# Biology of inhibitory immune receptors

Studies on CD200R and LAIR-1

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# Biology of inhibitory immune receptors

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## Biologie van inhibitoire immuunreceptoren

Studies aan CD200R en LAIR-1

(met een samenvatting in het Nederlands)

Proefschrift

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door

***Eva Suzanne Katrijn Rijkers***

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*The gods did not reveal from the beginning*
*All things to us; but in the course of time*
*Through seeking, men found that which is better.*

*But as for certain truth, no man has known it,*
*Nor will he know it; neither of the gods,*
*Nor yet of all the things of which I speak.*
*And even if by chance he were to utter*
*The final truth, he would himself not know it;*
*For all is but a woven web of guesses.*

Xenophanes

**Aan mijn ouders**

| | |
|---|---|
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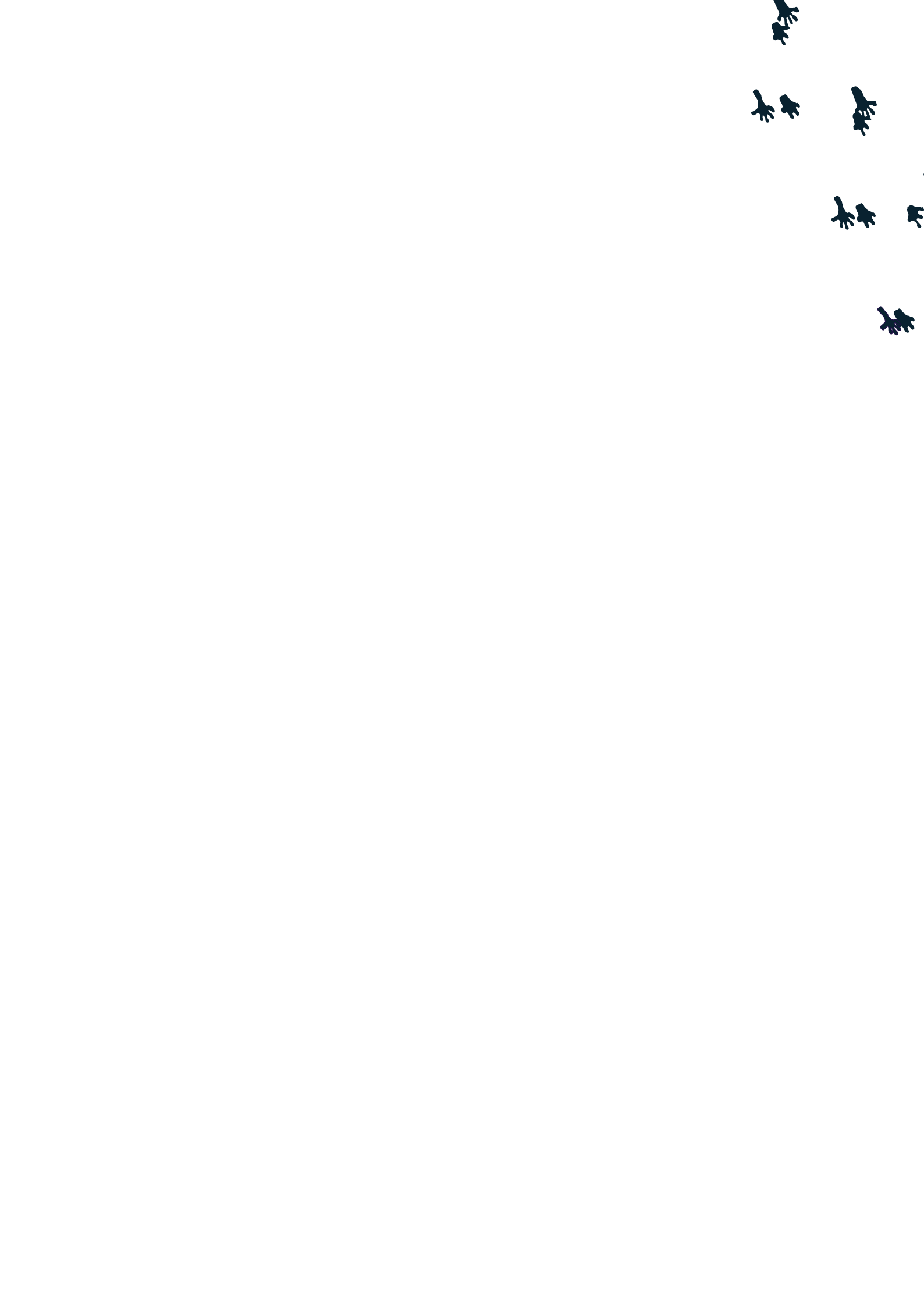
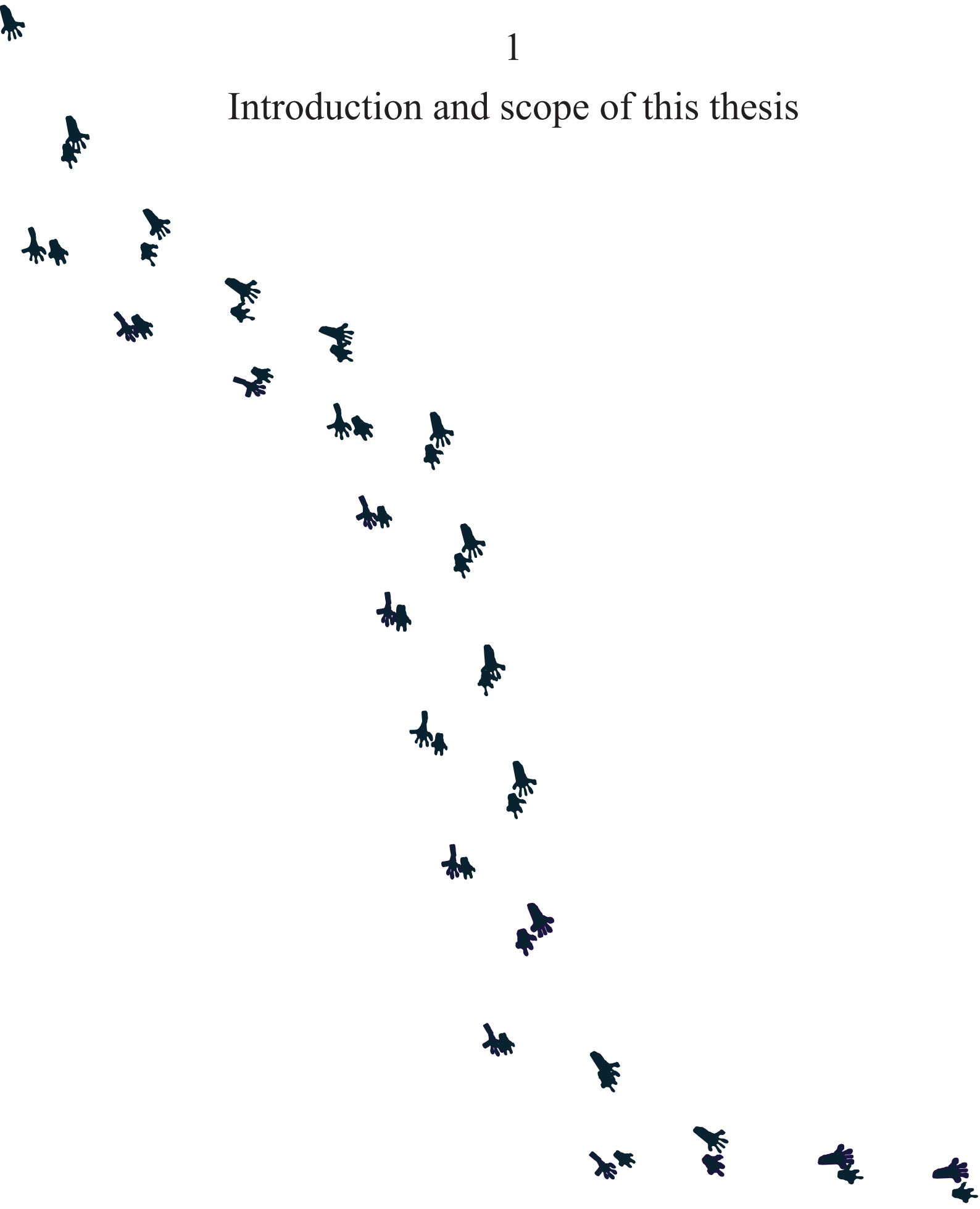


# 1

# Introduction and scope of this thesis



**Opposing signals regulate immune balance**

The primary function of the immune system is to protect the body against pathogens. Therefore, immune cells need to be adequately activated. However, immune responses should only be initiated when necessary and require termination once the causative agent is removed. Unwanted or prolonged activation of the immune system could result in uncontrolled inflammation and/or auto-immunity, leading to organ damage. The immune system needs to be in constant balance and for this purpose, immune cells express a set of inhibitory receptors to balance activating signals [1].

Initiation of immune responses requires activating signals to immune cells. In addition to activating signals from cytokine receptors or pattern recognition receptors such as Toll-like receptors (TLRs), activating signals can also be relayed via Immunoreceptor Tyrosine-based Activation Motifs (ITAMs). ITAMs are found in the intracellular tail of activating immune receptors or in chains associating to the transmembrane domain of receptors, and are characterised by the amino acid sequence YxxL/I$x_{(6\text{-}12)}$YxxL/I, where x denotes any amino acid (reviewed in [2]). Receptors signalling via ITAMs include the T-cell receptor (TCR), the B-cell receptor (BCR) and activating Fc receptors. Upon crosslinking of the receptor, the tyrosines in the ITAM are phosphorylated by Src family kinases and activating signal transduction molecules such as protein tyrosine kinases are recruited. Signals from these activating receptors may induce proliferation, differentiation, or execution of effector functions such as cytokine production [3].

Different mechanisms of immune regulation exist that are necessary for proper function of the immune system. Failure of immune cells to undergo apoptosis leads to lymphoproliferative disease or autoimmunity [4;5]. In addition, inhibitory cytokines such as IL-10 and TGF-ß are crucial in maintaining homeostasis and controlling inflammatory responses [6;7], whereas regulatory T cells are involved in maintaining immunological self-tolerance and prevent autoimmunity (reviewed in [8;9]). Furthermore, signals from inhibitory receptors can set a threshold for activation, downmodulate an ongoing response or altogether prevent an inappropriate immune response (reviewed in [1]). The importance of these receptors is illustrated by the phenotype of mice deficient for a specific inhibitory immune receptor. For example, mice deficient in the inhibitory receptor for IgG (FcγRII$^{-/-}$ mice) are prone to develop auto-immune disease. FcγRII$^{-/-}$ mice on a C57BL/6 genetic background spontaneously develop auto-antibodies and die of auto-immune glomerulonephritis [10], indicating that the inhibitory FcγRII is critical in maintaining peripheral tolerance to auto-antigens. In addition, several human auto-immune diseases are associated with polymorphisms in genes encoding inhibitory immune receptors. For example, two different types of polymorphisms in the gene for the human inhibitory Fc receptor, FcγRIIb, are associated with the auto-immune disease systemic lupus erythematosus [11;12].

**Immune inhibition is mediated by ITIM-bearing and non-ITIM bearing receptors**

During the last decade, much attention has been focussed on inhibitory immune receptors. Most inhibitory immune receptors contain one or more immunoreceptor tyrosine-based inhibitory motifs (ITIMs), defined as the amino acid sequence I/V/L/SxYxxV/L. ITIMs were first described in the intracellular domain of FcγRIIb [13;14]. Since then, it has become widely accepted that inhibitory immune receptors signal via phosphorylation of the tyrosine in the ITIM, which leads to recruitment of phosphatases such as SHP-1, SHP-2 or SHIP. These phosphatases can then dephosphorylate activating molecules, leading to suppression of the activation signal [15].

Most immune cells express several inhibitory receptors. The function of these inhibitory receptors is not redundant, as is shown in studies using mice deficient for several B-cell inhibitory receptors (reviewed in [15;16]): mice knockout for different receptors show different phenotypes. FcγRII, CD22 and Paired Immunoglobulin-like Receptor (PIR)-B deficient mice all have hyperresponsive B cells, but whereas FcγRII and CD22 deficient mice produce auto-antibodies, PIR-B deficient mice do not [10;17;18]. This indicates that different inhibitory receptors expressed on the same cell have non-redundant roles, underlining the importance of the individual inhibitory receptors.

