosomal tubulation. A. Formation of tubules is triggered by the initial bending of membranes by either (from left to right) altering membrane lipid composition, inducing asymmetrical lipid distribution between juxtapositioned monolayers, or creating domains by establishing diffusion barriers. **B.** Tubules are extended and stabilized by (from left to right) membrane-bound scaffolds that either imprint their shape into the membrane, deeply insert amphipathic helices, or simply by protein crowding, or by support or pulling forces enabled by the cytoskeleton. **C.** Membrane fission mediated in ATP-dependent and independent manner by members of the dynamin-super family.

Elongation or stabilization of tubules

An example of such PtdIns-binding proteins, are some members of the Bin/Amphiphysin/Rvs (BAR) domain protein superfamily. While this BAR domain protein superfamily lacks a consensus sequence motif, all members contain the banana-shaped BAR domain that typically form homo or heterodimers with the aid of complementary positioned charged residues in the hydrophobic dimerization pocket. This BAR domain has an arced structure with positively charged residues at its concave side that associates with membranes. The membrane-associated BAR domains can oligomerize into stable helices. *In vitro* studies on liposomes show that the arced structure allows them to bind curved membrane. By insertion of amphipathic helices[22], scaffolding[27], or crowding[28] (Figure 1B), BAR domain protein can stabilize pre-existing and/or induce extensive membrane curvatures[29].

The BAR protein Endophilin A1 induces different membrane shapes by changing its conformation upon phosphorylation. Endophilin A asymmetrically displaces lipids from the outer leaflet by shallow insertion of its amphipathic helices, thereby forming small vesicles with high membrane curvature. Accordingly, deep insertion of the amphipathic helices and close interaction of membrane and BAR domain drives tubule formation[22]. Of note, BAR domains appear somewhat flexible: the SNX9-BAR domain was shown to adopt two different arced structures in solution[29]. Whether this means that BAR domain proteins are less-specific in recognizing and binding distinct curved membranes is yet unknown.

Actin and other cytoskeleton components can assist in supporting tubular transport intermediates in various ways. Long actin filaments can stabilize tubular endosomes (Figure 1 C). Indeed, actin-associated β2-adrenergic receptor$^+$ tubular endosomes are much less dynamic than Tfn$^+$ tubules, and actin inhibition decreases TEN tubules by 25%[30]. Moreover, the cytoskeleton allows motor proteins to apply mechanical force to pull and extend the membrane, or alternatively drive membrane deformations directly upon polymerization[16]. Indeed, an intact cytoskeleton is pivotal to recycling of membrane proteins to the cell surface via ETEN from both LE and ERC[5, 31].

Scission of endosomal tubular transport intermediates

A second group of proteins that was brought forward to possibly mediate the induction or stabilization of endosomal tubulation is the Eps15 homology domain (EHD) containing protein family. *In vitro* liposome assays demonstrated that all 4 mammalian EHD proteins are capable of tubule formation by forming oligomeric ring-like structures. EHDs have been linked to a number of Rab proteins through their association with mutual effectors and thereby have a coordinating role in endocytic trafficking[32]. EHD proteins harbor a nucleotide-binding domain, which ATPase activity is stimulated by membrane association. ATP activity allows oligomerization and tubule formation *in vitro* by EHD2 protein[33]. However, EHD proteins show ~70% sequence similarity with Dynamin. Dynamin is known for scission of budding endosomes in ATP-independent manner. Therefore, it

was hypothesized that EHD proteins are perhaps involved in membrane fission *in vivo*. Recently, the ability of each EHD protein to tubulate or vesiculate recycling ETEN was assessed by reconstituting semi-permeabilized cells with purified EHD proteins. These data indeed showed that EHD1, EHD2, and EHD4, but not EHD3, are directly involved in scission of ETEN, similar as Dynamin for budding endosomes during endocytosis[34].

Putative mechanisms underlying ETEN formation that emanates from ERC in human DCs.

We demonstrated that ETEN arise from the ERC upon cognate interaction between human DC and antigen-specific $CD8^+$ T cells. More specifically, beads cross-linking class I MHC and/or ICAM-1, but not CD45, mimic $CD8^+$ T cells in respect to induction of Tfn^+ ETEN[5]. It is known that cross-linking ICAM-1 and/or HLA-A2 leads to an increased association between these molecules and recruitment of Src kinases[35]. Inactive Src localizes to perinuclear endosomes, whereas active Src localizes to site of stimulated integrin receptor[36]. Whereas Tfn is known to recycle via various pathways, recycling of Src kinases is limited to two pathways of which only one overlaps with Tfn recycling. This pathway is recently demonstrated by J. Reinecke *et al*. and involves the molecules MICAL-L1 and EHD1[37].

Are MICAL-L1 and EHD1 related to Tfn^+ ETEN? First considering EHD1, this protein is best known as a marker for elongated endosomal tubules emanating from the ERC in HeLa cells[38, 39], which resemble the Tfn^+ ETEN in human DCs[5]. Similar to Tfn^+ ETEN in human DCs, these EHD1-positive tubules require an intact microtubule cytoskeleton. EHD1-knock out embryonic fibroblasts exhibit delay in Tfn recycling to the plasma membrane with accumulation of Tfn in the ERC, thereby confirming EHD1's role in Tfn recycling. In addition, EHD1 was found to associate with ETEN containing both Arf6 and class I MHC. Overexpression of EHD1 enhances class I MHC recycling[38].

When considering MICAL-L1 next for its possible relationship to Tfn^+ ETEN, it has a calponin homology (CH), LIM, proline-rich (PxxP) region including a NPF motif, and coiled-coil domains. The CH domain of MICAL-L1 shares high similarity to the CH domains identified in various actin-associated proteins. MICAL-L1 interacts with various important regulators of Tfn recycling, including Rab8[40] and a few BAR domain proteins, such as Syndapin 2 (Synd2). Synd2 has a BAR, NPF, and SH3 domains[39]. Recently it was demonstrated that MICAL-L1, Synd2, and EHD1 decorate the same endosomal tubules[39, 40]. The membrane interaction of both proteins is stabilized by binding to eachother and EHD1. Moreover, depletion of EHD1 does not affect tubular localization of MICAL-L1 or Synd2[40], while depletion of MICAL-L1 or Synd2 does decrease tubular localization of the other proteins[39]. Thus, MICAL-L1 and/or Synd2 may recruit EHD1 to pre-existing tubules decorated with MICAL-L1 and Synd2. Synd2 harbors a BAR domain, therefore we believe this molecule is driving the elongation of recycling endosomes. *In vitro* Synd2 has shown to form tubules. Interestingly, Synd2 prefers to tubulate PA-containing liposomes and

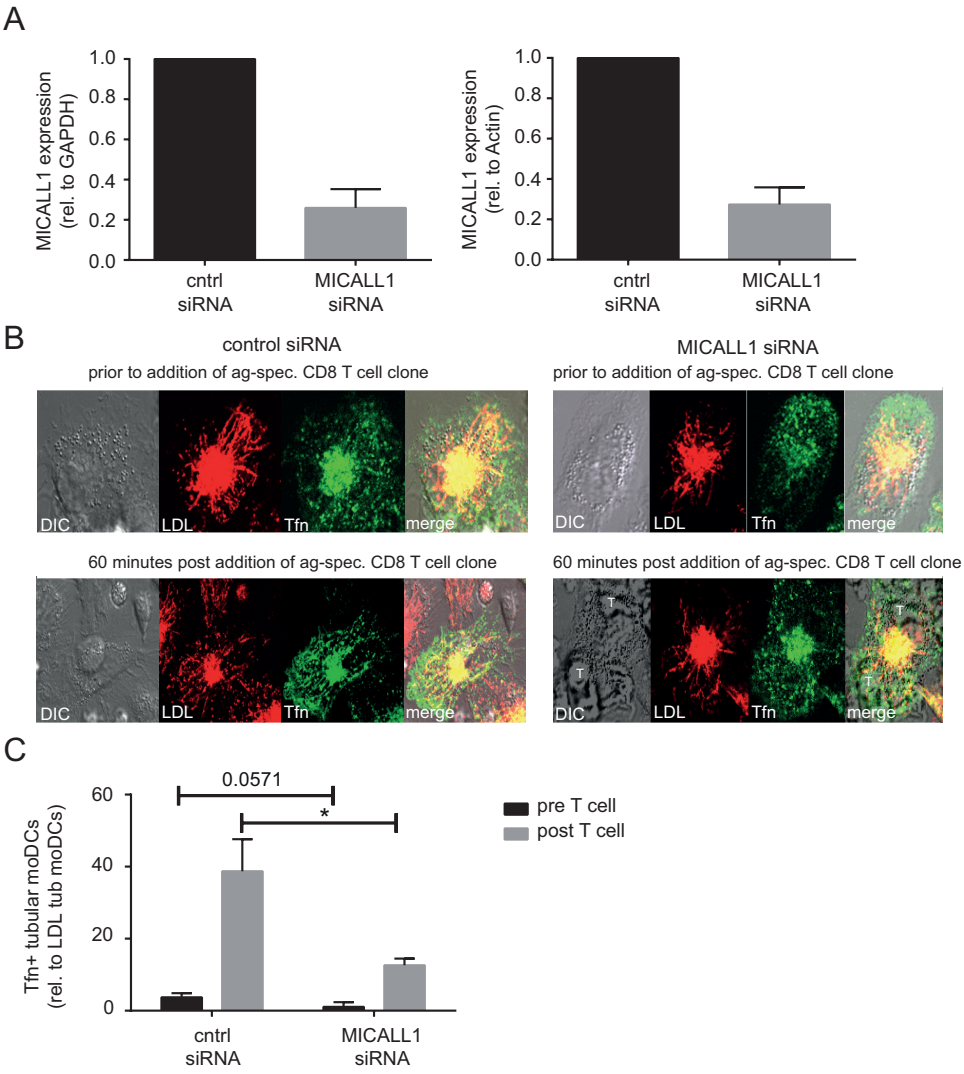

Figure 2. MICALL1 is required for Tfn-positive endosomal tubules formation in human dendritic cells.

A. MICAL-L1 RNA expression is efficiently silenced after 36hrs by 3 unique 27mer siRNA targeting MICAL-L1 (gray bars) and not by scrambled control siRNA (black bars). B. confocal image of stimulated moDCs (200 ng/ml LPS, 5ug/ml polyI:C, and 3ug/ml pp65 antigen) 36 hours after 10nM siRNA treatment (left, control siRNA; right, MICALL1 siRNA). Prior to (upper part) and 60-80 minutes post (lower part) addition of antigen-specific CD8+ T cells. Red depicts LDL, Green Transferrin, and yellow co-localisation of LDL and Tfn. C. The percentage of stimulated moDCs (200 ng/ml LPS, 5ug/ml polyI:C, and 3ug/ml pp65 antigen) with LDL+ endosomal tubules expressing tubular Tfn+ endosomes upon antigen-specific CD8+ T cells, 36 hrs after indicated siRNA treatment (10 nM scrambled or MICALL1-targeting siRNA).Data represents mean ± S.E. of three independent experiments. Two-tailed, Mann-Whitney U test; *, $p < 0.05$.

PA generated by Arf6 is pivotal to Tfn recycling[41]. Further investigation shows that Arf6 inhibition decreases MICAL-L1 tubule association[13]. The coiled-coil of MICAL-L1 and BAR-domain of Synd2 prefers to associate with PA$^+$ membranes. Therefore, it seems that the local generation of PA by Arf6 may recruit MICAL-L1 and Synd2 to initiate ETEN formation. In its turn, tip-to-tip or wedge loop-mediated lateral interaction would enable Synd2 to induce endosomal tubulation[42].

MICAL-L1 expression required for Tfn$^+$ elongated tubular endosomal network (ETEN)

We considered that impairment of Syndapin function interferes with clathrin-dependent endocytosis[43] and that Tfn is taken up in a clathrin-dependent manner. As a candidate protein that facilitates the formation of Tfn$^+$ ETEN, we therefore decided to investigate the role of MICAL-L1 and not Syndapin. One way to examine MICAL-L1 function in Tfn ETEN, would be to interfere with MICAL-L1 endogenous function by overexpressing mutant MICAL-L1. However, in a computational model Stachowiak *et al* demonstrated that membrane bending can be solely driven by protein crowding via steric pressure[28]. Hence, we decided to knock-down the expression of MICAL-L1 by siRNA treatment of human DCs. MICAL-L1 knock-down was shown in HeLa cells to be effective to decrease MICAL-L1 RNA expression previously[40].

Human monocyte-derived DCs were treated with control or MICAL-L1 targeting siRNA by electroporation at day 4, according to protocol of Hobo *et al*[44]. 36 hours later, MICAL-L1 RNA expression was decreased by 75% as determined by quantitative PCR (Figure 2A).

To assess MICAL-L1 function in either induction or stabilization of Tfn$^+$ ETEN, we pulsed both scrambled and MICAL-L1-siRNA treated moDCs for 4 h with 3µg of our model antigen pp65, in the presence of 200 ng/ml LPS and 5µg/ml poly(I:C). Hereafter LE and ERC (30 min, 37 ºC) are stained with 20µg/ml DiI-LDL and 5µg/ml Alexa Fluor 647-conjugated Tfn, respectively. Vesicle-to-tubule transformation was stimulated by co-culture of antigen-specific (NLVPMVATV) CD8$^+$ T cells for 60-80 minutes[5, 45]. We next analysed labelled moDCs for ETEN formation by live cell confocal microscopy prior and post addition of antigen-specific CD8$^+$ T cells. The fraction of moDCs that exhibit late endosomal tubulation (as scored by DiI-LDL fluorescence) showing Tfn$^+$ ETEN were determined by two independent investigators as published[5]. Representative pictures are shown in figure 2B.

MICAL-L1 siRNA but not control siRNA treatment of moDCs resulted in a significant reduction in moDCs with Tfn$^+$ ETEN from 40% to approximately 17% (Fig. 2 C). Thus, Tfn$^+$ ETEN in human DCs require MICAL-L1 expression in our experimental setup. This suggests that the endosomal Tfn$^+$ tubules emanating from ERC in human DCs are indeed induced or stabilized by MICAL-L1.

As observed[5], most LDL and Tfn tubular structures are overlapping (fig 2B, left panel). Therefore, we wondered whether LDL$^+$ and Tfn$^+$ tubules were two different compartments

or are perhaps two distinct tubules walking across the same microtubule. We could not investigate this by confocal microscopy, as the resolving power is insufficient. However, we here show that LDL$^+$ elongated tubules persist while Tfn$^+$ tubules disappear upon depletion of MICAL-L1. Therefore, it seems that both LDL$^+$ and Tfn$^+$ tubules are distinct compartments. Whether both Tfn$^+$ and LDL$^+$ tubules emanate from the same endosomal compartment is still possible, as various studies have shown that distinct populations of membrane tubules may arise from the same membrane[10]. This is possible because BAR domain proteins that cannot oligomerize do not colocalize at the same sorting tubule. This corroborates with the observation that Tfn$^+$ and β2-adrenergic receptor$^+$ tubules emanating from the same early endosomal compartment have distinct biochemical and kinetic properties: the β2-adrenergic receptor$^+$ tubular endosomes are more stable than Tfn$^+$ TEN tubules and have endosome-associated actin[30]. It is also reported that different BAR domain proteins recruit distinct motor protein complexes[46]. Therefore it seems that distinct tubule coats enable the rise of unique tubular transport intermediates, allowing for independent regulation of endosomal cargo transport.

In summary, research on the cell biological processes that underlie endosomal remodelling towards elongated tubular structures is now being applied to human DCs. The consequences of such remodelling to endosomal surface-directed transport of peptide/MHC class II and peptide/MHC class I complexes is not yet fully understood. Mouse work supports a role for late endosomal tubular remodelling in Class II MHC-mediated CD4$^+$ T cell activation[6], work that requires translation into human dendritic cell research. Future directions furthermore should include study of the role of molecules that corroborate ETEN, such as –but not limited to- MICAL-L1 and EHD1, in antigen cross-presentation and CD8$^+$ T cell stimulation. Clarification of endosomal sorting, remodeling and transport mechanisms may prove pivotal to the development of future human dendritic cell-based vaccines.

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Chapter 6.

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

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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 with 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 $CD8^+$ 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. FcɣR-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 (1×10^5 cells/ml, irradiated with 70 Gy) and PBMCs from three healthy donors (1×10^6 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 50 mM 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^{o}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 using a LSM 710 Confocal microscope (Zeiss). 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

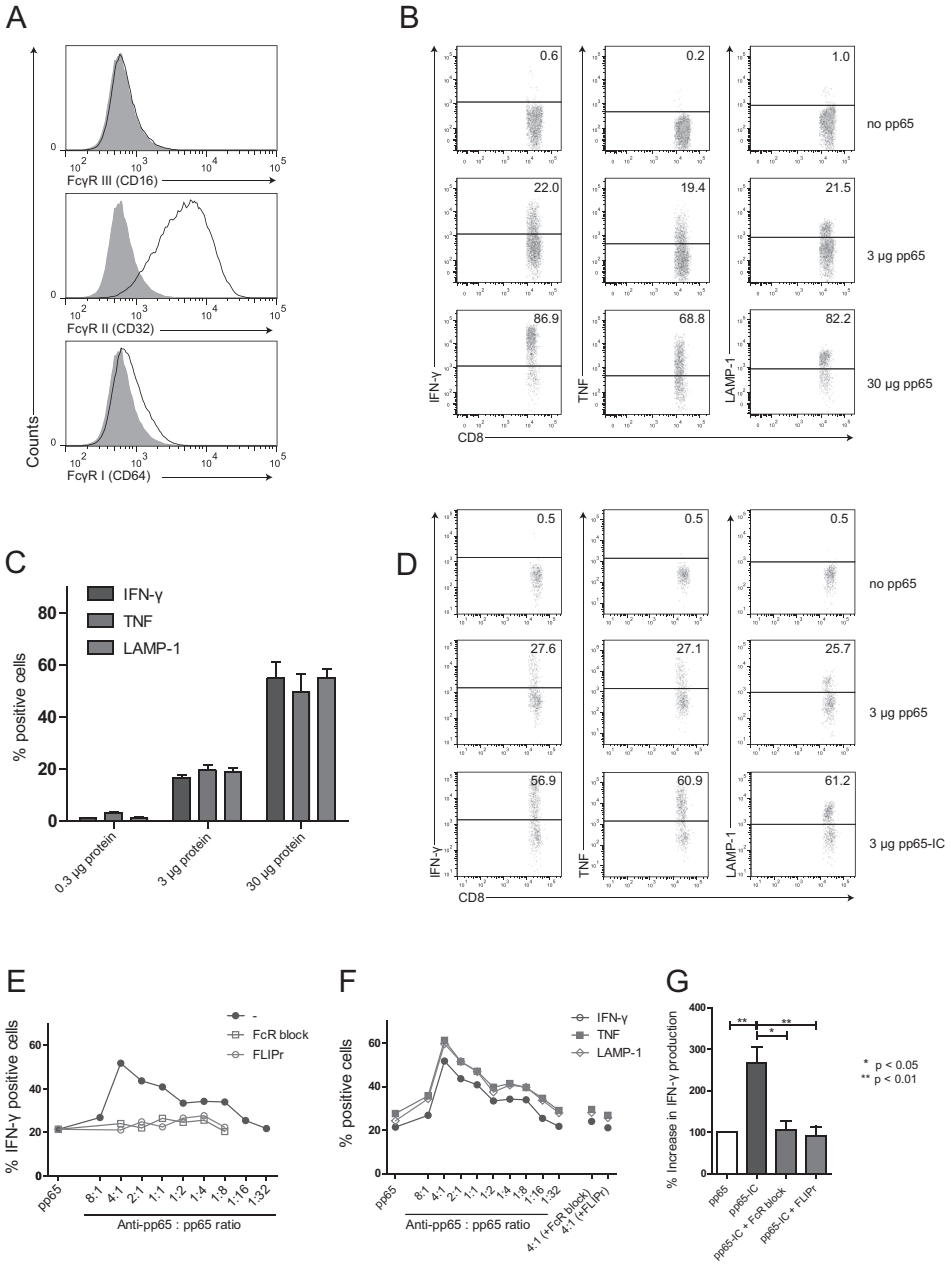

Figure 1: Human MoDCs express FcγRs that facilitate antigen cross-presentation

(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).

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 under 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-presentation 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

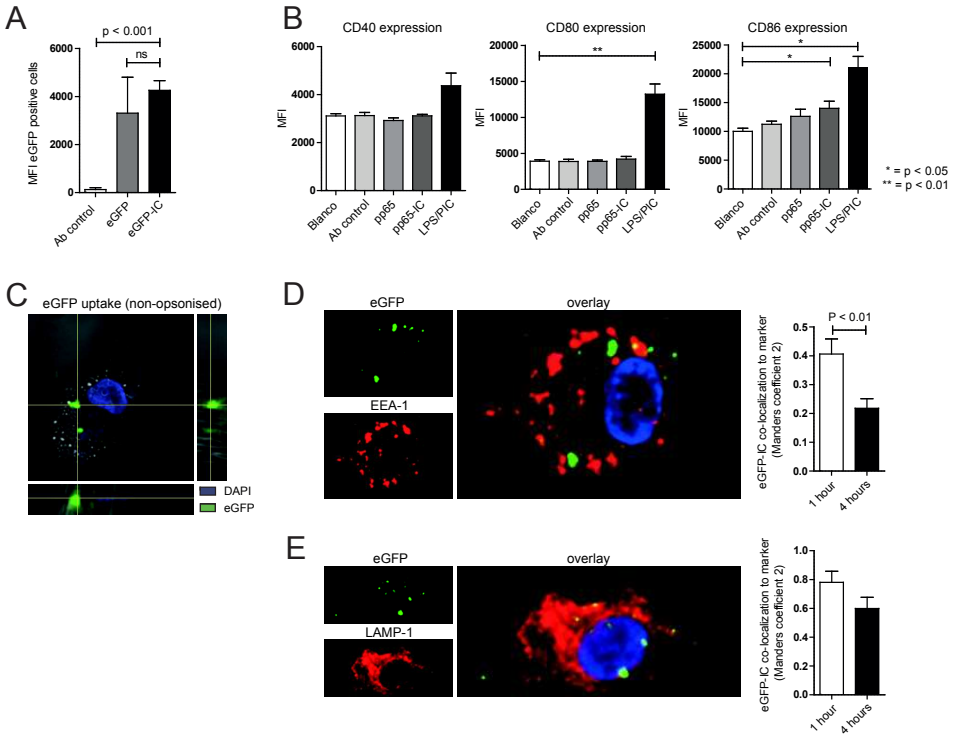

Figure 2: Enhanced cross-presentation of immune-complexes is not due to increased antigen uptake or DC maturation

(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).

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-1^{+} late endosomal compartments. Thus, immune complexes localize to early

and late compartments of the endosomal pathway in human MoDCs.

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 proteasome (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).

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 (50 μM), lactacysin (100 μM) or epoxomicin (10 μM) significantly reduced cross presentation of A2/NLVPMVATV complexes, as measured by IFNγ production by HCMV $pp65_{495\text{-}503}$–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 $pp65_{495\text{-}503}$–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 $pp65_{495\text{-}503}$–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

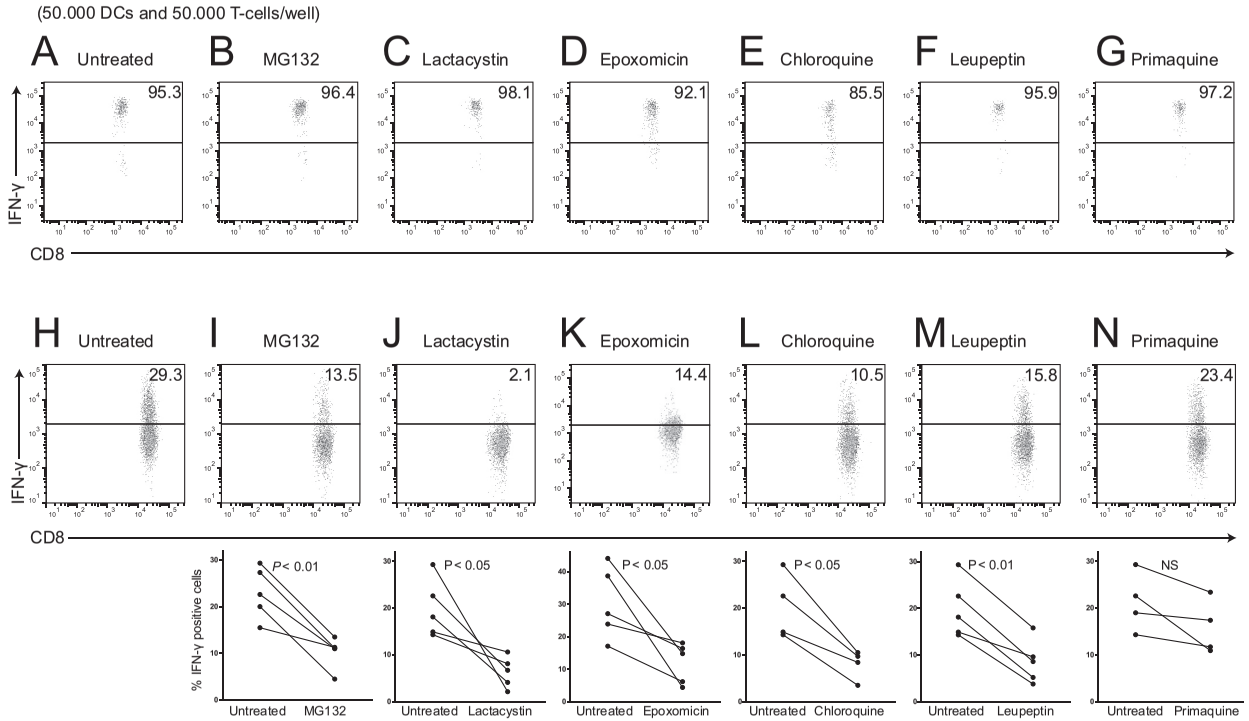

Figure 3: Cross-presentation of FcγR-targeted viral antigen requires antigen processing in both the endosomal pathway and by the Proteasome

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, 50 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.

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 FcγRs 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

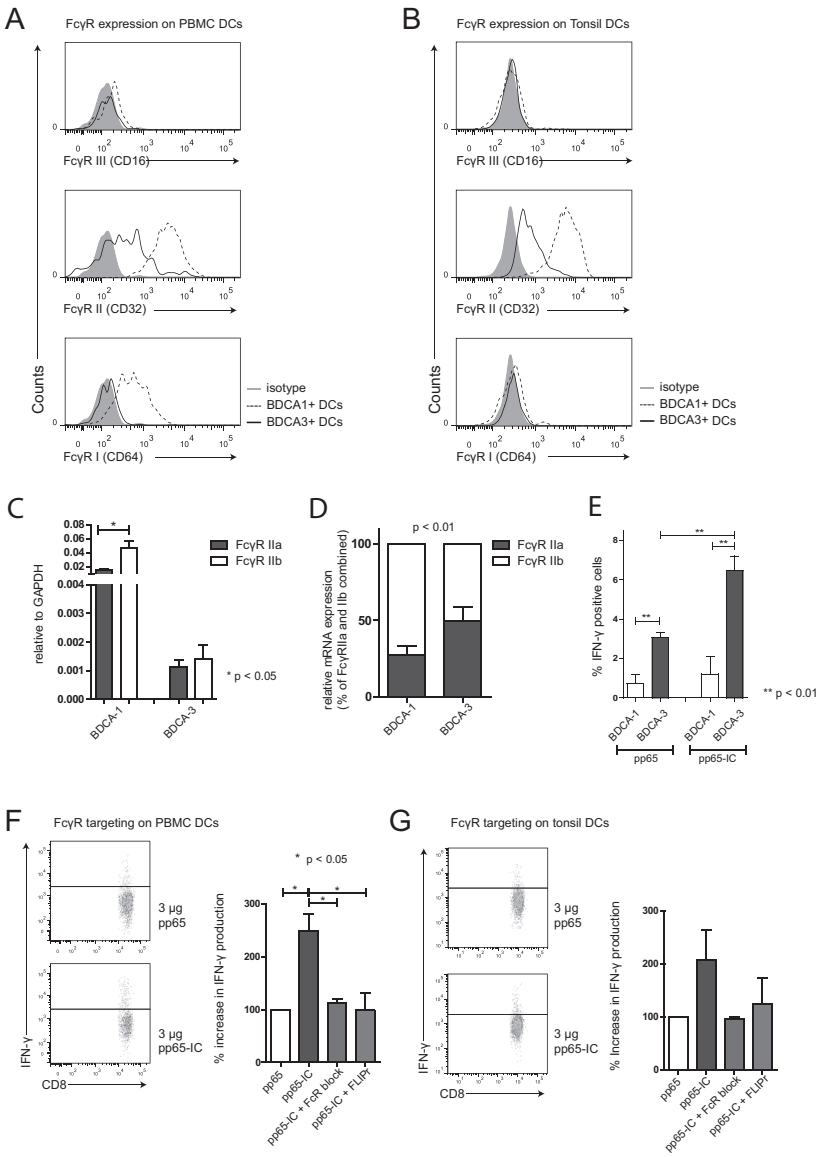

Figure 4: FcγRs on human BDCA-3$^+$ contribute to antigen cross-presentation

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).

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 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.

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

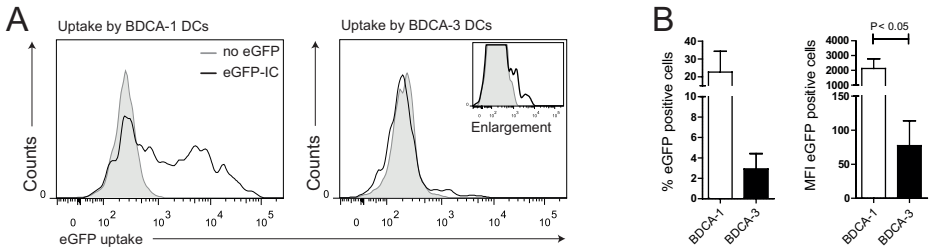

Figure 5: BDCA-1$^+$ rather than BDCA-3$^+$ DCs internalize immune-complexed antigen

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).

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 (50 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.

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.

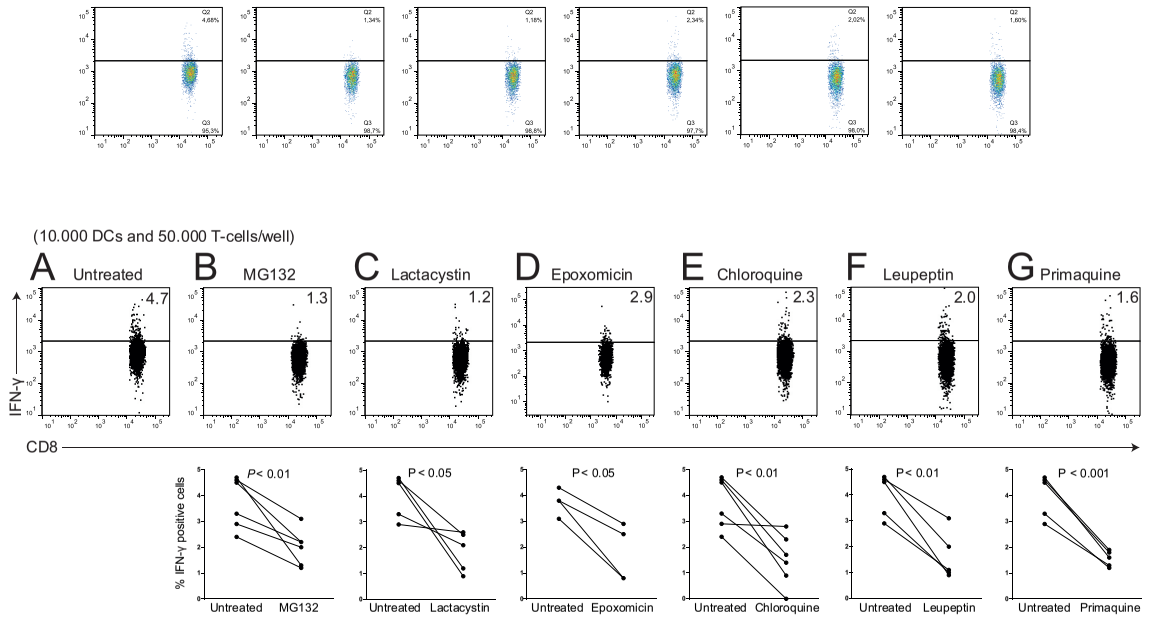

Figure 6: Cross-presentation of FcγR-targeted antigen by human BDCA-3⁺ DCs requires antigen processing in both the endosomal pathway and by the proteasome

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, 50 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.

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.

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Supplemental Figures

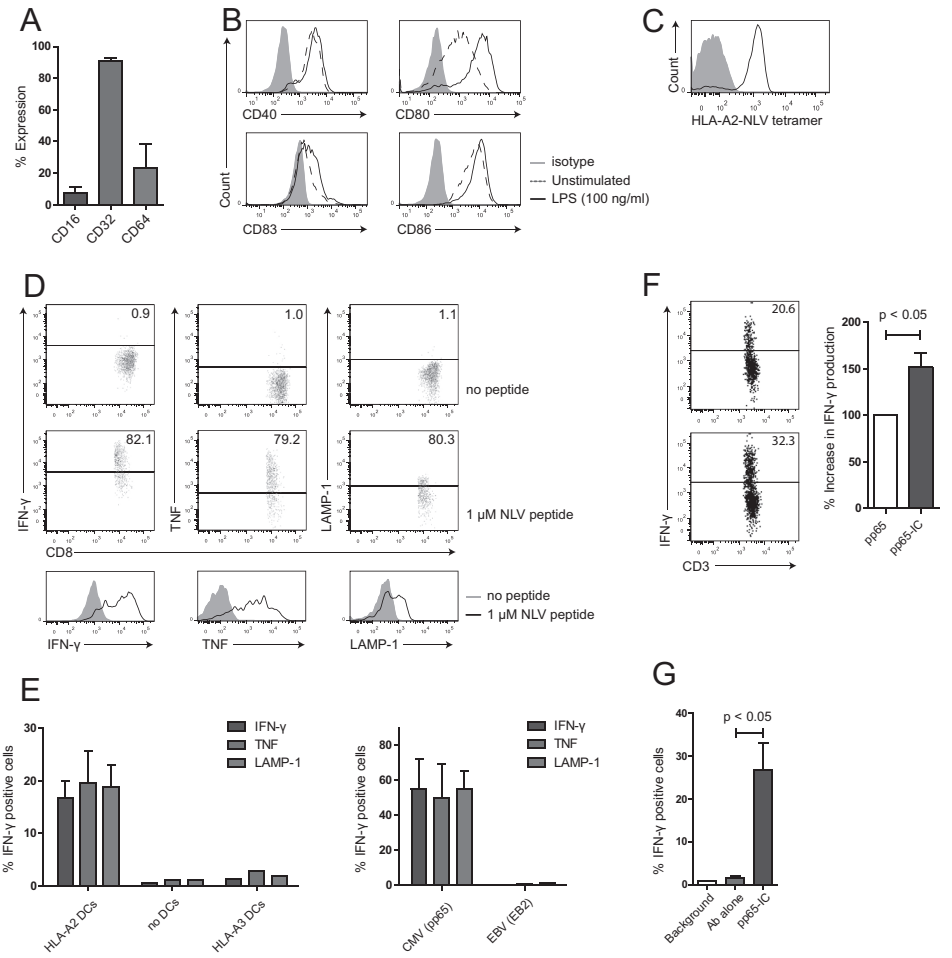

Figure S1: Human MoDCs are of intermediately matured phenotype and express FcγRs that facilitate antigen cross-presentation to A2/NLVPMVATV-specific CD8+ T-cell clones.