Although ITIMs are regarded as a general feature of inhibitory receptors [1], immune inhibition probably is not solely mediated by recruitment of phosphatases to ITIMs. For example, a small group of inhibitory receptors that do not signal via ITIMs exists. Most well-known representative of this group is CTLA-4, which is an inhibitor of T-cell activation. CTLA-4 contains two tyrosine residues that bind PP2A and SHP-2 (reviewed in [19-21]), through which it likely mediates its inhibitory function. However, its signalling mechanism remains controversial. Another example is CD5, an inhibitor of BCR and TCR signalling, which does contain two putative ITIMs but these are dispensable for its function [22]. In addition, CD200R is an inhibitory immune receptor that does not contain ITIMs [23].

Furthermore, crosslinking of different ITIM-bearing receptors can have differential effects that are not solely explained by their recruitment of phosphatases (reviewed in [15]). Thus, it is likely that, in addition to phosphatases, other molecules function to relay signals from ITIM-bearing receptors. However, this has not been systematically investigated.

**LAIR-1 inhibits immune cells via its interaction with collagens**

LAIR-1 (Leukocyte-associated Ig-like receptor-1 or CD305) is a type 1 transmembrane glycoprotein containing a single extracellular Ig-like domain and two intracellular ITIMs [24]. It is expressed on most peripheral blood mononuclear cells, including NK cells, T cells, B cells, monocytes, eosinophils and dendritic cells (DCs), as well as the majority of thymocytes and $CD34^+$ haematopoietic progenitor cells [24-26]. Furthermore, LAIR-1 is differentially

expressed on T [27;28] and B [29] lymphocytes and is inducibly expressed on peripheral neutrophils [25].

Crosslinking of LAIR-1 with antibodies in vitro can inhibit the function of various immune cells. For example, LAIR-1 crosslinking on human NK cells inhibits target cell lysis by both resting and activated NK cells [24;30]. In addition, LAIR-1 crosslinking inhibits the cytotoxic activity of effector T cells upon stimulation with αCD3 antibodies [27;28;31]. Furthermore, LAIR-1 crosslinking prevents proliferation and induces apoptosis in human myeloid leukaemia cell lines and primary leukaemias [32;33]. These data indicate that LAIR-1 is a broadly expressed inhibitory immune receptor that may regulate many aspects of immune cell function.

Recently, collagens have been identified as ligands for LAIR-1 and binding of LAIR-1 to collagens is dependent on the hydroxyproline residues characteristic for collagens [34]. Crosslinking of LAIR-1 by collagens results in inhibition of the function of LAIR-1-transfected mast cell and T-cell lines [34] and primary human T cells [28]. Because both collagens and LAIR-1 are widely expressed, interactions between these molecules may occur at many stages of an immune response or during maintenance of homeostasis and may be important in maintaining a threshold for immune cell activation.

Upon crosslinking of LAIR-1, the tyrosines in the intracellular ITIMs become phosphorylated and recruit SHP-1 and SHP-2 as downstream effectors that mediate inhibition of activation [35]. LAIR-1 requires phosphorylation of both ITIMs for full inhibitory function [35]. However, it is unknown whether the interaction with SHP-1 and SHP-2 is sufficient for full LAIR-1-mediated immune cell inhibition or whether other downstream signal transduction molecules may play an additional role in LAIR-1 signalling.

**CD200R inhibits immune cells after crosslinking by CD200**

CD200 (formerly OX2) was identified more than 25 years ago [36] as a membrane glycoprotein and a member of the immunoglobulin superfamily (IgSF). CD200 is expressed on thymocytes, activated T cells, B cells, DCs, endothelial cells and neurons. CD200 has a short intracellular tail devoid of any known signalling motifs [37] and it functions as a ligand for CD200R (previously OX2R) [23]. CD200 and CD200R are structurally closely related to each other and both genes are located on a small region of human chromosome 3q12-13 [38]. In addition, the genes are similarly positioned in the mouse genome, indicating that the genes likely have arisen by gene duplication [39]. Interestingly, the CD200 and CD200R genes are in close proximity to the homologous IgSF molecules CD80 and CD86, to which CD200 and CD200R are also structurally related [39]. The interaction between CD200 and CD200R is, similar to other interactions between proteins of the IgSF, mediated through the N-terminal Ig-like domains [40].

Characterization of CD200R was performed more than 15 years after the identification of

CD200 [23;41]. CD200R does not contain ITIMs in its intracellular tail, but it does contain three intracellular tyrosine residues, one of which is located in an NPXY motif [23]. In contrast to the broad distribution of CD200, CD200R expression is much more restricted. It is expressed on myeloid cells [41], such as macrophages, granulocytes and DCs but recently has also been found on T cells [39;42]. Its function remained elusive until mice deficient for CD200 were generated and studied [43]. Notably, $CD200^{-/-}$ mice displayed functional phenotypic changes only in cells expressing CD200R, indicating that CD200 itself does not have signalling capacity, but serves as a ligand for CD200R. In the absence of CD200R signalling, mice are more susceptible to induction of auto-immune diseases and show an enhanced immune response to an inflammatory stimulus [43-45]. Also, CD200R signalling may be implicated in regulating Th2 cell function [46] and in graft acceptance [47;48].

Direct evidence that CD200R is an inhibitory receptor comes from in vitro studies. Crosslinking of CD200R results in decreased degranulation and cytokine secretion of human and mouse mast cells [49;50]. In addition, CD200R crosslinking on mouse DCs induces production of the enzyme indoleamine-2,3-dioxygenase (IDO), that promotes immunosuppression of T cells through tryptophan catabolism [51]. Recently, crosslinking of CD200R was found to inhibit αCD3-induced proliferation of dendritic epidermal T cells [42], indicating a functional role for CD200R expression on lymphocytes. In addition, CD200 is upregulated on apoptotic cells [52], suggesting a role for CD200R in restriction of inflammation around apoptotic sites.

Thus, CD200R signalling plays an important role in many physiological processes in both inflammation and auto-immunity. However, when the research described in this thesis started, the signalling pathways employed by CD200R were unknown.

**CD200R has an extended family**

Several receptor families exist that contain both inhibitory and activating members. For example, inhibitory killer cell immunoglobulin-like receptors (KIRs) have a large intracellular domain containing ITIM motifs, whereas activating KIR family members do not contain a large intracellular domain. Instead, activating KIRs pair with the activating signal transduction molecule DAP12 via a positively charged amino acid in their transmembrane region [53-55]. Activating KIRs are thought to have developed in response to pathogens producing ligands for inhibitory receptors [56].

Also, activating counterparts of the inhibitory CD200R have been described [39;57] (Figure 1). In humans, one gene was described and named hCD200RLa. Additional mouse genes include mCD200RLa and mCD200RLb, which contain a positively charged lysine in their transmembrane regions, through which they bind DAP12. mCD200RLc and mCD200RLe, which is only expressed in some mouse strains, were also predicted to bind DAP12. An additional sequence named mCD200RLd was incomplete and may not result in protein expression. Whether

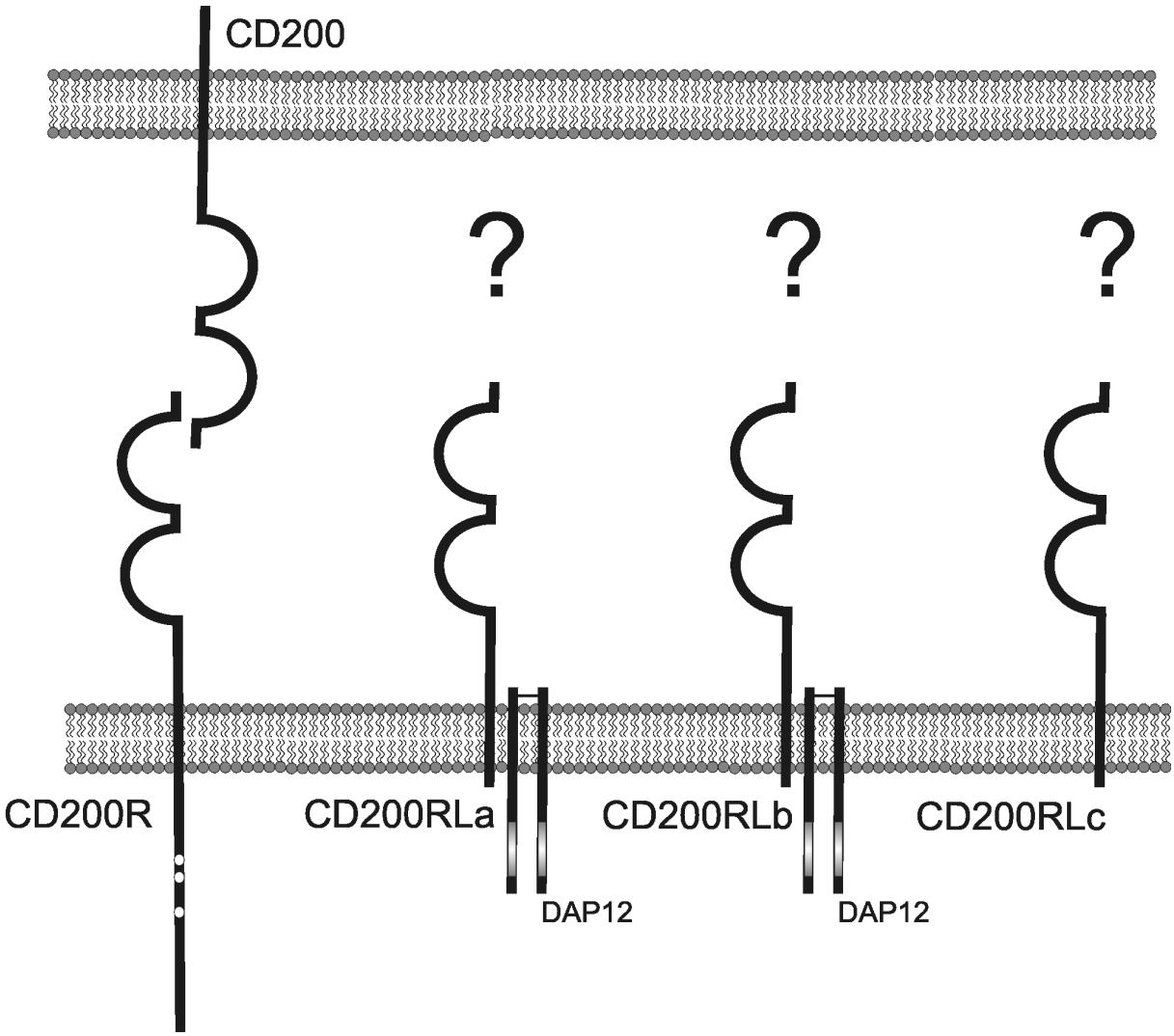



**Figure 1. The CD200R family**
The mouse CD200R family (shown here) consists of the inhibitory CD200R and three structurally related membrane glycoproteins, CD200R-like (CD200RL)a-c. In addition, a cDNA sequence named CD200RLd is incomplete and may not result in protein expression (not depicted), and CD200RLe is found in some mouse strains (not depicted). CD200 specifically interacts with CD200R through the N-terminal domains. Specific ligands for the CD200RL molecules have not yet been identified. The human CD200R gene family (not shown) consists of CD200R the closely related CD200RLa. In both mice and humans, CD200R acts as an inhibitory immune receptor, whereas the CD200R-like homologues are thought to be activating receptors. CD200RLa and CD200RLb interact with DAP12 and this interaction is predicted for CD200RLc. Tyrosines in the intracellular CD200R domain are depicted as white circles, ITAMs in DAP12 are depicted as shaded grey boxes.

these activating family members bind CD200 has been controversial [58], but recent evidence indicates that CD200 can not bind CD200RLa, CD200RLb, CD200RLc and CD200RLe [59] and the ligands for these molecules are currently unknown. It is speculated that, similar to activating KIRs, the activating CD200RL molecules have evolved as a mechanism to recognize bacterial or viral components [59]. In this thesis, CD200R refers specifically to the inhibitory CD200R.

**Immune inhibition during inflammation**

During inflammation, inhibition of immune cells is required to restrict damage. For example, immune responses against influenza virus are not solely beneficial but can result in pathology. Indeed, infection of T-cell deficient mice with influenza virus resulted in decreased lung pathology compared to wild type mice [69], indicating that T cells, next to clearing the infection, may also cause pathology. In addition, an over-active immune response causes local tissue damage and systemic illness through the induction of a cytokine storm [70]. Recently, development of severe disease after infection with H5N1 influenza virus was found to be due to immune-mediated lung pathology [71-73]. In addition, recent studies with the reconstructed 1918 pandemic influenza virus strain showed severe immune-mediated pathology after infection of mice and non-human primates with this strain [74-76]. Therefore, adequate restriction of the immune response is critical in influenza infection to prevent pathology. It is interesting to speculate that inhibitory immune receptors may contribute to the restriction of immune responses during influenza infection.

**Scope of this thesis**

Different inhibitory immune receptors use various signal transduction pathways to modulate different activating signals in immune cells. To study the differential effects of signal transduction pathways used by different inhibitory immune receptors, we set up a yeast tri hybrid assay to determine which molecules associate to the intracellular tails of LAIR-1 and CD200R. We report that LAIR-1 can function independently of phosphatases and recruits Csk (chapter 2). In chapter 3, we studied which signalling pathways are employed by CD200R to exert its inhibitory function. We report that all three tyrosines are required for full CD200R function and, using the yeast tri hybrid system, we identify Dok-1 and Shc as molecules associating to the intracellular CD200R tail (chapter 3). Since Dok-1 and Dok-2 are implicated in the control of myeloid leukaemia development, we investigated the myelopoiesis of $CD200^{-/-}$ mice (chapter 4) but found that this is not altered. Chapter 5 describes the differentially regulated CD200R expression on human and mouse T and B lymphocytes. To study the role of CD200R signalling during the immune response against a pathogen, we infected $CD200^{-/-}$ mice with influenza. We report that $CD200^{-/-}$ mice can not cope with influenza infection and develop a severe, and often lethal, T-cell mediated disease. These results are described in chapter 6. In chapter 7 finally, the results presented in this thesis are summarized and discussed.

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# 2

# Leukocyte-Associated Ig-like Receptor-1 has SH2-domain containing-phosphatase independent function and recruits C-terminal Src Kinase

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**Abstract**

Most inhibitory receptors in the immune system contain one or several Immunoreceptor Tyrosine-based Inhibitory Motif (ITIM)s and recruit the SH2 domain-containing phosphatases SHP-1, SHP-2 and/or SHIP, which are generally believed to be essential for the inhibitory function. However, it has not been systematically investigated whether ITIM-bearing receptors may exert their function through alternative interactions. Here we describe that Leukocyte-Associated Ig-like Receptor (LAIR)-1 has inhibitory function in DT40 chicken B cells that lack both SHP-1 and SHP-2. In addition, we found that LAIR-1 did not recruit SHIP upon phosphorylation. Thus, LAIR-1 can function independent of SH2 domain-containing phosphatases and must recruit at least one other signaling molecule. Using a yeast-tri-hybrid system, we found that phosphorylated LAIR-1 bound the C-terminal Src kinase (Csk). The interaction required the SH2 domain of Csk and phosphorylation of the tyrosine in the N-terminal ITIM of LAIR-1. We propose that Csk is an additional player in the regulation of the immune system by ITIM-bearing receptors.

## Introduction

An appropriate response of the immune system depends on a balance between activating and inhibitory signals. Inhibitory receptors play an important role in the regulation of immune cells. This is illustrated by the fact that mutations in inhibitory receptors or in the molecules through which they signal, are associated with autoimmune disease [1]. Most inhibitory receptors in the immune system contain one or several Immunoreceptor Tyrosine-based Inhibitory Motif (ITIM)s. This motif has the consensus sequence (I/V/L/S)-x-Y-x-x-(L/V/I), where x represents any amino acid [2]. Upon engagement, ITIM-bearing receptors become phosphorylated and recruit the SH2 domain-containing inositol phosphatase SHIP and/or the SH2 domain-containing tyrosine phosphatases SHP-1 and SHP-2. The phosphatases subsequently dephosphorylate and thereby inactivate key molecules involved in cellular activation [2, 3]. It is widely believed that the recruitment of SH2 domain-containing phosphatases is required for the inhibitory function of ITIM-bearing receptors. However, several studies, including our previous work on Leukocyte-Associated Ig-like Receptor (LAIR)-1, suggest that the phosphatases are not the sole down-stream effectors of ITIM-bearing receptors.

LAIR-1 is an ITIM-bearing receptor that is broadly expressed in the immune system [4] and functions as an inhibitory receptor on NK cells, T cells and B cells [4-9]. In addition, LAIR-1 inhibits the differentiation of peripheral blood precursors towards dendritic cells [10] and induces apoptosis in myeloid leukemia cells [11, 12]. LAIR-1 is phosphorylated by Src family kinases and recruits SHP-1 and SHP-2 [4, 13-15]. Recently, we identified a mouse homologue of LAIR-1, but unlike human LAIR-1, it does not recruit SHP-1. Still it has similar inhibitory capacity [16]. In addition, a human LAIR-1 mutant that lacks the N-terminal tyrosine does not detectably bind SHP-1 or SHP-2 but still has inhibitory function [15]. Taken together, this suggests that LAIR-1 may also recruit other molecules.

Here we show that LAIR-1 has inhibitory capacity in SHP-1 and SHP-2 deficient DT40 chicken B cells and does not recruit SHIP. This indicates that, in contrast to the established dogma, LAIR-1 has inhibitory function independent of SH2 domain-containing phosphatases. Therefore we searched for other LAIR-1-interacting proteins. Using a yeast-tri-hybrid screen, we have found that C-terminal Src kinase (Csk) interacts with phosphorylated LAIR-1. As Csk inactivates Src family kinases [17, 18], it may be a novel player in the regulation of immune responses by ITIM-bearing receptors.

## Results

*LAIR-1 has inhibitory function independent of SHP-1 and SHP-2*

LAIR-1 recruits both SHP-1 and SHP-2 [4, 13-15]. To determine the contribution of each phosphatase in LAIR-1 signaling, we investigated the function of LAIR-1 in the chicken B cell

line DT40. DT40 mutants have been generated that lack SHP-1, SHP-2 or both phosphatases ([19] and Figure 1A).

We stably transfected the DT40 cells with a mFcγRIIb/hLAIR-1 chimera that contained the extracellular and transmembrane domains of mFcγRIIb and the intracellular domain of LAIR-1. The mFcγRIIb/hLAIR-1 chimera can be co-ligated to the BCR by crosslinking the BCR with mouse IgM anti-chicken IgM in combination with intact rabbit Ig anti-mouse IgM, which will also bind to the FcγRIIb moiety of the chimera through the Fc part. No co-ligation of the chimera occurs when rabbit $F(ab')_2$ anti-mouse IgM is used as a secondary antibody [20, 21]. The expression of the mFcγRIIb/hLAIR-1 chimera was similar on all clones (Figure 1B).

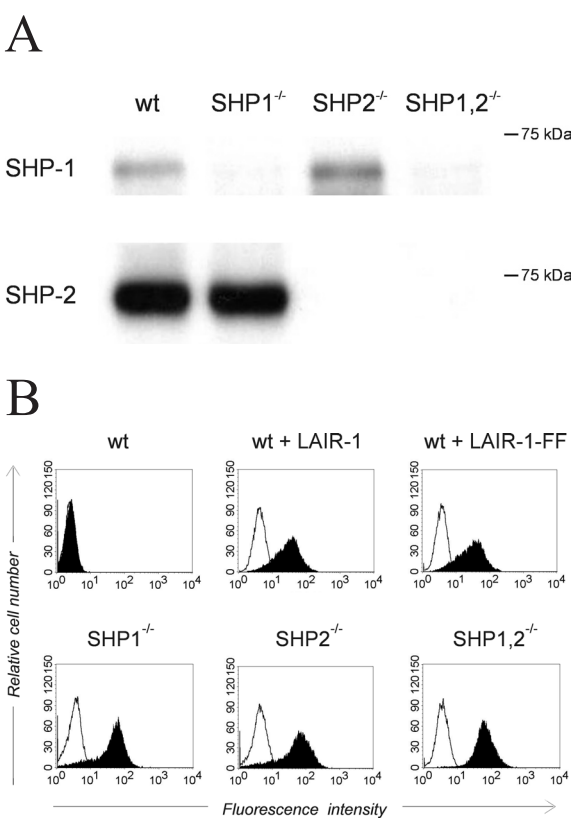


**Figure 1. Generation of DT40 clones stably expressing the mFcγRIIb/hLAIR-1 chimera.**
A. Expression of the phosphatases in the different DT40 cell lines was determined by immunoprecipitation of SHP-1 from pervanadate-treated cells and Western blot analysis using anti-phosphotyrosine antibodies (upper panel) and by Western blot analysis of whole lysates using anti-SHP-2 antibodies (lower panel).
B. Expression of the mFcγRIIb/hLAIR-1 chimera was analyzed by staining the DT40 clones with FITC-conjugated anti-mFcγRIIb antibodies (solid histograms) or isotype control (open histograms). Upper panels: untransfected DT40 (wt), wild type DT40 transfected with the mFcγRIIb/hLAIR-1 chimera (wt + LAIR-1), wild type DT40 transfected with the mFcγRIIb/hLAIR-1 chimera containing tyrosine-to-phenylalanine mutations (wt + LAIR-1-FF). Lower panels: SHP-1, SHP-2 or double deficient cells transfected with the mFcγRIIb/hLAIR-1 chimera.

Using this system we found that signaling by LAIR-1 inhibited BCR-induced calcium mobilization (Figure 2A). The inhibitory function depended on the LAIR-1 ITIMs, as mutation of the tyrosines within these ITIMs abrogated the inhibition of calcium mobilization (Figure 2B). We next investigated the contribution of each phosphatase to LAIR-1 mediated inhibition. In DT40 cells that were deficient for either SHP-1 or SHP-2, LAIR-1 inhibited the calcium mobilization to a similar extent as in wild type cells (Figure 2C and D), suggesting that SHP-1 and SHP-2 have redundant functions in LAIR-1 signaling. Surprisingly, LAIR-1 still had an inhibitory effect on BCR-induced calcium mobilization in cells lacking both phosphatases (Figure 2E), indicating that LAIR-1 has a phosphatase independent inhibitory function.

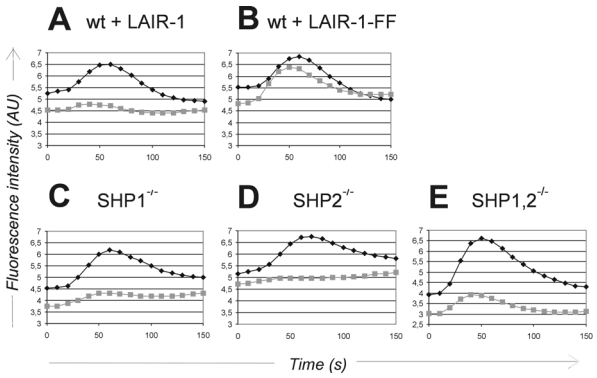


**Figure 2. LAIR-1 inhibits BCR-induced calcium mobilization in phosphatase deficient DT40 cells.**
DT40 transfectants were assayed for calcium mobilization upon BCR cross-linking with (grey squares) or without (black diamonds) co-ligation of the mFcγRIIb/hLAIR-1 chimera.
A, B. Wild type DT40 cells transfected with the mFcγRIIb/hLAIR-1 chimera (wt + LAIR-1) or the mFcγRIIb/hLAIR-1 chimera containing tyrosine-to-phenylalanine mutations (wt + LAIR-1-FF).
C, D, E. SHP-1, SHP-2, or double deficient DT40 cells transfected with the mFcγRIIb/hLAIR-1 chimera.
The results shown are representative of three independent experiments. Similar results were obtained with another set of independent clones.