(**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 were A2/NLVPMVATVtetramer 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.

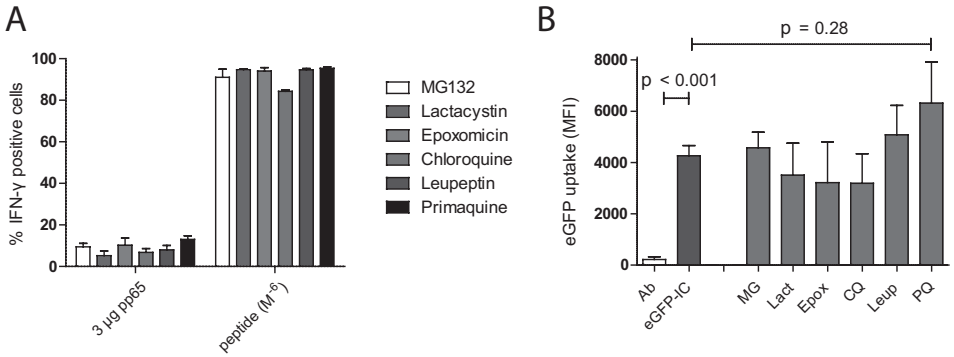

Figure S2: Human MoDCs retain antigen presenting capacity after culture in the presence of selective inhibitors.

(**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 $CD8^+$ Tcells 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).

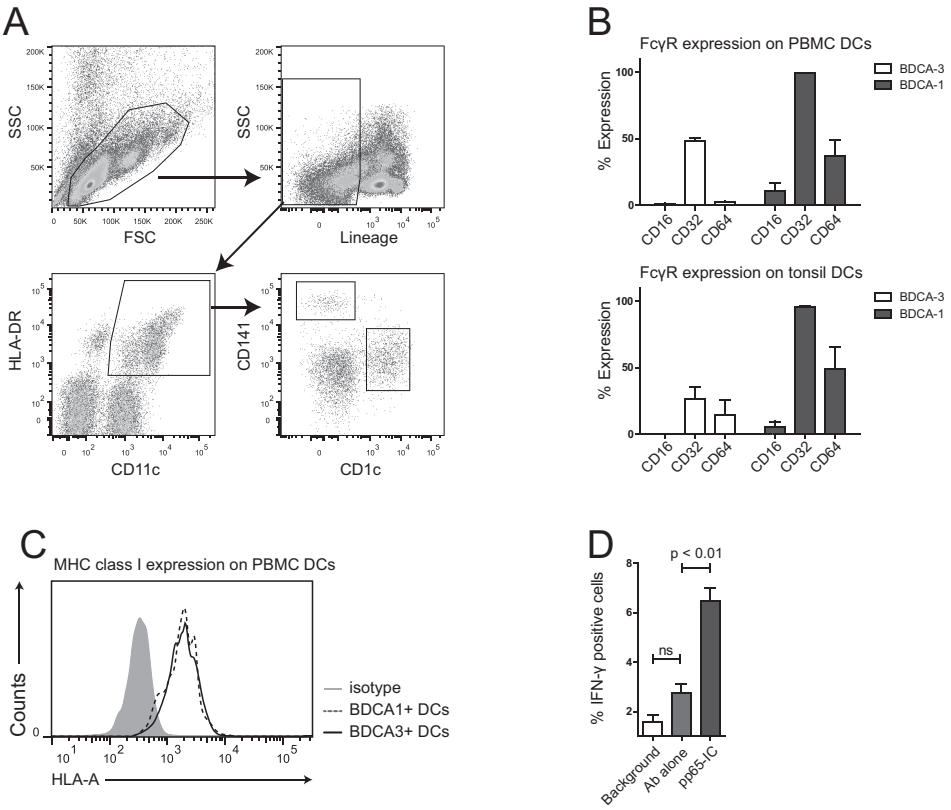

Figure S3: Human BDCA-3+ and BDCA-1+ DCs express FcγRs that contribute to antigen cross-presentation.

(**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.

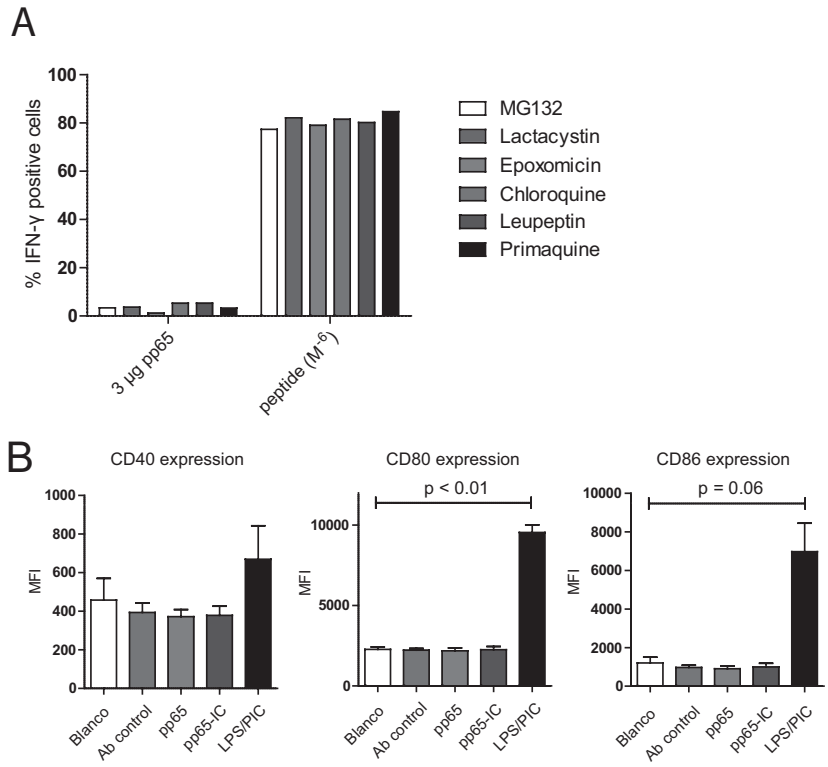

Figure S4: Human primary BDCA-3+ DCs retained antigen presenting capacity after culturing in the presence of specific blockers.

(**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).

PART II

Common Variable Immunodeficiency: dysregulated B cell activation and antigen presentation.

Chapter 7.

B–cell Defects in Common Variable Immunodeficiency: BCR Signaling, Protein Clustering and Hardwired Gene Mutations.

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Abstract

Common variable immunodeficiency (CVID) is the most frequently diagnosed symptomatic primary immunodeficiency. CVID develops as a consequence of absence or malfunction of proteins involved with immunoglobulin production by plasma and memory B-cells. The last decade has brought us clarification of several genetic predispositions to the development of CVID. Despite considerable effort, however, for eighty-five percent of CVID patients, disease etiology remains undefined. We propose that in subsets of patients, CVID may involve defective assembly of protein complexes, which is crucial for example for B cell activation upon antigen triggering of the B cell receptor/co-receptor complex. Such defective protein-protein interactions may not be uncovered by standard gene sequencing methods, and may involve epigenetic or post-transcriptional regulation. In this review, we summarize recent developments in CVID research and propose additional approaches to the clarification of etiology of CVID patient groups, necessary for development of tailored treatment options.

Introduction

Humoral immunodeficiencies encompass the majority of primary immunodeficiency diseases and clinically manifest themselves primarily by recurrent bacterial infections of the respiratory tract.1 The spectrum of antibody deficiencies is broad and ranges from asymptomatic IgA deficiency to severe infections in agammaglobulinemia patients. At an estimated prevalence of 1 per 25,000 Caucasians, Common Variable Immunodeficiency (CVID) is the most frequent antibody deficiency that requires treatment[2,3]. Apart from recurrent infections, CVID patients have increased propensity to non-infectious disease-related complications, including autoimmune disorders, lymphoproliferative disease, enteropathy and hematological malignancies.[4] Since its first description in 1953,[5] medical interventions have reduced the severity of CVID-related morbidity and mortality, especially by prevention of infections. A major step forward in this respect was made with the introduction of immunoglobulin replacement therapy.[6] However, this treatment does not ameliorate the noninfectious complications as described above, inevitably meaning that to date, CVID remains a debilitating disease with considerable morbidity. Despite continuous and intense investigations, the etiology of CVID remains incompletely understood. CVID is diagnosed in a heterogeneous group of patients with B-cell dysfunction, which may be the result of several unrelated genetic causes. The multigenetic base together with paucity of homogeneity in the CVID patient population makes etiologic research a convoluted endeavor, causing the majority of immunologic studies to be descriptive rather than mechanistic in nature. To date, these studies have mostly been focused on B- and T-cell phenotype, expression of surface molecules involved in activation an proliferation of B cells, and associations between cell subsets and clinical phenotypes.[7,8] The latter finding facilitated the development of CVID classifications aimed to identify subgroups of patients with overlapping disease etiology, with similar risk profiles for the development of disease-related complications, or patients in need of comparable treatment regimens.[9-12] In the past decade, significant progress has also been made in the identification of monogenetic defects, albeit in a modest fraction of the patient population. These defects include the transmembrane activator and CAML interactor (TACI)[13,14] CD19[15] and inducible costimulator (ICOS).[16] Mechanistic investigations were initiated with focus on distinct cell populations related to CVID development.[17-19] For example, Rakhmanov *et al*. discovered that human CD21low B cells resemble phenotypically the murine B cell subset called B-1 cells. As B-1 cells are an autoreactive subset, this finding thus provided possible insight in the increased incidence of autoimmunity in CVID patients characterized by high numbers of these B-1 cell-like B cells.[20] Lastly, subsets of CVID patients exhibit defects in B cell receptor (BCR)-mediated calcium signaling and in Toll-like receptor (TLR) signaling pathways.[21,22] Here, we focus on insights from recent studies that now imply B cell activation defects in the development of CVID.

ABROGATION OF TNF RECEPTOR PREASSEMBLY MAY LEAD TO A CVID PHENOTYPE.

Oligomerization is an important featureof TNF receptors

The TNF receptor superfamily consists of cell urface receptors that are critical for lymphocyte

development and function[23] and include p60 and p80 (TNFα receptor I and II, respectively), CD40, CD27, Fas/CD95, TACI24 and B Cell Activating Factor Receptor (BAFFR). The majority of TNF receptors are transmembrane glycoproteins containing three extracellular cysteine-rich domains (CRDs). As a conserved characteristic, these receptors display ligand-independent self-assembly into homo-trimeric complexes[25,26] via the preligand-binding assembly domain (PLAD).[27] The relevance of this trimerization domain is demonstrated via dominant-negative heterozygous mutations that associate with several human diseases.[26]

The clinical relevance of TNF receptor preassembly was confirmed by findings in autoimmune lymphoproliferative syndrome (ALPS), a severe condition caused by mutations in the *Fas* gene. Since receptor trimerization is necessary for normal function and signaling of TNFR members, heterozygous mutations may more readily show a disease phenotype than heterozygous mutations in non-oligomeric proteins. In a subgroup of CVID patients, the disease has been attributed to dominant mutations in TACI that preclude normal function. Defective TACI function can be caused by interference with receptor oligomerization, as will next be discussed.

TACI defects in CVID

The first genetic defect in the TNFR superfamily elucidated in CVID was the *TNFRSF13B* gene encoding TACI.[13,14] TACI is expressed on monocytes[28], dendritic cells[29], and peripheral B cells. Its known main functions are the instigation of isotype class-switching, mediation of immunoglobulin production in response to type II T-independent antigens and negative regulation of B cell homeostasis. TACI has two CRDs[24]; CRD1 is responsible for ligand-independent preassembly into TACI trimers[30], while CRD2 is the binding site for its ligands, a proliferation-inducing ligand (APRIL)[31] and BAFF.[32-34] TACI associates into trimeric complexes during assembly in the endoplasmic reticulum.30 Additionally, TACI is displayed as oligomers on the B cell membrane. TACI mutations were reported in CVID and involve inability for TACI to form oligomers or to bind ligands.[13,14] Heterozygous C104R and A181E TACI mutations were described that were not found in any of the 50 healthy controls.[13] The C104R mutated version of TACI no longer binds BAFF. In some patient B cells, stimulation with APRIL did not elicit isotype class-switching. Homozygosity with respect to S144X and C104R mutations resulted in loss of TACI function.[14] The C104R mutation is located in CRD1; C104R thus has a dominant-negative effect by abrogating oligomerization of TACI monomers.[30] A181E is located in the transmembrane domain, where it introduces a negative charge in the normally uncharged membrane-spanning

region. The A181E TACI mutation alters both constitutive and ligand-induced NF-κB signaling, possibly due to preclusion of normal receptor assembly by polar residues within the TACI transmembrane regions.[35] The impact of the A181E mutation was confirmed in a mouse model; its murine equivalent A144E abolished constitutive and ligand-induced TACI clustering, leading to disrupted signaling and impaired TACI-dependent B cell functions.[36] The role of TACI in CVID was reconsidered when several mutations were identified in the healthy population; TACI was redefined as a susceptibility gene, rather than a disease-causing monogenetic defect.[37,38] To date, heterozygous C104R, A181E and arguably C204insertionA sequence variants constitute risk factors for CVID. A multicenter study detected TACI mutations in 50 hypogammaglobulinemic patients (8.9%), of which two were homozygous, 41 heterozygous and 7 compound heterozygous mutations.[11] Heterozygous mutations in C104R, A181E or I87N were found in 2% of the healthy controls.[39] Although at a much lower rate than heterozygous TACI mutations, asymptomatic patient family members could carry bi-allelic mutations as well, complicating elucidation of the exact role of TACI in CVID. Apart from being a susceptibility gene for the development of CVID, heterozygous TACI mutations also predispose to autoimmunity and lymphoid hyperplasia in CVID, but immune and clinical phenotypes remain diverse.[40]

BAFFR and other TNFR defects in CVID

The ligands for TACI can bind two additional known receptors: B cell maturation antigen (BCMA)[41,42] and BAFFR. BAFFR is widely expressed by all B cells except bone marrow plasma cells.[43,44] Upon binding of BAFF, BAFFR transmits survival signals and thereby positively regulates B cell survival. A homozygous deletion within the BAFFR encoding *TNFRSF13C* gene was described in two related individuals, causing a partial deletion of the BAFFR transmembrane region, thereby abolishing BAFFR expression.[45] As a result, development and homeostasis of B cells in follicles and marginal zones were impaired, and memory B cells were low. IgA responses remained normal and T cell-independent responses against polysaccharides were abrogated. Nonetheless, the clinical phenotype of the affected individuals was not as severe as one would expect based upon these immunological features. One individual displayed characteristic recurrent lower respiratory tract infections, but no autoimmunity, and was not diagnosed with CVID until the age of 57. Of note, his sibling had an unremarkable medical history until the age of 70, showing that BAFFR deficiency does not inevitably lead to overt immunodeficiency. Furthermore, DNA samples from 49 families consisting of at least two related CVID patients and 50 sporadic patients were sequenced for the occurrence of mutations in the genes encoding APRIL and BCMA. No mutations were found.[46] Deficiency of CD27, a TNFR family member expressed on memory B cells and on T cells was recently identified in a patient with a medical history of chronic active Epstein-Barr virus infection, hypogammaglobulinemia and abnormal T-cell dependent B cell responses.[108]

In conclusion, heterozygous alterations in TNFR family members may predispose to the development of CVID, but should not be considered as disease-causing monogenetic defects. Homozygous and compound heterozygous alterations in the genes encoding these receptors presumably cause a stronger association with CVID, but a small number of healthy carriershave been described.

HYPOGAMMAGLOBULINEMIA DUE TO HOMOZYGOUS MONOGENETIC DEFECTS IN THE BCR/CO-RECEPTOR COMPLEX

Signaling threshold depends on assembly of the B cell receptor with its co-receptor Complex

Reorganization of membrane-expressed receptors is now established as being pivotal to lymphocyte activation.[47] Until recently, the widely accepted model for antigen-specific activation of the B cell receptor (BCR) involved crosslinking of multiple monomeric BCRs by antigen. A new view of BCR-mediated activation is based on the demonstration of autoinhibited BCR oligomers on resting B cells that upon activation acquire an open, antigen-receptive conformation; a dissociation-activation model.48 While appealing, it is too early for the new model to have gained experimental support and substantial intellectual traction. As both models support, cognate antigen binding by BCRs induces signaling of the BCR via the associated Igα/Igβ heterodimer, that both contain immunoreceptor tyrosine-based activation motifs (ITAMs).[49-51] Src-family kinases Fyn, Blk and Lyn are thereby recruited, Bruton's tyrosine kinase (Btk) is phosphorylated, and phospholipase Cγ2 is activated, which in turn facilitates the rapid release of calcium from the endoplasmic reticulum into the cytoplasm.[52-56] As a consequence, a sustained calcium entry into the cell occurs via plasma membrane associated store-operated calcium entry (SOCE) channels, which is necessary for full stimulation of B cells that results in plasma cell differentiation and immunoglobulin secretion.[57] Antigen-mediated signaling is enhanced by simultaneous binding of antigen to the BCR co-receptor complex. The BCR co-receptor complex consists of CD21 (CR2, complement receptor 2), CD19, CD81, CD225 and possibly other molecules. Complexes of antigen bound by the large cleavage products of C3 (i.e., C3d and C3b) can therefore cross-link the BCR and CD21 co-receptor. CD21 is laterally associated with CD19 and upon antigen ligation, the cytoplasmic CD19 tail is phosphorylated, resulting in binding and signal propagation via Src family kinases. Thus, BCR association with the co-receptor complex augments antigen-mediated signaling.[58,59]

Monogenetic defects in CVID and agammaglobulinemia

A small number of CVID patients with homozygous mutations in genes related to BCR signaling have been identified. These genetic defect all result in defective BCR co-receptor complex formation, and thus result in overlapping clinical phenotypes (table 1). First, four patients were found to have a mutated *CD19* gene, resulting in premature

stop codons and the deletion of the cytoplasmic tail of CD19.15 B cells were present, but expression of CD19 (and CD21) was marginal to absent. $CD27^+$ memory B cells and CD5+ B cells were decreased and BCR-mediated activation was impaired, as was deducted from *in vitro* stimulation experiments. The antibody response of these patients to rabies vaccination was suboptimal. One patient was identified having a mutation in the *CD81* gene, resulting in a complete CD81 protein deficiency. This patient, whose clinical features included hypogammaglobulinemia and nephropathy, showed absent CD19 surface expression, impaired responses upon BCR stimulation and reduced counts of memory B cells.60 The first human CD21 deficiency has also recently been described.61 And finally, human CD20 deficiency was documented to result in impaired T cell-independent antibody responses and hypogammaglobulinemia.[62] These data support the recently identified lateral membrane association of CD20 to the BCR.[63-65] B cell receptor signaling defects were found in antibody deficiencies related to CVID, such as X-linked agammaglobulinemia (XLA). XLA is a humoral immunodeficiency in which mutations in the *Btk* gene result in negligent numbers of peripheral B cells and paucity of immunoglobulins.[66-68] Mutations in μ heavy chain[69], BLNK70, Igα[71,72] and Igβ[73,74] are responsible for a resembling agammaglobulinemic phenotype. These findings indicate that molecules of the BCR signaling cascade are pivotal for B cell development and homeostasis, and for subsequent immunoglobulin production. The severity of immunodeficiency is mainly dependent on the consequence of the mutation on protein function and its location within the BCR signaling cascade. BCR proximal signaling defects can result in an early B cell developmental block and agammaglobulinemia, while downstream defects induce an immunodeficiency characterized by the presence of B cells, but with a variable degree of abnormal differentiation and function.

Mechanistic defects in B cell receptor signaling

Besides monogenetic defects, BCR-related mechanistic alterations were identified in subgroups of CVID patients. BCR triggering-induced calcium mobilization was used as a read-out for early BCR-mediated activation and was decreased in a subgroup of CVID patients, corresponding to class Ia of the Freiburg classification.[21] Patients in this group are characterized by a severe reduction in class-switched memory B cells and the expansion of innate-like CD21low B cells, and are prone to develop CVID-related complications.10 Calcium mobilization defects were independent of constitutional differences in the B cell compartment. The authors hypothesized the defect to be at the level of the B cell plasma membrane and suggested a link with inhibitory sialic acid-binding immunoglobulin-like lectin (SIGLEC) receptor CD22. In our pediatric CVID cohort, we confirmed these calcium signaling alterations, although they were not associated with a particular CVID classification.(our unpublished observations). Upon BCR crosslinking, CVID B cells exhibit a considerable reduction in tyrosine phosphorylation of relevant molecules compared to normal.[75] Moreover, the fusion of unaffected mouse plasma membrane vesicles into

the plasma membrane of CVID B cells restored phosphorylation and immunoglobulin production in these patients, suggesting the presence of an early signal transduction defect located in the B cell plasma membrane.[75] Others have described impaired BCR-mediated B cells effector functions, including defective upregulation of costimulatory molecules (CD70, CD86)[76,77] and decreased immunoglobulin production.[78] Although these data still require genetic support, their cumulative cell biology-based results imply a significant role of an aberrant BCR pathway in CVID.

FUNCTIONAL BUT NOT GENETIC DEFECTS IN TOLL-LIKE RECEPTORS IN CVID

Toll-like receptor signaling, a synopsis

TLRs recognize pathogen-associated molecular patterns (PAMPs) and play crucial roles in early, innate host defense against invading pathogens. TLRs are differentially expressed on dendritic cells, monocytes and B cells, and dependent on the type of TLR and likely availability of ligand, localization is at the cell surface (TLR1, TLR2, TLR4, TLR5, TLR6) or on endosomal membranes (TLR3, TLR7 and TLR9).[79] Intracellularly, TLRs have a conserved region of about 200 amino acids, known as the Toll/IL-1R (TIR) domain, as this domain is shared with the IL-1 receptors. The TIR domain is essential for subsequent cytoplasmic signaling. Upon ligation, TLRs undergo dimerization and conformational changes in order to allow recruitment of downstream signaling molecules. The TIR domain associates with myeloid differentiation primary-response protein 88 (MyD88), which in turn recruits IL-1R-associated kinase 4 (IRAK4).[80,81] Subsequently, IRAK1 is attracted, phosphorylated by IRAK4 and TNFR-associated factor 6 (TRAF6) next associates with the phosphorylated IRAK1. This complex eventually allows NF-κB to translocate to the nucleus and induce the expression of its target genes.[82] In addition, there are MyD88-independent pathways of TLR signaling not addressed here. (A full description of TLR signaling pathways falls outside the scope of this review). TLRs generally form homo-dimers, but selective members can hetero-dimerize with other TLRs as well (e.g. TLR2-6, TLR1-2) and as such expand may require clustering with other molecules; e.g.,the lipopolysaccharide (LPS)/LPS-binding protein (LBP) complex binds to CD14, enabling LPS/CD14 to associate with TLR4. TLR expression appears related to the developmental stage of the B cells; TLR6, TLR7, TLR9 and TLR10 are highly expressed on memory B cells, but low to undetectable in naive B cells. However, BCR stimulation may upregulate TLR expression in naive B cells.[84] TLRs play a role in B cell proliferation, differentiation and class-switch recombination.[85]

TLR signaling defects in CVID B cells

Considering their role in B cell function, TLRs are relevant research candidates in CVID. TLR9 is highly expressed on memory B cells and binds to unmethylated CpG dinucleotides, a strong natural bacteria-associated ligand. Expression of TLR9 was decreased in CVID B cells. B cell TLR9 stimulation by CpG caused defective upregulation of CD86 and activation-induced cytidine deaminase (AID) mRNA, and decreased production of IL-6,

Table 1: A selection of CVID-associated proteins known to require oligomerization for normal function.

Deficiency	Mutation	Oligomerization	Phenotype								Reference
			Total B cells	Transitional B cells	Memory B cells	IgM levels	IgG levels	IgA levels	Thymus-dependent vaccination response	BCR-mediated calcium response	
BCR signaling											
CD19	Cytoplasmic tail	Required for binding multiple cytoplasmic proteins (Lyn, Vav, PLCy2, Grb2, and p85 subunit of PI3-K.	Normal	Normal	Low	Decreased	Low	Decreased	Decreased	Impaired	Van Zelm et al. [15] Kanegane et al. [108]
CD20	Splice mutant causing abolished expression	Lateral association with BCR-complex.	Normal	Unknown	Low	Normal	Low	Normal	Normal	Normal	Kuijpers et al.[62]
CD81	Cryptic splice site mutation that cause abolished expression	Complex formation with CD19, CD21, and CD225; essential for lowering activation threshold.	Normal	Low	Low	Normal	Low	Decreased	Impaired	Impaired	Van Zelm et al.[60]
TNFR superfamily											
TACI	C104R: CRD1 A181E: transmembrane-domain	C104R: CRD1 required for oligomerization A181E: required for receptor clustering in plasma membrane, essential for downstream signalling.	C104R: slightly increased A181E: low	Normal	Normal	Normal	Now	Now	Mice: Normal Human: Unknown	Normal	Castigli et al.[13] Salzer et al.[14] Garibyan et al[30] Salzer et al[39]; Own unpublished observations
BAFFR	Transmembrane domain	Precluding BAFFR expression.	Low	Increased	Low	Low	Almost absent	Normal	Normal	Unknown	Warnatz et al[45]
TLR family											
TLR 7	No mutations identified	Oligomerization for ligand binding.	Unknown	Unknown	Unknown	Normal	Decreased	Decreased	Decreased	Unknown	Yu et al.[22]
TLR 9	No mutations identified	Oligomerization for ligand binding.	Unknown	Unknown	Unknown	Normal	Decreased	Decreased	Decreased	Unknown	Yu et al.[22] Cunningham-Rundles et al.[86]

IL-10, IgG and IgA.86 The defects were more pronounced when combined TLR9 and BCR-triggering was performed: CD86 upregulation and B cell proliferation were markedly reduced in CVID patients upon stimulation with CpG and anti-IgM antibody-mediated BCR crosslinking.[86,87] Second, defects were described in the related endosomal RNA-binding TLR7. Stimulation with synthetic ligands loxoribine (TLR7) and CL097 (TLR7/8) inefficiently induced B proliferation and differentiation in CVID. Immunoglobulin production and AID mRNA upregulation were defective. These deficiencies were partly reversed by addition of IFNα, which is normally produced by plasmacytoid dendritic cells upon TLR7 ligation.[22] Alterations were not B cell restricted, but were also observed in CVID plasmacytoid dendritic cells.[22]

Taken together, defects in TLR signaling may play a substantial role in the pathogenesis of CVID. However, it remains uncertain whether this is an intrinsic molecular defect, or an extrinsic defect that involves interference with other pathways, including BCR signaling. We propose that the oligomerization of receptors or association of protein involved with B cell activation is probably involved. To date, genetic defects have not been identified although CVID patients have been screened; no mutations or polymorphisms were found in the *TLR9* gene86 or its promoter.[88]

PROSPECTS

Multiple genetic and mechanistic defects related to B cell surface receptors in CVID were described in the last few years. That surface receptors were first addressed as candidates for B cell dysfunction in CVID is rational from a mechanistic point of view, as receptors provide the first contact with the extracellular environment and initiate all sequential B cell processes. Concomitantly, they are of importance in early B cell development and selection.[89] Although the molecular discoveries provide insight in the mechanistic alterations in CVID, they provide a diagnosis in only a fraction of the patient population. CVID is variable as the name suggests and the etiology of CVID remains a conundrum in the majority of patients. In this regard, several problems are encountered. First, although monogenetic defects have been identified, they usually represent only sporadic cases, mostly originating from consanguineous families. Their number remains low, in particular when taking into account the extensive screening efforts that has been performed on numerous patients for candidate genes related to B cell development and function.[46,90] Second, the plasticity of the immune system and the consequent redundancy for most of its molecules is clearly beneficial for the maintenance of general health, but may hamper the search for disease-causing mutations. Even mutations in relevant genes that lead to immunodeficiency in some individualsdo not necessarily cause a clinically overt CVID phenotype in others. This could be related to differences in penetrance or epigenetic regulation of gene expression.[91] The recently expanding interest in epigenetics indicates the potential importance of alterations of posttranslational modification state of proteins, which may change degradation or activation of the affected protein. An

alternative hypothesis is the influence of supplementary genetic variations between patients and asymptomatic siblings. As we anticipate that screening approaches that cover the entire genome for potential variations will yield a wealth of new CVID-associated genes, we believe that mechanistic cell biological studies may narrow down the defective proteins within affected B cells. However, former studies indicate that it is extremely challenging to link mechanistic defects to a genetic base in this heterogeneous patient population.[21,22] The interplay between different receptors is robust and complex. TLR signaling is at several levels intermingled with BCR signaling. BCR stimulation induces the upregulation of TLRs on naive B cells.[21,84] BCR/TLR crosslinking can occur simultaneously by immune complexes such as in systemic lupus erythematosus.[92,93] Thus, a BCR defect will negatively influenceTLR signaling and *vice versa*. Furthermore, TLR signaling is linked with TACI and BAFFR pathways;[94-96] He *et al.* moreover recently showed that TACI contains a conserved motif which binds adaptor MyD88, and thus employs the TLR pathway via a TIR-independent route.[97] Taking into account these linkages in signaling pathways and the fact that CVID is multifactorial, it is plausible that for instance heterozygous TACI mutations in combination with a (minor) TLR signaling-related gene defect will lead to a CVID phenotype (whereas asymptomatic TACI mutant siblings have no additional genetic defect). Extended fundamental knowledge regarding the receptor pathway signaling interplay, in combination with precarious selection of patients based on their clinical and immunological features for further screening, will facilitate the identification of additional defects in CVID. Taken together, we believe that the approach of genetic screening for candidate genes may not yield many novel genes in CVID patients, nor will isolated functional studies as currently performed be able to identify molecular defects. Ideally, a combination of these approaches should be performed, by investing in screening methods for target genes involved in basic functional (predominantly B cell-intrinsic) mechanisms. For such a modified screening approach, the investigation of surface receptor clustering, by combination of live cell confocal visualization techniques, flow cytometry and immunoprecipitation/blotting techniques, we consider an interesting candidate mechanism for two reasons. First, as mentioned above and elsewhere, oligomerization is crucial for receptor function. Defective oligomerization impacts on functional quality, even in the presence of perfectly functional monomers, by means of dominant-negative interference; the C104R mutation in TACI provides an example for this situation. Relevant B cell surface receptors need dimerization or oligomerization in order to transduce signals. Therefore, an alternative possibility is that the intracellular association is defective; recruitment and hence interaction of adaptor proteins with the receptor may be impaired, eventually resulting in signaling defects and consequent defects in antibody class switch recombination or other more overt CVID consequences. Second, there is clinical evidence that dysfunctional receptor clustering, due to defective cytoskeleton rearrangements causes severe immunodeficiency disorders. This is demonstrated by at least two diseases: Wiskott-Aldrich syndrome

(WAS) and DOCK8 deficiency. WAS is a rare X-linked primary immunodeficiency complicated by thrombopenia and eczema, due to loss-of-function mutations in the Wiskott-Aldrich syndrome protein (WASP).[98] WASP regulates actin cytoskeleton reorganization by stimulation of ARP2-ARP3 mediated actin polymerization and has an expression restricted to hematopoietic cell lineages. As a result, WASP deficiency hinders immunological synapse formation in response to antigen receptor stimulation[99,100], affecting T cell and arguably B cell activation and thus contributing to the immunodeficiency.[100-103] Recently, mutations were found in the gene encoding dedicator of cytokinesis 8 protein (DOCK8)104 in patients with a previously undefined combined immunodeficiency or atypical autosomal recessive hyper IgE syndrome.[105,106] All patients suffered from recurrent respiratory tract infections and extensive cutaneous viral infections, and many developed virus-related cutaneous dysplasia. Immunological features included T and B cell lymphopenia, eosinophilia, defective $CD8^+$ T cell proliferation and impaired antibody responses. DOCK8 is a Rac/Cdc42 GTP-exchange factor (GEF), a regulator of cytoskeleton rearrangement. DOCK8 is thought to have a relatively specialized role in adaptive immunity. Indeed, DOCK8 mutated mouse B cells were unable to form marginal zone B cells and had diminished affinity maturation, due to poor survival of these cells. The defects were caused by impairment in immunological synapse formation; mutant B cells were unable to cluster ICAM-1 into a peripheral supramolecular activation cluster (SMAC).[107] These findings indicate that the organization of the immunological synapse, including receptor oligomerization, is critical for B cell subset survival. The described examples of primary immune deficiencies clearly demonstrate the importance of synapse formation and membrane rearrangements for lymphocyte function. Possibly, other molecules involved in these rearrangements are affected in CVID; this idea is supported by the observation of defective expression of Vav, another member of the GEF family, and a subsequent impaired actin reorganization in CVID T cells.[17] In conclusion, the elucidation of molecular defects in CVID remains a challenge and requires especially now, the continuous joint efforts of clinical and basic researchers with immunological, cell biological and genetic backgrounds. Etiologic research should be extended by exploring mechanistic and functional lymphocyte defects in precariously selected patients. However, the approach remains limited by the current knowledge regarding the molecular basics of those basic mechanisms in B cells. Of particular interest are mechanisms that limited to B cells or adaptive immunity, as CVID patients in general have no other tissue problems. Subdividing patients based on phenotypic and functional B cell characteristics may facilitate and enhance the success rate of revealing novel defects in this complex disorder.

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Chapter 8.

Dysfunctional BLK in Common Variable Immunodeficiency perturbs B cell proliferation and ability to elicit antigen–specific $CD4^+$ T cell help.

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Abstract

Common Variable Immunodeficiency (CVID) is the most prevalent primary antibody deficiency, and characterized by defective generation of high-affinity antibodies. Patients have therefore increased risk to recurrent infections of the respiratory and intestinal tract. Development of high-affinity antigen-specific antibodies involves two key actions of B-cell receptors (BCR): transmembrane signaling through BCR-complexes to induce B-cell differentiation and proliferation, and BCR-mediated antigen internalization for class-II MHC-mediated presentation to acquire antigen-specific $CD4^+$ T-cell help.

We identified a variant (L3P) in the B-lymphoid tyrosine kinase (BLK) gene of 2 related CVID-patients, which was absent in healthy relatives. BLK belongs to the Src-kinases family and involved in BCR-signaling. Here, we sought to clarify BLK function in healthy human B-cells and its association to CVID.

BLK expression was comparable in patient and healthy B-cells. Functional analysis of L3P-BLK showed reduced BCR crosslinking-induced Syk phosphorylation and proliferation, in both primary B-cells and B-LCLs. B-cells expressing L3P-BLK showed accelerated destruction of BCR-internalized antigen and reduced ability to elicit CD40L-expression on antigen-specific $CD4^+$ T-cells.

In conclusion, we found a novel BLK gene variant in CVID-patients that causes suppressed B-cell proliferation and reduced ability of B-cells to elicit antigen-specific $CD4^+$ T-cell responses. Both these mechanisms may contribute to hypogammaglobulinemia in CVID-patients.

Introduction

Common variable immunodeficiency (CVID) is the most common primary immunodeficiency (PID), with an estimated prevalence of 1 in 25.000-50.000 adult Caucasians. CVID patients are characterized by defective generation of high affinity antibodies by B cells and therefore suffer from recurrent infections of the respiratory and intestinal tract[1-3].

The vast majority of mature B cells recirculate through the blood and the follicular regions of the lymphoid tissues. These quiescent naive B cells require sustained expression of functional B cell receptors (BCRs) to transduce tonic signals for their survival. B cells may capture encountered antigen with their specific BCR that is composed of the Igα/Igβ dimer. Productive BCR-antigen engagement initiates a signaling cascade that starts with the activation of tyrosine kinases from the Src family. Src kinases phosphorylate the ITAM motif of Igα and Igβ, leading to the recruitment and subsequent activation of the Spleen tyrosine kinase (Syk). Ultimately this leads to functional B cell activation and clonal expansion. For the development of high affinity antigen-specific antibodies, BCR expression and signaling is additionally necessary to internalize antigen into endosomes for processing by endosomal proteases. Antigen-derived peptides thereby generated are presented as antigen-specific peptide/class II MHC complexes to acquire antigen-specific $CD4^+$ T cell help in form of cytokines and co-stimulatory molecules, such as IL-4 and CD40L respectively. Therefore it is not surprising that in patients showing characteristics of CVID, monogenetic defects are found in genes relevant to BCR signaling or the elicitation of $CD4^+$ T cell help, including CD19[4], CD20[5], CD81[6], or CD27[7], CD40L[44] and ICOS-L[8].

Using our recently developed Primary Immunodeficiency (PID) targeted Next-Generation Sequencing-based approach[9], we screened patients with CVID of unknown origin for (putative) PID-associated gene variants. Here we report a Leucine to Proline replacement at position 3 in B lymphoid tyrosine kinase (BLK). BLK was initially thought to be expressed solely in B-lineage cells[12], but is now known to be also expressed in both human and mouse pancreatic β-cells[13], in murine plasmacytoid dendritic cells[45], and is required for the development of T cells and IL-17-producing γδ T cells in mice[14]. BLK belongs to the Src family of tyrosine kinases that phosphorylate Igα subunit of BCR signalling complex[15,17]. We sought to clarify BLK function in human B cells and its association to CVID. To this end, we researched the effect of L3P-BLK gene variant expression on BLK function and B cell function in primary CVID-patient B cells and immortalized B-LCLs. Unlike in mice, where to a certain extent functional redundancy exists between Src kinases[10;41;42], in human B cells the L3P-BLK mutation has functional consequences to BCR signaling. We propose that BLK disease variants may contribute to CVID disease pathology by perturbing B cells to proliferate and adequately elicit $CD4^+$ Tcell help, known to support B cells class-switching of Ig heavy chain regions and eventually the sufficient production of high affinity IgG and IgA antibodies.

Methods

Patients and healthy donors

The index patient, his parents, and his two sisters were included in this study. Adult volunteers were healthy employees of the University Medical Center Utrecht. This study was approved by the institutional review board, and informed consent was obtained.

Targeted Next-Generation Sequencing

The Next-Generation Sequencing is targeting 170 PID-related (IUIS[2]) and >350 putatively PID-related genes[9]. We used both targeted array-based and in-solution enrichment combined with a SOLiD sequencing platform and bioinformatics analysis, as described previously[9]. Subsequently, the selected variant was validated with Sanger sequencing. Amplicons were bidirectly sequenced with the Big Dye Terminator version 3.1 cycle sequencing kit and an ABI 3730 DNA Analyzer (Life Technologies). Sequences were compared with reference sequences by using Mutation Surveyor (SoftGenetics). The prevalence of the BLK gene variant was determined in the dbSNP, 1000genomes, and GoNL exome databases.

B cells overexpressing B Lymphoid tyrosine Kinase variants.

The CVID-associated mutation of BLK was inserted in pWZL-Neo-Myr Flag-BLK (Plasmid 20430, Addgene) by site-directed mutagenesis according to manufacturers protocol (Qiagen) using primers (Sigma-Aldrich): BLK Fwd1: CACCTGGATGAAGACAAGCA and BLK Rev1: CCTTCCGACCCTGTGATCTA. Packaging cells (Phoenix-Ampho) were transfected with gag-pol (pHIT60), env (pCOLT-GALV), and pWZL-Neo-Myr Flag-BLK wildtype or disease-associated variant, using Fugene6 (Promega). The produced virus particles were applied to freshly thawed B Lymphoblastoid Cell Lines from 4 different healthy donors. After 1 week of selection, B-LCLs were used in experiments.

Quantitative PCR

Freshly isolated PBMCs or cultured B-LCLs overexpressing BLK disease-associated or wildtype variant were lysed and total mRNA was isolated using Tripure isolation reagent (Roche Diagnostics) according to the manufacturer's instructions. RNA concentrations were measured by spectrophotometer and equalized for all samples prior to reverse transcription using an iScript cDNA synthesis kit (Biorad). Primers were mixed with IQ SYBR green supermix (BioRad). The detection run started at 95°C for 10 min, followed by 45 cycles of 95°C for 15s and 60°C for 1 min. Assays were performed in duplicate or triplicate as 15µl reactions in 96well plates using C1000 Thermal Cycler (BioRad). Results were normalized to the endogenous GAPDH and Actin mRNA. The following primers were used: GAPDH Forward 5'-GTCGGAGTCAACGGATT-3'; GAPDH Reverse 5'-AAGCTTCCCGTTCTCAG-3'; Actin Forward 5'-CATGTACGTTGCTATCCAGGC-3';

Actin Reverse 5'-CTCCTTAATGTCACGCACGAT -3; BLK Forward 5'-CACCTGGATGGAAGACAAGCA-3'; BLK Reverse 5'-CCTTCCGACCCTGTGATCTA-3' (All Sigma-Aldrich).

Flow cytometry and functional assays

Isolate PBMCs by Ficol-plaque and let them rest for at least 2hours at 37C. Stimulate rested PBMCs or equal amount of B-LCLs (BLK-wt and L3P variant) for 0, 4, 10, 30, 90, 120, and 240 min. with goat anti-human IgM and IgG F(ab')2 fragments (5µg/ml). This is followed by fixation with 1.3% EM grade paraformaldehyde (Electron Microscopy Technologies) for 5 min. at RT. These cells were washed and taken up in FACS buffer (PBS complemented with 1% Bovine Serum Albumin (BSA, Roche) and 0.1% Sodium Azide). Extracellular CD20 (Pacific Blue, Biolegend) and IgM or IgG (PE, Fab fragments, Invitrogen) were stained (RT, 30 min.) prior to permeabilization with ice-cold methanol (4C, 5 min.). Cells are rehydrated and washed with PBS+ 1% BSA, and phosphorylated Syk was stained (PECy7, cl17A1P-ZAP70, BD Bioscience, RT, 20min.)

Goat anti-human IgG and IgM F(ab')2-fragments (Invitrogen) were conjugated to EZ-link Sulfo-NHS Biotin according to manufactures protocol (Thermo Scientific). Purified Tetanus Toxoid (RIVM, the Netherlands) or DQ-BSA (Life Technologies) was conjugated to Lightening-link streptavidin according to manufactures protocol (Novus Biologicals). Biotinylated anti-IgM or anti-IgG F(ab')2 fragments are complexed overnight to streptavadin-conjugated DQ-BSA or Tetanus Toxoid in 2:1 or 4:1 (*w:w*) ratio.

For $CD4^+$ T cell activation assay, these complexes were incubated for 4 hours to PBMCs or B-LCLs. Followed by overnight incubation with tetanus toxoid-specific $CD4^+$ T cell clones. Antigen-specific CD154 expression on $CD3^+$ $CD4^+$ T cells was determined by staining with CD154 (Pacific Blue, Biolegend), CD3 (APC, BD Bioscience), and CD4 (FITC, eBioscience). For antigen degradation assay, anti-IgM/G DQ-BSA complexes are administered to B-LCLs expressing either L3P- or common BLK variant and put on ice at indicated timepoints. BCR-targeted antigen destruction was determined by Flow cytometric analysis of the MFI emitted by processed DQ-BSA per B-LCL.

BCR complex molecules and co-stimulatory molecules were determined by flow cytometric analysis upon staining PBMCs or B-LCLs with following antibodies: CD19, CD21, CD81, CD40, and CD54 (all from BD Bioscience); CD20, CD86, HLA-DR, CD154, streptavadin-APC (all Biolegend).

B cell proliferation assay

B-LCLs proliferation was determined by the dilution rate of Cell Tracer Violet (Invitrogen). To this end, 1x10^6 B-LCLs expressing either BLK variants are stained with 2µM Cell

Tracer Violet for 10 minutes at 37C. Staining and excess Cell tracer Violet is removed by spinning B cells down in 5x volume Fetal Calf Serum (FCS). Cells were maintained in a humidified incubator at 37 °C with 5% CO_2 for 4 days in RPMI 1640 medium with 1% (v/v) PenStrep (Invitrogen), 1% (v/v) GlutaMAX (Invitrogen), and 10% (v/v) FCS. Mean Fluorescent Intensity was determined on the Flow cytometer at day 1, 2, 3, 4.

Statistical analysis

Flow cytometry data were collected on FacsCanto II (Becton Dickinson) 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 (GraphPad Software, Inc, La Jolla, California).

Results

Case report of index patient and family

The index patient presented in our hospital at age 7, with a history of severe recurrent pulmonary infections from the age of 8 months onward, requiring frequent hospitalizations. He responded well to antibiotics (iv and orally) and bronchodilatory medication. As part of the diagnostic trajectory a High Resolution Computed Tomography (HRCT) was made showing small airway disease. Laboratory investigations showed hypogammaglobulinemia with persistent low levels of IgG (5g/L), low IgA levels (0.2g/L), and relatively high IgM levels (5.6g/L) (Table 1). The patient was vaccinated according to the national vaccination program in the Netherlands, however showed insufficient vaccination responses for Haemophilus Influenza B (Hib), Meningococci type C, and pneumococcal polysaccharide antigens 4-6 weeks after re-vaccination. Based on these findings the patient was diagnosed with CVID[1] and treated with monthly infusion of intravenous immunoglobulin (Ig), and antibiotic prophylaxis.

To study potential causes of the antibody deficiency, flow cytometric analysis was performed on peripheral blood of the patient. This showed normal numbers of monocytes ($0{,}4x10^9$/L), lymphocytes ($2{,}47x10^9$/L) and NK cells (11%), but slightly reduced B cell numbers (Table 2). Within the B cell compartment, IgG memory B cells were in the low normal range (2.6% of B cells), but IgM (5.6%) and IgA (0.2%) were significantly reduced[11]. There were no signs for autoimmunity or lymphoproliferative disease.

The patient's father has a disease history comprising of recurrent respiratory tract infections and episodes of bacteraemia upon small skin lesions. Moreover, he has relative low IgM (0,28g/L) and IgG (6,65g/L) levels (shown in Table 1). In contrast to both the index patient and his father, the mother, and two daughters show no clinical symptoms related to antibody deficiency. The mother was excluded from functional experiments, because she received immunosuppressive therapy for recently diagnosed ulcerative colitis.

Table 1: Serum antibody titers of L3P-BLK carrying individuals in comparison to age-matched controls.

Immunoglobulins		patient (L3P-BLK)	reference values	father (L3P-BLK)	reference values
IgM-total	g/L	0.39	0.28-1.9	0.28	0.40-2.3
IgA-total	g/L	0.42	0.54-2.5	1.2	0.7-4.0
IgG-total	g/L	5	5.20-14.3	6.65	7.00-16.0
IgG1	g/L	3.4	3.5-9.1	4.1	4.9-11.4
IgG2	g/L	0.62	0.85-3.30	1.91	1.50-6.40
IgG3	g/L	0.34	0.20-1.04	0.20	0.20-1.10
IgG4	g/L	0.02	0.03-1.58	0.05	0.08-1.40
Post-vaccination: serum antibody titers		**patient (L3P-BLK)**	**father (L3P-BLK)**	**reference values**	
Meningococci C	mg/ml	0.33	<0.24	>1mg/ml	
HiB-BL	µg/mL	0.32	0.50	>1µg/ml	
PnPS1-BL	µg/mL	0.16	0.38	>1µg/ml*	
PnPS3-BL	µg/mL	0.22	3.2	>1µg/ml*	
PnPS4-BL	µg/mL	0.10	0.049	>1µg/ml*	
PnPS5-BL	µg/mL	0.26	0.072	>1µg/ml*	
PnPS6B-BL	µg/mL	0.27	0.061	>1µg/ml*	
PnPS7F-BL	µg/mL	0.72	>40	>1µg/ml*	
PnPS9V-BL	µg/mL	0.43	0.18	>1µg/ml*	
PnPS14-BL	µg/mL	1.4	4.5	>1µg/ml*	
PnPS18C-BL	µg/mL	4.9	0.051	>1µg/ml*	
PnPS19F-BL	µg/mL	1.2	0.38	>1µg/ml*	
PnPS23F-BL	µg/mL	0.13	0.082	>1µg/ml*	

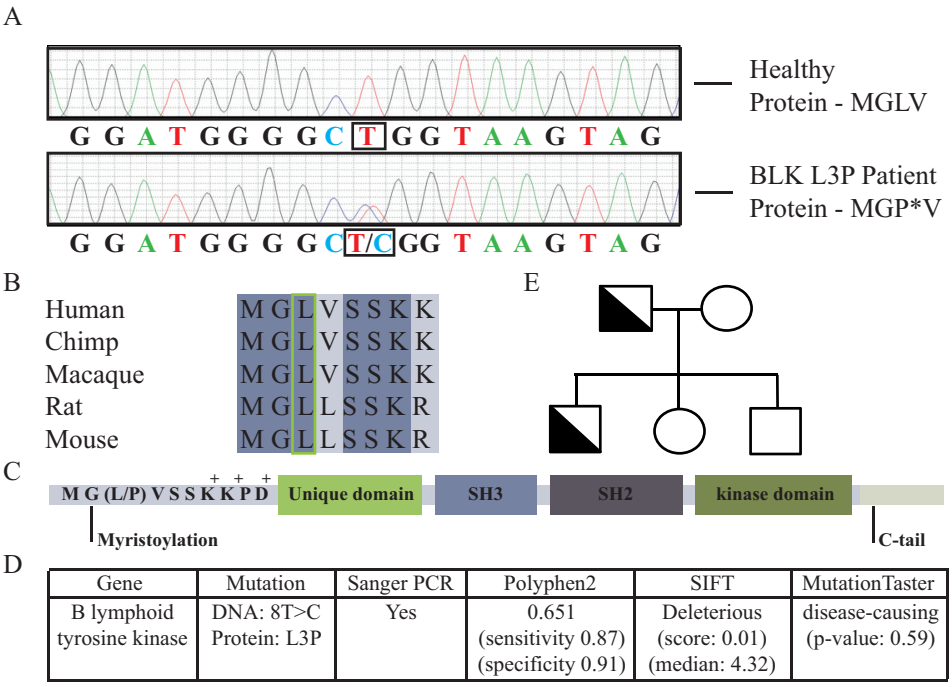
**: >6 years of age, a normal response is defined as IgG reponses of >1µg/ml in at least 8/11 pneumococcal polyssacharide serotypes measured.*

Screening of Common Variable Immunodeficiency-associated candidate genes uncovers a L3P point mutation in the BLK gene.

Regular genetic analysis of the CVID patient revealed no mutations in known CVID-associated genes. Hence, we exploited our recently developed Primary Immunodeficiency (PID)-targeted Next-Generation Sequencing-strategy based on 170 PID-related (IUIS[2]) and 350 candidate PID-genes. This strategy allows detection of point mutations with a sensitivity and specificity >99% in covered regions[9], and revealed that both CVID patients but not their healthy relatives have a heterozygous point mutation (NM_001715.2 c.8T>C) in the B cell lymphoid tyrosine kinase (BLK) gene. No mutations were found in the other known or candidate PID-genes present on the chip[9]. The point mutation in BLK was subsequently confirmed by Sanger sequencing, as shown in Figure 1A. The L3P-BLK gene variant is unique to these CVID patients, with healthy family members not carrying the mutation (Figure 1E) and it not being present in the dbSNP, the 1000 genome or Dutch population specific GoNL databases. Moreover the residue changed by the mutation is a highly conserved Leucine at the third position to a Proline (Figure 1B and 1C), predicted to be potentially damaging by PolyPhen-2, SIFT and Mutation Taster (Figure 1D).

We determined by quantitative analysis that BLK RNA expression is not significantly different in human peripheral blood $CD19^+$ B cells of healthy individuals and our CVID patients carrying L3P-BLK gene variant (figure 2A), but is absent in monocyte-derived

dendritic cells (DCs) and human CD4⁺ T cells in circulation (our data not shown). Additionally, we seem to observe similar levels of BLK protein in human peripheral blood CD19⁺ B cells of healthy individuals and our index CVID patients (supplemental Figure 1A). Thus, L3P mutation affects BLK function rather than BLK haploinsufficiency. Together this data prompted us to perform functional studies.



D

Gene	Mutation	Sanger PCR	Polyphen2	SIFT	MutationTaster
B lymphoid tyrosine kinase	DNA: 8T>C Protein: L3P	Yes	0.651 (sensitivity 0.87) (specificity 0.91)	Deleterious (score: 0.01) (median: 4.32)	disease-causing (p-value: 0.59)

Fig 1. Classification of the novel L3P mutation in B lymphoid tyrosine kinase of Common Variable Immunodeficiency (CVID) patients.

A. Confirmation of heterozygous mutation L3P in B lymphoid tyrosine kinase of CVID patient by Sanger PCR. **B.** The mutated Leucine indicated is an amino acid conserved amongst species. **C.** The mutation occurs at third position, between a myristoylated Glycine residue and a charged amino acid cluster. **D.** Three bioinformatics models predict a deleterious effect of the mutation on BLK function. **E.** Family tree showing CVID diagnosed L3P-BLK carrying patients (father and son) and non-carrying healthy family members.

Reduced Syk phosphorylation upon BCR crosslinking in L3P-BLK patient B cells.

In B cells, BLK protein associates with Igα upon activation[16], which it can phosphorylate upon antigen-induced BCR-crosslinking[17]. However, experiments executed in COS cells using an overexpressed mutated L3C-BLK earlier demonstrated that BLK-mediated Igα phosphorylation was blocked[15], suggesting that in analogy, L3P-BLK in human cells may have a functional defect at phosphorylation. We tested this possibility by measuring Syk phosphorylation, the immediate downstream consequence of Igα phosphorylation. We induced BCR-crosslinking by addition of anti-IgM and IgG-F(ab')2 fragments, fixed

PBMCs at indicated time points, and measured levels of phosphorylated (p)Syk in $CD20^+$ B cells by flow cytometry as previously performed by others[18]. Instead of anti-IgM/G antibodies, anti-IgM/anti-IgG F(ab')2 fragments are used. This approach ensure specific BCR targeting and eliminate non-specific binding with Fc receptors. In healthy controls, BCR crosslinking induces increased amounts of pSyk within 2 minutes, which corroborates with previous data[18]. In contrast, in L3P-BLK carrying CVID patient B cells pSyk levels are induced later and remain at 50% lower levels in comparison to primary B cells of healthy individuals (Figure 2C). Surface expression of B cell (co)receptor complex molecules CD19, CD20, CD21, and membrane-bound IgM, is similar on B cells from individuals carrying L3P- and common BLK variant (Figure 2D). Thus, BCR-mediated signaling rather than B cell (co)receptor complex components expression levels appear affected in L3P-BLK carrying B cells of our CVID patients.

Table 2: Lymphocytes in peripheral blood of L3P-BLK carrying individual in comparison to age-matched controls.

Lymphocyte subset		patient (L3P-BLK)	reference values
$CD3^+$	(% within Ly)	76	57-76
$CD3^+CD4^+$ T-cells	(% within Ly)	46.1	29-46
$CD3^+CD8^+$ T-cells	(% within Ly)	24.5	19-34
$CD19^+$ B-cells	(% within Ly)	11	12-26
NK cells	(% within Ly)	11	6-21
$IgM^+IgD^+CD27^-$ naïve B-cells	(% within B-cells)	74	52-73
$CD27^-CD21^{int}CD38^{++}CD10^+IgM^{++}$ trans. B-cells	(% within B-cells)	9	2.9-23.8
IgD^-CD27^+IgM memory B-cells	(% within B-cells)	5.6	6.5-22.2
IgD^-CD27^+IgG memory B-cells	(% within B-cells)	2.6	1.5-8.8
IgD^-CD27^+IgA memory B-cells	(% within B-cells)	0.2	1.3-6.1

Reduced Syk phosphorylation upon BCR-crosslinking in L3P-BLK-overexpressing B cell lines.

In B-LCLs, that are immortalized by Epstein-Bar virus (EBV), EBV-derived Latent Membrane Protein 2a (LMP2A) drives activation of α- and β-chains of the BCR[19]. Similarly as the BCR, LMP2a associates with Src family of tyrosine kinases[20]. This capacity of B-LCLs allows us to study the effect of L3P-BLK variant expression on B cell signaling and function. To this end, we drove overexpression of either L3P- or common BLK variant in 3 separately derived B lymphoblastoid cell lines (B-LCLs) by retroviral transduction. To enable comparison, we selected clones within each B-LCL cell line with similar overexpression levels of either L3P- or common BLK protein as determined by quantitative PCR (Figure 2B) and Western blot (Supplemental Figure 1B).

To address whether L3P mutation in BLK is solely responsible for the reduced levels of Syk phosphorylation observed in the primary $CD19^+$ B cells of our CVID patients (Figure 2C), we analyzed B-LCLs overexpressing BLK and L3P-BLK for relative levels of pSyk similarly as described above. In analogy of the L3P-BLK primary B cell data, also B-LCLs that overexpress L3P-BLK exhibit reduced Syk phosphorylation without affecting expression levels of the B cell (co)receptor molecules CD19, CD20, CD21, and membrane-bound IgG

(Figure 2E and 2F). Thus, the L3P-BLK variant suppresses BCR crosslinking-induced Syk phosphorylation, when compared to the common BLK protein.

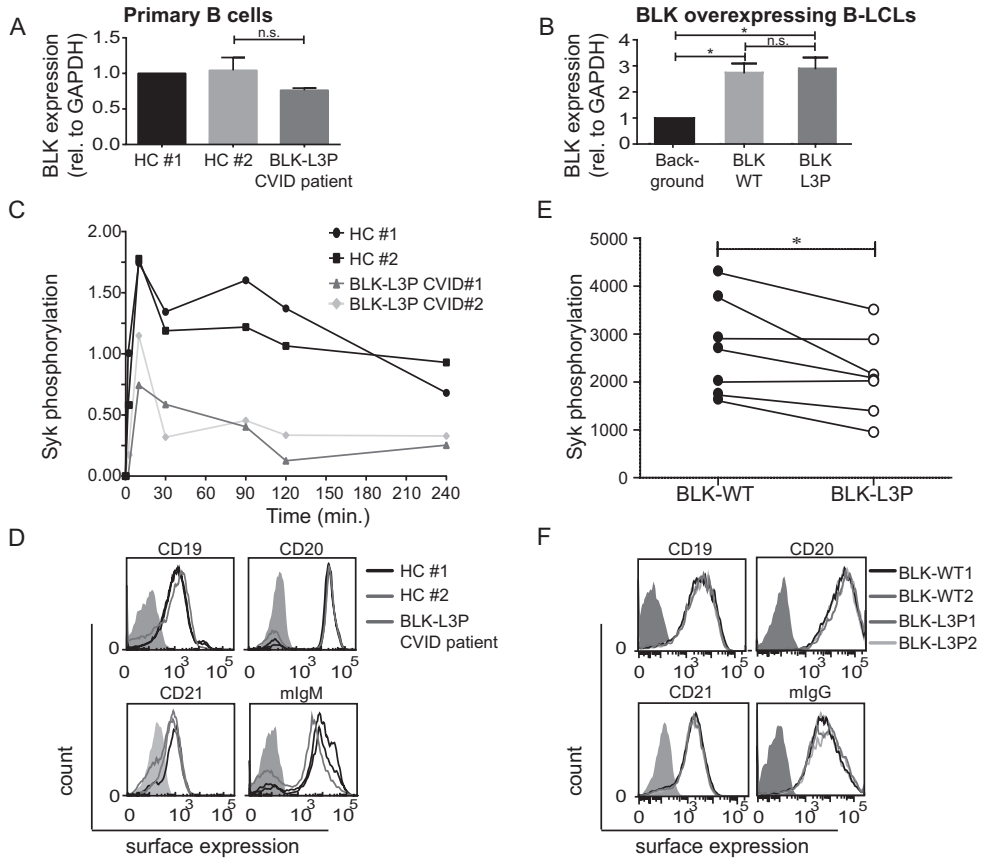


Fig 2. Reduced Spleen tyrosine kinase (Syk) phosphorylation upon B cell receptor crosslinking of CVID (L3P-BLK) patient CD20⁺ B cells.

Quantitative PCR demonstrates that heterozygous L3P-BLK CVID patient comparable levels of BLK mRNA (p=0.25) in **A.** CD19⁺ B cells relative to healthy controls or **B.** BLK overexpressing B-LCLs. Data of 3 independent experiments represented as mean +/- SEM. **C**. B cell receptors are crosslinked by incubation of PBMCs from CVID (L3P-BLK) patient or healthy controls with excess amounts of anti-IgM and IgG F(ab')2 fragments for the indicated duration (0, 2, 10, 30, 90, 120, and 240 minutes). At the end of incubation the cells are fixed and surface-stained with CD20 antibody. Subsequently, the cells are permeabilized with methanol and stained for intracellular presence of phosphorylated Syk (pSyk), followed by flow cytometric analysis on live (FSC/SSC) CD20⁺ B cells. Representative of 3 independent experiments, presented as mean fluorescent intensity levels of phosphorylated Syk over time in CD20⁺ B cells of healthy controls (Black), CVID (L3P-BLK) patient (Red), and CVID patient #2 (Blue) also heterozygous for L3P-BLK mutation. **D.** Expression of B cell (co)receptor molecules CD19, CD20, CD21, and membrane-bound IgM as B cell receptor (mIgM) in CD20⁺ B cells of healthy controls (Black and Grey) and CVID (L3P-BLK) patient (Red). Representative of 3 independent experiments. Filled grey graphs are non-stained negative controls. **E.** LMP2a driven phosphorylation of Syk in B-LCLs overexpressing either L3P- or common BLK variant in simultaneously executed experiments. **F.** Expression of B cell (co)receptor

molecules CD19, CD20, CD21, and membrane-bound IgM as B cell receptor (mIgM) in B-LCLs overexpressing L3P-BLK (Red) or common BLK variant (black/grey). Representative of 3 independent experiments. Grey filled graphs are non-stained B-LCLs. *P-value <0.05, **P-value <0.01, Two-tailed Wilcoxon-signed rank test.

L3P-BLK variant negatively affects tonic signaling-dependent B cell proliferation.

B cell proliferation requires tonic BCR signaling via Src family kinases to which BLK belongs[17]. We considered this finding in light that our L3P-BLK CVID index patient shows reduced numbers of circulating $IgD^{-}CD27^{+}IgM$ and $IgD^{-}CD27^{+}IgA$ memory cells (Table 2). Therefore, we investigated whether and to which extend L3P-BLK may affect B cell proliferation. We stained B-LCLs overexpressing either the L3P-BLK or common BLK variant with Cell Tracer Violet at day 1, to assess the B cell proliferation rate. During 4 days, cell tracer violet fluorescence was monitored using flow cytometry (Figure 3A). Already after the first day until the last day, B-LCLs overexpressing L3P-BLK have a small but significant delay in B cell proliferation when compared to common BLK carrying B-LCLs (Figure 3B). All together, these data support that L3P-BLK has less proficiency than the common BLK variant to transduce tonic BCR signaling towards B cell proliferation, which may contribute to the reduced B cell number phenotype in the L3P-BLK CVID patient.

B cell receptor endosomal routing upon antigen binding is altered in B-LCLs that overexpress L3P-BLK compared to the common BLK variant.

While superfluous in tonic BCR signaling, Syk kinase function is considered essential for the propagation of antigen-induced B cell signaling (reviewed in[46]). To investigate whether the L3P-BLK defect to elicit Syk phosphorylation propagates to downstream defects, we determined the fate of BCR-targeted antigen upon endocytosis, using DQ-BSA complexed to anti-IgG F(ab')2 fragments (Figure 3E). DQ-BSA is a model antigen that becomes fluorescent upon proteolytic degradation in acidic late endosomal compartments. After BCR-mediated uptake of anti-IgG/DQ-BSA complexes in B-LCLs, we observed accelerated degradation of DQ-BSA in L3P- than common BLK expressing B-LCLs (Figure 3C and 3D). Similarly, our CVID patient B cells shows faster degradation of specfiically BCR-targeted DQ-BSA in comparison to non-targeted DQ-BSA or $CD19^{+}$ B cells derived from healthy individuals (Figure 3F).

Internalization of BCR complexes upon crosslinking with nti-aIgG antibodies seems unaffected in B-LCLs expressing either BLK variant (supplemental figure 2), which corroborates with unaltered expression of B cell (co)receptor complex components on B cell surface (Figure 2F). All together, it seems that the L3P-BLK variant when compared to the common BLK directs the sorting of BCR-internalized antigen/IgG complexes towards proteolytic active, degradative endosomal compartments.

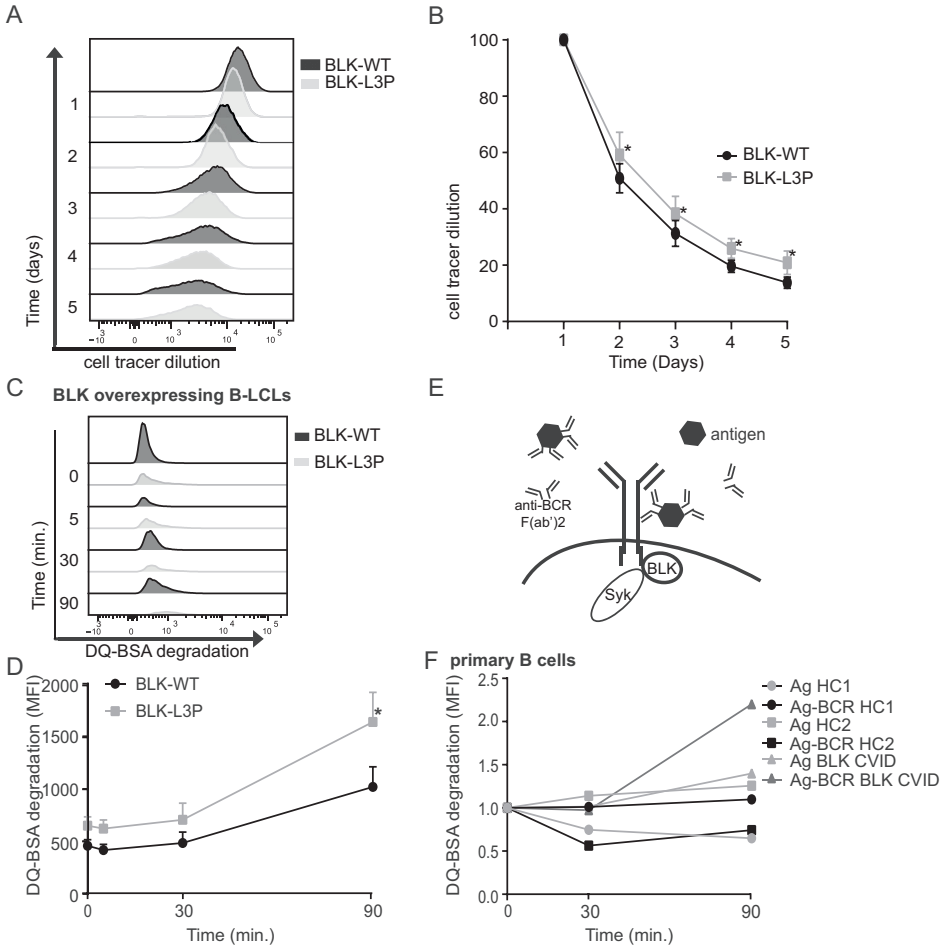


Figure 3. L3P-BLK has diminished ability to transmit tonic and ligand-induced B cell receptor signals.