SHP-1 and SHP-2 exert their inhibitory function by dephosphorylation of key molecules involved in cellular activation [22]. We therefore investigated the effect of LAIR-1 mediated signaling on BCR-induced tyrosine phosphorylation. Crosslinking of the chicken BCR resulted in the phosphorylation of a number of proteins, which was inhibited upon co-ligation of the mFcγRIIb/hLAIR-1 chimera (Fig 3, left panel). Again, the inhibitory function depended on phosphorylation of the ITIMs, as the LAIR-1 mutant lacking functional ITIMs had no effect on tyrosine phosphorylation (Figure 3, middle panel). However, in the absence of SHP-1 and SHP-2, co-ligation of the wild type mFcγRIIb/hLAIR-1 chimera still reduced BCR-induced tyrosine phosphorylation. Thus, the ITIMs of LAIR-1 are able to recruit at least one other molecule that mediates the inhibition of protein tyrosine phosphorylation and cellular activation.

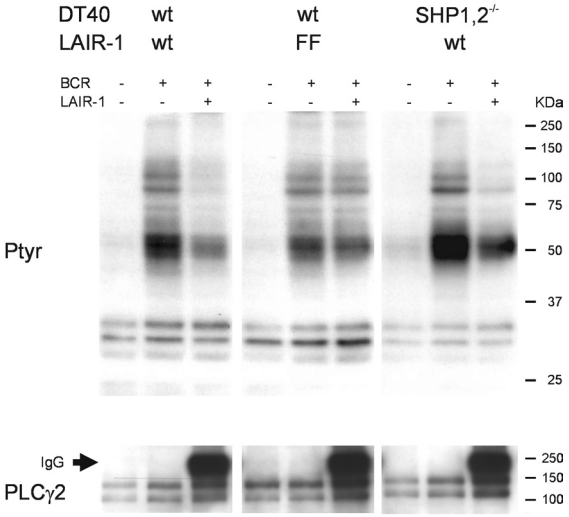


**Figure 3. SHP-1 and SHP-2 are not required for LAIR-1 mediated inhibition of tyrosine phosphorylation.**
Wild type (wt) and phosphatase deficient DT40 cells transfected with the mFcγRIIb/hLAIR-1 chimera or the mFcγRIIb/hLAIR-1 chimera containing tyrosine-to-phenylalanine mutations (FF) were left untreated, stimulated through the BCR alone or stimulated through the BCR with co-ligation of the mFcγRIIb/hLAIR-1 chimera (LAIR-1) for 1 minute. Cells were lysed immediately and Western blotting was performed using anti-phosphotyrosine antibodies. Staining for PLCγ2 was used as a loading control. The results shown are representative of three independent experiments.

*LAIR-1 does not recruit SHIP*

An alternative molecule involved in the signaling of several ITIM-bearing receptors is the 5'-inositol phosphatase SHIP. To investigate whether SHIP binds to phosphorylated LAIR-1, we transfected 293T cells with FLAG-tagged LAIR-1 (Figure 4A). FLAG-tagged human FcγRIIb was used as a positive control [23, 24]. The cells were treated with pervanadate to induce extensive phosphorylation of the receptors [15], and the FLAG-tagged proteins were immunoprecipitated. While a significant amount of SHIP co-immunoprecipitated with FcγRIIb, no SHIP was found in association with LAIR-1 (Figure 4B). In agreement with this observation, the mFcγRIIb/hLAIR-1 chimera inhibited BCR-induced calcium mobilization in SHIP deficient DT40 cells (Figure 4C and 4D), while co-ligation of wild type FcγRIIb has no inhibitory effect in these cells [20]. Thus, we conclude that SHIP does not play a role in LAIR-1 mediated signaling.

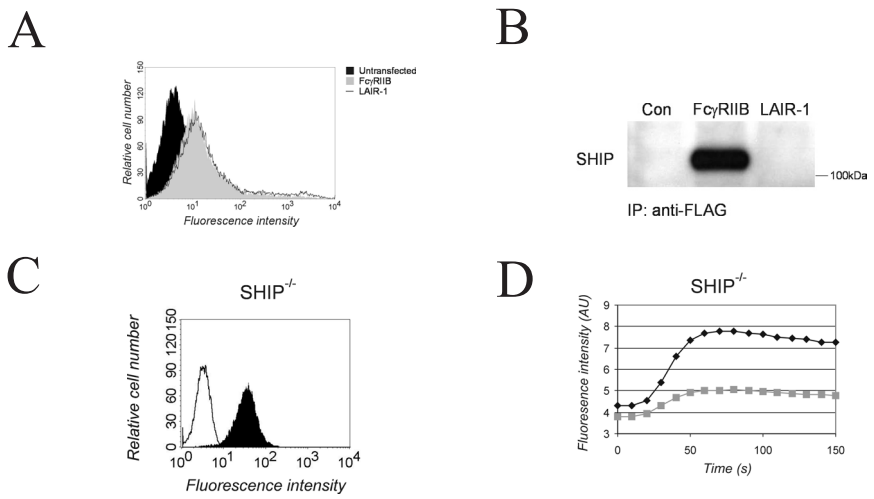


**Figure 4. SHIP does not bind to LAIR-1.**
A. 293T cells (black histogram) and 293T cells transfected with FLAG-tagged FcγRIIb (grey histogram) or FLAG-tagged LAIR-1 (open histogram), were stained with anti-FLAG antibodies followed by phycoerythrin (PE)-conjugated goat anti-mouse antibodies and analyzed by flow cytometry.
B. The cells described in A. were treated with pervanadate and the phosphorylated receptors were immunoprecipitated using anti-FLAG coated beads. Western blotting was performed with anti-SHIP antibodies.
C. SHIP-deficient DT40 cells transfected with the mFcγRIIb/hLAIR-1 chimera were stained with FITC-conjugated anti-mFcγRIIb antibodies (solid histogram) or isotype control (open histogram).
D. The cells were assayed for calcium mobilization upon BCR cross-linking with (grey squares) or without (black diamonds) co-ligation of the mFcγRIIb/hLAIR-1 chimera. The results shown are representative of three independent experiments. Similar results were obtained using another clone.

*Human and mouse LAIR-1 bind Csk*

To identify proteins that selectively bind to phosphorylated LAIR-1, we performed a yeast-tri-hybrid screen with a human fetal brain library using the intracellular tail of human LAIR-1 as bait. We used the Ylck4.1 strain, in which expression of Lck can be induced, enabling the identification of phosphotyrosine dependent interactions. Protein interactions were detected by the expression of the ß-galactosidase reporter gene. We identified two clones that were positive in the reporter assay. Both clones contained a segment of Csk carrying the SH2-domain and the kinase domain (amino acids 84-450). This Csk fragment bound to LAIR-1 in a phosphorylation-dependent manner, as the yeast cells did not show ß-galactosidase activity when grown on media containing methionine, which suppresses Lck expression. The intracellular tail of mouse LAIR-1 (mLAIR-1) also bound Csk in yeast (Figure 5A).

To confirm the interaction of Csk with LAIR-1 in eukaryotic cells, we performed co-immunoprecipitation studies in Rat Basophilic Leukemia (RBL) clones stably expressing human LAIR-1 or a human LAIR-1/mouse LAIR-1 chimera. Both molecules were selectively

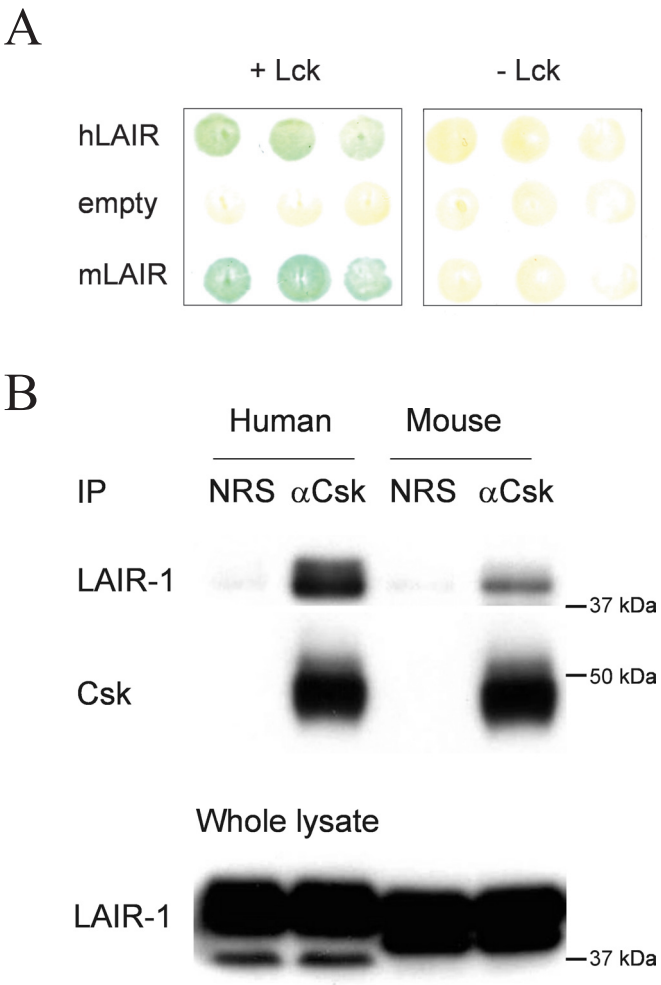


**Figure 5. Csk binds to phosphorylated LAIR-1.**
A. Ylck stably transfected with either Gal4BD-human LAIR-1 (human) or Gal4BD-mouse LAIR-1 (mouse) was transfected with Gal4 AD-CskΔSH3 and grown on selective medium in the absence (+Lck) or presence (-Lck) of methionine for 48 hours. The yeast was transferred to a nylon membrane and assayed for Gal4-promotor activity using a standard ß-galactosidase assay. Yeast stably transfected with Gal4BD alone (empty) was used as a negative control.
B. RBL cells stably transfected with human LAIR-1 or a human LAIR-1/mouse LAIR-1 chimera (mLAIR) were lysed and immunoprecipitation was performed with normal rabbit serum (NRS) or rabbit anti-Csk antibodies. Immune complexes were separated by SDS-PAGE and Western blotting was performed with anti-LAIR-1 and anti-Csk antibodies. Equal amounts of whole cell lysates were loaded to confirm LAIR-1 expression. The results shown are representative of three independent experiments.

co-immunoprecipitated using anti-Csk antibodies (Figure 5B). As the interaction between LAIR-1 and Csk is phosphorylation dependent in yeast, our results suggest that there must be constitutive LAIR-1 phosphorylation in RBL cells.

Indeed, we have found that in unstimulated RBL cells, although barely detectable, there is phosphorylation of LAIR-1. This is sufficient for a low level of SHP-1 recruitment, which requires the phosphorylation of the ITIMs ([15] and data not shown). As shown below, Csk did not interact with the LAIR-1 mutant in which both tyrosines were mutated, indicating that the interaction with Csk also requires phosphorylation of LAIR-1 in RBL cells.

*The interaction of Csk with LAIR-1 requires the SH2 domain of Csk and the tyrosine residue in the N-terminal ITIM of LAIR-1*

Csk consists of a SH3 domain, a SH2 domain and a kinase domain. The Csk fragment that was obtained in the yeast-tri-hybrid screen contained the SH2 domain and the kinase domain. As the interaction of Csk with LAIR-1 is phosphotyrosine dependent, we investigated whether Csk binds to LAIR-1 through its SH2 domain. We therefore transformed yeast cells, stably

expressing the Gal4 binding domain-LAIR-1 fusion protein, with Gal4 activation domain fusion proteins of either full length Csk, Csk lacking the 83 N-terminal amino acids containing the SH3 domain (CskΔSH3) or Csk lacking the SH2 domain (CskΔSH2). As shown in Figure 6A, full length Csk and CskΔSH3 bound to LAIR-1, while CskΔSH2 did not. This indicates that indeed the SH2 domain of Csk binds to phosphorylated LAIR-1.