A and B. B-LCLs overexpressing either L3P- or common BLK variant are stained with cell tracer violet and B cell proliferation was determined by dilution of cell tracer violet MFI measured by flow cytometry each day. Data of 3 independent experiments, represented as mean +/- SEM. **C.** DQ-BSA becomes fluorescent when cleaved. DQ-BSA degradation was measured by flow cytometry as MFI increase. **D and E.** Streptavid-modified DQ-BSA is complexed in 4:1 ratio with biotinylated anti-IgM and anti-IgG to target to BCR. These anti-BCR/DQ-BSA complexes are administered to B-LCLs overexpressing L3P- or common BLK variant, or **F.** in primary $CD19^{+}$ B cells derived from healthy volunteers (squares and circles) or BLK-L3P carrying CVID patients (triangles). Representative (F) of Data (D) of 3 independent experiments, represented as mean +/- SEM. *P-value <0.05, **P-value <0.01, Two-tailed Wilcoxon-signed rank test.

L3P-BLK variant obstructs B cell receptor-mediated antigen presentation to antigen-specific $CD4^{+}$ T cells.

Could the rapid degradation of antigen-BCR complexes modulate the ability of L3P-BLK B cells to function as antigen presenting cells? The generation of high-affinity IgG and IgA

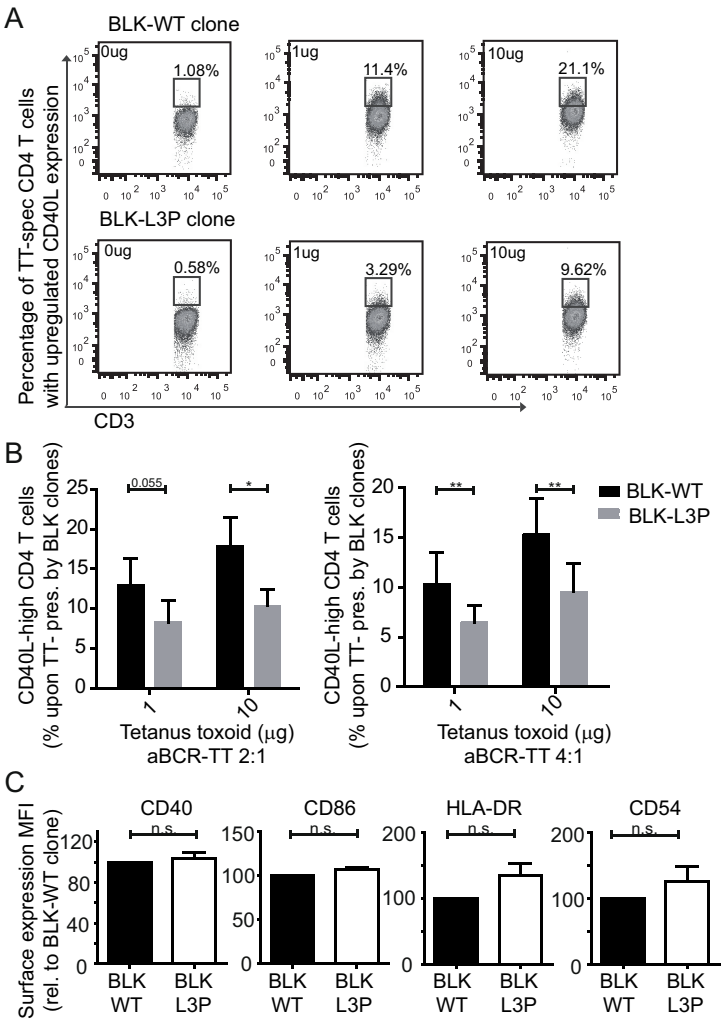


Fig 4. Decreased capacity of L3P-BLK to facilitate B cell receptor-mediated HLA-DR/peptide presentation to CD4⁺ T cells.

Tetanus toxoid (TT) protein is complexed with anti-IgG F(ab')2 fragments in ratio 1:2 or 1:4 for 6 hours at 4°C (using streptavidin/biotin conjugation). Paired human B-LCLs expressing common or L3P-BLK variant are loaded (4hrs, 37°C) with 0, 1, or 10 μg anti-IgG-TT complexes. Next, human TT peptide/MHCII-specific CD4⁺ T cells are added for co-culture with the B-LCLs (1:1 ratio, O/N, 37°C). B-LCL mediated activation of antigen-specific CD4⁺ T cells was measured by analysis of induced CD154 (CD40L) expression. **A.** CD40L staining in absence of exogenous antigen is set as background levels for both B-LCLs expressing common BLK (upper panel) or L3P-BLK (lower panel). Representative of 3 independent experiments. **B.** L3P-BLK expressing B-LCL cells (grey) are less able to induce activation of antigen-specific CD4⁺ T cells (CD40L high phenotype) compared to common BLK-expressing B-LCL cells (black). Experiments performed on B-LCLs of three independent donors, in at least three independent experiments. Data represented as mean +/- SEM. **C.** Expression of L3P-BLK does not affect expression of class II MHC molecules (HLA-DR) and co-stimulatory molecules (CD40, CD86, and CD54) in B-LCLs. Data of 3 independent experiments, represented as mean +/- SEM. *P-value <0.05, **P-value <0.01, Two-tailed Wilcoxon-signed rank test.

antibodies requires class II MHC-mediated antigen presentation by B cells to invoke T cell help and CD40 binding by CD40L upregulation on antigen-triggered T cells[23]. Indeed, Syk signaling participates in BCR-mediated antigen processing and presentation of human B cells[21;22]. Considering both L3P-BLK CVID patients are hypogammaglobulinemic and have low post-vaccination titers elicited by T cell-dependent antigens, we hypothesized that L3P-BLK protein may be less capable to facilitate class II MHC antigen presentation by B cells, when compared to the common BLK protein. To address this question, we generated Tetanus Toxoid (TT)-IgM/G immune complexes by incubating biotinylated anti-IgM/anti-IgG F(ab')2 fragments to streptavidin-conjugated TT in 2:1 or 4:1 (*w:w*) ratio overnight(Figure 3B)[24]. We administered BCR-targeted TT-Ig complexes to paired B-LCL lines expressing either L3P-BLK or the common BLK for 4 hours (37°C), followed by overnight co-culture with TT-specific $CD4^+$ T cells. We determined stimulation levels of TT-specific $CD4^+$ T cells by measure of upregulated CD40L, using flow cytometry (Figure 4A). The fraction of $CD4^+$ T cells expressing high levels of CD40L increases in antigen dose-dependent manner by both L3P- or common BLK variant expressing B-LCLs. However, antigen-driven activation of the TT-specific $CD4^+$ T cells is significantly suppressed in co-cultures using B-LCLs expressing L3P-BLK compared to common BLK variant (Figure 4A, 4B). These data support that L3P-BLK obstructs antigen-specific B cell-mediated antigen presentation to $CD4^+$ T cells. Considering that the recruitment of $CD4^+$ T cell help in form of CD40-CD40L engagement is required to generate high affinity antibodies, these data clarify an unexpected role for B cell-expressed L3P-BLK in the elicitation of $CD4^+$ T cell help, with relevance to CVID disease pathology.

After 4 hours of incubation of TT-anti IgG F(ab')2 complexes with B-LCLs overexpressing L3P- or common BLK variant, we measured the surface expression levels of CD40, CD86, class II MHC (HLA-DR), and CD54 (ICAM-1). Surface expression of these markers was comparable, as determined by flow cytometry (Figure 4C). This unaltered display of HLA-DR and co-stimulatory molecules suggest that the L3P mutation in BLK does not affect overall antigen-induced BCR-crosslinking dependent B cell activation. Thus, the L3P-BLK variant obstructs BCR-signaling and sorting of BCR-internalized antigen towards endosomal compartments conductive to Class II MHC-mediated antigen presentation. These data clarify a previously unrecognized role of human BLK through Syk signaling in the support of antigen processing and peptide/Class II MHC presentation by B cells.

Discussion

The selection and expansion of antigen-specific B cells to become functional Ig-secreting plasma cells and generate memory B cells requires BCR-signaling and antigen-specific $CD4^+$ T cell help, most notably through CD40L-CD40 engagement[25-27]. Molecules involved in the Ig isotype class-switching and eventual production of large amounts of high affinity antibodies include RAG[28], AID[29], and UNG[30]. Additional B and T cell receptor

signaling molecules are involved: CD20[5;31], CD21[32], CD81[6], ICOS[8], and CD40L[33]. Therefore it is not surprising that mutations affecting genes encoding these proteins are found in human patients that suffer from primary immunodeficiencies, including CVID[2]. We here report the functional analysis of a CVID-associated variant in the protein BLK, a Src-kinase family member that serves early downstream of the BCR. We describe a defect in BCR-triggering-induced Syk phosphorylation by the CVID-associated L3P-BLK variant. Analysis of this CVID-related BLK mutant moreover allowed us to clarify a role for BLK in B cell proliferation and BCR-mediated antigen presentation to elicit activation of antigen-specific $CD4^+$ T cells in humans.

As member of the Src kinase family, BLK shares a conserved tyrosine kinase domain with other family members. While mouse-based experiments suggested that the catalytic activity of Src kinases is redundant[10;41;42], we found that in human B cells this is not the case. Specificity is supported by tissue-specific expression and subcellular localization. Myristoylation, at the second amino acid of BLK, is necessary for its membrane localization and kinase activity. Exchange of the third amino acid of BLK for a cysteine has earlier been shown to abrogate its ability to phosphorylate Igα[15]. Hence, our identified mutation of the third amino acid of BLK into Proline may cause defective myristoylation and thereby affect localization of BLK to lipid bilayers. However, the retroviral construct we used to overexpress BLK and L3P-BLK in B-LCLs has incorporated an additional myristoylation-tag distal from the L3P mutation, thereby relieving the requirement of the myristoylation motif around the L3P mutation. Thus, while we did find a functional defect in B-LCLs overexpressing L3P-BLK, the defect in patient cells may be even more severe due to obstruction of myristoylation-mediated subcellular membrane localization. Of note, besides myristoylation, additional motifs support the anchorage of Src kinases to membranes, including palmitoylation[15,39]. In Src, and probably BLK, several basic amino acids appear to interact with inner leaflet membrane phospholipids that are acidic[39]. Such electrostatic interaction-based association is rather sensitive to disruption, supporting the possibility that a L3P mutation could have effects on localization and thereby function. Attempts to address this possibility directly, by visualization of the subcellular localization of BLK and L3P-BLK in primary B cells from patients and healthy controls, were unfortunately unsuccessful due to the relative small cytosol volume present in primary B cells.

In mice, functional redundancy between Src kinase family members is apparent as a single Src kinase member-knockout mice has no or subtle deficiencies[10;41;42]. Only when several Src family members are deleted, in double or triple knock outs, major defects are observed; SRC/YES and SRC/FYN are lethal whereas HCK/FGR double knock-out mice are immune-compromized[10]. In the human, Src-kinase family members are less superfluous [43]. Especially BLK seems to be non-redundant in function, as BLK is the only Src kinase family member that is able to phosphorylate and subsequently associate with co-transfected Igα and Igβ chimeras *in vivo*[17]. We believe this to be the reason that the

CVID-associated BLK mutation has functional consequences.

Diminished B cell proliferation and T cell help is associated with reduced numbers of class-switched memory B cells and defective production of high affinity antibodies, as showed for CD20[2;31], CD21[32], CD81[6], ICOS[8], and CD40L[34] deficient CVID patients. In addition, selective CVID patient T cells have a reduced T cell responses to tetanus toxoid, even though primary allo-stimulation of the same T cells was normal in CVID patients[35]. Moreover, reduced $CD4^+$ T cell numbers are reported in several CVID patients[37]. All these data support that defective elicitation of $CD4^+$ T helper cell help may contribute or even cause pathology in a subset of CVID patients. In line with this, our CVID patients that also show reduced numbers of class-switched memory B cells and defective production of high affinity antibodies carry a L3P-BLK variant that distort BCR signaling required for B cell proliferation and recruitment of T cell help. We propose that dysfunctional BLK variant underlies CVID disease pathology by perturbing B cell proliferation and elicitation of antigen-specific $CD4^+$ T cell help. Further research should be aimed to determine the proportion of CVID patients that harbor defects in BLK or other early B cell activation-related signaling molecules, and how gene defects overall relate to distinct B cell functions as antigen presenting cells and Ig-secreting plasma cells.

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Supplemental figures

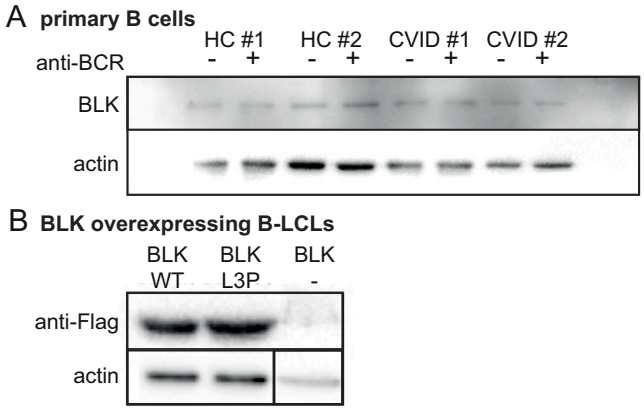

Figure S1. BLK protein expression. (A) Expression of endogenous BLK protein in primary CD19⁺ B cells of healthy or L3P-BLK CVID patients, or (B) common and L3P variant construct-derived BLK protein, as determined by western blots.

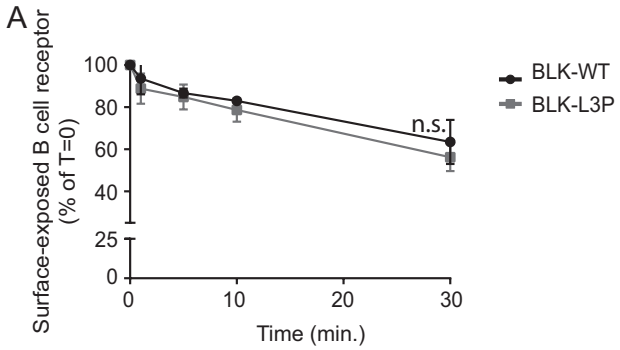

Figure S2. Ligand-induced B cell receptor internalization. (A) Surface exposed B cell receptor upon cross-linking with anti-IgG antibodies over time on B-LCLs expressing either L3P- or common BLK variant. *P-value <0.05, **P-value <0.01, Two-tailed Wilcoxon-signed rank test.

Chapter 9.

Defective calcium signaling and disrupted CD20–B cell receptor dissociation in Common Variable Immunodeficiency Disorders.

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Abstract

BACKGROUND: B cells of patients with common variable immunodeficiency (CVID) disorders display impairment in production of immunoglobulin class-switched antibodies, which is possibly contributed to by defects in early B-cell activation. On resting B cells, B-cell receptors (BCRs) are organized in oligomers that are signaling inactive. Their triggering by cognate antigen causes the lateral reorganization of BCRs and associated proteins into signalosomes, resulting in BCR-activated calcium entry. In resting cells the B-cell surface antigen CD20 is associated with the BCR but dissociates on signalosome formation.

OBJECTIVE: We sought to determine whether CD20 dissociation from the BCR during early B-cell activation might contribute to the development of CVID disorders.

METHODS: We evaluated BCR signalosome formation, internalization, and signaling in primary B cells of pediatric patients with CVID disorders and healthy control subjects.

RESULTS: In many pediatric patients with CVID disorders, B cells exhibit significant deficits in BCR triggering-mediated calcium entry in the cytosol, which correlates with impaired plasmablast differentiation in vitro. These alterations did not originate from upregulation of CD22 or defects in calcium channels and did not involve gene mutations in phospholipase Cγ2 or Bruton tyrosine kinase. Instead, B cells from patients with CVID disorders exhibited reduced BCR dissociation from CD20. BCR or CD20 cross-linking induced less BCR internalization, and antibody-mediated CD20 triggering elicited less BCR downstream signaling, as measured based on secondary fluxes.

CONCLUSIONS: We propose that CD20 dissociation from the BCR signalosome is pivotal to BCR-mediated calcium mobilization in the cytosol. Defects in CD20/BCR signalosome conformation might predispose to the spectrum of CVID disorders.

Introduction

Common variable immunodeficiency (CVID) is a heterogeneous immunodeficiency characterized by defective antibody production.[1] Sporadic gene mutations have been described,) but disease etiology remains unknown for 90% of patients.[3] Deficiency of CD81,[4] CD21[5], and CD20[6] led to CVID-like disorders with hypogammaglobulinemia and antibody synthesis deficits. Mutations in the BCR co-receptor complex, combined with abrogated B cell effector functions, suggested the possibility of defective B cell activation in CVID or CVID-like disorders.

B cells express up to 120,000 inactive B cell receptors (BCR).[7] Ligation of cognate antigen induces the reorganization of signaling active BCR oligomers, or signalosomes, that provoke elevation of intracellular calcium ion concentrations.[8] Binding of the co-receptor complex (CD19, CD21, CD81 and associated proteins) results in CD19 phosphorylation and recruitment of Src-family tyrosine kinases and PI-3-kinase. This results in activation of phospholipase Cɣ2 (PLCɣ2), which via generation of inositol 1,4,5-trisphosphate 3 (IP_3) and binding to IP_3 receptors at the endoplasmic reticulum (ER) membrane facilitates calcium release from ER stores into the cytoplasm. Consequently, calcium enters the cell via store-operated calcium entry channels. We proposed that early defects in BCR-mediated calcium signaling may cause B cell dysfunction in CVID disorders. We investigated CD20 that is almost exclusively expressed on B cells.[9] While its role in B cell function is not fully clear, CD20 is a direct regulator or component of a calcium channel.[10;11] CD20 forms homo-oligomers that physically associate with the BCR,[12] and CD20 antibody treatment induces BCR-mediated signal transduction, including calcium flux and resulting in similar transcription patterns as BCR triggering.[13;14] Following BCR ligation, CD20 oligomers dissociate and remain on the cell surface, while the BCR and its accessory molecules are endocytosed.[12;15] CD20 deficiency causes CVID-like disease.[6] Furthermore, B cells of a subgroup of CVID patients exhibit defects in calcium influx induced by BCR signaling[5].

We here report defective BCR-mediated calcium signaling in B cells of numerous pediatric CVID disorder patients. Afflicted patients exhibited a reduction in cytosolic calcium mobilization upon BCR crosslinking. Rates of calcium mobilization in fresh CVID disorder B cells correlated with B cell differentiation efficiency *in vitro*. While it had been known that B cell activation induces plasma membrane dissociation of the BCR from the CD20 for induction of calcium fluxes, this phenomenon had not yet been associated with a human disease. We show defects in BCR internalization and calcium mobilization in our cohort of CVID disorders when induced by crosslinking of either CD20 or BCR. Thus, in selected patients, CVID disorders may involve defects in dissociation of the BCR from surface-retained CD20 during BCR-mediated B cell activation.

Materials and methods

Patients and healthy donors

Forty-two pediatric patients (mean age 12 years, range [4 -18]) with CVID disorders, fourteen healthy children (9 yrs [4-13]) and 23 adult volunteers (29 yrs [23-40]) were included; detailed information is available in the Online Repository. This study was approved by the institutional review board and informed consent was obtained.

Patients

Of the 42 patients, 23 were diagnosed with CVID according to the ESID criteria.[E1] All but two newly diagnosed patients received immunoglobulin replacement therapy. Nineteen did not completely meet these CVID criteria and were therefore diagnosed with 'CVID-like' disease; defined as selective antibody deficiency (defined as the inability to produce protective titers of specific antibodies upon vaccination to polysaccharide and/or to recall antigens), combined with low IgA, low IgM, and/or low IgG subclass levels, or a solitary decreased IgG. 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 (e.g. iatrogenic or due to enteral protein loss) were ruled out in all patients. Previous studies have shown that pediatric CVID-like patients as described above are clinically and immunologically (e.g. B and T cell phenotype characterization) comparable to definite CVID patients.[E2;E3] As the current flow cytometry-based CVID classifications are not applicable to children,[E2] we did not attempt to classify them. Five children had however already developed disease-related complications as described by Chapel *et al.*[E4] These were enteropathy, autoimmune hemolytic anemia (3x), idiopathic thrombocytopenic purpura (3x), autoimmune nephritis, hepatosplenomegaly with extensive lymphadenopathy, lymphoid interstitial pneumonia, systemic lupus erythematosus and vitiligo.

Ten healthy pediatric controls were asked to donate blood when elective surgery was performed. Leftover peripheral blood (1 mL) was used from four healthy pediatric controls of another approved study in our lab. All adult volunteers were healthy employees of the University Medical Center Utrecht.

Genetic analysis of PLCG2 and BTK

Granulocytes were isolated from peripheral blood with the use of Ficoll gradient centrifugation and subsequently genomic DNA was isolated using the autopure kit (Qiagen). Exon 22 of the *PLCG2* gene (NM_002661.2) and exon 18 of the *BTK* gene (NM_000061.2) were directly sequenced after PCR on an ABI 3100 automated sequencer (PE Applied Biosystems, Foster City, CA, USA) and a 3130XL genetic analyzer using the Applied Biosystems BigDye Terminator v1.1 cycle sequencing kit according to the manufacturer's protocol. Analysis was done using the SeqScape v2.5 (Applied Biosystems) and Mutation Surveyor software (Softgenetics, LLC, State College, PA, USA).

PCR conditions and primer sequences are available upon request.

B cell cultures

PBMC were thawed quickly, washed twice and resuspended in RPMI (Gibco) containing 10% fetal calf serum, 1% penicillin and streptavidin and 1% glutamine in a 24-well plate for 24 or 48 hours in with following stimuli: PMA (50ng/mL) and ionomycin (2ug/mL, Calbiochem, San Diego, CA) as a positive control, anti-IgM F(ab')$_2$ fragments (20 ug/mL, Jackson Immunoresearch, West Grove, PA) and anti-IgM F(ab')$_2$ fragments (10ug/mL) with IL-4 (200 U/mL, Immunotools, Friesoythe, Germany). After 24 hours, cold FACS buffer was added and cells were washed twice. After Fc receptor blocking with mouse serum, cells were stained for CD19, CD80 and CD86 (all Becton Dickinson) for 30 minutes at 4 ºC. After washing twice, cells were acquired on FACS Canto II Flow Cytometer and analyzed using FACS Diva software (both Becton Dickinson). After 48 hours, cells were lysed and total mRNA was isolated using Tripure isolation reagent (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions, and reverse using an iScript cDNA synthesis kit (Biorad, Hercules, CA). Primers were mixed with IQ SYBR green supermix (BioRad). The detection run started at 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min. Assays were performed in triplicate on a C1000 Thermal Cycler (BioRad). Results were normalized to endogenous GAPDH mRNA and the unstimulated sample ($2^{-\Delta\Delta CT}$). The following primers were used: GAPDH Forward: 5'-GTCGGAGTCAACGGATT-3'; GAPDH Reverse: 5'-AAGCTTCCCGTTCTCAG-3'; NFAT Forward: 5'-GCAGAGCACGGACAGCTATC-3'; NFAT Reverse 5'-GGGCTTTCTCCACGAAAATGA-3' (All Sigma-Aldrich).

Flow cytometry and functional assays

Besides previously described phenotyping,[16;17] peripheral blood mononuclear cells (PBMC) were stained with antibodies to CD19, CD20, CD21, CD22, CD27, CD38, CD45 (all Becton Dickinson), IgM F(ab')$_2$ and Igλ F(ab')$_2$ (Southern Biotechnology Associates, Birmingham, AL). Samples were acquired on a FACS Canto II Flow Cytometer and analyzed using FACS Diva software (both Becton Dickinson).

For calcium flux assays, one million fresh PBMC were loaded directly with 4 µM Fluo-3, 10 µM Fura Red (Invitrogen, Carlsbad, CA) and later, CD19-APC was added. Cells were washed twice and resuspended in Hank's balanced salt solution or alternatively, Ca^{2+}-free phosphate buffered saline, both supplemented with fetal calf serum (FCS). After measuring baseline cytosolic calcium levels, 20 µg/mL anti-IgM F(ab')$_2$ fragments (Jackson ImmunoResearch, West Grove, PA) were added and calcium influx was measured for 5 min. Subsequently, 2 µg/mL ionomycin (Calbiochem, San Diego, CA) was added. To study CD20, PBMC were incubated with 10 µg/mL anti-CD20 mAb rituximab[(18)] (Roche Diagnostics, Mannheim, Germany) for 30 min. Epstein-Barr virus (EBV) transformed lymphoblastoid B cell lines (LCL) were generated.[19] ER Ca^{2+} storage capacity was assessed

with ionomycin (0.1µg/mL) or thapsigargin (10 µM, Santa Cruz Biotechnology, Santa Cruz, CA) in Ca^{2+}-free medium. B lymphocyte differentiation assays were performed as described previously.[16]

Confocal microscopy

Control and CVID disorder LCL were sorted for high expression of IgM and CD20. Two million B cells were adhered on a coverslip coated with 1% alcian blue (Klinipath bv, Geel, Belgium). Cells were stained at 0°C for IgM BCR and CD20 with 1:100 anti-human IgM-Cy3 $F(ab')_2$ fragments (Jackson ImmunoResearch) and 1:10 anti-human 2H7-Alexa Fluor 647 (BioLegend, San Diego, CA). Cells were washed 3 times and resuspended in 1640 RPMI without phenol red, containing 10% FCS and 10 mM HEPES buffer. Cells were kept at 0°C and imaged on a 710 Laser Scanning Microscope Meta (Carl Zeiss MicroImaging GmbH, Germany). BCR stimulation was initiated by quickly increasing the temperature to 37°C and supplementing additional anti-IgM. Imaging was continued for 30 min at 37°C. Using ZEN 2009 Software, the percentage of colocalization was calculated as described previously.[15]

Statistical analysis

Differences in nonparametric data were assessed by comparing the medians of groups using Mann-Whitney *U* tests or Kruskal-Wallis tests, followed by post hoc Dunn's comparisons. The effect of αCD20 was assessed with paired t-tests and correlations were calculated with Pearson correlation. Tests were performed two-tailed. Data were analyzed using SPSS 15.0 for Windows (SPSS Inc.; Chicago, IL). Areas under curve were calculated using Graphpad Prism 5.0 software (La Jolla, CA).

Results

Pediatric CVID disorder B cells are reduced in memory subsets but display a normal surface phenotype

CVID and CVID-like children had lower percentages of memory B cells than both control groups: $CD27^+IgM^-$ Ig class-switched memory B cells, median ±interquartile range, healthy adults 14.6% ±15.8; healthy children 7.1% ±5.3; CVID disorders 4.4% ±4.5, p=0.01; $CD27^+IgM^+$ memory cells, healthy adults 25.5% ±23.4; healthy children 11.2% ±13.3; CVID disorders 7.9% ±9.1, p=0.001. No differences were observed in $CD38^-CD21^{low}$ B cell populations between patients and controls, although in two patients with active autoimmune disease we noticed expansion of $CD21^{low}$ cells. (Fig S1) CD19 and IgM BCR expression on individual B cell subpopulations were comparable between patients and controls (Fig S2, A); IgM expression was slightly increased on $CD27^+IgM^+$ B cells in CVID disorders compared to healthy children (Fig S2, *B*). We observed no significant differences in CD20 and CD22 expression. (Fig S2, *C and D*) In line with previous studies, we found no

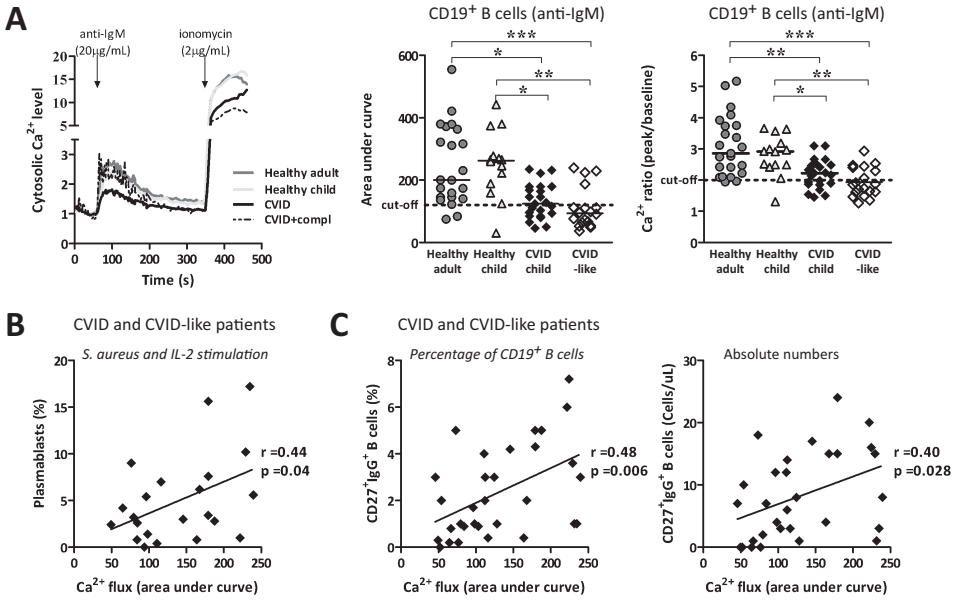

Figure 1: Decreased calcium mobilization in CVID disorders B lymphocytes.

A, Ca^{2+} mobilization kinetics (i), area under curve (ii) and ratios (iii). Cut-off levels are at a level above which 90% of the healthy controls are positive. **B,** B cell differentiation correlated with Ca^{2+} mobilization in CVID disorders. **C,** Percentages (i) and absolute (ii) numbers of IgG^+ memory B cells correlated with BCR-mediated calcium influx in age-corrected CVID disorders (n=30). Pearson's correlations. * p<0.05; ** p<0.01; *** p<0.001.

significant differences between CVID and CVID-like patients.[16] In conclusion, all subjects had a substantial IgM^+ B cell population that displayed a normal surface phenotype.

Impaired BCR crosslinking-induced calcium mobilization and defective B cell differentiation in vitro in CVID disorder B cells

We analyzed the ability of fresh B cells to mobilize calcium upon activation. Baseline calcium levels of B cells were similar in all groups. Upon αIgM $F(ab')_2$-mediated BCR crosslinking, B cells from both CVID and CVID-like children exhibited reduced calcium mobilization, as measured by area under curve (AUC). (Fig 1, *A* AUC median ±interquartile range, healthy adults 200 ±221; healthy children 263 ±120; CVID 124 ±95; CVID-like 93 ±69, p<0.001) Initial peak influx was mainly decreased, while further kinetics patterns appeared unaffected. Indeed, calcium ratios (peak/baseline)[5] were also decreased compared to controls (calcium ratio healthy adults 2.9 ±1.5; healthy children 2.9 ±0.8; CVID 2.2 ±0.6; CVID-like 1.9 ±0.8, p<0.001). We could not classify patients with a decreased calcium flux into certain subgroups, but noticed that patients with autoimmunity or lymphoproliferation had relatively high calcium mobilization. (Fig 1, A and S1, B) Since there were no differences between CVID and CVID-like patients, they were combined in further experiments and referred to as 'CVID disorders'.

Restimulation of αIgM $F(ab')_2$-treated B cells with ionomycin, which acts directly on

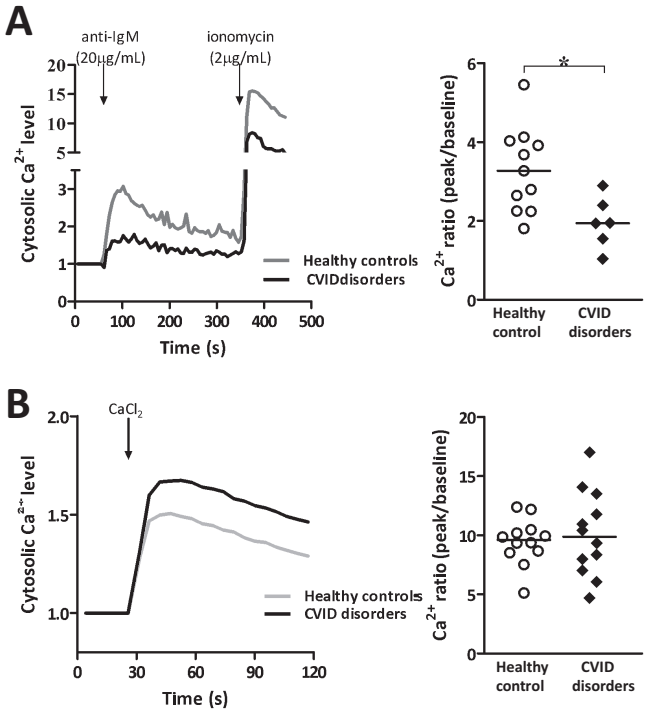

Figure 2: Plasma membrane calcium channels function in CVID disorders B cells

A, ER Ca^{2+} release upon anti-IgM in calcium-depleted medium; averaged kinetics plot (i) and Ca^{2+} ratios (ii) in B cells of 11 controls and 6 CVID disorders patients. B, $CaCl_2$ addition induces Ca^{2+} influx in patient and control LCL (both n=12). Lines indicate medians. Mann-Whitney *U* test, * p<0.05

IP_3 receptors, induced sub-optimal subsequent calcium mobilization in CVID B cells. (calcium ratio healthy adults 17.2 ±4.8; healthy children 19.4 ±6.6; CVID 11.5 ±5.5; CVID-like 10.4 ±10.0, p<0.001) In contrast, $CD19^-$ CVID disorder lymphocytes showed fluxes comparable to control $CD19^-$ lymphocytes (data not shown).

We asked whether decreased calcium mobilization could lead to subsequent B cell defects. BCR-mediated calcium signals correlated significantly with *in vitro* differentiation into plasmablasts upon stimulation with IL-2 and *Staphylococcus Aureus* Cowan strain I antigen, which engages the BCR (amongst other receptors), and elicits a T-independent B cell response,[20-22](Fig 1, *B*,) and with proportions of IgG^+ memory B cells. (Fig 1, *C* and table EI) BCR-mediated upregulation of costimulatory molecules and nuclear factor of activated T cells (NFAT) gene transcription[23] was comparable between patients and controls. (Fig S3) Thus, CVID B cells exhibit a selective deficit in BCR-mediated calcium mobilization, which positively correlates with decreased plasmablast differentiation *in vitro* and class-switched memory B cell formation or maintenance *in vivo*, although these findings do not necessarily establish a causal relation.

Defective calcium mobilization is not caused by defects in plasma membrane channels, ER calcium storage release, and BCR signaling molecules Btk or PLCγ2

Plasma membrane calcium channel function was evaluated. Lacking exogenous calcium influx, AUC (and calcium ratios) remained significantly lower in CVID disorders compared to controls (Fig 2, *A*, AUC controls 276 ±124; CVID disorders 83 ±223, p=0.02). Calcium-depleted CVID disorder LCL generated from CVID patients showed similar transmembrane calcium influx upon $CaCl_2$ supplementation as did control LCL (Fig 2, *B,* AUC control 37 ±19; CVID disorders 47 ±53, p=0.3).

To investigate ER calcium release, LCL were treated with ionomycin or thapsigargin (Fig S4). Thapsigargin inhibits SERCA pumps, which maintain ER calcium stores.[24] Both treatments led to a similar pattern of calcium release into the cytosol between patient and control LCL. To survey the function of BCR signaling cascade molecules, relevant tyrosine phosphorylation sites of *BTK*[25] and *PLCG2*[26;27] were sequenced in sixteen patients exhibiting reduced calcium ratios, but no mutations were found (see Online Repository). Taken together, protein function of Btk and PLCγ2, as well as ER calcium homeostasis and plasma membrane channel function appeared intact in our CVID disorder B cells. Henceforth, we focused on immediate BCR activation events in the plasma membrane.

Contribution of CD20 to activation-induced calcium mobilization defect in CVID disorder B cells

BCR and CD20 constitutively partly colocalize at the B cell plasma membrane (Fig 3, *A* and [15]). CD20 mAb treatment specifically increased cytosolic calcium levels in healthy and CVID disorders B cells after 30 minutes (Fig 3, *B*). Additionally, CD20-specific mAb ligation caused internalization of the BCR/co-receptor complex, as noticed by significant reduction in surface expression of Igλ light chains, CD21 and CD19, but not CD45, at 37°C but not at 4°C (Fig 3, *C* 37°C*,* and data not shown).

We next asked whether defective lateral dissociation of BCR from CD20 could explain defective calcium mobilization in CVID disorders. B lymphocytes were pre-treated with CD20-specific mAb for 30 min to induce CD20 crosslinking-induced B cell activation, as shown previously and in figure 4B.[13;28] The effectiveness in BCR/CD20 dissociation was measured by inducing a secondary calcium flux upon BCR stimulation. We found that healthy but not patient B cells were hyporesponsive to subsequent BCR triggering, reflecting that initial elicitation of the BCR-signaling pathway by anti-CD20 was productive in healthy but not CVID disorder B cells (Fig 4, *A*; healthy control calcium ratio only αIgM 2.2 ±1.1; with αCD20+αIgM 1.3 ±0.35, p=0.004; CVID disorders only αIgM 1.8 ±0.47; αCD20+αIgM 1.4 ±0.61, p=0.16). Direct triggering of CD20 exhausted subsequent receptor-mediated calcium fluxes in healthy but not patient B lymphocytes. Further, CD20-mAb decreased surface expression of CD19 in controls (CD19 MFI*10^3 without αCD20 4.78 ±1.65, with αCD20 2.00 ±1.07, p<0.001), but not in patients (without αCD20 2.87 ±1.24, with αCD20 1.68 ±0.56, p=0.08) (Fig 4, *B*).

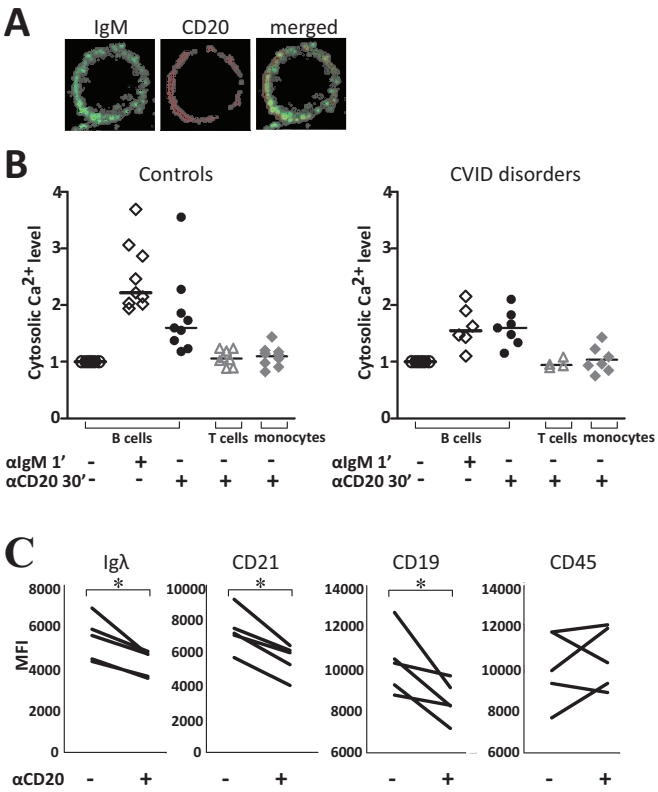

Figure 3: Anti-CD20 induces calcium influx and BCR/co-receptor internalization

A, Colocalization of IgM *(green)* and CD20 *(red)* in resting B cells. **B,** Anti-CD20 triggers a specific Ca^{2+} flux in healthy (i) and CVID disorder (ii) B lymphocytes. **C,** Anti-CD20 treatment of healthy B cells leads to selective internalization of molecules clustered in the BCR/co-receptor complex. Paired t-test, * $p<0.05$.

We hypothesized that CVID disorder pathogenesis may involve defects in the activation-induced disassembly of CD20 from the BCR signalosome,[12] and visualized CD20 and BCR plasma membrane mobility upon BCR crosslinking on IgM^+ LCL cells. Upon BCR-crosslinking, dissociation and subsequent internalization of the BCR was observed, while CD20 remained at the cell surface.[12;15] This process seemed less effective in the CVID disorder cells (Fig 4, *C*, and Fig E5). In conclusion, we show that calcium signaling is disturbed in CVID disorder B cells, and can be contributed to defective dissociation of the BCR from CD20 at the plasma membrane. Our findings suggest defective kinetics of the disassembly of BCR signalosomes to be a contributing factor in the development of CVID disorders.

Discussion

We describe a selective defect in calcium mobilization in B lymphocytes observed in numerous pediatric CVID disorder patients, which correlates with abrogated plasmablast

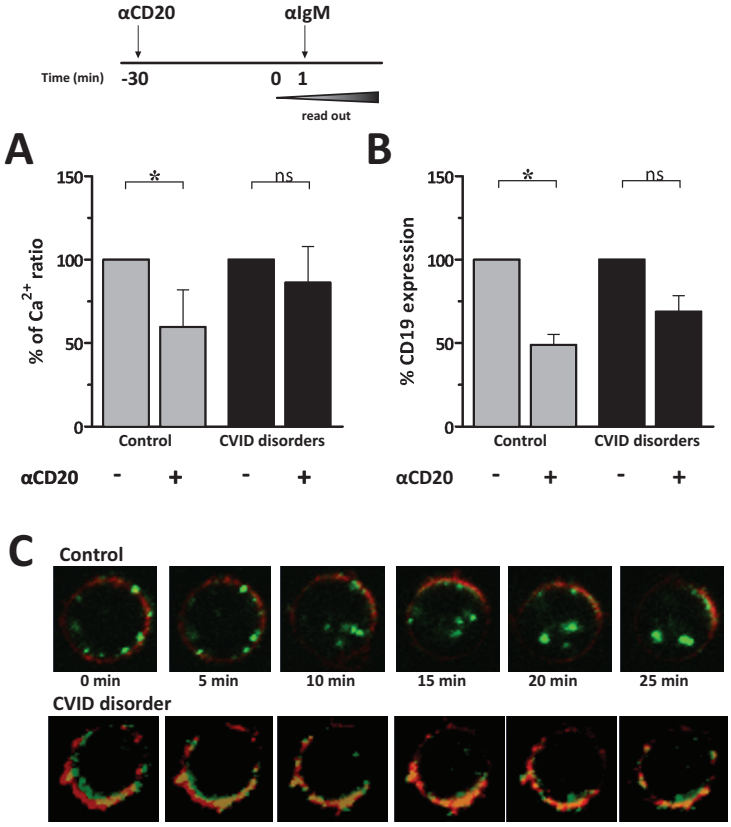

Figure 4: Anti-CD20 does not exhaust BCR-mediated calcium mobilization in CVID disorders

A-B, Anti-CD20 pre-treatment decreases Ca^{2+} mobilization *(A)* and CD19 surface expression *(B)* in primary B cells of 10 controls and 8 patients. Mean ± SEM. Paired t-test, * $p<0.05$ **C,** IgM BCR *(green)* and CD20 *(red)* dissociate upon anti-IgM $F(ab')_2$ stimulation of control and CVID disorder LCL.

development *in vitro* and decreased numbers of class-switched memory B cells *in vivo.* We show that CD20 dissociation from the BCR/co-receptor complex on B cells is required for activation-induced calcium signaling and propose that failing of this process may contribute to development of CVID disorders.

Calcium fluxes are pivotal for lymphocyte function, and cytosolic calcium levels are tightly regulated. Calcium signaling influences cell-fate choices by differential regulation of transcription factor pathways including NFAT and nuclear factor kappa B (NFκB).[23;29;30] We found that decreased calcium mobilization correlated with decreased B cell differentiation into plasmablasts upon BCR stimulation, and with decreased fractions of IgG^+ memory B cells in peripheral blood. These findings to date cannot establish a causal relation, and other factors may be involved. Nevertheless, they suggest an important role for BCR-mediated calcium mobilization in the development or maintenance of end-stage B cells. Defective B cell activation could explain the B cell abnormalities characteristic of CVID disorders. B cell calcium mobilization was defective in a larger proportion of

our pediatric cohort than was previously described in an adult CVID population, and we did not find an increased expression of the inhibitory B cell co-receptor CD22.[5] Calcium fluxes were measured in fresh unfractionated $CD19^+$ B cells. We asked whether decreased calcium fluxes in CVID patients could be explained by differences in composition of the B cell compartment but confirmed that CVID patients have a relatively larger IgM^+ B cell compartment than healthy controls.[16;17] Moreover, calcium mobilization was reduced in all post-transitional B cell subpopulations in adult CVID,[5] and our decreased Ca^{2+} ratios did not correlate with percentages of IgM^+ B cells (our unpublished observations).

We tested plasma membrane calcium channel and ER calcium storage capacity upon cellular activation, and found them in CVID B cells to be comparable to controls. These features are conserved between lymphocytes and defects would likely cause a more severe, combined immunodeficiency.[31;32] Nevertheless, we cannot completely exclude the possibility that sporadic CVID disorder patients have smaller defects in less pertinent or yet unidentified channels, or in BCR-specific phosphotyrosine kinases. As key residues of PLCγ2 and Btk were not mutated in calcium signaling-affected cells, it is unlikely that these will be etiologic factors in a considerable proportion of the CVID population. Complete dysfunction of such molecules usually leads to negligent numbers of peripheral B cells and agammaglobulinemia,[33-35] which was not seen in this cohort.

The plasma membrane undergoes dynamic remodeling during B cell activation.[15;36] We hypothesized this remodeling to be defective. CD20 homo-oligomers associate with the BCR in resting cells,[15;28;37] but BCR stimulation causes dissociation of the BCR from CD20.[(12)] Direct triggering of CD20 induces calcium flux, which requires association of CD20 with the BCR, and is mediated via the BCR signaling.[13;14] CD20 ligation on healthy B cells triggered a calcium flux that exhausted the BCR signaling cascade; ensuing BCR-triggering scarcely induced subsequent signaling. These findings support recent work, describing that αCD20 pre-treatment results in a time-dependent inhibition of the BCR signaling cascade in healthy human B cells.[18] In contrast, in CVID disorder B cells, αCD20 pre-treatment triggers an initial calcium flux, but does not yield complete BCR-mediated signaling, as further residual calcium signaling can be elicited when BCRs are restimulated after 30 min (Fig. 5).

We propose that upon CD20 and/or BCR triggering, BCR-CD20 signalosomes induce downstream signaling as described above and elsewhere, followed by rapid dissociation at the cell surface, where CD20 remains while the BCR/co-receptor complex is endocytosed,[12;15] and triggering calcium signaling in the cytosol.[13;18] We first stimulated with anti-CD20 and subsequently added anti-IgM. We chose this order, as anti-CD20 treatment requires more time than anti-IgM to elicit calcium fluxes, as measured in real time by flow cytometry. However, as both antibodies trigger the same signaling route, either order of (re)stimulation should yield similar results. Our data suggests this synergistic mechanism to be impaired in a subgroup of CVID disorder patients, rendering B cells less capable to resolve CD20/BCR oligomers, resulting in reduced BCR/co-receptor

internalization and decreased calcium signaling. It remains to be identified which genes are involved in this process.

In conclusion, we found a calcium mobilization defect in B lymphocytes of children with CVID disorders, which we propose results from altered dissociation of the BCR from CD20. Other additional signalosome-associated proteins may be involved. Further studies are required to address the molecules involved in CD20-associated BCR-receptor signaling, the underlying genetic defects leading to the CVID disorder phenotypes, for elucidation of possible intervention points for B cell activation targeted therapy.

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Supplemental Figures.

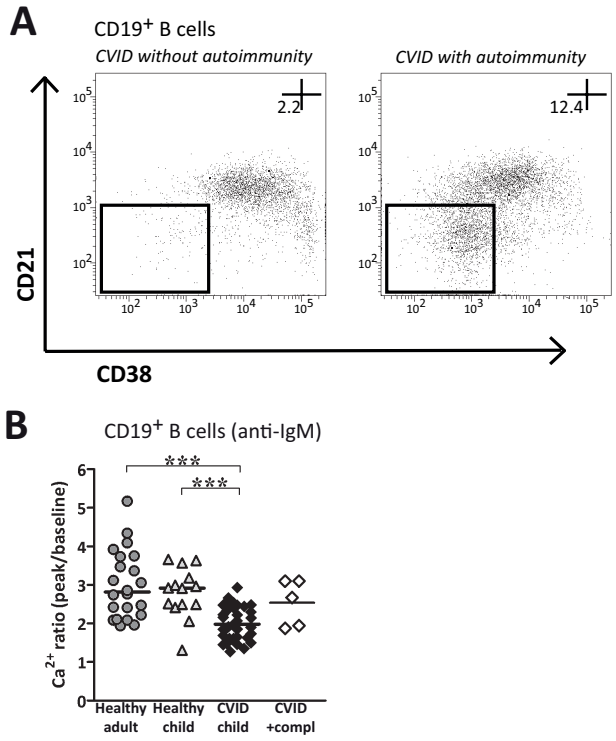

Fig S1. Characteristics of patients with CVID disorders with disease-related complications.

A, Autoreactive $CD21^{-}CD38^{-}$ B-cell numbers are increased in patients with CVID with autoimmune complications *(right)* compared with numbers seen in patients without autoimmunity *(left)*. **B,** Patients with CVID disorders with autoimmune or lymphoproliferative complications had greater calcium mobilization than patients without these complications. Kruskal-Wallis with *post hoc* Dunn comparisons: $***P < .001$.

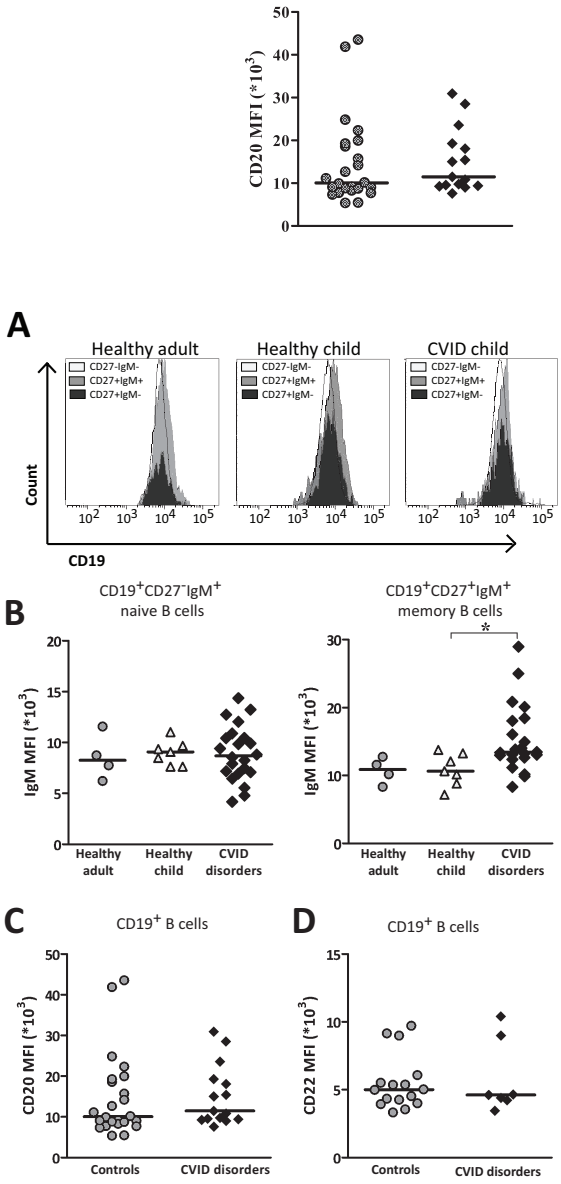

Fig S2. Expression of B-cell surface molecules in patients with CVID disorders.

A, CD19 expression in 5 adults, 10 healthy children, and 34 children with CVID disorders. **B,** Mean fluorescence intensity **(MFI)** of IgM on naive B cells and memory B cells. **C** and **D,** CD20 (Fig E2, **C**) and CD22 (Fig E2, **D**) expression in patients and control subjects. Kruskal-Wallis with **post hoc** Dunn comparisons: ∗**P** < .05.

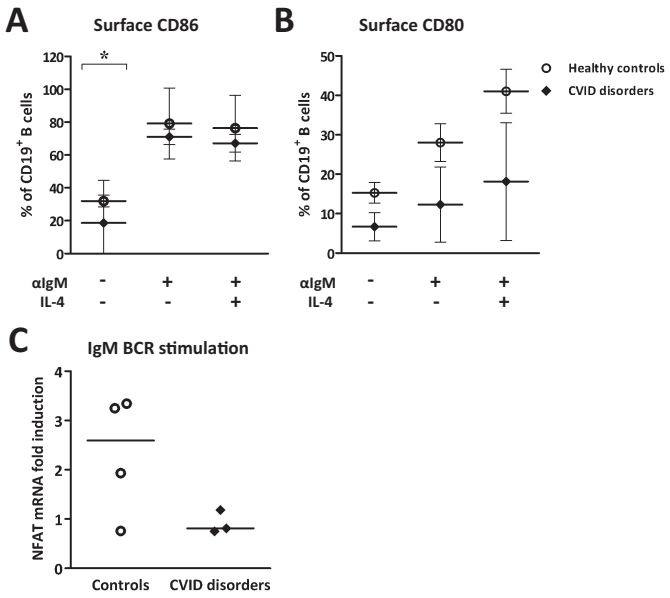

Fig. S3. BCR-mediated upregulation of costimulatory molecules.

Percentages of $CD80^+$**(A)** and $CD86^+$**(B)** B cells after 24 hours of culture. Means ± SEMs of 16 control subjects and 7 patients. Mann-Whitney **U** test: ∗**P** < .05. **C,NFAT** gene transcription in PBMCs of 4 control subjects and 3 patients after 48 hours of stimulation with anti-IgM $F(ab')_2$ fragments.

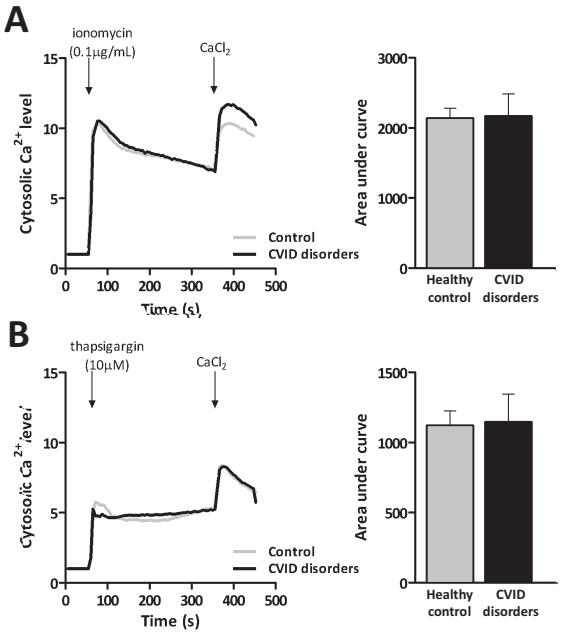

Fig. S4. ER calcium mobilization and storage capacity in B lymphocytes from patients with CVID disorders.

Kinetics of ER storage Ca^{2+} release after ionomycin stimulation **(A)** or thapsigargin depletion **(B)** in calcium-depleted medium, as demonstrated by an averaged kinetics plot **(left)** and AUC **(right)** in LCLs from 6 control subjects and 6 patients with CVID disorders. **Bar graphs** represent means ± SEMs.

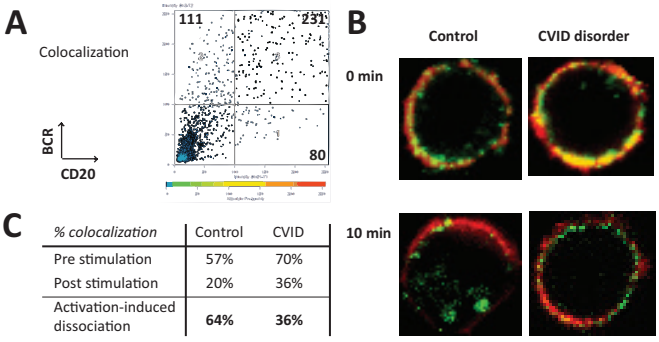

Fig. S5. Impaired BCR-induced BCR/CD20 lateral dissociation in patients with CVID disorders.

A, Colocalization was calculated by dividing double-positive pixels (BCR/CD20) by the positive pixels within the BCR image. **B,** LCLs from control subjects **(left)** and patients with CVID disorders **(right)** before **(upper panel)** and after **(lower panel)** BCR stimulation. **C,** Percentage of dissociation on BCR triggering based on greater than 25 cells per donor in 2 experiments.

Chapter 10.

General discussion.

Antigen presentation plays a central role in the initiation of adaptive immune responses, leading to antigen-specific and long-lasting immune protection. In fact, antigen presentation underlies our current vaccination strategies. The widespread success of classical (humoral immunity-based) vaccination illustrates the strength of immune protection by the initiation of adaptive responses. These achievements stimulated the use of vaccination strategies also in other fields, such as anti-viral and anti-tumor therapy. To this end, strategies such as dendritic cell (DC)-vaccinations are explored. In an autologous setting, DC vaccination has showed some beneficial effects[1,2]. However, in the last 15 years over 100 clinical trials with DC vaccination strategies involving more than 1000 patients were performed of which most have been unsuccessful (**chapter 2**). For example, DCs laden with HCMV-derived pp65 induced pp65-specific $CD8^+$ T cells responses in only 5 out of 24 patients receiving allogeneic stem cell transplantation, with risk to HCMV[3]. However, this lack of clinical effectiveness for most DC vaccination strategies should not be interpreted as a 'dead end', instead it emphasizes the need for further research and optimization.

A multitude of approaches to improve DC vaccination strategies are currently under investigation. This thesis aims to contribute to optimization of vaccination particularly, by increasing our understanding of endosomal processes involved in antigen presentation.

Endosomal compartments are central to antigen presentation.

In general, there are two ways to introduce antigenic protein in a cell-based vaccination strategy. Either nucleic acids (DNA/RNA) are introduced that enables endogenous expression of antigens, or exogenous antigens are administered to the cells. The latter is regarded more efficient as a number of studies have shown that immunization of mice by DCs pulsed with tumor antigens can prime a tumor-specific cytotoxic $CD8^+$ T cell response and protective anti-tumor immunity [4–6]. Hence, most DC vaccination strategies to date administer exogenous antigen (**chapter 2**). Administration of exogenous antigen results in class II MHC or antigen cross-presentation. For antigen presentation, in short, peptide-class I or II MHC complexes are presented in appropriate context by antigen presenting cells (APCs) for activation of antigen-specific T cells. This requires antigen uptake into intracellular transport vesicles named endosomes, followed by antigen degradation into appropriately sized peptides, incorporation into peptide-receptive MHC molecules, and finally presentation of these complexes in context of co-stimulatory molecules. In case of class II MHC, antigenic peptides are formed by limited proteolysis of endosomal cargo by endosomal proteases[7]. Peptide-receptive class II MHC are most of the time newly synthesized class II MHC molecules complexed with an invariant chain (Ii), that is progressively removed by proteolytic activity. Subsequently, HLA-DM catalyzes the loading of antigenic peptides into the class II MHC groove[8]. In professional APCs, such as immature DCs, late endosomes (LEs) are seen as the major contributor of peptide-class II MHC complexes as they contain 60% of cellular class II MHC molecules[9]. LEs with

accumulated class II MHC molecules are named MIICs. This compartment is observed as multivesicular bodies surrounded by delimiting membranes. They are part of the normal array of LEs and lysosomes comparable to those in non-APCs[10]. However, next to class II MHC, they also accumulate HLA-DM and their pH is optimal for most endosomal proteases. Interfering with endosomal processes hampers antigen presentation. For example, interfering with pH-sensitive endosomal processing by adding strong base Chloroquine inhibits antigen cross-presentation[11]. This demonstrates the crucial role endosomes play in antigen presentation as shown in **chapter 6** of this thesis[12]. In addition to LE/MIICs also lysosomes[13] and recycling endosomes[14,15] have been implicated in class II MHC peptide loading.

For antigen cross-presentation, formation of class I MHC-peptide complexes is a bit more multifaceted as it may obtain antigen derived of proteasome-dependent or proteasome-independent pathways in distinct cellular organelles. As described in the thesis introduction, classical class I MHC peptide loading occurs in the ER in a TAP/proteasome-dependent pathway. As the bulk of proteasome substrates consists of polypeptides that are rapidly degraded after synthesis (half life <10 min), these polypeptides are the principal source of viral and self-peptide ligands for class I MHC antigen presentation[16,17]. Especially defective ribosomal products appear to be a major source of rapidly degraded polypeptides, enabling immune surveillance of infectious pathogens and tumors[17].
In parallel, exogenous antigen-derived peptides can also be loaded onto class I MHC molecules. This process, named antigen cross-presentation, enables a cytotoxic $CD8^+$ T cell immune response even when APCs are not infected by the pathogens. Introducing exogenous antigen in the ER may occur directly by ER-phagosome fusion[18] or indirectly via endosome-to-cytosol and subsequent TAP-mediated antigenic peptide translocation into ER[19,20]. However, so far no mechanism has been identified to restrict ER peptide-loading to exogenous antigen-derived peptides. This implies that a small amount of antigenic peptides during infection has to compete with numerous self-peptides for the limited amount of class I MHC molecules[21], rendering ER-located class I MHC antigen peptide loading ineffective. In contrast, class I MHC peptide loading inside endosomes or phagosomes allows selected loading of endosome/phagosome-residing antigens, thereby limiting presentation of self-peptides and potentiating exogenous antigen peptide loading[22]. In agreement, a large portion of class I MHC molecules resides in endosomes[23].
In contrast to class II MHC presentation, antigen-cross presentation may occur in early endosomes (EEs) as well as LEs. The endosomal/vacuolar pathway of cross-presentation can be independent on proteasome function and rather depend on endosomal proteases to generate appropriate-sized peptides in LEs[24]. In addition, a pH of 5.0, such as in LE, is enough to destabilize pre-existing peptide-class I MHC complexes and to generate peptide-receptive class I MHC[25]. This allows formation of peptide-class I MHC complexes

in LE. As for example observed by Basha *et al;* independent of TAP and in a chloroquine-sensitive manner[26]. TAP and associated tapasin catalyzes peptide binding to class I MHC, similar as HLA-DM does for class II MHC. TAP enables class I MHC cross-presentation from early/recycling endosomes in murine DCs[22], human IFN-DCs[27], or phagosomes in murine DCs[18]. Alternatively, a proteasome-dependent pathway with phagosome-to-cytosol and subsequent TAP-dependent cytosol-to-phagosome re-entry in a Myd88-dependent manner has been reported[22,28]. In conclusion, endosomes are crucial to both class II MHC and Class I MHC-mediated antigen cross-presentation. The class I and II MHC peptide loading in both early and late endosomes probably contributes to presentation of diverse and broad peptide repertoire to antigen-specific T cells.

Uniqueness of endosomes.

Burgdorf *et al*. provided supporting data that TAP was recruited to certain EEs[22], suggesting that not every endosome contributes in a similar extent to antigen cross-presentation. Indeed, endosomes are a rather heterogeneous population. Not only do they differ in endosomal maturation stage, e.g. EE and LE, but even within EE or LE population distinct populations exist in parallel. For example, a dynamic and a more static population of EEs co-exist. The dynamic population of EE matured rapidly into LE and showed a rapid fusion towards lysosomes removing material for class II MHC antigen presentation[29], whereas "static" endosomes displayed less proteolytic activity which putatively protect antigens from excessive antigen destruction[30,31]. However, certain general mechanisms apply on these heterogeneous endosomes that creates similar compartments specialized in peptide-loading. In part this occurs by recruitment of key components to specific endosomes, such as peptide-receptive MHC molecules and antigenic cargo.

Recruitment of peptide receptive MHC molecules into antigen processing endosomal compartments.

As outlined in **chapter 3**, the major route of endosomal class I MHC delivery is constitutive endocytosis from the plasma membrane[32]. At first, an evolutionary-conserved tyrosine residue mediates delivery of class I MHC-peptide complexes from plasma membrane into lysosomes in a clathrin-dependent manner[26]. Secondly, two or three conserved lysine residues[33] in the cytoplasmic domain of class I MHC molecules may drive lysosomal delivery when poly-ubiquitinated as induced by Kaposi's sarcoma-associated herpesvirus K3[34]. Thirdly, class I MHC as well as class I MHC-like molecule CD1a can be internalized in a clathrin- and dynamin-independent pathway that targets both molecules into an Arf6- and Rab22-dependent endosomal recycling route[35–37].

Finally, Ii was recently also shown to complex with class I MHC molecules and drive class I MHC from ER to endolysosomal compartments in splenic murine DCs[38]. Basha *et al*.

demonstrates that this allows endolysosomal class I MHC peptide loading and crucial for DC-mediated cross-priming of $CD8^+$ T cells in an anti-viral response[38].

In case of class II MHC, newly synthesized class II MHC binds Ii. These class II MHC-Ii complexes are transported via trans-Golgi Network (tGN) directly[39] or indirectly via plasma membrane[40] to endosomal compartment. Two sorting motifs present in Ii direct tGN-to-endosome class II MHC translocation with the aid of AP1. In case class II MHC-Ii complexes are expressed at plasma membrane, AP2 drives rapid internalization of class II MHC-Ii complexed by clathrin-mediated endocytosis[41]. Both pathways deliver class II MHC-Ii complexes in EE (Tfn^+) before these complexes reach MIICs, allowing entrance of class II MHC-Ii complexes into recycling endosomes[13]. In addition, the C-tail of Ii is capable of inducing endosome vacuolation and fusion by an unknown mechanism[42]. Interestingly, Ii C-tails have been found on both EE and LE, thus their interactions could possibly cause endosome populations to mix[43].
In general to get peptide-receptive class II MHC complexes, multiple endosomal proteases cleave off C-terminal parts of Ii until CLIP is left in the peptide-binding groove of class II MHC. This is true for all class II MHC isoforms, however, the extent to which peptide loading is blocked by (cysteine) protease inhibitors varies widely among murine class II MHC isoforms[44]. Upon cleavage of Ii, HLA-DM subsequently catalyzes peptide loading onto class II MHC-CLIP complexes mainly in MIICs. However, small amounts of HLA-DM have been observed in EEs and plasma membranes of human B cells and immature DCs[45]. As presentation of an recycling class II molecules-dependent antigen was reduced in HLA-DM^- B cells in comparison to their HLA-DM^+ counterpart, it shows that HLA-DM catalyzes peptide loading in EE/REs[46]. However, this occurs in an antigen or B cell line specific manner as also HLA-DM independent antigen presentation in EE of other human B cell lines is reported[14].

Additionally, destabilized class II MHC-peptide complexes may exchange their loaded peptide for another one and re-locate to the plasma membrane. While class II MHC-Ii complexes are taken up in an AP2- and clathrin-dependent manner, class II MHC-peptide complexes are internalized in AP2-, clathrin- and dynamin-independent pathway from the plasma membrane. Using confocal microscopy on human DCs, Walseng *et al.* showed that class II MHC-peptide complexes are delivered in elongated tubular early/recycling endosomes in human DCs, whereas class II MHC-Ii complexes were destined for perinuclear vesicles and excluded from these tubular endosomes[47]. Class II MHC-peptide containing elongated recycling tubules show similar morphology and dynamics as our reported Tfn^+ elongated tubular network (ETEN). In **chapter 5** we demonstrated that the formation of Tfn^+ ETEN requires sufficient expression of MICAL-L1. MICAL-L1 is known to link Arf6 and Rab35 in endosomal recycling and interacts with EHD1. Walseng *et al*. provides additional data that supports the idea that Tfn^+ ETEN and class II MHC-peptide

complex-positive tubules are the same compartment, as HA-Arf6, GFP-Rab35 and GFP-EHD1 associated with these class II MHC-peptide complex-positive ETEN in HELA-CIITA cells[47]. However, we demonstrated that Tfn$^+$ ETEN formation occurs within 30 minutes of cognate T cell contact and involves ligation of HLA-A2 and ICAM-1 by T cell-expressed T cell receptor and LFA-1, respectively (**Chapter 4**). In contrast, Walseng *et al*. shows class II MHC-peptide complexes in tubular endosomes almost immediately upon administration of anti-HLA-DR mAb L243, which visualizes class II-MHC peptide complexes and thereby indirectly the tubules. Anti-HLA-DR mAb L243 is shown to drive activating cross-linking of class II MHC[48]. Therefore it is tempting to speculate that activation causing cross-linking of class II MHC causes the formation of Tfn$^+$ ETEN similarly as cross-linking of class I MHC drives Tfn$^+$ ETEN formation.