We have previously shown that the ITIMs of LAIR-1 contribute differentially to the recruitment of SHP-1 and SHP-2 [15]. To investigate which tyrosine is required for the interaction with Csk, we performed co-immunoprecipitations in RBL cells stably expressing wild type LAIR-1 (wt) or mutants of LAIR-1 with tyrosine-to-phenylalanine mutations in the N-terminal ITIM (FY), the C-terminal ITIM (YF) or both (FF). The expression level of each LAIR-1 mutant was similar [15]. The mutant in which the C-terminal ITIM was mutated still bound Csk, while

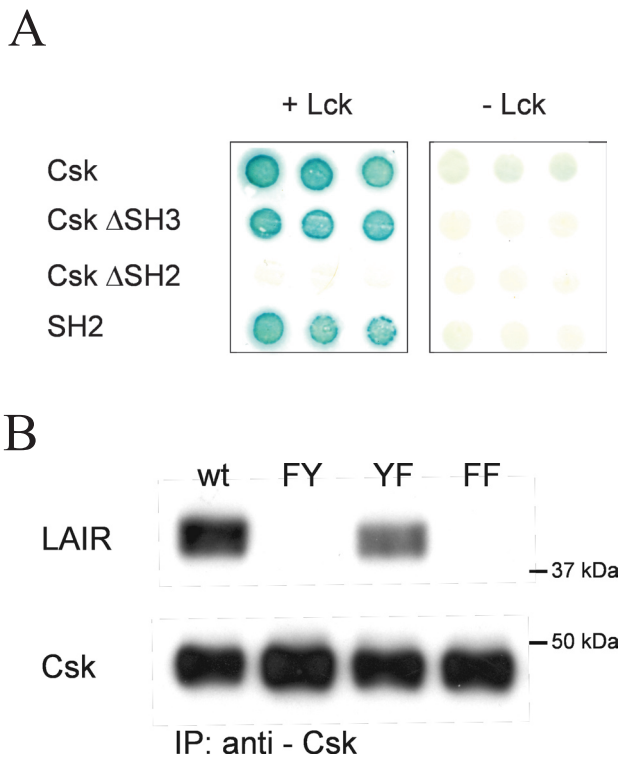


**Figure 6. The interaction of Csk with LAIR-1 requires the SH2 domain of Csk and the N-terminal tyrosine of LAIR-1.**
A. Ylck stably transfected with Gal4BD-human LAIR-1 was transfected with Gal4 AD-Csk constructs containing either full length Csk, CskΔSH3, CskΔSH2 or the SH2 domain alone and grown on selective medium in the absence of methionine (+Lck) or in the presence of methionine (-Lck) for 48 hours. The colonies were assayed for Gal4-promotor activity using a standard ß-galactosidase assay.
B. RBL cells stably expressing either wild type (wt) LAIR-1 or LAIR-1 mutants containing tyrosine-to-phenylalanine mutations in the N-terminal ITIM (FY), C-terminal ITIM (YF) or both ITIMs (FF) were lysed and immunoprecipitation was performed with anti-Csk antibodies. Immune complexes were separated by SDS-PAGE and Western blotting was performed with anti-LAIR-1 and anti-Csk antibodies.

mutation of the N-terminal ITIM abrogated Csk binding (Figure 6B). Thus, the SH2 domain of Csk binds to the phosphorylated tyrosine residue in the N-terminal ITIM of LAIR-1.

## Discussion

Many ITIM-bearing receptors recruit SHP-1 and SHP-2 upon phosphorylation and it is generally thought that these phosphatases are necessary for their inhibitory function. Here we investigated the role of these phosphatases in LAIR-1 mediated signaling. Surprisingly, we found that

LAIR-1 inhibited BCR-induced calcium mobilization and tyrosine phosphorylation in DT40 cells lacking both SHP-1 and SHP-2. This is in contrast to previous studies with other ITIM-bearing receptors that recruit both SHP-1 and SHP-2: Killer cell Ig-like Receptor (KIR), Platelet Endothelial Cell Adhesion Molecule (PECAM)-1 and CD66a are no longer effective in SHP-1 and SHP-2 deficient DT40 cells, while the inhibitory function of Paired Ig-like Receptor (PIR)-B is strongly reduced [19-21, 25]. The inhibitory function of LAIR-1 in the absence of SHP-1 and SHP-2 is not due to recruitment of SHIP, since we did not find an interaction between SHIP and LAIR-1 under the conditions in which SHIP does bind to FcγRIIb. This is in accordance with the fact that neither ITIM of LAIR-1 contains a leucine at the Y+2 position, a residue that is essential for the recruitment of SHIP to FcγRIIb [26]. In addition, LAIR-1 function was not affected in SHIP deficient DT40 cells. Thus, we conclude that LAIR-1 has an inhibitory function independent of SH2 domain-containing phosphatases and that there must be at least one other protein that can associate with LAIR-1 and inhibit cellular activation.

Using a yeast-tri-hybrid system, we found that phosphorylated LAIR-1 bound to Csk. This interaction was mediated by the SH2 domain of Csk and required phosphorylation of the N-terminal ITIM of LAIR-1. Mutants of LAIR-1 that lack the N-terminal ITIM did not bind Csk, which may be explained by the limited phosphorylation of these mutants [15], or by the specificity of the SH2 domain of Csk for the N-terminal tyrosine.

Although in the last few years several Csk interacting molecules have been identified (listed in [27]), there is no specific consensus sequence that predicts binding of Csk. Most Csk-interacting proteins do not contain ITIMs. The ITIM-containing SHP-2 Interacting Transmembrane adaptor protein (SIT) binds Csk, but via a tyrosine residue outside the ITIM [28]. However, previously, it was reported that Signal Regulatory Protein (SIRP)α and Ig-like Transcript (ILT)-2 bind Csk [29, 30]. In contrast, KIR3DL1 was reported not to recruit Csk [31]. In accordance with these reports, we found in the yeast-tri-hybrid system that Csk also bound to FcγRIIb and SIRPα , but not to KIR3DL1 and KIR2DL2 (data not shown). Thus, Csk appears to interact with several, but not all ITIM-bearing receptors. It remains to be investigated what determines whether a particular ITIM-bearing receptor recruits Csk. Since the sequence of the N-terminal ITIM of LAIR-1, VTYAQL, is also present in KIR3DL1, which does not bind Csk, it is likely that not the ITIM consensus sequence, but another motif that includes the same tyrosine, is involved in Csk binding.

Csk is a kinase that phosphorylates Src family kinases at the C-terminal inhibitory tyrosine residue [17, 18]. Disruption of the Csk gene in mice results in embryonic death, indicating that Csk plays an essential role in development [32]. By overexpression of Csk it has been shown that Csk negatively regulates signaling by the T Cell Receptor (TCR) and FcεRI [33, 34], while downregulation of Csk by RNAi lowers the threshold for TCR signaling [35]. In addition, mice in which the Csk gene is deleted in granulocytes develop multifocal inflammation and are hyperresponsive to LPS, indicating an essential role for Csk in setting an activation threshold in

granulocytes [36]. Taken together, these studies show that Csk has an important control function in immune cells.

A major step in the understanding of the function of Csk was the identification of Csk Binding Protein/Phosphoprotein Associated with Glycosphingolipid-enriched microdomains (Cbp/PAG), a membrane-associated protein that is localized to lipid rafts [37, 38]. In resting T cells, Cbp serves as an anchor for Csk at the cell membrane, where it can act on Src family kinases and sets a threshold for T cell activation [37, 38]. Our data suggest that Csk may also regulate cell function by binding to an ITIM-bearing receptor. So far, we have been unable to identify and unambiguous effect of Csk on LAIR-1 function, using either RNA interference or overexpression of Csk mutants to modify Csk function. This may be due to the fact that LAIR-1 itself is phosphorylated by Src family kinases [15]: therefore, by inactivating Src family kinases, Csk may also indirectly affect the function of LAIR-1.

In conclusion, we have found that LAIR-1-mediated inhibition does not solely depend on SHP-1 and SHP-2. In addition, we found that Csk interacts with phosphorylated LAIR-1. Further research should elucidate the role of Csk in the function of LAIR-1 and possibly other ITIM-bearing receptors.

## Materials and methods

*cDNA constructs*

Human LAIR-1 and LAIR-1 containing tyrosine-to-phenylalanine mutations in the ITIMs have been described before [4, 15]. The mFcγRIIb/hLAIR-1 chimera containing the extracellular and transmembrane domain of mFcγRIIb and the intracellular domain of LAIR-1 (amino acids 187-289) was generated by polymerase chain reaction as previously described for the mFcγRIIb/PECAM-1 chimera [21] and cloned into the pMx expression vector. Human FcγRIIb cDNA has been described before [39]. N-terminal FLAG-tagged FcγRIIb and LAIR-1 were generated by cloning the cDNA to the 3' site of the FLAG sequence in the pMx vector (generated at the DNAX Research Institute, Palo Alto, CA). cDNA encoding human Csk was generated by Neet et al. [40]. cDNA encoding Csk lacking the SH2 domain (amino acids 80-163, CskΔSH2) or the SH2 domain of Csk alone (amino acids 77-172) were generated by polymerase chain reaction.

*Cell lines and culture*

The DT40 chicken B cell line and SHP1$^{-/-}$, SHP2$^{-/-}$, SHP1,2$^{-/-}$ and SHIP$^{-/-}$ DT40 cells have been described before [19, 20] and were purchased from RIKEN Cell Bank (Tsukuba Science City, Japan). The DT40 cells were grown on RPMI 1640 media (Gibco, Paisley, UK) supplemented with 10% fetal calf serum (Integro, Dieren, The Netherlands), 1% chicken Serum

(Sigma), 4 mM glutamine (Gibco, Paisley, UK), 50 µM ß-mercaptoethanol and antibiotics. To generate DT40 cells expressing the mFcγRIIb/hLAIR-1 chimera the cells were transfected by electroporation. The cells were grown on selective media and cloned by the limiting dilution method, 24 hours after transfection. To confirm expression of the chimera, the cells were stained with FITC-conjugated anti-mFcγRIIb 2.4G2 antibodies or FITC-conjugated isotype control (Pharmingen BD) and analyzed by flow cytometry.

RBL-2H3 is a rat basophilic leukemia cell line [41]. RBL transfectants stably expressing either wild type LAIR-1, LAIR-1 mutants or a hLAIR-1/mLAIR-1 chimera have been described previously [15, 16]. Human embryonic kidney 293T cells were obtained from American Type Culture Collection (Manassas, VA). 293T cells and RBL cells were grown in RPMI 1640 media (Gibco, Paisley, UK) supplemented with 10% fetal calf serum (Integro, Dieren, The Netherlands) and antibiotics.

*Antibodies*

Polyclonal anti-chicken SHP-1 antibodies were a generous gift of Dr. T. Kurosaki (Kansai Medical University, Moriguchi, Japan). Polyclonal anti-SHP-2 and PLCγ2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The PY20 anti-phosphotyrosine antibody was purchased from BD Transduction Laboratories (Franklin Lakes, NJ). Mouse anti-chicken IgM mAb M4 was purchased from Southern Biotechnology Associates (Birmingham, AL). Intact and F(ab')2 rabbit anti-mouse IgM were obtained from Zymed (San Francisco, CA). Polyclonal anti-SHIP antibodies were kindly provided by Dr. J. Cambier (University of Colorado Health Sciences Center, Denver, CO). Monoclonal mouse IgG1 antibody directed against human LAIR-1, 8A8, has been described before [16]. For immunoprecipitation polyclonal rabbit anti-Csk antibodies were used (purchased from Santa Cruz Biotechnology, Santa Cruz, CA). For Western blot monoclonal anti-Csk antibody (BD Transduction Laboratories) was used. Horse Radish Peroxidase (HRP)-conjugated rabbit anti-mouse antibody (DAKO) or HRP-conjugated goat anti-rabbit antibody (Pierce) were used as secondary antibodies in Western blotting.

*Calcium mobilization assay*

DT40 cells ($10^7$/ml) were loaded with 3 µM Fluo 4-AM (Molecular Probes, Eugene, OR) in PBS for 20 min at 37°C. The cells were washed and resuspended in PBS supplemented with 1 mM $MgCl_2$ and 1 mM $CaCl_2$ . Triggering of the B cell receptor (BCR) with or without co-ligation of the mFcγRIIb/hLAIR-1 chimera was performed as described previously [20, 21]. Co-ligation of the mFcγRIIb/hLAIR-1 chimera is achieved by cross-linking the FcγRIIb moiety of the chimera by the Fc part of the intact antibody used to cross-link the BCR, while $F(ab')_2$ fragments do not cross-link the chimera. Briefly, the cells were incubated with intact or $F(ab')_2$ rabbit anti-

mouse IgM (final concentrations 9.5 µg/ml and 6.3 µg/ml respectively) for 5 minutes at room temperature. Mouse IgM anti-chicken IgM was added (clone M4, final concentration 25 µg/ml) and the fluorescence intensity was measured with 10 second-intervals in the Fluoroskan Ascent (Thermo Labsystems, Franklin, MA) with excitation by 485 nm and emission at 527 nm.