How can cross-linking drive internalization and incorporation of class II and putative class I MHC peptide complexes in Tfn$^+$ ETEN? Although data is not shown, Walseng *et al.* observed that truncation of the entire cytosolic domain of class II MHC α and β did not alter kinetics of cross-linked class II MHC-peptide complexes their endosomal trafficking and incorporation in the elongated tubules. This eliminates the involvement of any MHC-intrinsic signalling or ubiquitination motifs. Class I MHC and CD1a are internalized independent of clathrin and dynamin[35,37]. Clathrin and Dynamin-independent cargo internalization involves CLICs, which are ring or tubular invaginations of the plasma membrane that lack an electron-dense coat. Recently, it was demonstrated that Galectin-3, secreted from the cytoplasm as a monomer, binds to complex N-glycans of cargo such as integrin β1, oligomerizes into pentamers, and cross-links cargo into clusters on the plasma membrane. The clustering drives membrane bending, generating ring and tubule CLICs, through a process that does not require signalling motifs but requires energy and the actin cytoskeleton[49]. Whether cross-linking of class I or II drives them in these CLICs is not determined yet, but it is likely as integrin β1 and class I MHC are known to share a clathrin-independent internalization pathway that targets them both into a similar Arf6-dependent recycling route[35–37,50].

In conclusion, class I and II MHC-peptide complexes that are internalized via a clathrin- and dynamin-independent pathway, probably can be targeted into Tfn$^+$ ETEN by their initial clustering at the cell surface. On one hand this directs MHC-peptide (pMHC) complexes away from LE and possible lysosomal degradation. Therefore, this rapid recycling back to the plasma membrane enables longer surface expression of particular pMHC complexes. On other hand, in particular cases it enables incorporation of new peptides in the MHC molecule. This seems to occur in an antigen receptor-dependent manner[22], but further investigation is required.

Alternatively, signalling motifs in Ii bound to class I and II MHC, signalling motifs in C-tail of class I MHC, or MHC poly-ubiquitination, drives clathrin-dependent internalization that destines MHC molecules to LE compartment. In LE, MHC is either degraded upon

LE-lysosome fusion or rescued from degradation and can form pMHC complexes.
The extensive amount of viruses that interfere with MHC routing and degradation illustrates how important MHC routing is for efficiency of antigen presentation, as pointed out in this thesis (**chapter 3**). Further research into how particular pMHC complexes are targeted away from degradation, such as incorporating them into Tfn$^+$ ETEN, could be crucial for optimizing DC vaccination strategies.

Recruitment of cargo into antigen processing endosomal network.

For efficient antigen presentation, in addition to recruitment of sufficient peptide-receptive class I or II MHC molecules, antigens must be efficiently taken up to provide sufficient amount of antigenic peptides. While antigen may be internalized by receptor-dependent or independent pathways, receptor-dependent uptake has been demonstrated to be more efficient, especially for low concentrations of cargo[51]. To allow rapid antigen internalization, cells express a variety of receptors that either recognize antigen directly or indirectly via plasma components, such as complement or antigen-specific antibodies. An example of direct antigen recognition is the CLEC9A (DNGR1) receptor, that directly recognizes and internalizes necrotic cells for antigen cross-presentation[52]. In **chapter 6**, we demonstrated that antibody-opsonized antigen is internalized more efficiently than non-opsonized antigen by BDCA-1$^+$ DCs (**Fig.5. chapter 6**). This is in analogy with two studies. First, Kim *et al.* who showed that antibody-opsonization of *E.coli* enhances its uptake into murine DCs[53]. Secondly, Regnault *et al*. demonstrated that FcyR-signalling allows robust CD8$^+$ T cell activation with low antigen concentrations[54].
Interestingly, we showed that BDCA-1$^+$ internalized the most immune-complexes, but that nevertheless BDCA-3$^+$ DCs activated more CD8$^+$ T cells by cross-presentation of opsonized antigens. This illustrates that the efficiency of antigen cross-presentation is not only influenced by the amount of antigen uptake, but potentially also by the route of antigen presentation. As an example, phagocytosis of S. *pyogenes* results in presentation of $M5_{17\text{-}31}$ epitope by recycling class II MHC molecules, while macropinocytosis of S. *pyogenes* results in presentation of $M5_{306\text{-}319}$ epitope on newly synthesized class II MHC in MIICs[55].
One way to affect the route of antigen presentation is by targeting antigens to distinct antigen presentation compartments. Indeed, Montfoort *et al.* showed that FcyR-mediated internalization of immune-complexed OVA resulted in prolonged storage (>4 days) of antigen inside mature DCs. This enabled long-lasting cross-priming of cytotoxic CD8$^+$ T cells. The antigens were stored in a LAMP1$^+$, electron dense, non-MIIC endosomal compartment[56]. Similarly, merocytic DCs stored tumor cell-associated materials in non-acidic compartments for prolonged periods. This enabled sustained antigen presentation and induction of type I IFN by the merocytic DCs[57].

Interestingly, we observed that antigen opsonization did not significantly increase both antigen uptake by BDCA-3$^+$ DCs or their DC maturation **(chapter 6**). Together this suggests that it is not the amount of antigen or context of antigen presentation but rather efficient formation or transport of class I MHC-peptide complexes that improve cross-presentation. This corroborates with our experimental data in **chapter 4 and 8**, which demonstrated that the endosomal compartment can be modulated after antigen recognition via B cell receptor (BCR) and Toll-like receptor (TLR) signalling respectively.

B cell receptor signalling regulates endosomal processes pivotal to endosomal class II MHC processing:

The B cell receptor (BCR) is comprised of a plasma membrane immunoglobulin coupled to the Igα–Igβ (CD79a-CD79b) dimer, which both contain immunoreceptor tyrosine-based activation motifs (ITAMs). Upon antigen binding, BCR signalling initiates with ITAM-tyrosine residues that become rapidly phosphorylated by Src family kinases such as BLK[58]. This results in recruitment and activating phosphorylation of spleen tyrosine kinase (Syk). Subsequently, Syk-binds ubiquitin ligase c-Cbl that mediates signalling-dependent BCR ubiquitination[59]. This instigates clathrin-dependent uptake of BCR-antigen complexes[60], thereby enabling presentation of antigens present in low concentrations[61]. Defective BCR signalling such as in Xid mice, which lack Syk downstream mediator Btk, results in diminished BCR internalization and BCR-mediated antigen processing and presentation[62]. Moreover, Xid mice exhibit differential endosome-directed BCR trafficking[62]. This trafficking aberrance feature corroborates with observations in murine B cells with disrupted Syk function, showing that Syk function is crucial for endocytic trafficking to H2-DM$^+$ compartment and their ability to form class II MHC-peptide complexes from BCR-internalized antigens[63]. Accordingly, we showed in chapter 8 that expression of a mutant (L3P) BLK results in diminished BCR-mediated Syk activation, altered BCR endosomal trafficking, and diminished BCR-mediated antigen processing and presentation. Thus BCR signalling is crucial for proper endocytic trafficking. The disruption in endocytic trafficking is associated to a failure of Syk-deficient cells to properly reorganize their actin cytoskeleton in response to BCR engagement[63]. In agreement, preliminary data on BLK-L3P expressing B-LCLs show decreased F-actin remodelling upon BCR signalling. Therefore, it would be interesting to investigate the role of actin in endosomal trafficking. In addition to the crucial role of BCR signalling in antigen targeting to endosomal compartment, BCR signalling is shown to initiate remodelling of discrete peripheral vesicular LEs into a massive central cluster. This cluster is essentially composed of LEs with concentrated antigen, class II MHC and accessory molecules[64]. This partially depends on reversible down regulation of cathepsin S activity by BCR signalling, which causes diminished proteolytic removal of Ii and leads to transient accumulation of Ii-class II MHC complexes in these LEs. In addition, BCR cross-linking induces acidification of LEs, thereby affecting endosomal protease activity that biases towards a certain peptide

repertoire[65]. Possibly this explains the rapid degradation of DQ-BSA in BLK-L3P expressing human B cells. In parallel, BCR-antigen complex uptake rate seems unaltered in our CVID patient B cells. Thus this is the first model in which signalling for BCR internalization and further downstream endosomal processes are uncoupled. Clearly, endosomal BCR signalling is regulating endosomal antigen degradation in B cells.

How is the L3P mutation interfering with BLK-mediated Syk activation upon BCR stimulation? Quantitative PCR and preliminary western blot data suggest that L3P does not interfere with BLK expression, which made haploinsufficiency unlikely. Is non-functional BLK-L3P in competition with endogenous functional BLK and BLK has a non-redundant role in human BCR signalling, or does BLK-L3P has a dominant-interfering effect? In mice, most Src kinases have redundant function. Mutations in more than one Src kinase family member is often necessary in order to reveal a requirement for these kinases in particular systems. Indeed, BLK knock-out mice do not show major defects in B cell development and B cell activation[66]. Recently a slight effect was reported in the development of splenic B1 B cells (Wu *et al*. JI 2012). Similarly, Fyn/lck double and no single knockout mice show severe T cell defects[67]. However, expression of dominant-interfering mutants against single Src family members has demonstrated involvement in specific receptor signalling[68]. In contrast, in human B cells only BLK and not Fyn, Lyn, Syk, Hck was able to phosphorylate and subsequently associate with co-transfected Igα and Igβ chimeras *in vivo*. This suggests that in human B cells, BLK may have a non-redundant BCR signalling function. As the L3P mutation occurs in a region involved in membrane localization and not per se in competition for CD79A, this suggest that a dominant-interfering effect at the BCR is not likely.

Toll-like receptor signalling regulates endosomal processes pivotal to endosomal class II MHC processing:

Toll-like receptors (TLRs) recognize conserved macromolecules of microbial origin and can distinguish them on the basis of their specificities[69]. Upon ligand engagement, TLRs are efficiently internalized by themselves or in cooperation with other dedicated antigen uptake receptors[70]. In conventional DCs, TLR signalling temporarily upregulates internalization machinery before most general internalization machinery is shut down[71]. Later on after TLR stimulation, primarily (antigen-specific) receptor-mediated endocytosis continues to capture and internalize antigens[72].

TLR signalling can also modulate endosomal processes involved in both class II MHC and antigen cross-presentation[73]. TLR signalling modulates phagosomal maturation that is required for processing of Ii. NADPH oxidase (NOX2) activity may be regulated by TLR-downstream adaptor MyD88[74]. NOX2 activity in early phagosomes in DCs results in sustained production of low levels of reactive oxygen species, causing active and maintained alkalinisation of phagosomal lumen. This affects pH-sensitive endosomal protease activity required for Ii processing. Therefore, both murine and human

DCs lacking NOX2 show enhanced phagosomal acidification and increased antigen degradation, resulting in impaired cross-presentation[31,75]. This endosomal modulating role of TLR signalling is crucial and non-redundant as antigens in phagosomes that did not encounter TLR ligands were not presented to antigen-specific $CD4^+$ T cells[76].
Additionally, TLR stimulation shortly boosts protein synthesis in DCs, before most protein synthesis is suppressed in mature DCs[77]. This causes a reduction in substrates for classical class I MHC presentation. However, class I and II MHC synthesis continues[78] and both proteasome and TAP activity are increased after TLR engagement[79], thus stimulating presentation of exogenous presentation by class I and II MHC.

Antigen receptor cross-talk.

Although expression levels of BCRs, TLRs, and FcRs may differ between immune cell types, their cross-linking always results in phosphorylation and activation of spleen tyrosine kinase (Syk). Syk is required for efficient activation and internalization of both BCR and FcR in B cells[80,81]. **In chapter 8** we demonstrated that diminished Syk phosphorylation accompanies disrupted antigen presentation of BCR-internalized antigen, putatively with defective actin remodelling involvement (our unpublished data) and as shown before[63]. Similarly, Syk-deficient cells showed a complete block in FcR-mediated particle uptake due to a block in membrane fusion, a subsequent step to actin polymerization[82]. Moreover, Syk-inhibitors have demonstrated that B cell activation via both BCR and/or TLR signalling requires Syk. In addition, a recent article showed that Syk is required for BAFF-signalling driven B cell survival[83]. Therefore, it seems that Syk acts downstream of many innate receptors.
Another recent study shows that Syk has opposing regulatory roles in TLR4-mediated TRAF3 and TRAF6 signalling pathways[84]. The authors identified several domains of Syk that may interact with TRAF3, TRAF6, and other TLR signalling adaptors such as TAK1, and TBK1. This suggests that Syk may act as a common regulator of various TLR responses.
Interestingly, DCs that lack FcγR have an increased TLR response[85]. Moreover, Syk-mediated BCR signalling is required for optimal TLR9 responses[86]. Therefore, it seems that Syk balances various innate receptor signalling and thereby may fine-tune the immune response. In support is our notion of a supposedly compensatory mechanism occurring in a CVID sibling carrying dysfunctional TACI, e.g. a BAFF-related moIecule. The asymptomatic sibling showed enhanced TLR9 expression and signalling, whereas the symptomatic sibling carrying the same mutation was not[122]. It would be interesting to investigate whether diminished BCR-mediated Syk phosphorylation increases sensitivity of TLR-induced Syk phosphorylation in our BLK-L3P CVID patients.
This antigen receptor cross-talk is also very interesting in the context of disease. For example, in rheumatoid arthritis both FcR and TLR can be simultaneously stimulated by immune-complexes and TLR ligands, respectively. A recent report showed that human M2 macrophages, which are usually anti-inflammatory, elicit pro-inflammatory cytokines

(TNFα, IL-1β, and IL-6) and stimulate Th17 T cell proliferation upon simultaneous stimulation of FcR and TLR[123].

The extent of Syk phosphorylation could putatively be used as a diagnostic marker for PIDs with TLR, FcR, BAFF, or BCR signalling defects, such as CVID patients. Especially in combination with Phosflow, a flow cytometry-based technique that allows tracking of multiple intracellular signalling molecules in the immune system at a single-cell level[87]. Using Syk as a drug target is less likely to occur in the short term as this was not successful thus far in clinical settings[88].

The effect of cell subsets:

We already summarized in **table 1** of **chapter 2** that expression of similar receptors drives distinct antigen presentation outcomes in mice or men. Similarly, differences in expression of antigen recognition receptors affects the outcome of antigen presentation. Receptor expression varies based on cell subset, activation status, and physiological location. For example, naïve human B cells express TLR1, 6, 7, 9, 10, and activation via BCR, cytokines or CD40, elevates TLR1, 6, 7, 9, 10 expression levels[89] and induces TLR4 expression[90].

In the case of DC subsets, there has always been a broad distinction between myeloid DCs (mDCs), monocyte-derived DCs (moDCs), and plasmacytoid DCs (pDCs) (**Figure 1**). The pDCs are typically known as major producers of type I interferons, whereas moDCs are especially important in secondary immune responses. In parallel, murine myeloid DCs were divided into $CD8\alpha^+$ and $CD8\alpha^-$. $CD8\alpha^-$ mDCs are more specialized in Class II MHC and $CD4^+$ T cell responses, in contrast to $CD8\alpha^+$ mDCs that are specialized in class I MHC/cross-presentation towards $CD8^+$ T cell responses. In late 2010, three groups independently identified a human counterpart of the mouse $CD8\alpha^+$ cross-presenting DC, referred to as BDCA-3^+ or $CD141^+$ DCs[91–93]. These groups showed that the BDCA-3^+ or $CD141^+$ DCs are superior in cross-presentation of cell-associated antigens in comparison to all other DC subsets[91–93]. This seems to be mainly due to expression of XCR1 and CLEC9A[93]. In addition, we described in **chapter 6** that BDCA-3^+ DCs are better cross-presenters of exogenous immune-complexed antigen than BDCA-1^+ DCs, possibly because BDCA-3^+ DCs express more stimulatory FcγRIIa than inhibitory FcγRIIb in comparison to BDCA-1^+ DCs. The BDCA-3^+ or $CD141^+$ DCs also have higher levels of TLR3 expression than BDCA-1 and are superior in antigen cross-presentation when polyI:C (TLR3-ligand) is present[92]. However, Mittag *et al.* showed that although $CD141^+$ DC antigen cross-presentation increased upon polyI:C administration, the levels of $CD8^+$ T cell activation was not significantly higher than blood CD1b/c^+ DCs[94]. This shows that TLR3 expression is relevant, but not the only determinant for superior antigen cross-presentation.

In addition to BDCA-1^+ DCs, also BDCA-1^+or $CD16^+$ DC and pDCs are capable of antigen cross-presentation in humans[94,95]. More and more examples in the literature demonstrate that BDCA-3^+ DC are not the best cross-presenting DC subset in every situation. Especially

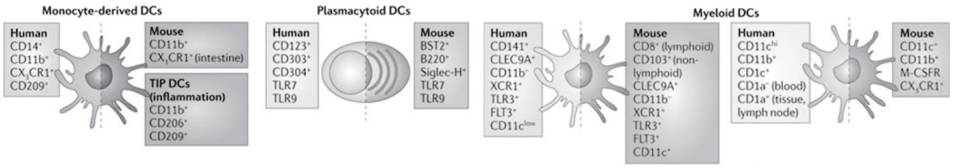

Figure 1. Dendritic Cell subsets (Adapted from Collin *et al.*[125]).

Four major dendritic cell subsets are identified in both humans and mice. From left to right: inflammation induced monocyte-derived DCs, plasmacytoid DCs, and myeloid DCs divided in classical CD8$^-$(mouse)/CD1c$^+$(human) DCs and CD8$^+$(mouse)/CD141$^+$(human) DCs.

in the case of exogenous soluble antigen, it is not clear which DC subset is the best cross-presenter. For example, Tel *et al*. showed that pDCs cross-presented soluble antigen as efficiently as myeloid DCs, but pDCs continued presenting long peptides over 100 hours and were therefore better cross-presenters[95]. In contrast, Bachem *et al*. showed that all DC subsets except pDC could cross-present soluble exogenous antigen[91]. These findings illustrate that rather than targeting one selective DC subset, we should aim for efficient antigen presentation in multiple DC subsets for vaccination optimization.

Endosomal network as cell-surface directed transport machinery for antigen presentation.

To be able to engage antigen-specific TCRs for T cell activation, pMHC complexes formed intracellularly need to be present at the APC-surface. The intracellular location where pMHC complexes are formed will dictate the trafficking route that is taken. Peptide loading within the ER, as for class I MHC, results in transport via the biosynthetic pathway to the cell surface, although some ER-generated class I MHC-peptide complexes may be targeted to endosomal compartments for a second peptide loading via CD74-dependent pathway[38].

It is apparent that pMHC complexes can be formed throughout the endosomal compartment. In murine cells it has been clearly demonstrated using pMHC complex recognizing antibodies, that pMHC class II complexes transfer via LE vesicles and tubules towards the cell surface[96–98].

Using the recycling inhibitor Primaquine, it has been shown that presentation of some viral epitopes requires recycling class II MHC molecules. This suggests that EE formed class II MHC-peptide complexes may be transported to the cell surface via recycling endosomes[99].

Similarly, Primaquine causes accumulation of class I pMHC complexes in EE[22], and hampers consequential CD8$^+$ T cell activation in our cross-presentation model (**chapter 4**). Together, this suggests that EE-formed class I pMHC complexes are recycled to the cell surface for presentation. LE-to-cell surface trafficking of class I or class II pMHC in

human APCs has not been properly investigated, due to a lack of suitable inhibitors or putative knock-down target proteins. However, a genome-wide multidimensional RNAi screen has identified Arf7 as a regulator of LE-to-surface transport of class II pMHC complexes[100], thereby enabling future investigation into this process in human APCs.

Apparent role for Elongated Tubular Endosomal Network (ETEN) in antigen presentation.

LE-emanating ETEN are shown to transport class II MHC and costimulatory molecules towards the cell surface, which coincides with a drastic increase of class II MHC surface expression[101,102]. As multiple class II pMHC complexes are necessary to fully activate $CD4^+$ T cells, it is believed that these tubules therefore contribute to the efficiency of antigen-specific T cell activation. However, these LE tubules arise within ten minutes whereas most cargo reaches LE at a later time point. Therefore the first wave of class II pMHC complexes delivered by endosomal tubules at the plasma membrane of DCs is probably not carrying antigenic peptides. In addition, upon inflammatory stimulation the surface-expressed pMHC class II complexes consist of ~50% and 75% of newly synthesized complexes, at 4 or 8 hours post stimulation respectively[103]. This suggests that the first wave of pMHC class II complexes delivered during the formation of endosomal LE tubules is rapidly internalized upon DC surface arrival and thus unlikely to play a crucial role in antigen-specific T cell activation at that moment. However, this first wave will re-enter into the endosomal compartment, which is now significantly altered by the presence of LE tubules, which are observed even at 8 hours post stimulation (**chapter 4).** However, ten Broeke *et al*. show that a predominant fraction of antigen-laden class II MHC complexes that is stably expressed at the plasma membrane by mature DC is synthesized after exposure to inflammatory stimuli, illustrating that these endosomally formed class II pMHC complexes are not stably expressed on DCs matured by LPS stimulation. This gives rise to two different populations of class II pMHC complexes pools: one that is stably expressed on the surface, and another one that remains predominantly in endosomal compartments. The contribution of both pools to antigen-specific T cell activation has not yet been determined. Ten Broeke *et al.* concluded that endosomally stored class II MHC does not contribute to antigen presentation by DCs at inflammatory conditions[103]. However, they did not investigate the contribution of endosomally stored class II pMHC complexes in inflammatory conditions upon encounter with antigen-specific T cells. That LPS and T cells induce distinct endosomal remodelling is known, since in murine DCs LPS-induced LE-emanating tubules are in average 5µm whereas T cell-induced tubules are 15-20µm in length. Various research groups have illustrated how bidirectional signalling during DC-T cell interaction is pivotal. Examples are tolerization of DCs upon encounter with regulatory T cells[104], licensing of DCs by cognate $CD4^+$ T cells to herald $CD8^+$ T cell immunity after human allogeneic umbilical cord blood transplantation[124], and our data demonstrating $CD8^+$ T cell-induced ETEN in human DCs at inflammatory conditions

(**chapter 4**).

Interestingly, neither Tfn$^+$ nor LDL$^+$ ETEN polarize extensively towards the DC-T cell contact zone. Instead, usually one or two endosomal tubules appear to stay polarized to the DC-T cell contact zone. Perhaps these observations illustrate the necessity of human DCs to engage multiple T cells simultaneously. In the situation that DCs engage multiple T cells, endosomally stored pMHC complexes can be rapidly delivered at different sites of the DC via endosomal tubules. We have for example observed endosomal tubules shifting 45 degrees within a millisecond, which resulted in an approximately surface transported distance of 25µm/ms in a DC with average diameter of 10µm. This is significantly more expeditious than the reported lateral mobility of class II MHC at the DC surface of 0.18µm/s[105]. Moreover, pMHC complexes and costimulatory molecules are delivered together and form clusters at the cell surface[97]. In addition, surface-delivered pMHC complexes form small (<100 nm) microclusters and are stabilized in cholesterol-containing microdomains at the cell surface. Cholesterol depletion inhibits the stability of these clusters as well as the ability of DCs to function as APCs[106], illustrating the potency of these membrane microdomains and endosomal surface delivery.

Defective antigen presentation in Common Variable Immunodeficiency:

Primary immunodeficiencies (PIDs) are individually rare but collectively diverse genetic defects that influence the development and/or function of immunity. They are inherited diseases of the immune system caused by disease-associated gene variants that result in diverse clinical manifestations. They are 'experiments by nature' and have taught us valuable lessons of human immunology (**chapter 7**).

Common variable immunodeficiency (CVID) is the most frequently diagnosed symptomatic PID. CVID develops as a consequence of absence or malfunction of proteins involved with high affinity antigen-specific immunoglobulin production by plasma and memory B cells (**chapters 8 and 9**). Genetic defects are observed in both B and T cells[107]. Central in the development of high affinity antigen-specific antibodies are BCRs. BCRs are capable of antigen recognition, followed by transmembrane signalling to induce B cell differentiation, and antigen internalization for processing followed by MHC class II-mediated presentation to acquire antigen-specific CD4$^+$ T cell help. Monogenetic defects in genes relevant to BCR signalling are found in CVID(-like) patients, CD19[108], CD20[109], CD81[110], respectively. Moreover, monogenetic defects are found in genes of T cells essential for T cell help required for class-switching to high affinity antibodies, CD27[111], CD40L[112] and ICOS[113], respectively. This demonstrates that BCR signalling and defective T cell help underlies CVID pathology in subsets of CVID patients.

As we know that BCR signalling is involved in the modulation of endosomal processes underlying endosomal class II MHC processing, you would expect reports on CVID patients with defective antigen processing or presentation towards CD4$^+$ T cells. However, all but

one described cases in literature show that it is the T cells that are impaired in their response towards B cell presented antigen[114,115]. The one report observed a decreased B cell CD40-expression[116], which potentially may affect B cell antigen presentation. However, this observation is disputed by an earlier report[117].

In **chapter 8** we show that expression of BLK-L3P in human immortalized B cells accelerates antigen degradation and diminishes B cell-mediated antigen presentation. Unfortunately, due to HLA-mismatch we were not able to demonstrate *in vitro* that the patient's B cell-mediated antigen presentation is diminished. However, we demonstrate similar accelerated antigen degradation between BLK-L3P B cell line and patient B cells. Therefore, we propose that L3P mutation in B lymphoid tyrosine kinase (BLK) hampers BCR-mediated antigen processing and therefore underlies CVID of our index patients. Whether more CVID patient B cells have an accelerated degradation or defective endosomal processing is unknown. It is my belief that impaired B cell-mediated antigen presentation towards $CD4^+$ T cells is not only limited to our index CVID patients. For example, a few CVID patients are described with defective TLR signalling[118–120]. As TLR signalling is essential in endosomal processes underlying antigen processing and presentation, either directly or possibly indirectly via the TLR/BCR synergistic pathway[121], it is highly likely that these CVID patients also harbour altered antigen presentation capabilities contributing to CVID pathology.

In conclusion.

At the moment, pre-clinical studies mainly focus on optimization of DC vaccination by identification of DC subsets or antigen formulations that elicit most potent priming of antigen-specific T cells (**chapter 2**). Accordingly, we investigated and demonstrated in this thesis that FcγR-targeted antigen delivery can potentiate antigen-specific $CD8^+$ T cell activation by human monocyte-derived DCs and BDCA-3^+ DCs[11]. These BDCA-3^+ or $CD141^+$ DCs, characterized by CLEC9A and XCR1 expression, were initially believed to be superior in antigen cross-presentation in comparison to other DC subsets. However, in the following years their superior role was disputed by other groups. At present, the existence of a superior antigen cross-presenting human DC subset is considered unlikely. Hence, we also investigated the more abundant subset of BDCA-1^+ DCs. We could not potentiate BDCA-1^+ DCs cross-presentation by FcγR-targeted antigen delivery, possibly due to different expression levels of stimulatory FcγRIIA and inhibitory FcγRIIB expression on distinct DC subsets. At least no negative effect on BDCA-1^+ DCs antigen cross-presentation was observed. Here we encountered a common problem with antigen receptor targeting, as receptors are variably expressed between cell types, cell subsets, differentiation and developmental stages, and species. Additionally, receptor isoforms or cross-talk may alter receptor-signalling unknowingly. These issues might complicate the usage of receptor-targeted antigen delivery for (DC) vaccination optimization. In addition, the enhancing effect of FcγR-targeted antigen delivery to antigen cross-

presentation in our model is relatively modest. Hence, we sought additional ways to improve vaccination strategies. In this thesis we discovered and investigated three novel cell-biological processes that may be required for efficient antigen presentation. At first, we showed that TLR and/or antigen-specific $CD8^+$ T cell contact induced tubulation of endosomal structures that accompanied antigen cross-presentation in human DCs[98]. Secondly, we demonstrated the necessity of MICAL-L1 expression in the antigen-specific $CD8^+$ T cell-induced tubulation of Tfn^+-endosomes (**Chapter 5**). The conservation of endosomal remodelling amongst species allows for a more broad application in diverse cell subsets and species although future research is required to clarify a potential beneficial contribution of endosomal tubulation to antigen (cross) presentation. For example, increasing antigen processing within EEs would target specifically recently internalized exogenous antigen. This may be achieved by TLR-ligand inclusion in antigen formulation as Burgdorf *et al.* showed in murine DCs[22]. Concomitant cross-linking cell-surface MHC molecules may direct them into and instigate EE-emanating ETEN. This in turn is associated with increased MHC cell-surface directed trafficking and directs antigen away from full degradation, potentiating antigen presentation. Thus, it is my hope that the insights gained here can be implemented in the development of future (DC) vaccinations.

Thirdly, we showed the requirement of functional BCR-signalling molecule BLK for efficient BCR-mediated antigen presentation by human B cells, as a previously unrecognized cause of CVID. Although further research is required to determine the proportion of CVID patients that harbour defects in antigen presentation capacity, our research opens new possibilities for future stratification and more targeted treatment of CVID patients.

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Chapter 11.

Summary

Samenvatting

List of publications

Curriculum Vitae

Dankwoord

Summary

Initiation of adaptive immune responses requires presentation of antigens to antigen-specific lymphocytes. Considering conventional T cells, antigen presentation is the process by which a protein antigen is presented in the form of short peptide fragments embedded within class I and class II Major Histocompatibility Complexes (MHC). Antigen presenting cells (APCs) present peptides that are generated by proteolysis of exogenous antigens in class II MHC/peptide complexes. In parallel, class I MHC complexes display peptides derived from endogenous antigen, generated by proteasomes and additional peptidases to confer immunity. In a third pathway, the capture of exogenous antigens somehow enter the class I MHC/peptide presentation pathway inside APCs, thereby eliciting a $CD8^+$ T cell response. This process is called antigen cross-presentation; a pivotal mechanism for the development of the specific $CD8^+$ T cell response against tumors and viruses that do not infect APCs.

Both class II antigen presentation and antigen cross-presentation is initiated by internalization of an antigen into an endosome. In class II antigen presentation, antigens are proteolytically processed into small peptides by endosomal proteases. In the case of antigen cross-presentation, antigens can be processed either by endosomal proteases or cytosolic proteasomes and peptidases. Hereafter, peptides are loaded into peptide-receptive MHC molecules, which are present throughout the endosomal network and in the endoplasmic reticulum (ER). Eventually, peptide-MHC complexes are transported via endosomes to the cell surface for presentation. Hence, the highly dynamic intracellular transport network of endosomes is key to antigen presentation and elicitation of following adaptive immune responses.

The work in this thesis is aimed to clarify endosomal processes that are pivotal to antigen-specific lymphocyte activation. Such knowledge may lead to improvements in cellular vaccination strategies, and as demonstrated, may elucidate dysfunctional mechanisms underlying primary immunodeficiencies.

Regulating antigen cross-presentation in human dendritic cells.

The widespread success of vaccination has stimulated the use of vaccination strategies in other fields, such as anti-viral and anti-tumour therapy. To this end, focus is placed on exploring cellular vaccination strategies, whereby immune cells are educated and exposed to a selected antigen outside the body prior to introduction to the patient. All APCs, especially dendritic cells (DCs) are equipped with specialized machinery that promote an effective display of peptide-MHC complexes, rendering them the most potent stimulators of T cells, even capable at stimulating naïve T cells to become effector cells. Hence, DC-vaccination strategies has been explored, primarily in an autologous setting where DC vaccination has showed some beneficial effects.

However, our literature overview in **chapter 2** shows the importance of optimizing DC

vaccination strategies. Over a 100 clinical trials with DC vaccination strategies were performed and most of which have been unsuccessful. We elaborate on distinct DC subsets in mice and men, and their known capacity for antigen cross-presentation. Although most DC subsets can cross-present antigens as peptide-class I MHC complexes under certain conditions, it seems that the recently identified subset of BDCA3$^+$ (CD141$^+$) DCs is specialized in this process and therefore of particular interest for DC vaccination strategies.

In **chapters 2 and 3** we review recent literature regarding cell-biological mechanisms of antigen cross-presentation as it is extensively investigated to date. Depending on the model system used, two major pathways of cross-presentation are proposed. In the cytosolic pathway, the antigen is endocytosed and requires endosome-to-cytosol translocation for degradation by proteasomes. The vacuolar pathway relies on endosomal proteases for antigen processing and is therefore proteasome and endosome-to-cytosol translocation independent. The vacuolar pathway additionally requires the endosomal recycling network for acquisition of peptide-receptive class I MHC molecules and transport of peptide-class I MHC complexes to the cell surface. Many pathogens interfere with the endosomal transport network as an immune evasion strategy, illustrating the endosomal network is important in the adaptive immune response. However, not much is known about endosomal logistics of antigen cross-presentation. This triggered us to research endosomal behaviour during DC function, leading to the results presented in chapter 4.

In **chapter 4** we used live cell fluorescent microscopy on human monocyte-derived DCs to study endosomal behavior upon activation. We made use of fluorescent cargo DiI-LDL and Transferrin-Al647 that is incorporated, and thus specifically stain, distinct endosomal compartments, respectively LE and early/recycling endosomes. We showed that activation of human dendritic cells (DCs) by LPS stimulation causes the remodelling of LE (LDL$^+$) endosomes leading to formation of an elongated tubular endosomal network, e.g. ETEN. This corroborates with earlier studies in murine DCs, thereby showing conservation of this mechanism between mice and man. Indeed, earlier electron microscopic studies showed that LE-tubules extend towards the periphery with small transport vesicles protruding towards the plasma membrane at the tubule tips. This data suggest that activation-induced LE tubules represent transport intermediates for cell surface-directed transport.

However, the vast majority of endosome-cell surface transport normally occurs via endosomal recycling compartments (ERCs). ERCs are known to form short tubular transport intermediate structures. One apparent question was whether ETEN is derived from ERC-derived membranes. Therefore, we studied Transferrin (Tfn)-positive ETEN in human DCs upon DC activation. In contrast to LE, Tfn$^+$ ETEN did not arise upon LPS stimulation, but did so upon subsequent cognate interaction between antigen-presenting DC and antigen-specific CD8$^+$ T cells. These Tfn$^+$ elongated endosomal tubules

emanate from a juxta-nuclear region where the ERC is located. Further support that ERC contributes to ETEN came from use of the recycling inhibitor primaquine: Tfn^+ tubules were lost when human DCs were briefly treated with a low dose of primaquine 30 minutes prior to T cell contact. This data suggests that these induced tubular structures are indeed involved in cell-surface directed transport, which is crucial for DCs to function as antigen presenting cell. In more detail, we demonstrate the requirement for ligating class I MHC and ICAM-1 molecules by T cell-expressed T cell receptor and LFA-1 for Tfn^+ ETEN formation. Finally, we used selective reversible inhibitors to disintegrate microtubules or inhibit endosomal recycling. Both inhibitor treatments abolishes tubular ERCs, which coincides with reduced antigen-dependent $CD8^+$ T cell activation. This underscores the importance of this tubular cell-surface directed transport to adaptive immune activation. In the absence of inhibitor treatment, we established that both LDL^+ and Tfn^+ ETEN are stable for at least 6 hours in human DCs. Since tubules are not the energetically favoured conformation of lipid bilayers in soluble aqueous surroundings, we hypothesized that there should be a concerted action of several factors that model and stabilize these endosomal tubules. In **chapter 5** we first review literature reporting on molecular mechanisms involved in initiation, stabilization, elongation, and scission of membrane tubules. One of the molecular mechanisms is the EHD1 and MICAL-L1 dependent recycling of Src kinase upon integrin receptor stimulation via a Tfn^+ elongated tubular endosomal network. Using a siRNA-based knock-down approach we demonstrate that MICAL-L1 is necessary for ETEN remodeling originating from ERC in human DCs. Reduced MICAL-L1 expression does not significantly interfere with LPS-induced remodelling of LE in human DCs, allowing us to investigate ERC-emanating ETEN function. Future directions should include studies focussing on molecules that corroborate ETEN, such as –but not limited to- MICAL-L1 and EHD1, in antigen cross-presentation and $CD8^+$ T cell stimulation.

As discussed in chapter 2, receptor-mediated uptake can direct an antigen towards distinct antigen presentation pathways. It has been demonstrated in mice that Fcγ Receptors (FcγRs), expressed a.o. DCs, skew antigen toward antigen cross-presentation. In **chapter 6** we examined cross-presentation of FcγR-targeted antigen in human DCs. We show that FcγR-targeting of antigen (using IgG-opsonised antigen) resulted in increased $CD8^+$ T cell activation following antigen cross-presentation. Using several pharmaceutical inhibitors, we obtained data that support that cross-presentation of FcγR-targeted antigen requires both cytosolic and vacuolar pathways of antigen cross-presentation in monocyte-derived DCs, as well as in primary $BDCA\text{-}1^+$ and $BDCA\text{-}3^+$ DCs. Interestingly, $BDCA3^+$ DCs showed an increase in cross-presentation upon antigen delivery to the FcγR, showing it can be exploited to human DC vaccination strategies. $BDCA\text{-}3^+$ DCs its superiority in cross-presentation of FcγR-targeted antigen in comparison to $BDCA\text{-}1^+$ DCs could not be explained by increased antigen-uptake as $BDCA\text{-}1^+$ rather than $BDCA\text{-}3^+$ DCs internalize

immune-complexed antigen. Putatively, relative higher levels of the stimulatory isoform FcγRIIa on BDCA-3$^+$ may explain these findings.

Dysregulated B cell activation and antigen presentation.

In **Chapter 7** we describe combined disorder called 'Common Variable Immunodeficiency (CVID), which is the most frequently diagnosed symptomatic primary immunodeficiency. CVID develops as a consequence of absence or malfunction of proteins involved with (high-affinity) immunoglobulin production by plasma and memory B cells. The last decade has brought us clarification of several genetic predispositions to the development of CVID. Development of high affinity antigen-specific antibodies involves at least two downstream actions of the B cell receptor (BCR): transmembrane signaling through BCR-complexes to induce B cell differentiation, and antigen internalization for class II MHC-mediated presentation that recruits antigen-specific CD4$^+$ T cell help. Therefore it is not surprising that in patients showing characteristics of CVID, monogenetic defects are found in genes relevant to B cell receptor signaling or T cell help, including CD19, CD20, CD81, or CD27 and ICOS-L.

Despite this considerable effort, in eighty-five percent of CVID patients, disease etiology still remains to be elucidated. In an attempt to identify unknown origin for disease-associated gene variants, we used a targeted Next-Generation Sequencing-based approach to screen genetically-undiagnosed CVID patients. In **chapter 8**, we report on two related CVID patients carrying a Leucine to Proline replacement at position 3 in the src kinase B lymphoid tyrosine kinase (BLK). Unlike in mice, where functional redundancy exists between Src kinases, we discovered that L3P-BLK abrogates early BCR signaling (e.g. Syk phosphorylation) required for B cell proliferation. Moreover, expression of L3P-BLK accelerates late endosomal/lysosomal BCR-antigen complex delivery, causing destruction of the antigen. Together these events reduce antigen cross-presentation as shown by decreased BCR-mediated HLA-DR-restricted antigen-specific activation of CD4$^+$ T cells. Thus, defective BCR signalling and interdependent antigen presentation underlies CVID disease in some cases. Whether more CVID patients harbour defective antigen presentation is unknown to date, but **chapter 9** shows disturbed early BCR signaling in a significant proportion of pediatric CVID(-like) patients. We demonstrate that in these pediatric CVID patients there is a disruption in calcium signaling. The calcium defect correlates with disturbed plasmablast differentiation *in vitro* and appears to occur upstream in the BCR signaling cascade, since Ca^{2+} influx via plasma membrane channels and both storage and depletion of $Ca2^+$ from the ER was normal. This disturbed early BCR signaling did not originate from upregulation of CD22 and did not involve gene mutations in PLCγ2 or Btk. Instead, CVID disorder B cells exhibited reduced BCR dissociation from CD20. BCR or CD20 crosslinking-induced less BCR internalization and antibody-mediated CD20 triggering elicited less BCR downstream signaling as measured by secondary $Ca2^+$ fluxes. Therefore, it seems that defects in CD20/BCR signalosome

conformation predispose to CVID.

In conclusion, this thesis describes the pivotal role of the endosomal network in initiation of adaptive immune responses. Regulating key endosomal processes by interfering a.o. with ETEN formation, antigen (Fcγ) receptor-targeting, or src kinase (BLK) function may contribute to an improvement in future therapeutic intervention strategies contributing to patient survival.

Samenvatting

Voor het activeren van de specifieke “adaptieve” afweerreactie is er presentatie van antigenen aan antigeen-specifieke lymfocyten nodig. Bij conventionele T-cellen is antigeen presentatie het proces waarbij een eiwit-antigeen wordt gepresenteerd in de vorm van korte peptidefragmenten in klasse I of II Major Histocompatibility Complex (MHC) moleculen. Normaliter presenteren antigeen-presenterende cellen, afgekort APCs, peptiden die afkomstig zijn van exogene antigenen in klasse II MHC. Daarentegen worden peptidefragmenten afkomstig van endogene antigenen, die afgebroken zijn door proteasomen en peptidases, gepresenteerd in klasse I MHC moleculen. In een derde mechanisme worden exogenen antigenen opgenomen op zo’n manier dat ze in klasse I MHC moleculen terechtkomen, waardoor ze $CD8^+$ T-cellen kunnen activeren. Dit proces heet antigeen kruis-presentatie en is erg belangrijk om $CD8^+$ T-cellen te activeren tegen tumoren en virussen die APCs niet infecteren.

Zowel klasse II antigeen presentatie als antigeen kruis-presentatie begint met de opname van exogene antigenen in intracellulaire blaasjes, genaamd endosomen. Voor klasse II antigeen presentatie worden antigenen afgebroken in peptidefragmenten door proteases die aanwezig zijn in de endosomen. In het geval van antigeen kruis-presentatie kunnen de antigenen worden afgebroken door deze endosomale proteasen, maar ook door cytosolaire proteasomen en peptidases. De peptiden worden hierna geladen in peptide-ontvankelijke MHC moleculen, die aanwezig zijn in het endoplasmatisch reticulum (ER) en het netwerk van endosomen. Uiteindelijk worden de peptide-MHC complexen getransporteerd met behulp van endosomen naar het celoppervlak voor antigeen presentatie. Hieruit blijkt wel hoe belangrijk het dynamische intracellulaire transportnetwerk van endosomen is voor antigeen presentatie en de daaruitvolgende activatie van de adaptieve afweerreactie.

Het werk in dit proefschrift is uitgevoerd om meer inzicht te verkrijgen in endosomale processen die belangrijk zijn voor antigeen-specifieke T-cel activatie. Zoals wij laten zien kan de opgedane kennis opheldering verschaffen van ontregelde processen die onderliggend zijn aan primaire immuundeficiënties. Daarnaast zou het kunnen dat de nieuwe verkregen inzichten bijdragen tot verbetering van cellulaire vaccinatie strategieën.

Regulatie van antigeen kruis-presentatie in humane dendritische cellen

Het succes van klassieke vaccinaties heeft het gebruik van soortgelijke technieken gestimuleerd in andere velden, zoals anti-virus en anti-tumor therapieën. Momenteel wordt er voornamelijk geconcentreerd op het gebruik van cellulaire vaccinatie strategieën. Hierbij worden afweercellen blootgesteld en “getraind” voor geselecteerde antigenen buiten het lichaam, waarna ze weer worden teruggebracht in de patiënt.

Alle APCs, maar voornamelijk dendritische cellen (DCs), hebben speciale mechanismen

in zich die effectieve antigeen presentatie mogelijk maken. Daarom zijn deze cellen het best in het activeren van T-cellen. De DCs kunnen zelfs naïeve T-cellen stimuleren tot "effector" T-cellen. Daarom wordt er veel onderzoek gedaan naar DC-vaccinaties. Tot nu toe is er enkel in een autologe setting een voordelig effect van DC vaccinaties op patiënten beschreven.

Ons literatuuroverzicht **in hoofdstuk 2** laat het geringe succes van DC-vaccinaties tot nu toe zien: er zijn namelijk meer dan 100 klinische studies met DC vaccinaties uitgevoerd en de meeste ervan waren niet succesvol. Daarom is het erg belangrijk om DC vaccinaties verder te optimaliseren. Verder wijden we in hoofdstuk 2 uit over verschillende DC soorten in muis en mens en hun capaciteit van antigeen kruis-presentatie. Hoewel de meeste DC soorten in specifieke omstandigheden antigeen kunnen kruis-presenteren, lijkt het erop dat de BDCA3$^+$ (CD141$^+$) DCs het meest gespecialiseerd zijn voor het kruis-presenteren van antigen. Daarom zijn de BDCA-3$^+$ DCs erg interessant voor toekomstige DC vaccinatie strategieën.

In **hoofdstuk 2 en 3** bespreken we literatuur die recentelijk gepubliceerd is over celbiologische mechanismes van antigeen kruis-presentatie. Afhankelijk van het onderzoeksmodel dat gebruikt wordt, zijn er twee mechanismen van antigeen kruis-presentatie die het meest voorkomen. In het "cytosolaire" model wordt het antigeen opgenomen in endosomen en heeft het vervolgens verplaatsing van endosoom naar het cytosol nodig voor afbraak bij cytosolaire proteasomen. Het "vacuolaire" model is afhankelijk van endosomale proteases voor afbraak van antigeen in peptides en is daarom proteasoom en endosoom-naar-cytosol translocatie onafhankelijk. Hiernaast is het "vacuolaire" model afhankelijk van het endosomale recycling netwerk voor het verkijgen van peptide-ontvankelijke klasse I MHC moleculen en transport van peptide-klasse I MHC complexen naar het celoppervlak. Verscheidene pathogenen verstoren het endosomale transportnetwerk om de afweerreactie te ontwijken. Dit geeft aan hoe belangrijk het endosomale netwerk is in het aanzetten van de pathogeen-specifieke afweerreactie. Echter, er is weinig bekend over de logistiek van endosomen die betrokken zijn bij antigeen kruis-presentatie. Dit is de reden dat we het gedrag van endosomen tijdens het functioneren van DCs wilden onderzoeken. Dit onderzoek heeft geleid tot de resultaten van hoofdstuk 4.

In **hoofdstuk 4** gebruiken we fluorescentie microscopie op levende humane DCs die afkomstig zijn van monocyten (moDCs) om het endosomale gedrag te bestuderen tijdens DC activatie. We maken gebruik van fluorescerende LDL en Transferrine die beiden worden vervoerd in specifieke endosomale compartimenten: respectievelijk "late" en "vroege/recyclende" endosomen. We laten zien dat activatie van humane moDCs door LPS leidt tot vervorming van late endosomen (LE) blaasjes in verlengde buizen (ETEN). Dit komt overeen met eerdere studies in DCs van muizen, dus dit mechanisme is bewaard gebleven tijdens de evolutie van muis naar mens. Eerder onderzoek in de muis met elektronenmicroscopie heeft laten zien dat de LE-buisjes zich uitrekken tot

net onder het celoppervlak. Net onder het celoppervlak vormen de buisjes weer kleine transportblaasjes die vervolgens met het celoppervlak samensmelten om de vracht hier af te leveren. Daarom lijkt het erop dat activatie-geïnduceerde LE buizen een tussenvorm zijn in endosomaal transport naar het celoppervlak.

Echter, normaal gesproken vind celoppervlak-gericht transport plaats door het endosomale recycling compartiment (ERC), welk erom bekend is kortere buisjes te vormen. Daarom vroegen we ons af of ETEN uit het ERC gevormd worden. Om deze vraag te beantwoorden, hebben we transferrine (Tfn)-postieve ETEN bestudeerd in humane DCs na activatie. In tegenstelling tot LE blaasjes hervormen de Tfn^+ blaasjes zich niet in lange buizen (ETEN) na stimulatie met LPS. Pas na interactie met antigeen-specifieke $CD8^+$ T cellen vormden zich ook Tfn^+ ETEN in DCs. Tfn^+-ETEN ontspringen uit een regio net naast de celkern, waar het ERC ligt. Bovendien bewijzen experimenten met de recycling remmer Primaquine dat Tfn^+-ETEN ontstaan uit het ERC: Tfn^+ buizen verdwijnen namelijk na het gebruik van Primaquine.

Verder onderzoek van ons laat zien dat het binden van klasse I MHC en ICAM-1 van de moDCs met de T-cel receptor en LFA-1 nodig is voor de vorming van TFN^+-ETEN. Vervolgens gebruikten we reversibele remmers die microtubuli afbreken of endosomale recycling remmen, het geen de Tfn^+ buizen deed verdwijnen. De verdwijning van de Tfn^+ buisjes ging gepaard met een vermindering in antigeen-specifieke activatie van $CD8^+$ T-cellen door de DCs. Dit geeft aan hoe belangrijk het celoppervlak-gerichte transport via buisjes is voor activatie van de adaptieve afweerreactie.

In afwezigheid van de remmers kunnen we na minstens zes uur nog steeds de LDL^+ en Tfn^+ buizen waarnemen in humane DCs. Omdat de endosomale buizen energetisch gezien een ongunstige vorm in een waterige omgeving zijn, zijn er vermoedelijk één of meerdere moleculen die de endosomalen buizen vormen en stabiliseren. In **hoofdstuk 5** bespreken we moleculaire mechanismen die voor de initiatie, stabilisatie, elongatie en opknippen van de endosomale buizen zouden kunnen zorgen. Eén van de besproken moleculaire mechanismen is de EHD1 en MICAL-L1 afhankelijke recycling van src-kinases via Tfn^+ buizen in navolging van integrine receptor stimulatie. We laten zien dat MICAL-L1 nodig is voor Tfn^+ ETEN door MICAL-L1 expressie te verminderen met behulp van MICAL-L1 specifieke siRNAs. De vermindering van MICAL-L1 expressie heeft geen significant effect op LPS-geinduceerde LE buizen in humane DCs. We kunnen dus specifiek de functie van Tfn^+ ETEN onderzoeken in verdere studies. Naar mijn mening zou toekomstig onderzoek zich verder moeten concentreren op moleculen die ETEN beïnvloeden, zoals MICAL-L1 en EHD1. Daarnaast zou de rol van ETEN in antigeen kruis-presentatie en $CD8^+$ T-cel stimulatie verder onderzocht kunnen worden.

Zoals bediscussieerd in hoofdstuk 2 kan receptor-gemedieerde opname invloed hebben op welke antigeen-presentatie route genomen wordt door het opgenomen antigeen. In de muis is bewezen dat de Fcɣ receptoren (FcɣRs)-gemedieerde opname van antigenen, o.a. geëxpresseerd op DCs, leidt tot kruis-presentatie van de antigenen. In **hoofdstuk 6**

onderzoeken we antigeen kruis-presentatie van FcγR-gerichte antigenen in humane DCs. We laten zien dat het sturen van antigenen naar FcγRs door middel van IgG opsonisatie, resulteert in verhoogde $CD8^+$ T-cel activatie na antigeen kruis-presentatie. Met meerdere remmers laten we vervolgens zien dat kruis-presentatie van FcγR-gerichte antigenen zowel de "cytosolaire" en "vacuolaire" mechanismen gebruiken in humane moDCs, BDCA-1^+, en BDCA-3^+ DCs. Interessant is dat alle DCs, maar vooral de BDCA-3^+ DCs, een verbetering laten zien in hun capaciteit van antigeen kruis-presentatie na antigeen opname via FcγRs. De superioriteit van BDCA-3^+ DCs t.o.v. BDCA-1^+ DCs in antigeen kruis-presentatie van FcγR-gerichte antigen kan niet verklaard worden door verhoogde antigeen opname capaciteit, want juist de BDCA-1^+ DCs nemen meer antigenen op. Waarschijnlijk is de relatief hogere expressie van de stimulerende FcγRIIa variant in BDCA-3^+ ten opzichte van BDCA-1^+ DCs de verklaring voor de betere antigeen kruis-presentatie capaciteit.

Ontregeling van B cel activatie en antigeen presentatie.

In **hoofdstuk 7** beschrijf ik de ziekte "Common Variable Immunodeficiency" (CVID). Dit is de meest voorkomende gediagnostiseerde symptomatische primaire immuundeficiëntie. CVID ontwikkelt zich als gevolg van een afwezigheid of slechte werking van eiwitten die betrokken zijn bij de productie van antistoffen met een hoge affiniteit. In het laatste decennium zijn er verscheidene genetische predisposities voor de ontwikkeling van CVID opgehelderd. De ontwikkeling van antigeen-specificieke antistoffen met hoge affiniteit heeft tenminste twee acties nodig van de B-cel receptor (BCR): transmembraan signalering voor de stimulatie van B-cel differentiatie en antigeen opname voor klasse II MHC antigeen presentatie en daaruitvolgend aanwerven van $CD4^+$ T-helper cellen. Het is dus niet verrassend dat patiënten met CVID-achtige symptomen monogenetische defecten hebben in genen die betrokken zijn bij BCR signalering of het verkrijgen van $CD4^+$ T-helper cellen, zoals de CD19, CD20, CD81, CD27, en ICOS-L genen.

Ondanks een grote inspanning is de oorzaak of het ziektemechanisme van CVID nog niet opgehelderd in 85 procent van de CVID patiënten. In een poging om nieuwe oorzaken voor CVID te vinden, hebben we een nieuwe genetische diagnose techniek (targeted Next-Generation sequencing) toegepast op een aantal CVID patiënten met een onbekende oorzaak van hun CVID ziektebeeld.

In **hoofdstuk 8** beschrijven we twee gerelateerde CVID patiënten die een mutatie in het B-lymphoid tyrosine kinase (BLK) gen hebben, waardoor het BLK eiwit zijn derde aminozuur (een Leucine) verandert in een Proline. BLK behoort tot de familie van src-kinases, die van belang zijn in BCR signalering. In tegenstelling tot muizen, waar er een overlap in functie is tussen src-kinases, leidt de L3P mutatie in BLK tot vermindering van BCR signalering, in vorm van verminderde Syk fosforylering, welke nodig is voor B-cel celdeling. Daarnaast laten we zien dat de expressie van L3P-BLK leidt tot versnelde afgifte van BCR-opgenomen antigenen in late endosomen. In late endosomen worden

de antigenen vernietigd. Dit zorgt voor een vermindering in kruis-presentatie van het antigeen, wat resulteert in verminderde BCR-gemedieerde HLA-DR afhankelijke antigeen-specifieke activatie van $CD4^+$ T-cellen. We kunnen hieruit opmaken dat defecte BCR signalering en verbonden antigeen-presentatie in sommige gevallen een onderliggend ziektemechanisme kunnen zijn in CVIDers. Of er meerdere CVID patiënten zijn met defecte antigeen-presentatie is momenteel nog onbekend, maar in **hoofdstuk 9** laten we zien dat een significante hoeveelheid CVID kinderpatiënten een defect in BCR signalering hebben. We laten namelijk zien dat deze CVID kinderen een slechte calcium influx hebben na stimulatie van de BCR. Dit calcium defect verstoort differentiatie in plasmablasten *in vitro* en lijkt vroeg in de BCR signalerings cascade plaats te vinden, aangezien de calcium influx via de celoppervlakte kanalen en het opslaan en depleteren van ER-calcium normaal is. De verstoorde BCR signalering wordt niet veroorzaakt door de bekende oorzaken: toename in expressie van CD22, of mutaties in PLCɣ2 of Btk. We laten zien dat B-cellen van de CVID kinderpatiënten een verminderde dissociatie heeft tussen BCR en CD20 moleculen op het celoppervlak. BCR of CD20 stimulatie leidt tot verminderde BCR opname. Daarnaast leidt CD20 stimulatie tot minder sterke BCR signalering, gemeten als navolgende calcium fluxen. Hieruit concluderen we dat fouten in het CD20/BCR signaal complex onderliggend kunnen zijn aan CVID.

In conclusie, dit proefschrift beschrijft de belangrijke rol dat het endosomale netwerk heeft in de initiatie van adaptieve afweerreacties. Het reguleren van essentiële endosomale processen door onder andere het verstoren van ETEN formatie, het richten van antigenenen naar (Fcɣ) receptoren, of src-kinase (BLK) functioneren, kan bijdragen aan de verbetering van toekomstige therapeutische toepassingen en overlevingskansen van patiënten.

List of Publications

This thesis

Compeer EB, Janssen W, van Royen-Kerkhof A, van Gijn M, van Montfrans JM, Boes M. Dysfunctional BLK in Common Variable Immunodeficiency perturbs B cell proliferation and ability to elicit antigen-specific $CD4^+$ T cell help. *Oncotarget 2015 Mar 14;5.*

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Curriculum Vitae

Ewaldus (Ewoud) Bernardus Compeer was born on April 14th 1986 in Rotterdam, the Netherlands. In 2004, he completed secondary education at "De Ring van Putten" in Spijkenisse, with his major in Biology and Health. The same year, he started Biomedical Sciences at the University of Utrecht. His bachelor thesis and first internship as part of the master program "Immunity and Infection" (I&I), focused on endosomal requirements for effective Coronavirus infection. In 2008, Ewoud obtained grants from the Dutch Digestive Foundation, Trajectum, and the Dutch Kidney Foundation to research SLAM's modulation of endosomal lumen under supervision of Prof. Cox Terhorst at Harvard Medical School in Boston, USA. In 2010 he began his PhD training at the "Eijkman Graduate School for Infection and Immunity" at the UMC Utrecht in Utrecht, the Netherlands. He worked in the "Center for Molecular and Cellular Intervention" (CMCI), now part of "Laboratory of Translational Immunology" (LTI), in the laboratory of Dr. Marianne Boes. During this PhD program, he studied novel endosomal processes in human antigen presenting cells that are pivotal to activation of the adaptive immune system. The results obtained during this PhD project are presented in this thesis.

As member of the I&I educational board, PhD council and Board of Studies of Graduate School of Life Sciences, Ewoud has contributed to the education of Life Science master students and PhD candidates of Utrecht's University. Internationally he affected current education of Life Science PhD candidates as creator of the Graz-declaration and chair of "Quality of PhD education"-Lausanne as member of "Organization for PhD Education in Biomedicine and Health Sciences in the European System" (ORPHEUS).

In 2014, he started his post-doctorate under Dr. Jeremie Rossy and Prof. Katharina Gaus in the recently established ARC Centre of Excellence "Centre for Advanced Molecular Imaging" at the University of New South Wales in Sydney, Australia. Currently he is studying the connection between 2D surface T cell signalling microdomains and the 3D microarchitecture of the endosomal network at a nanoscale, enabled by novel 2D and 3D single-molecule and ultra-fast fluorescence microscopic techniques.

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Niek en Ed, lieve zusje en soon-to-be zwager, eigenlijk spreken we elkaar te weinig. Maar als we elkaar dan zien en spreken is het altijd gezellig (behalve als er een spelletje gespeeld word). Nicolien, ik vind het hartstikke leuk dat jij straks als paranimf naast me staat om me te ondersteunen op zo'n belangrijk moment in mijn leven. En ik kijk uit naar jou (eigenlijk jullie) grote moment. Ik zal er zijn, ongeacht welk vliegtuig, boot, of kano reis die ik moet ondernemen.

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Appendix.

SLAM is a microbial sensor that regulates bacterial phagosome functions in macrophages.

Scott B Berger*, Xavier Romero*, Chunyan Ma, Guoxing Wang, William A Faubion, Gongxian Liao, **Ewoud Compeer**, Marton Keszei, Lucia Rameh, Ninghai Wang, Marianne Boes, Jose R Requeiro, Hans-Christian Reinecker and Cox Terhorst.

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Abstract

Phagocytosis is a pivotal process by which macrophages eliminate microorganisms after recognition by pathogen sensors. Here we unexpectedly found that the self ligand and cell surface receptor SLAM functioned not only as a costimulatory molecule but also as a microbial sensor that controlled the killing of Gram-negative bacteria by macrophages. SLAM regulated activity of the NADPH oxidase NOX2 complex and phagolysosomal maturation after entering the phagosome, following interaction with the bacterial outer membrane proteins OmpC and OmpF. SLAM recruited a complex containing the intracellular class III phosphatidylinositol kinase Vps34, its regulatory protein kinase Vps15 and the autophagy-associated molecule beclin-1 to the phagosome, which was responsible for inducing the accumulation of phosphatidylinositol-3-phosphate, a regulator of both NOX2 function and phagosomal or endosomal fusion. Thus, SLAM connects the Gram-negative bacterial phagosome to ubiquitous cellular machinery responsible for the control of bacterial killing.

Introduction

Diverse macrophage receptors act together to recognize bacteria via conserved structures on the bacterial surface and facilitate phagocytosis and/or signaling that initiates the innate immune response and triggers subsequent activation of adaptive immunity. These bacterial receptors include scavenger receptors, C-type lectins, integrins, Toll-like receptors (TLRs) and Siglec proteins, which recognize conserved bacterial moieties ranging from lipopolysaccharide (LPS) on Gram-negative bacteria to peptidoglycan and lipoteichoic acid on Gram-positive bacteria1. Many of these receptors are somewhat promiscuous, recognizing multiple ligands with varying degrees of specificity and affinity2. Whether and how these macrophage cell surface receptors, some of which enter the phagosome, control key steps in microbicidal functions, such as the production of reactive oxygen species or phagolysosomal fusion, is not well understood at present.
Receptors of the signaling lymphocyte-activation molecule family (SLAMF), encoded by Slamf1–Slamf9 in the mouse, are adhesion molecules on the surface of most hematopoietic cells that serve as costimulatory molecules that initiate distinct signal-transduction networks in T cells, natural killer cells and antigen-presenting cells3,4. Both functional and structural studies have demonstrated that the ectodomains of the SLAMF receptors are homophilic or self ligand receptors, except SLAMF2 (CD48), which uses both SLAMF4 (CD244) and CD2 as its counter-ligands. These receptors not only operate as costimulatory molecules in the adaptive immune system but also participate in lineage-commitment steps of hematopoiesis and natural killer T cell development, as well as in the functional regulation of natural killer cells, neutrophils, dendritic cells, macrophages and platelets3. In addition, SLAM (SLAMF1; CD150) is a receptor for measles virus5. As most SLAMF receptors are expressed on the surface of myeloid cells, we decided to examine the role of SLAMF receptors in innate immune responses other than natural killer cell functions. This idea was supported by several studies of the role of SLAM receptors in responses to bacteria or bacterial components6,7. In this report we show that SLAM (SLAMF1) had a role in innate immune responses to inoculation with Escherichia coli or an attenuated Salmonella typhimurium strain negative for production of the Salmonella pathogenicity island 2 type III secretion system SseB protein (SseB−) by regulating bacteriocidal activity in the phagosome of macrophages. After interaction with E. coli, SLAM entered the bacterial phagosome, where it mobilized a ubiquitous enzyme complex involved in organelle fusion through the production of phosphatidylinositol-3-phosphate (PtdIns(3)P)8. Because PtdIns(3)P also regulates the activity of the NADPH oxidase (NOX2) complex in phagosomes, SLAM-deficient macrophages killed inefficiently. Thus,SLAM is not only a self ligand and a receptor for measles virus but also a bacterial sensor that, like some TLRs, regulates intracellular enzyme activities involved in the removal of Gram-negative bacteria.

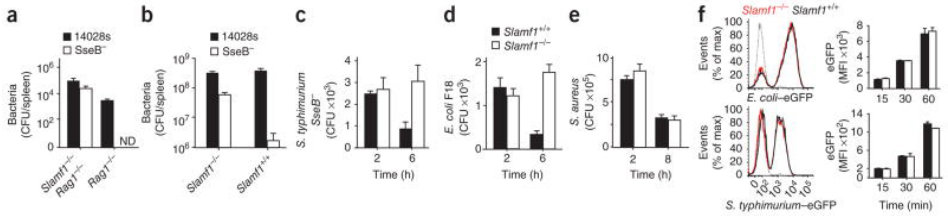

Figure 1. SLAM controls *in vivo* and *in vitro* killing of Gram-negative bacteria by mouse macrophages.

(**a**,**b**) Bacteria in the spleens of Slamf1−/− Rag1−/− and Rag1−/− BALB/c mice (**a**) or Slamf1−/− and Slamf1+/+ BALB/c mice (**b**) 48 h after intraperitoneal injection of virulent S. typhimurium 14028s or attenuated S. typhimurium SseB−. CFU, colony-forming units; ND, not detectable. Data are representative of four independent experiments (mean and s.d.). (**c**–**e**) Killing of bacteria by peritoneal macrophages from Slamf1+/+ and Slamf1−/−BALB/c mice exposed to S. typhimurium SseB− (**c**), E. coli F18 (**d**) or S. aureus (**e**), assessed by gentamycin assay. Data are representative of five independent experiments (mean and s.d.). (**f**) Uptake of bacteria by Slamf1+/+ (red solid lines) or Slamf1−/− (black solid lines) BALB/c peritoneal macrophages incubated at 37 °C with E. coli–eGFP or S. typhimurium–eGFP or by Slamf1+/+ macrophages incubated for 60 min at 4 °C with the bacteria (dotted lines). Right, mean fluorescence intensity (MFI). Data are representative of three independent experiments (mean and s.d.).

RESULTS

Inefficient killing by SLAM-deficient macrophages

As SLAM is expressed on the surface of macrophages and because SLAM is involved in the regulation of cytokine secretion by human and mouse macrophages and dendritic cells3,7, we set out to evaluate a possible role for SLAM in innate immune responses. We therefore inoculated mice with double knockout of SLAM and recombination-activating gene 1 (Slamf1−/− Rag1−/− mice) with a 1:1 mixture of the attenuated S. typhimurium SseB− strain9 and virulent wild-type S. typhimurium strain 14028s. At 48 h after infection, the double-knockout mice cleared neither bacteria, as judged by strain-selective bacterial counts in the spleen (Fig. 1a). In contrast, Slamf1+/+ Rag1−/− mice cleared the attenuated strain but not the wild-type bacteria (Fig. 1a). This weakened innate immune response of the double-knockout mice was reflected in the impaired clearing of the same attenuated S. typhimurium SseB−strain by Slamf1−/− mice but not SLAM-sufficient wild-type mice (Fig. 1b).

The inefficient responses of the mutant mice to bacterial inoculation were caused by defective killing of nonopsonized S. typhimurium SseB− by Slamf1−/− macrophages, as determined by gentamicin killing assays (Fig. 1c). Whereas killing of E. coli was also impaired (Fig. 1d), we observed no defect in the response to Gram-positive S. aureus (Fig. 1e).

The differences in macrophage killing could not be attributed to an obvious defect in bacterial uptake because we found no difference between mutant and wild-type killing at early time points of the gentamicin assay (Fig. 1c,d). In addition, we found

no difference between wild-type and mutant macrophages in a cytofluorometry-based phagocytosis assay with S. typhimurium SseB− or E. coli expressing enhanced green fluorescent protein (eGFP; Fig. 1f). We excluded the possibility of developmental defects (Supplementary Fig. 1) or an influence of the genetic background of the mutant mice (Supplementary Fig. 2) because Slamf1+/+ and Slamf1−/− macrophages had no difference in their expression of cell surface markers (Supplementary Fig. 1) and because the killing of E. coli by Slamf1−/− C57BL/6 (B6) and Slamf1−/− BALB/c macrophages was equally impaired (Supplementary Fig. 2). The latter finding was not unexpected, as both Slamf1−/− BALB/c and Slamf1−/− B6 mice contain the SLAMF haplotype II Slamf1–Slamf7 locus3, derived from the original 129 embryonic stem cells7 (Supplementary Figs. 3 and 4). Thus, the cell surface receptor SLAM positively regulates the killing of Gram-negative bacteria by macrophages.

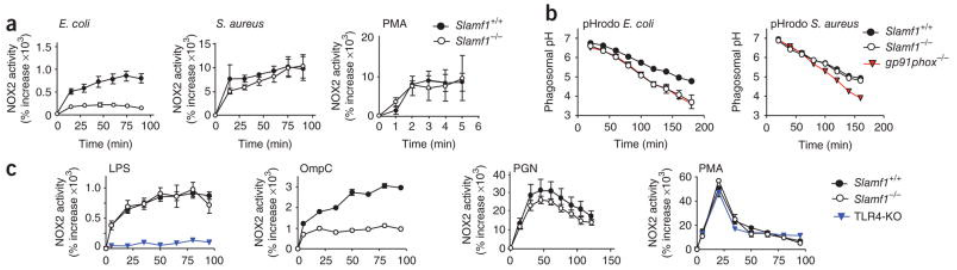

Figure 2. Defective NOX2 activity in primary macrophages derived from SLAM-deficient mice.
(**a**) NOX2 activity in Slamf1+/+ and Slamf1−/− BALB/c peritoneal macrophages stimulated for 0–100 min with E. coli F18, S. aureus or PMA, assessed with lucigenin. Data are representative of five independent experiments (mean ± s.d.). (**b**) Phagosomal pH of Slamf1+/+, Slamf1−/− and gp91phox−/− B6 primary macrophages loaded for 0–200 min withpHrodo-coated E. coli or S. aureus, analyzed by flow cytometry. Data are representative of three independent experiments (mean ± s.d.). (**c**) NOX2 activity in primary macrophages in response to LPS, purified OmpC, peptidoglycan (PGN) or PMA, assessed with lucigenin. TLR4-KO, TLR4-deficient (strain del/Jtht; C3H). Data are representative of five independent experiments (mean ± s.d.).