*Yeast-tri-hybrid assay*

The yeast strain Ylck4.1 [42] and the pYTH9B vector were generously provided by Dr K. Fuller (Glaxo Smith Kline, UK). cDNAs encoding the intracellular parts of human and mouse LAIR-1 (amino acids 187-287 and 165-263 respectively) were cloned in frame to the DNA binding domain of Gal4 in the pYTH9B vector. Stable transformants were generated by integration of the constructs into the Trp locus.

For screening, yeast cells stably expressing the BD-hLAIR-1 fusion protein were transformed with 50 µg human fetal brain library in the pACT2 vector (BD Biosciences) using a lithium acetate/Tris EDTA/polyethylene glycol protocol [42]. Transformants were grown on selective media containing 50 mM 3-amino-1,2,4-triazole (Sigma) for 10-14 days. Large colonies were re-streaked on selective plates, grown for 48 hours and filter-lifted on Hybond-N membranes (Amersham). ß-Galactosidase activity was determined by a freeze-thaw fracture assay. Yeast clones that turned blue in this assay were considered as possible positives. Library DNA was recovered by mechanically disrupting the yeast cells with acid-washed glass beads. To exclude false positives, the recovered library DNA was transformed into yeast cells that expressed the DNA binding domain of Gal4 alone. The proteins that interacted selectively with LAIR-1 were then identified by automated DNA sequencing of the library DNA.

For Csk interaction studies in yeast, full length Csk, CskΔSH2 and the SH2 domain were cloned in frame to the activation domain of Gal4 in the pACT2 vector (BD Biosciences). 1 µg of these constructs or the Csk construct obtained in the screen (CskΔSH3) was transformed in yeast cells expressing the DNA binding domain/hLAIR-1 or DNA binding domain/mLAIR-1 fusion protein. Three colonies were spotted on media and grown in the presence or absence of methionine for 48 hours. Interactions were identified by the ß-galactosidase assay as described above.

*Immunoprecipitation and Western blot analysis*

To assess the expression of SHP-1 in the DT40 cell lines, the cells were treated with 50 µM pervanadate and lysed in TNE lysis buffer (50 mM Tris-HCl, pH 8, 1% Nonidet P-40, 2 mM EDTA, 0.02% sodiumazide) supplemented with protease inhibitors (Complete Mini EDTA-free protease inhibitor cocktail tablets, Roche, Mannheim, Germany) and 1 mM phenylmethylsulfonyl fluoride. Protein A/G PLUS-Agarose beads (Santa Cruz Biotechnologies,

Santa Cruz, CA) were coated with anti-chicken SHP-1 antibody and immunoprecipitation was performed for 90 minutes in the presence of 0.5% BSA. Immune complexes were washed three times with lysisbuffer supplemented with 1 mM phenylmethylsulfonyl fluoride and boiled in non-reducing Laemmli sample buffer. Western blotting was performed and phosphorylated SHP-1 was detected by anti-phosphotyrosine antibodies. To assess the expression of SHP-2, the cells were directly lysed in Laemmli sample buffer and equal amounts of cell lysate were analyzed by Western blotting.

To investigate the LAIR-1 mediated effects on BCR-induced tyrosine phosphorylation, DT40 cells were incubated with intact or $F(ab')_2$ rabbit anti-mouse IgM, followed by mouse IgM anti-chicken IgM as described for the calcium mobilization assay and left at 37 °C for 1 minute. The cells were immediately washed in ice-cold PBS containing 250 μM orthovanadate and lysed in non-reducing Laemmli sample buffer.

For the identification of LAIR-1/Csk interactions, RBL cells stably transfected with LAIR-1 or LAIR-1 mutants were washed in PBS and 25 x $10^6$ cells were lysed in TNE lysis buffer. Immunoprecipitation was performed as described above using polyclonal anti-Csk antibodies.

To investigate binding of SHIP to LAIR-1 293T cells were transfected with FLAG-tagged LAIR-1 or FcγRIIb using FuGENE 6 (Roche) as transfection agent. To confirm expression of the FLAG-tagged receptors the cells were stained with M2 anti-FLAG antibody (Sigma) followed by phycoerythrin (PE)-conjugated goat anti-mouse IgG (Southern Biotechnology Associates) and analyzed by flow cytometry. After 48 hours the cells were treated with 50 μM pervanadate and lysed in Triton lysisbuffer (containing 1% Triton X-100, 10 mM Tris-HCl, pH 7.5, 150 mM NaCl and 0.02% sodiumazide) supplemented with protease inhibitors as described above. Immunoprecipitation was performed as described before [15] using precoated beads with anti-FLAG antibodies (Sigma). The samples were boiled in reducing Laemmli sample buffer.

Proteins were resolved by SDS-polyacrylamide gel electrophoresis and transferred to Immobilon-P membranes (Millipore, Bedford, MA). Western blot analyses were performed with anti-phosphotyrosine, anti-SHP-2, anti-PLCγ2, anti-SHIP, 8A8 anti-LAIR-1 or monoclonal anti-Csk antibodies, followed by HRP-linked secondary antibodies. Proteins were detected by enhanced chemiluminescence (Amersham, Little Charfort, UK).

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# 3

# CD200R-mediated inhibition of FcεR-induced degranulation requires at least two functional tyrosines

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**Abstract**

In contrast to most inhibitory immune receptors, which bear one or more immune receptor tyrosine based inhibition motifs (ITIMs), few inhibitory receptors do not signal via ITIMs. One of these receptors is CD200R, which is reported to be mainly expressed on cells of the myeloid lineage. The interaction between CD200R and its ligand CD200 is important in downregulation of cellular responses. CD200R does not contain ITIMs, but it does contain one NPXY motif, as well as two single tyrosines in its cytoplasmic tail. We studied the involvement of these three tyrosines in the capacity of CD200R to inhibit FcεRI-mediated degranulation of rat basophilic leukaemia cells. Degranulation could be inhibited by the wild type CD200R tail but not by a mutant in which all tyrosines are replaced with phenylalanines. Thus, tyrosine phosphorylation is an important step in CD200R signaling. Furthermore we demonstrate that CD200R needs all three tyrosines for full inhibition of degranulation but that none of the individual tyrosines is essential for inhibition. Finally we show that PTB-domain containing adapter proteins Shc and Dok-1 associate with CD200R.

## Introduction

The signaling motif ITIM is regarded as a general feature of inhibitory receptors [1]. It is widely accepted that inhibitory immune receptors signal via phosphorylation of the tyrosine in the ITIM, which leads to recruitment of phosphatases such as SHP-1, SHP-2 or SHIP. These can then dephosphorylate activating molecules, leading to abrogation of the activation signal. However, it has been shown recently that also C-terminal Src kinase (Csk) can bind to ITIM-bearing receptors [2;3], suggesting that immune inhibition is not solely mediated by recruitment of phosphatases to ITIMs. In addition, the existence of a small group of non-ITIM bearing inhibitory receptors shows that in some cases ITIMs are altogether expendable. The most well-known representative of this group is CTLA-4, which is an inhibitor of T cell activation, and contains two tyrosines that bind PP2A and SHP-2 (reviewed in [4;5;6]). This interaction is likely to mediate its inhibitory function, although the precise mechanism of action remains controversial. Another example is CD5, an inhibitor of B cell receptor signaling, which does contain two putative ITIMs but these are dispensable for its function [7].

CD200R, like CTLA-4, is an inhibitory immune receptor that does not contain ITIMs [8]. The ligand for CD200R is CD200, a widely expressed cell surface molecule of the Ig superfamily that has only a short intracellular tail, which does not contain any signaling motifs [9]. In contrast to the broad distribution of CD200, CD200R expression is much more restricted. It is expressed mainly on myeloid cells [10], such as macrophages, granulocytes and dendritic cells but recently has also been found on T cells [11].

Deletion of the CD200 gene in mice results in enhanced susceptibility to auto-immune diseases as collagen-induced arthritis (CIA) and experimental autoimmune encephalomyelitis (EAE) [12]. Thus, the inhibitory CD200R is an important signaling molecule in the down-modulation of myeloid responses to challenge. More recently, a role for CD200R cross-linking in induction of immunosuppressive IDO-activation in plasmacytoid DCs was described [13]. Also, CD200R signaling may be implicated in regulating Th2 cell function [14]. Direct evidence that CD200R is an inhibitory receptor comes from in vitro studies showing that ligation of CD200R results in decreased degranulation of and cytokine secretion by mast cells [15] and monocytes/ macrophages [16].

Although CD200R lacks ITIMs, it does contain three tyrosines, of which the most membrane-distal (tyrosine 298) is located in an NPXY motif. NPXY motifs are conserved signaling motifs, present in a broad range of receptors, which include members of the low density lipoprotein (LDL) receptor family, such as LDLR and LDL receptor-related protein LRP, and integrin β subunits [17;18]. Tyrosine-phosphorylated NPXY motifs bind to adaptor proteins containing PTB domains, thereby propagating the signal from the receptor [17;19]. For example, autosomal recessive hypercholesterolemia protein (ARH) binds the NPXY motif of the LDL receptor via its PTB motif. The interaction is disrupted by a mutation in the PTB

domain of ARH [18].

Previously it was demonstrated that the intracellular tyrosines in CD200R can be phosphorylated after pervanadate treatment, but their relative contribution to the inhibitory signals remains unresolved [8]. We have used tyrosine to phenylalanine mutants to study the relative role of the tyrosines in CD200R signaling. Also, by employing a yeast tri hybrid screening method, we have identified Shc and Dok-1 as molecules interacting with the tyrosine-phosphorylated intracellular tail of CD200R. Our data demonstrate that CD200R needs all three tyrosines for full inhibitory function and that Shc and Dok-1 may not be the sole mediators of CD200R function.

## Materials and methods

*Cell lines and culture*

RBL-2H3 is a rat basophilic leukemia (RBL) cell line [20]. Human embryonic kidney 293T cells and RBL cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). All cells were cultured in RPMI 1640 (Gibco, Paisley, UK), supplemented with 10% FCS (Integro, Dieren, the Netherlands), 50 μM β-mercaptoethanol and antibiotics.

*cDNA constructs and transfectants*

A chimeric construct of extracellular human LAIR-1 (amino acids 1-160) and transmembrane and intracellular rat CD200R (amino acids 236-327) was cloned into pcDNA3.1/zeo (Invitrogen, Breda, the Netherlands). Also, tyrosine (Y) to phenylalanine (F) mutants of tyrosines 287, 290 and 298 in the intracellular rCD200R tail were generated with PCR-based mutagenesis, as well as the double and triple Y to F mutants. The mutants were cloned into the same vector and all sequences were confirmed by automated DNA sequencing.

Stable RBL transfectants were made by electroporation (GenePulser Xcell, Bio-Rad Laboratories), were selected using 50 μg/ml zeocin (Invitrogen, Carlsbad, CA) and were subcloned by the limiting dilution method. To assess the expression levels of the LAIR-1/CD200R chimera and mutants, cells were stained with anti-LAIR-1 DX26-phycoerythrin (PE) (BD Biosciences Pharmingen) [21] and measured by flow cytometry.

Transient 293T transfections were done using Fugene 6 Tranfection Reagent (Roche Diagnostics, Mannheim, Germany). 293T cells were used for analysis after 24 to 30 hours after transfection.

*Immunoprecipitations and western blotting*

For immunoprecipitations, Protein A/G PLUS-agarose beads (Santa Cruz Biotechnology) were

coated with anti-LAIR-1 mAb (DX26). Transfected cells were treated with 50 µM pervanadate, a phosphatase inhibitor, in PBS for 15 minutes at 37° or with PBS only. Cells were lysed using 1% Triton in Tris buffer (10 mM Tris, 150 mM NaCl, 0.02% azide, pH 7.5, Triton was freshly added), after which immunoprecipitations were carried out for 90 minutes in the presence of 0.5% BSA. Immunoprecipitates were washed with 0.1% Triton buffer and boiled in non-reducing Laemmli buffer. Proteins were resolved using SDS-PAGE and transferred to Immobilon membranes (Millipore, Bedford, MA). Western blot analysis was performed with 8A8 anti-LAIR-1 [21] and anti-phosphotyrosine (PY20, BD Transduction Laboratories), followed by rabbit anti-mouse Ig – Horse Radish Peroxidase (-HRP, from DAKO A/S, Glostrup, Denmark). Proteins were detected with enhanced chemiluminescence (Amersham, Little Chalfont, UK).