SLAM regulates NOX2 activity in *E. coli*–containing phagosomes

One of the key mechanisms by which phagocytes can kill bacteria uses the NOX2 enzyme complex, composed of the plasma membrane–bound proteins p22phox and gp91phox and the cytosolic proteins p40phox, p47phox, p67phox, Rac 1 and Rac 2, which are recruited to the membrane complex10. In macrophages, the active NOX2 enzyme in the phagosomal membrane is responsible for the reduction of O2 to O2−, which is further converted in the lumen of the phagosome to superoxide. By using the chemiluminescence detector lucigenin9 to detect the production of superoxide in the cell, we found that primary Slamf1−/− macrophages produced less reactive oxygen in response to E. coli than did wild-type macrophages (Fig. 2a and Supplementary Fig. 5). In contrast, NOX2 activity induced by S. aureus was not affected in the SLAM-deficient macrophages. Thus, SLAM is a positive regulator of NOX2 activity in macrophages. We

confirmed that with the finding that in response to E. coli, NOX2 activity was greater in Slamf1-transfected RAW264.7 mouse macrophages (Supplementary Fig. 6), which themselves are SLAM deficient.

To test the hypothesis that SLAM specifically regulates NOX2 activity in the lumen of Gram-negative phagosomes, we compared the pH of E. coli– and S. aureus–containing phagosomes in freshly isolated macrophages. The fluorescence assay we used is based on the principle that conversion of the reactive oxygen species that are produced by active NOX2 to HO2 and H2O2 consumes protons, which enter the phagosome via a proton pump11. Thus, impairment in the activity of the NOX2 enzyme would result in less proton consumption in the bacterial phagosome and the pH would be lowered more rapidly12. To test that idea, we used the pH-sensitive dye pHrodo13, coupled to the outer surface of either E. coli or S. aureus, to monitor the decrease in pH in bacterial phagosomes by cytofluorometry.Indeed, the E. coli–containing phagosomes acidified more rapidly in Slamf1−/− macrophages than in wild-type macrophages. In fact, acidification in the Slamf1−/− macrophage–derived phagosomes was similar to that of phagosomes from mice deficient in the gp91phox component of NOX2 (Cybb−/−; called 'gp91phox−/−' here; Fig. 2b). In contrast, acidification of the S. aureus–containing phagosomes took place at the same rate in Slamf1−/− and wildtype macrophages, whereas the pH was lower in the S. aureus–containing phagosomes from gp91phox−/− mice (Fig. 2b). Thus, the defective function of NOX2 in Slamf1−/− macrophages resided in the E. coli–containing phagosomes.

One plausible explanation for the specificity for E. coli could be that the impaired NOX2 response by Slamf1−/− macrophages involves an indirect response to lipopolysaccharide (LPS) due to a convergence of SLAM- and TLR4-induced signal-transduction networks. However, NOX2 activation by LPS was impaired only in TLR4-deficient macrophages, not in primary Slamf1−/− macrophages (Fig. 2c). Predictably, NOX2 responses by macrophages to peptidoglycan or the phorbol ester PMA were not affected by SLAM deficiency (Fig. 2c). We conclude that SLAM positively regulates the function of NOX2 in E. coli–containing phagosomes but not in S. aureus–containing phagosomes.

Delayed maturation in the absence of SLAM

Because lysosomal enzymes contribute to the elimination of bacteria by macrophages14, we assessed whether phagolysosomal maturation was dysregulated in the absence of SLAM.

For this, we transfected primary macrophages with LAMP-1 (an established lysosomal marker) conjugated to red fluorescent protein (RFP) and initiated phagocytosis of eGFPexpressing E. coli (E. coli–eGFP) or S. aureus (S. aureus–eGFP). When we evaluated the staining of E. coli–containing phagosomes with RFP-conjugated LAMP-1 by fluorescence microscopy, we observed a quantitative delay in the recruitment of LAMP-1 in Slamf1−/− macrophages relative to its recruitment in wild-type cells. In contrast, we

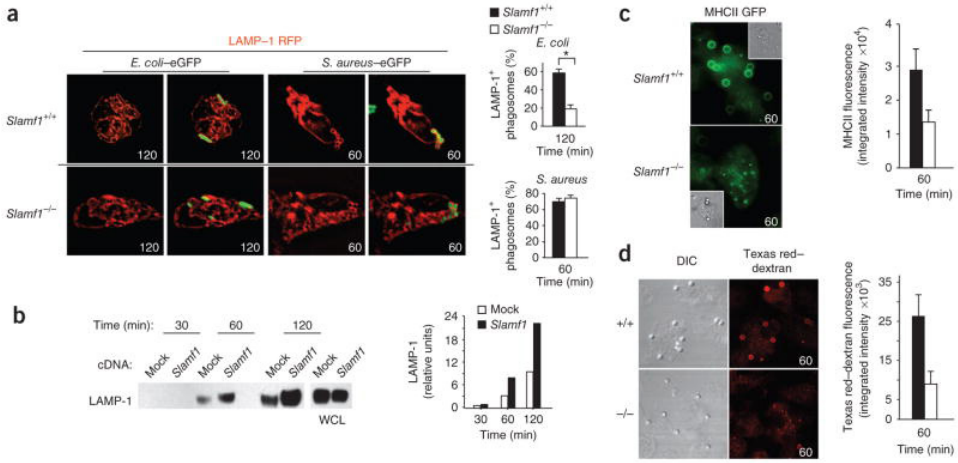

Figure 3. Impaired phagolysosomal maturation in Slamf1−/− macrophages. (a) Fluorescence microscopy of E. coli–containing phagosomes in primary macrophages transfected with RFP-conjugated LAMP-1, showing colocalization with E. coli–eGFP or S. aureus–eGFP. Right, quantification of LAMP-1+ phagosomes in the microscopy at left. (b) Immunoblot analysis of LAMP-1 in phagosomes isolated by sucrose-gradient flotation from RAW264.7 macrophages after phagocytosis by Slamf1- or mock-transfected RAW264.7 macrophages

of beads coated with E. coli outer membrane extract. WCL, whole-cell lysate. Right, quantification of LAMP-1 in the immunoblot at left. (**c**) Localization of MHC class II–eGFP (MHCII GFP) in phagosomes of primary macrophages from Slamf1+/+ or Slamf1−/− MHC class II–eGFP (B6) mice and 3-µm beads coated with E. coli outer membrane extract. (**d**) Entry of 3-µm beads coated with E. coli outer membrane extract into lysosomes loaded with Texas red–dextran. Right (**c**,**d**), quantification of fluorescence in the microscopy at left. Numbers in bottom right corners (**a**,**c**,**d**) indicate time (in min). Original magnification (**a–c**), ×60. DIC, differential interference contrast. Data are representative of three combined experiments (**a**) or at least three independent experiments (**b–d**) with at least 100 beads or 80 bacteria in each (error bars, s.d.).

found no difference in the recruitment of LAMP-1 to S. aureus–containing phagosomes in wild-type or Slamf1−/− macrophages (Fig. 3a). Thus, consistent with the impaired bacterial killing and diminished NOX2 activity, the maturation of phagosomes containing E. coli, but that of not those containing S. aureus, was affected by the absence of SLAM in macrophages.

We confirmed a role for SLAM in phagolysosomal maturation by analyzing phagosomes isolated by sucrose-gradient flotation15. We initiated phagocytosis by adding 3-µm polystyrene beads coated with a crude preparation of E. coli outer membrane extracts to mock-transfeted or Slamf1-transfected RAW264.7 macrophages. At various times (30, 60 or 120 min) after the initiation of phagocytosis, we isolated the organelles on the basis of bead buoyancy. Phagosomes purified from SLAM+ macrophages had more LAMP-1 at 60 and 120 min after the initiation of phagocytosis than did those isolated from mock-transfected macrophages (Fig. 3b).

In the next set of experiments we used a second phagolysosomal marker: eGFP-tagged major histocompatibility complex (MHC) class II. We compared phagosomal maturation

in macrophages from a reporter mouse in which the gene encoding MHC class II is replaced with a version that encodes MHC class II tagged with eGFP (MHC class II–eGFP)16 with that of the progeny of MHC class II–eGFP mice crossed with Slamf1−/− mice (Slamf1−/− MHC class II–eGFP mice). We allowed primary macrophages to phagocytose 3-µm polystyrene beads coated with a crude preparation of E. coli outer membrane extracts and monitored translocation to phagolysosomes by confocal microscopy. We detected considerably fewer MHC class II–positive phagolysosomes in Slamf1−/− MHC class II–GFP macrophages than in wild-type MHC class II–GFP macrophages 60 min after initiation of phagocytosis (Fig. 3c). However, we observed no difference 120 min after the initiation of phagocytosis (data not shown). This confirmed that trafficking of cargo to the phagolysosome was delayed in the absence of SLAM. As an alternative approach to determining the arrival of cargo in the phagolysosome, we loaded lysosomes with Texas red–dextran before initiating phagocytosis with coated 3-µm polystyrene beads. We quantified colocalization of the beads and Texas red at various time points by fluorescence microscopy. Whereas Slamf1+/+ macrophages contained coated beads in the dextran-loaded lysosomes 60 min after the initiation of phagocytosis, we detected only a small number of beads in Slamf1−/− lysosomes (Fig. 3d). The consequence of the absence of SLAM was already detectable at an earlier stage of phagosome maturation, as recruitment of the tethering molecule EEA1 and the small GTPase Rab5.GTP17,18 was delayed, as judged by quantitative fluorescence microscopy (Fig. 4). The specificity of the role of SLAM in bacterial phagosomal maturation was further emphasized by the finding that SLAM deficiency had no effect on the formation of transferrin-loaded recycling endosomes or the formation of low-density lipoprotein–endolysosomes, which are dependent on fusion events involving EEA1 (Supplementary Figs. 7 and 8). We observed the delay in phagosomal maturation, as judged by Rab5 localization, in primary macrophages from both Slamf1−/− BALB/c mice and Slamf1−/− B6 mice (Supplementary Fig. 9), and this defect was not caused by the impaired NOX2 function itself, because gp91phox-deficient and p40phox-deficient macrophages had a normal phagolysosomal maturation (data not shown). Together, the outcomes of these experimental approaches demonstrate that both NOX2 activity in the E. coli–containing phagosome and the progression of phagolysosomal maturation are positively controlled by the cell surface receptor SLAM.

Entry of SLAM into *E. coli*–containing phagosomes

Because of its effect on two key microbicidal phagosomal processes, we reasoned that any SLAM-dependent mechanism(s) would require entry of SLAM into the Gram-negative phagosome. To test our hypothesis, we first transfected SLAM-deficient RAW264.7 macrophages with cDNA encoding a fusion protein of SLAM and the red fluorescent protein mCherry (SLAM-mCherry) before phagocytosis of E. coli–eGFP or S. aureus–eGFP. Quantitative fluorescence microscopy demonstrated that SLAM-mCherry localized

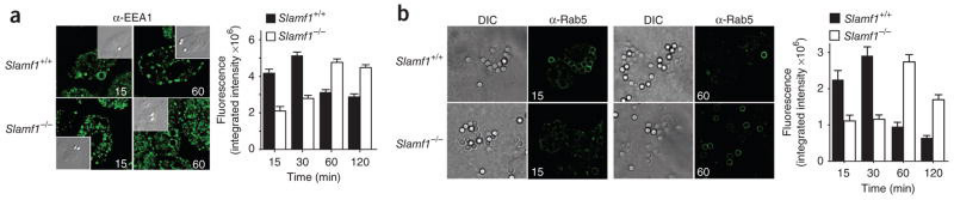

Figure 4.Delay in early phagosomal maturation in Slamf1−/− macrophages.

(**a**,**b**) Association of EEA-1 (**a**) or Rab5 (**b**) with phagosomes generated in primary macrophages by 3-μm beads coated with E. coli outer membrane extract; numbers in bottom corners indicate time (in min). α-, anti-. Original magnification, ×60. Right (**a**,**b**), quantification of fluorescence. Data are representative of at least three independent experiments (error bars, s.d.).

together with E. coli–eGFP but not with S. aureus–eGFP in transfectant cells (Fig. 5a). Furthermore, we also detected SLAM in phagosomes isolated from SLAM-transfected RAW264.7 macrophages (Fig. 5b). These observations demonstrate that SLAM enters the phagosome with the same degree of specificity as its regulation of microbicidal activity (Figs. 2 and 3).

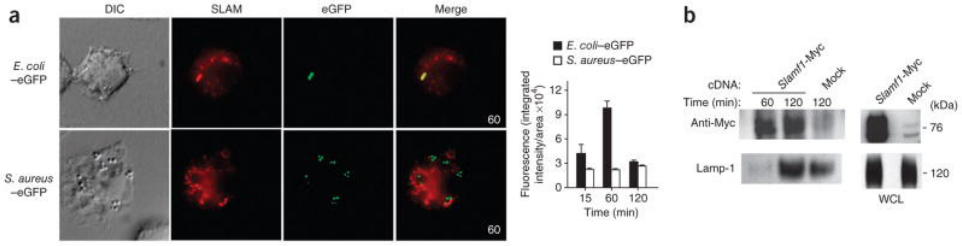

Figure 5. SLAM enters the E. coli–containing phagosome.

(**a**) Colocalization of SLAM and bacteria in RAW264.7 macrophages transfected with SLAM-mCherry and allowed to phagocytose E. coli–eGFP or S. aureus–eGFP for 0–120 min (time (in min), bottom right corners at left). Original magnification, ×60. Right, quantification of fluorescence at left. Data are representative of two independent experiments with a minimum of 40 bacteria per time point (error bars, s.d.). (**b**) Immunoblot analysis of phagosome isolates from RAW264.7 macrophages transiently transfected with cDNA encoding Myc-tagged SLAM (Slamf1-Myc) or mock transfected and allowed to phagocytose beads coated with E. coli outer membrane extract for 60 or 120 min. kDa, kilodaltons. Data are representative of at least three independent experiments.

A

SLAM recognizes bacterial OmpC and/or OmpF

Because the deletion of SLAM affected E. coli–related phagosomal events, we reasoned that SLAM recognizes a surface component of the bacterium. To test our hypothesis, we developed a sensitive signal-amplification assay in which we transfected a fusion protein of the mouse SLAM ectodomain and the intracellular region of human CD3ζ into Jurkat human T cells together with a luciferase reporter driven by the promoter of the gene encoding interleukin 2. With this cell-surface based assay we determined that SLAM recognized both E. coli and S. typhimurium SseB− (Fig. 6a). In contrast, S. aureus did not

induce a response above the background response caused by homophilic SLAM-SLAM interactions.

We confirmed the specificity of the direct interaction of SLAM with E. coli by two experiments: first, recognition of E. coli was abolished after removal of the N-terminal ectodomain (immunoglobulin V) of SLAM (Fig. 6b and Supplementary Fig. 10); and second, two monoclonal antibodies directed against SLAM19 blocked the response to E. coli (Fig. 6c and data not shown). Recognition of these Gram-negative bacteria was independent of LPS binding because Jurkat cells do not express TLR4. Furthermore, in response to E. coli, TLR4-deficient macrophages are not impaired in phagocytosis20 or NOX2 function9. Additionally, SLAM does not directly bind to LPS because NOX2 activation by LPS was identical in primary macrophages from wild-type and Slamf1−/− mice (Fig. 2c). To determine which component of the E. coli outer membrane might be involved in recognition by SLAM, we analyzed the crude E. coli membrane extract used for coating the polystyrene beads in the phagocytosis assays (Figs. 3–5). Nonquantitative mass spectrometry–based analyses indicated that the outer membrane porins were principal components of the preparation used (data not shown). We therefore determined whether SLAM recognized E. coli mutants lacking one or more of the E. coli outer membrane porins.

We found that an E. coli mutant (HN705) lacking both of the outer membrane porins OmpC and OmpF was not recognized by SLAM, whereas E. coli mutants lacking either OmpC or OmpF were partially recognized (Fig. 6d). In contrast, many other E. coli variants or mutants were recognized by SLAM (Supplementary Fig. 11). The most plausible explanation for these observations is that the membrane-distal SLAM ectodomain binds to one or more of the extracellular loops of OmpC and OmpF, which have similar sequences21 (Supplementary Fig. 12).

To evaluate the role of OmpC in a SLAM-dependent physiological process, we used purified OmpC to induce NOX2 activity in SLAM-deficient and SLAM-sufficient primary macrophages (Fig. 2c). Indeed, SLAM deficiency resulted in a lower NOX2 response to the purified protein, which indicated a relationship between SLAM function and this bacterial component. In contrast, NOX2 responses in macrophages to LPS, peptidoglycan, CpG (a TLR9 ligand) or PMA were not affected by the alteration of SLAM (Fig. 2c and data not shown). Additionally, we made use of the HN705 E. coli mutant doubly deficient in OmpC and OmpF to functionally confirm that these proteins were the targets of the SLAM receptor (Supplementary Fig. 13). As expected, SLAM-deficient macrophages had less NOX2 activity in response to wild-type E. coli (JM101). However, we observed no defect in response to the HN705 double mutant. Together these data demonstrate that SLAM itself is able to sense the molecular signature of Gram-negative bacteria by recognizing OmpC and most probably OmpF and as a consequence enters the phagosome, where it directs NOX2 activity and phagosomal maturation.

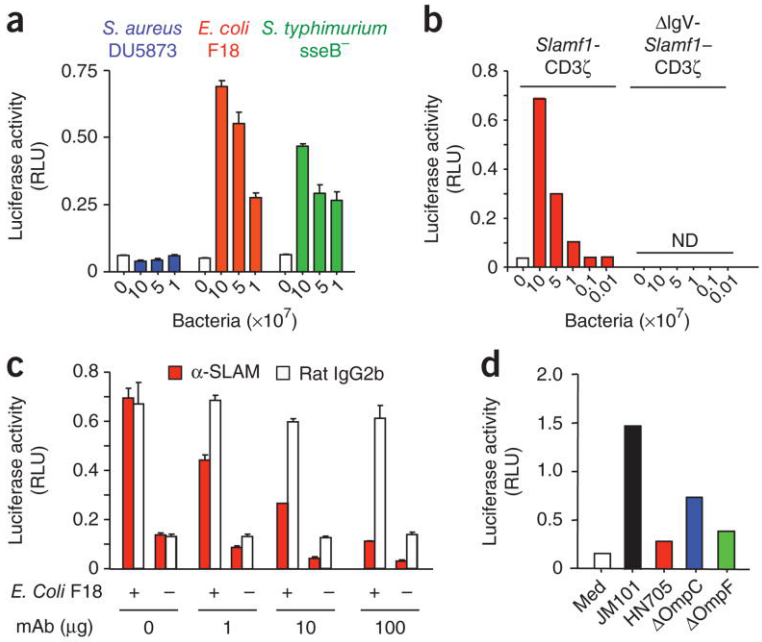

Figure 6. SLAM recognizes E. coli and S. typhimurium Sseb– but not S. aureus.

(**a**) Luciferase activity in Jurkat cells transfected with a fusion of SLAM and CD3ζ and a luciferase reporter (as described in Results), plus a renilla luciferase reporter, then exposed to heatkilled bacteria (top). (**b**) Luciferase activity in Jurkat cells transfected with the fusion in **a** (Slamf1-CD3ζ) or a fusion of SLAM ectodomain construct lacking the immunoglobulin V domain and CD3ζ (ΔIgV-Slamf1–CD3ζ), and luciferase reporters as in **a**, then exposed to heat-killed E. coli F18. (**c**) Luciferase activity in Jurkat cells transfected as in **a**, then left uninoculated (−) or inoculated with 10 × 108 E. coli F18 (+), followed by the addition of monoclonal antibody (mAb; amount, under graph) 9D1 to SLAM (α-SLAM) or rat immunoglobulin G2b isotype-matched control antibody (Rat IgG2b). (**d**) Luciferase activity in Jurkat cells transfected as in **a**, then exposed to medium alone (Med), wild-type E. coli (JM101), or JM101 E. coli mutants lacking both Omp C and Omp F (HN705) or lacking either OmpC (ΔOmpC) or OmpF (ΔOmpF). Data are representative of at least three independent experiments (error bars (**a**), s.d.).

SLAM regulates PtdIns(3)P production in phagosomes

A potential mechanism by which SLAM could control two seemingly disparate microbicidal functions of macrophages after uptake of E. coli (NOX2 activity and phagosomal maturation) involves PtdIns(3)P. First, PtdIns(3)P, located in the outer layer of the phagosomal lipid bilayer22,23, binds to the PX domain of the NOX2 subunit p40phox (NCF4), which is required for both assembly and stabilization of the NOX2 enzyme24. Second, PtdIns(3)P in the outer leaflet of the phagosome and endosome lipid bilayer interacts with the FYVE domain of the tethering molecule EEA1, a requisite step for successful phagosomal and endosomal maturation25.

To test the idea that absence of SLAM affects the amount of PtdIns(3)P in the outer leaflet of the phagosomal lipid bilayer, we transfected cDNA encoding a PtdIns(3)P-binding reporter into wild-type and SLAM-deficient primary macrophages. After initiating

phagocytosis with E. coli expressing the red fluorescent protein DsRed, we monitored the amount of PtdIns(3)P in E. coli–containing phagosomes by live-cell spinning-disc confocal microscopy. The production of PtdIns(3)P in the E. coli–containing phagosomes of Slamf1−/− macrophages was defective (Fig. 7a). We independently confirmed that result by quantitative fluorescence microscopy of the phagocytosis of 3-μm beads coated with an E. coli outer membrane extract. Whereas in wild-type peritoneal macrophages PtdIns(3)P peaked in phagosomes at 15 min after the initiation of phagocytosis (Fig. 7b), it reached its peak at 45 min after the initiation of phagocytosis in SLAM-deficient macrophages (Fig. 7b). We obtained the same result when we examined bone marrow–derived macrophages (data not shown). We did not detect any PtdIns(3)P by this analysis in the plasma membrane when we used 15-μm coated polystyrene beads, which cannot be phagocytosed (data not shown). Furthermore, the delay in appearance of PtdIns(3)P in the phagosomal lipid bilayer coincided with the delay in recruitment of the tethering EEA1 dimer (Fig. 4a), which depends on the binding of PtdIns(3)P to its FYVE domain26. In a biochemical experiment, we labeled primary macrophages with tritiated myoinositol, the PtdIns(3)P precursor. After the initiation of phagocytosis, we detected less PtdIns(3)P in SLAM-deficient macrophages than in wild-type macrophages, as determined by highpressure liquid chromatography (HPLC) analysis (Fig. 7c). In this experiment we measured new production of PtdIns(3)P, which represents enzyme activity in the phagosomes. The outcome of the lipid analysis therefore confirmed the delay in PtdIns(3)P production in Slamf1−/− phagosomes determined by the two fluorescence microscopy studies. Because the experiments reported above indicated that after entering the phagosome, SLAM must be a positive regulator of PtdIns(3)P production, we next used SLAM+ RAW264.7 macrophage transfectants. Quantitative fluorescence microscopy showed SLAM-enhanced mobilization of PtdIns(3)P to the phagosomes (Fig. 7d). We conclude from these experiments that in phagosomes, SLAM governs the amount of PtdIns(3)P in the membrane, which binds to p40phox to affect the function of NOX2, and to EEA1, which influences phagosome maturation.

SLAM interacts with the Vps34–Vps15–beclin-1 complex

The most likely enzyme to regulate PtdIns(3)P in the phagosome would be the class III phosphatidylinositol kinase Vps34, which resides exclusively in intracellular membrane compartments and is the sole kinase able to convert phosphatidylinositol into PtdIns(3)P8,27. Vps34, along with its regulatory protein kinase Vps15, is a critical regulator of endocytic sorting in yeast and mammalian cells8,28. However, Vps34 and Vps15 have been found to constitutively exist in a heterotrimeric complex along with the autophagy-associated molecule beclin-1 (Atg6), referred to as the ‘beclin 1–phosphatidylinositol-3-kinase complex’29,30. When this heterotrimeric complex is associated with the ultraviolet irradiation resistance–associated protein UVRAG, it generates PtdIns(3)P, which promotes the fusion of organelles31,32. We therefore set out to demonstrate

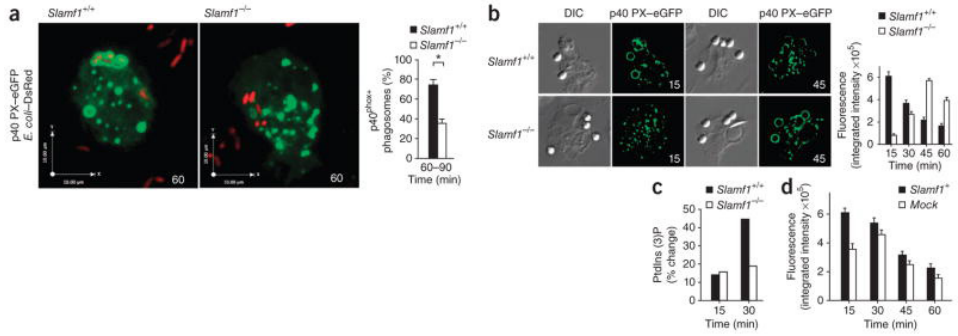

Figure 7. PtdIns(3)P production in phagosomes of primary macrophages is controlled by SLAM.
(**a**,**b**) Production of phagosomal PtdIns(3)P in E. coli–containing phagosomes of primary peritoneal macrophages transfected with reporter cDNA encoding an eGFP-tagged PX domain of p40phox (p40 PX–eGFP) and treated with DsRed-expressing E. coli (E. coli–DsRed; **a**) or beads coated with E. coli outer membrane extract (**b**). Numbers in bottom right corners indicate time (in min). Original magnification, ×60. Right, quantification of fluorescence in microscopy at left. (**c**) HPLC analysis of the production of PtdIns(3)P in primary macrophages labeled with 3H-tagged myoinositol and treated with beads coated with E. coli outer membrane extract. (**d**) PtdIns(3)P production in RAW264.7 cells stably expressing SLAM or a mock construct, transfected with eGFP-tagged p40 PX and treated with beads coated with E. coli outer membrane extract. Data are from three combined experiments (**a**) or are representative of at least three independent experiments (**b**,**d**) or two independent experiments (**c**) with at least 100 beads or bacteria per experiment (error bars,s.d.).

that SLAM interacts with this enzyme complex in intracellular membranes.
To assess whether SLAM directly interacts with the Vps34–Vps15–beclin-1 complex, we expressed all four proteins together in HEK293 human embryonic kidney cells. Coimmunoprecipitation and immunoblot analysis demonstrated that SLAM interacted with the Vps34–Vps15–beclin-1 complex (Fig. 8a). Although detailed mutational analyses are needed, the main interaction seemed to be between SLAM and beclin-1. The observation that the SLAM-specific adaptor EAT-2A enhanced the interaction with Vps34 (Fig. 8a) raised the possibility that binding of EAT-2 to SLAM might stabilize the protein interactions. When we used a mutant form of SLAM lacking the cytoplasmic tail, we found that neither Vps34 nor any of its associated components was coprecipitated (Fig. 8b). We confirmed the interaction of SLAM with the beclin-1-containing complex in phagosomes by fluorescence microscopy of RAW264.7 macrophages transfected with SLAM and GFP tagged beclin-1 (Fig. 8c). The outcome of these experimental approaches showed that the presence of SLAM led to more PtdIns(3)P in the lipid bilayer of the phagosome because SLAM recruited the Vps34–Vps15–beclin-1 enzyme complex. Thus, SLAM regulated bactericidal activity in the Gram-negative phagosome through the recruitment of this complex and subsequent phagosomal maturation and NOX2 activity (Supplementary Fig.14).

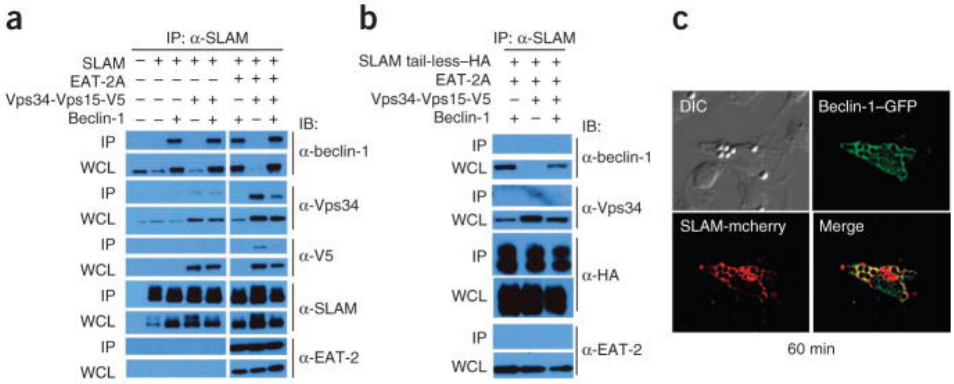

Figure 8. SLAM recruits the intracellular Vps34–Vps15–beclin-1 complex to the phagosome.
(**a**,**b**) Immunoassay of 293 cells transfected with various combinations of full-length SLAM (**a**) or hemagglutinin-tagged (−HA) tailless SLAM (**b**), EAT-2A, V5-tagged (−V5) Vps34-Vps15, and beclin-1; proteins immunoprecipitated (IP) from lysates with monoclonal antibody to SLAM, as well as whole-cell lysates (WCL), were analyzed by immunoblot (IB) with antibeclin-1, anti-Vps34, anti-V5, anti-hemagglutinin, anti-SLAM or anti-EAT2. (**c**) Microscopy of RAW264.7 cells transiently transfected with cDNA encoding GFP-tagged beclin-1 and SLAM-mCherry, then treated with beads coated with E. coli outer membrane extract (at a ratio of 10:1, beads/cells) and fixed after 60 min. Data are representative of six (**a**) or two (**b**,**c**) independent experiments.

DISCUSSION

Our experiments have demonstrated that in addition to being a costimulatory molecule, SLAM acts as a vital regulator in the innate immune defense against Gram-negative bacteria in macrophages by independently regulating two main bactericidal processes: phagosome maturation and the production of free radical species by the NOX2 complex. To regulate these microbicidal processes, SLAM first recognizes bacterial surface proteins embedded in the outer membrane of most Gram-negative bacteria33,34. Once SLAM engages the bacteria, it is actively dragged into the developing phagosome, where it is responsible for recruiting a complex containing Vps34, Vps15 and beclin-1 to the early phagosome. The recruitment of active Vps34 catalyzes the conversion of phosphatidylinositol phosphate to PtdIns(3)P, a key regulator of phagosomal maturation through the recruitment of EEA-1 via its FYVE domain and the production of reactive oxygen species by its recruitment of p40phox via its PX domain. This pathway, when intact, leads to the optimal and efficient elimination of Gram-negative species.

Bacteria are recognized by low-specificity, high-affinity receptors such as integrins, lectins and scavenger receptors that initiate the formation of the phagocytic synapse and intracellular signaling events. Our data have shown that SLAM is a bacterial receptor that recognizes the outer membrane proteins OmpC and OmpF. OmpC has become an area of interest in the study of inflammatory bowel disease because a subset of patients with Crohn's disease have relatively high concentrations of antibodies to OmpC35. OmpC and OmpF are highly homologous porins that are regulated in an extremely complex way

that involves a variety of growth conditions such as temperature, pH and osmolarity33. They fulfill many tasks that are crucial to bacterial homeostasis, including solute-protein translocation and structural integrity, and they are also thought to contribute to bacterial virulence. So far, no other receptors for OmpC or OmpF have been identified, to our knowledge, and only OmpA from Klebsiella pneumoniae has been described as binding to the scavenger receptors LOX-1 and SREC-I, which results in the activation of macrophages and dendritic cells in a TLR2-dependent way. The mechanism by which the interactions of SLAM with OmpC and OmpF is orchestrated is as yet undetermined. The Vps34–Vps15–beclin-1 complex has become the focus of much work in terms of its role in the macroautophagy (autophagy) process. Autophagy is a universal process by which a cell acts to degrade superfluous materials in the cytoplasm and organelles and reutilize them as vital elements of cell survival in response to cellular stress36. Additionally, in some cases, this process is also important in immune defense against pathogens37. This phenomenon is achieved through the formation of a double-membraned structure called the autophagosome that eventually fuses with the lysosome, a process similar to phagosomal maturation. Studies have shown that the Vps34–Vps15–beclin-1 complex also associates with UVRAG and that this complex, called the autophagy complex, is indispensable to the initiation and continuance of autophagy31,32,36. Thus, SLAM may support phagosome maturation, which itself is dependent on vesicular fusion events38, by borrowing from the ubiquitous autophagy machinery. There is also overlap between the elements used in phagosome maturation and autophagy, as TLRdependent triggering of phagocytosis recruits the autophagy proteins beclin-1 and LC3 to the phagosome39. Additionally, optimal activation of reactive oxygen species by the NOX2 complex is essential for the initiation of autophagy40, which makes SLAM a potential candidate for its induction. Whether SLAM is also a regulator of the autophagy process in the context of stress or immunity is an area that requires further investigation. We conclude that after phagocytosis of Gram-negative bacteria, SLAM connects the phagosome to an enzyme system that is present in every cell; thus, we have identified a previously unknown innate receptor function that tailors the immune response to Gram negative bacteria. Because we found that SLAMF6 (Ly108) also recognized E. coli (data not shown) but not S. aureus, and because SLAMF2 is one of the receptors for FimH, a lectin on the pili of some Enterobacteriaceae, we propose that the SLAMF receptors are another family of microbial sensors. Like other innate immune receptors, such as TLRs41, SLAMF receptors are promiscuous in that they can recognize many ligands; for example, SLAMF1 reacts with self proteins, measles virus proteins and bacterial proteins.

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