*RBL-2H3 degranulation assay*

The assay to measure degranulation of RBL cells has been described before [22]. Measurements were performed using triplicate cultures. The percentage of inhibition by the LAIR-1/ CD200R chimera and mutants was calculated as: percentage of inhibition = [(OD405 without CD200R crosslinking – OD405 with CD200R crosslinking) / (OD405 without CD200R crosslinking – OD405 spontaneous release)] * 100.

*Yeast tri hybrid*

The yeast strain Ylck4.1 [23] and the pYTH9B vector were generously provided by Dr. K. Fuller (Glaxo Smith Kline, UK). cDNA encoding the intracellular part of mouse CD200R (amino acids 260-326) was cloned in frame to the DNA binding domain (BD) of Gal4 in the pYTH9B vector. Alternatively, the intracellular parts of human and rat CD200R and the tyrosine mutants were used. Stable transformants were generated by integration of the constructs into the Trp locus.

For screening, yeast cells stably expressing the BD-mCD200R fusion protein were transformed with 50 µg human fetal brain library in the pACT2 vector (BD Biosciences) containing the GAL4 activation domain (AD) using a lithium acetate/Tris EDTA/polyethylene glycol protocol [23]. Transformants were grown on selective media containing 50 mM 3-amino-1,2,4-triazole (Sigma) for 10-14 days. Large colonies were re-streaked on selective plates, grown for 48 hours and filter-lifted on Hybond-N membranes (Amersham). ß-Galactosidase activity was determined by a freeze-thaw fracture assay. Yeast clones that turned blue in this assay were considered as possible positives. Library DNA was recovered by mechanically disrupting the yeast cells with acid-washed glass beads. To exclude false positives, the recovered library DNA was transformed into yeast cells that expressed the DNA binding domain of Gal4 alone. The proteins that interacted selectively with CD200R were then identified by automated DNA sequencing of the library DNA. To determine phosphorylation dependency of the interaction,

clones were tested for interaction in the presence or absence of the lck protein by adding or withholding methionine to the culture.

*Statistical analyses*

Nonparametric statistical tests were used since the data were not normally distributed. Differences between the groups were analysed using Mann-Whitney tests. A p-value smaller than 0.05 was considered statistically significant. All statistical analyses were performed using SPSS 12.0 (SPSS Inc, Chicago, IL, United States).

**Results**

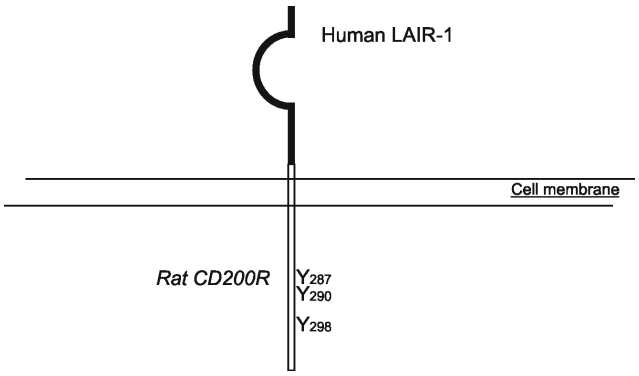
*Tyrosine 290 of CD200R is not phosphorylated when no other tyrosines are present*

To determine whether phosphorylated tyrosines play a role in CD200R signaling, we first investigated whether CD200R could be phosphorylated in our cell lines. Because we did not have an agonistic antibody to CD200R available which we could use in later studies, we constructed a cDNA encoding a chimeric protein containing the extracellular domains of human LAIR-1 and the transmembrane and intracellular domains of rat CD200R (Figure 1A). LAIR-1 is an inhibitory immune receptor of the immunoglobulin superfamily (IgSF) containing two intracellular ITIM motifs, for which agonistic antibodies are available [21]. The LAIR-1/ CD200R chimeric construct was either transiently transfected into 293T cells or stably into RBL cells. Treatment of the cells with the phosphatase inhibitor pervanadate resulted in tyrosine phosphorylation of the chimeric protein in both cell lines, indicating that kinases that can phosphorylate CD200R are present in these cells (Figure 1B).

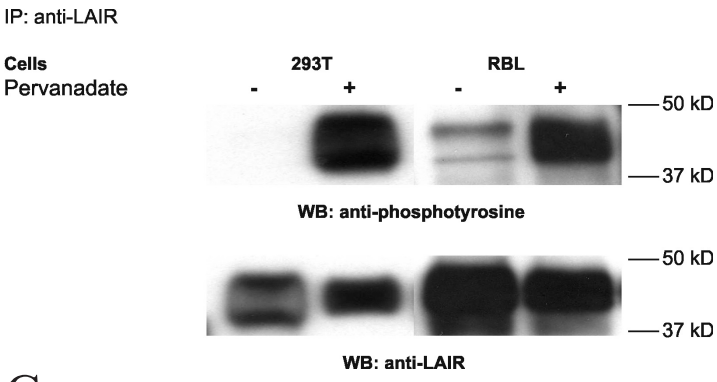
To study the role of the different tyrosines in the function of CD200R, tyrosine to phenylalanine mutants of all tyrosines in the intracellular tail were made in all possible combinations (Figure 1C). These mutants were also cloned in the context of the human LAIR-1/ CD200R chimera. The mutant constructs were transiently transfected into 293T cells, after which the cells were treated with pervanadate and the chimeric proteins immunoprecipitated to study tyrosine phosphorylation of the mutants (Figure 1D).

As expected, the wild type CD200R tail was tyrosine phosphorylated, whereas the mutant lacking all tyrosines, FFF, did not show phosphorylation. In addition, the FYF mutant was not phosphorylated at all, suggesting that the tyrosine at position 290 is not phosphorylated in wild type CD200R or that phosphorylation of other tyrosines is required for the phosphorylation of tyrosine 290. Compared to the wild type CD200R tail, the single mutants FYY and YYF and the double mutants FFY and YFF showed decreased tyrosine phosphorylation. We conclude that tyrosine 290 is not phosphorylated when no other tyrosines are present.

A




B




C

| | |
|---|---|
| MQP**Y**AS**Y**TEKS**NPLY**DTV | **YYY** |
| ---**F**--**Y**----**NPLY**--- | **FYY** |
| ---**Y**--**F**----**NPLY**--- | **YFY** |
| ---**Y**--**Y**----**NPLF**--- | **YYF** |
| ---**F**--**F**----**NPLY**--- | **FFY** |
| ---**F**--**Y**----**NPLF**--- | **FYF** |
| ---**Y**--**F**----**NPLF**--- | **YFF** |
| ---**F**--**F**----**NPLF**--- | **FFF** |

D

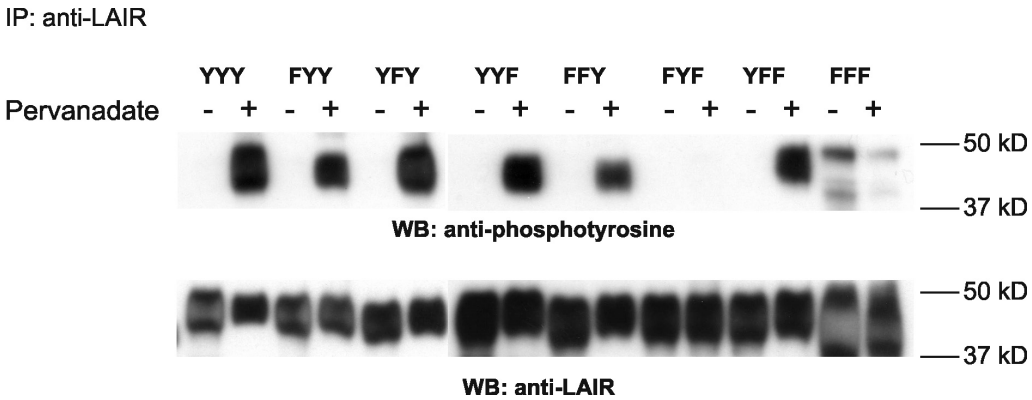



**Figure 1. Phosphorylation of CD200R and mutants after treatment with pervanadate**

A. Schematic representation of the LAIR-1/ CD200R chimera. Tyrosines in the intracellular CD200R domain are indicated. The most membrane-distal tyrosine (tyrosine 298) is located in an NPXY motif.

B. The LAIR-1/ CD200R chimera can be phosphorylated in 293T cells and RBL cells. The chimeric construct was transfected into 293T cells and RBL cells. After transfection, cells were treated with 50 µM pervanadate in PBS. Lysates were used for anti-LAIR-1 immunoprecipitation and analyzed on western blot for phosphotyrosine and total LAIR. The figure shown is representative of two independent experiments.

C. Schematic overview of the CD200R mutants. Represented are amino acids 284-301 of the cytoplasmic domain. Dashes indicate identical amino acids.

D. Phosphorylation of the CD200R mutants in 293T cells treated with pervanadate. 293T cells were transiently transfected with 1 µg of wild type (YYY) or the different mutant chimeric constructs. 30 Hours after transfection, cells were treated with 50 µM pervanadate in PBS. Lysates were used for anti-LAIR-1 immunoprecipitation and analyzed on western blot for phosphotyrosine and total LAIR. The figure shown is representative of four independent experiments.

*CD200R signaling is dependent on at least two intact tyrosines*

To study the contribution of the different tyrosines to CD200R signaling, we studied FcεRI-mediated degranulation of the rat basophilic leukemia cell line RBL-2H3 cells. This system is often used to study inhibition of FcεRI-mediated signals by ITIM-bearing receptors [24]. The LAIR-1/ CD200R wild type construct and the constructs containing the different tyrosine to phenylalanine mutants were stably transfected into the RBL-2H3 cell line and independent clones containing high expression levels of the chimeras were obtained (Figure 2A). Simultaneous cross-linking of the FcεRI with the LAIR-1/ CD200R chimera using anti-LAIR antibodies demonstrated that signaling by the wild type CD200R tail inhibited degranulation by approximately 55% (Figure 2B, left bar). Thus, CD200R was able to inhibit ITAM-mediated activation signals. The tyrosine mutants were evaluated for their ability to inhibit FcεRI-induced degranulation through cross-linking of the LAIR-1/ CD200R chimera (Figure 2B).

Crosslinking of the FFF mutant, which does not contain tyrosines and cannot be phosphorylated, resulted in only very modest inhibition of degranulation. This indicates that tyrosine phosphorylation is required for the inhibitory function of CD200R. None of the tyrosine mutants was able to inhibit the degranulation response to the same level as wild type CD200R. Mutants FYY and YFY were most efficient in inhibiting the degranulation (28%), while the YYF mutant could still inhibit the degranulation by about 21%. Similar to the FFF mutant, all three double mutants lacking two tyrosines only modestly inhibited the degranulation. Therefore, we conclude that CD200R requires at least two phosphorylated tyrosine residues to be able to inhibit cellular function and that no single tyrosine is absolutely essential for CD200R function.

*The intracellular tail of CD200R associates with Shc and Dok-1*

To evaluate which downstream molecules are important for transduction of the CD200R inhibitory signal, we have used a yeast tri hybrid assay to identify molecules specifically interacting with the phosphorylated CD200R tail. For this purpose, we used the Ylck4.1 strain, in which lck expression can be induced, allowing us to identify proteins specifically interacting with phosphorylated CD200R. We screened a human fetal brain library with intracellular mouse CD200R as bait. Protein interactions were detected using the β-galactosidase reporter gene. We identified five clones that were positive in the reporter assay. Four of these clones encoded the p64 isoform of N-Shc (amino acids 46 - 594), the other one encoded Dok-1 (amino acids 8 - 481).

The binding of both these molecules was phosphorylation dependent, as there was no interaction in yeast cells in which lck expression was suppressed (Figure 3A). Since both Shc and Dok-1 contain a PTB domain, we hypothesized that both of these molecules would bind to

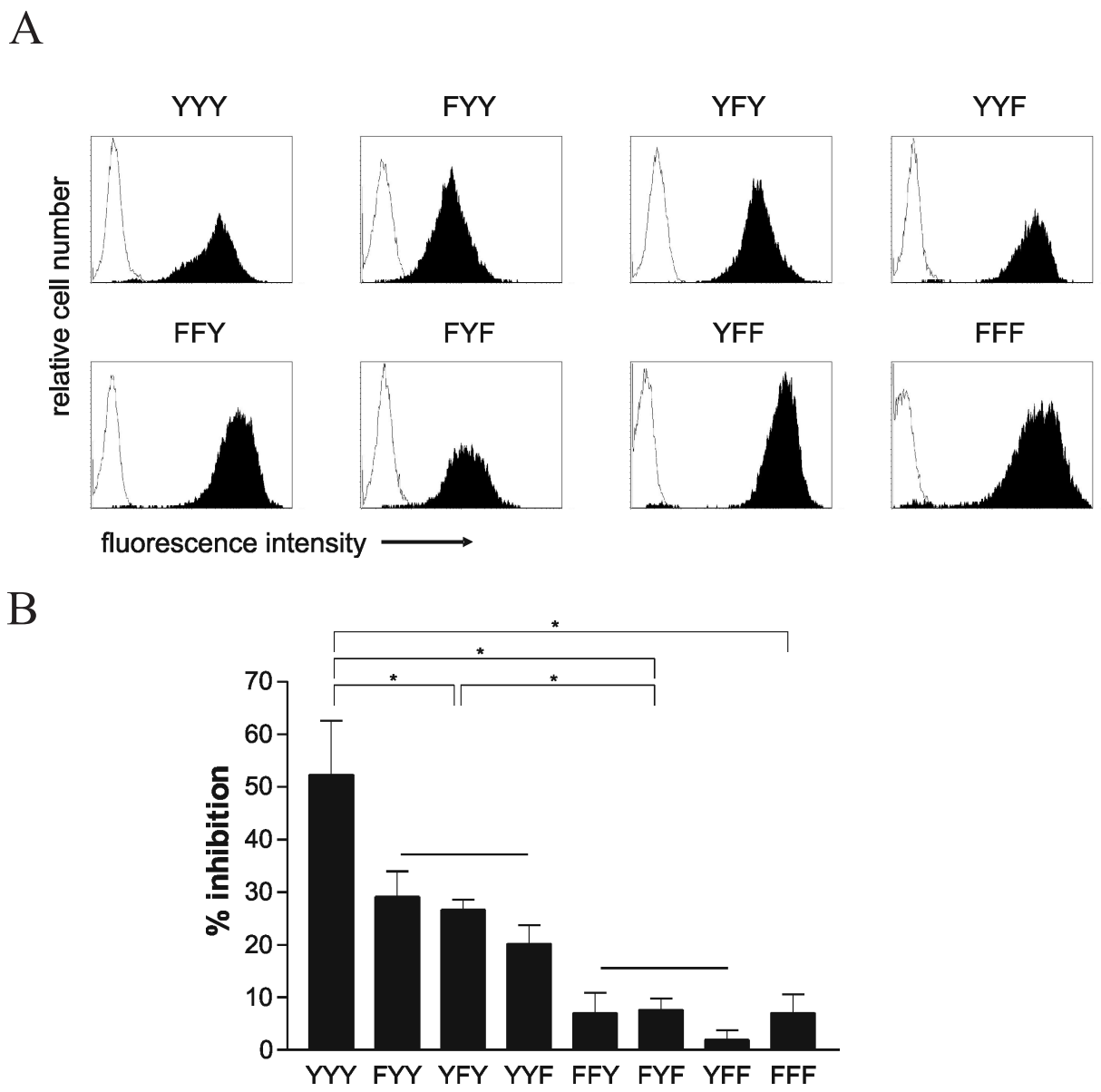
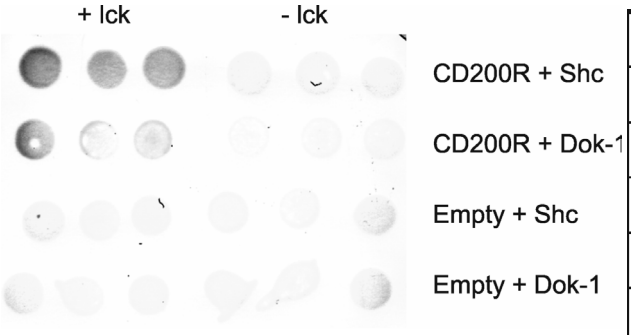



**Figure 2. Inhibition of degranulation of RBL cells by CD200R mutants**
A. The chimeric constructs were stably transfected into RBL cells using electroporation. After selection with zeocin and subcloning, expression of the construct was analysed using anti-LAIR (DX26; closed histograms). Open histograms depict isotype control staining. One of three representative clones per construct is shown.
B. Clones expressing high amounts of the different constructs were used to measure their ability to inhibit the FcεRI-induced degranulation of RBL cells. Cells were coated with IgE anti TNP and stimulated with BSA-TNP. To measure inhibition by CD200R, cells were coated with both IgE and anti-LAIR-1 (8A8) and were stimulated with BSA-TNP and GaM-F(ab')$_2$. Percentage inhibition was calculated by comparing the degranulation when anti-LAIR antibody was present with degranulation without anti-LAIR-1 (see Methods). Per mutant, at least 3 different clones were tested at least twice. Error bars indicate standard error of the mean (SEM). Mutants were tested for significant differences between each other. For clarity, significant differences are shown between groups (wild type, single mutants, double mutants, and triple mutant), since the members of each group do not differ significantly from one another. * indicates $p<0.05$.

tyrosine 298, which is located in an NPXY motif. To evaluate this hypothesis, human, mouse and rat CD200R and the tyrosine mutants of rat CD200R were checked for binding to Shc and Dok-1 using the yeast reporter assay. Unexpectedly, we found that Dok-1 only bound to the wild type proteins and to none of the tyrosine mutants (Figure 3B). Shc bound to none of the constructs in which tyrosine 298 was mutated, but did bind to mouse and rat wild type CD200R, as well as the YFY and FFY mutants (Figure 3B). Surprisingly, Shc did not bind to the FYY mutant and hCD200R.

A




| | + lck | - lck |
|---|---|---|
| CD200R + Shc | + | - |
| CD200R + Dok-1 | + | - |
| Empty + Shc | - | - |
| Empty + Dok-1 | - | - |

B

| | Dok-1 | Shc |
|---|---|---|
| mCD200R | + | + |
| hCD200R | + | - |
| rCD200R YYY | + | + |
| FYY | - | - |
| YFY | - | + |
| YYF | - | - |
| FFY | - | + |
| YFF | - | - |
| FYF | - | - |
| FFF | - | - |

**Figure 3. CD200R associates with Shc and Dok-1**
A. Ylck cells stably transformed with Gal4BD-mouse CD200R were transformed with Gal4 AD-Shc (Shc) or Gal4AD-Dok-1 (Dok) and grown on selective medium in the absence (+Lck) or presence (-Lck) of methionine for 48 hours. The yeast was transferred to a nylon membrane and assayed for Gal4-promotor activity using a standard ß-galactosidase assay. Yeast stably transformed with Gal4BD alone (empty) was used as a negative control. The figure shown is representative for two independent ß-galactosidase experiments. The table summarizes the obtained results. + denotes interaction (yeast turned blue), - denotes no interaction.
B. The recovered library DNA of Dok-1 or Shc was transformed into yeast cells stably expressing human, mouse or rat CD200R or one of the tyrosine mutants and interaction was determined in the presence of lck using the ß-galactosidase assay. + denotes interaction, - denotes no interaction.

**Discussion**

CD200R is a non-ITIM bearing inhibitory immune receptor that has three tyrosines in its intracellular tail, one of which is located in an NPXY motif. To evaluate the role of the intracellular tyrosines in CD200R signaling, we mutated all three tyrosines to phenylalanines. This completely abrogated phosphorylation of CD200R, and greatly reduced its ability to inhibit FcεRI-induced degranulation. This shows that CD200R can inhibit ITAM-mediated signals and that the tyrosines of CD200R mediate its inhibitory function. Little residual activity is left when all three tyrosines are mutated, suggesting some inhibitory capacity of CD200R outside the three tyrosines. However, to determine whether this is indeed the case, further research is needed, for example assessing the inhibitory capacity of a tailless mutant.

To determine the individual role of the different tyrosines in CD200R signaling, we studied the tyrosine phosphorylation and inhibitory capacity of the single and double tyrosine mutants in all possible combinations. After pervanadate treatment, phosphorylation of tyrosine 290 did not occur in absence of the other tyrosine residues. Analysis of the function of the YFY mutant revealed that it can still inhibit cellular function, but at a lower level than the wild type protein. This indicates that, although tyrosine 290 apparently can not be phosphorylated without the presence of the other tyrosines, it does play a role in signaling. Thus, we conclude that tyrosine 290 is only phosphorylated when one or both of the other tyrosines are phosphorylated. Alternatively, it could recruit adaptor molecules independently of its phosphorylation status.

Mutation of either tyrosine 287 or tyrosine 298 resulted in decreased phosphorylation and in decreased inhibition of cellular function. The decrease in inhibitory capacity of both mutants was comparable to that of the YFY mutant, indicating that all three tyrosines contribute to the inhibitory function of CD200R equally.

Using a yeast tri hybrid screen, we found Shc and Dok-1 to bind to CD200R in a specific and phosphorylation-dependent manner. Our experimental set-up also demonstrates that the Src-family kinase lck is able to phosphorylate CD200R. While these studies were being performed, Zhang et al reported binding of Shc and Dok-1 (and Dok-2) to phosphopeptides of the intracellular tail of CD200R [25], providing an independent confirmation of our data.

Shc is an adaptor protein involved in many processes (reviewed in [26]). Its main function is to activate the Ras signaling pathway via an interaction with Grb2 and Sos. Also, it binds SHIP through an NPXY-PTB domain interaction [27;28]. This is thought to sequester Shc away from its activating function, resulting in inhibition of cellular function. However, the exact role of this complex still needs to be determined [26]. Our association data in yeast indicated binding of Shc to tyrosine 298 in the NPXY motif of CD200R. Unexpectedly, Shc did not seem to bind to human CD200R or the FYY mutant, although we could not determine the expression level of these constructs in the yeast cells. We and others [25] have not been able to confirm the interaction between Shc and CD200R in mammalian cells. Thus, whether Shc plays a role in

CD200R function remains to be elucidated.

The second protein we identified that associates with phosphorylated CD200R is Dok-1. Dok-1 was first described as a RasGAP-binding protein and a target of the tyrosine kinase Abl [29;30;31]. Dok-1 inhibits the Ras signaling pathway, which is an important activation pathway downstream of, for example, the B cell receptor (BCR) and FcεRI [32;33;34]. Dok-1 is also a negative regulator of LPS signaling [35] and of leukemia development, together with Dok-2 [36;37]. Dok-1 recruits RasGAP, which transforms active RasGTP to inactive RasGDP, although this is probably not the only mechanism through which Dok-1 can inhibit many activation signals [38]. Dok-1 contains a PTB domain through which it can interact with the tyrosine in an NPXY motif. However, preliminary association studies in yeast showed that mutation of either one of the tyrosines in CD200R abolished Dok-1 binding. Therefore, several steps may be required for Dok-1 binding.

The binding of Dok-1 and Dok-2 to the intracellular tail of CD200R was confirmed in mammalian cells by Zhang et al [25;39]. They found the interaction to be phosphorylation-dependent and mutation of tyrosine 290 in CD200R did not influence association of Dok-1.

Since tyrosine 298 is located in an NPXY motif and can be phosphorylated, we expected that this tyrosine would play a crucial role in CD200R signaling. However, all single tyrosine to phenylalanine mutants showed a similar reduction in inhibitory function. Surprisingly, Zhang et al find that mutation of the second tyrosine does not affect function, but that mutation of the first or the third tyrosine completely abrogates inhibition of mast cell degranulation [39]. These differences can be explained by the fact that they use transfected primary mouse mast cells that endogenously express wild type CD200R. Although the authors claim that the endogenous CD200R does not respond to their crosslinking antibody because of the lower expression level, it might make the system less sensitive. Also, they use another activating signal, namely CD200RLa, which is one of the activating family members of CD200R that associate with DNAX activating protein 12 (DAP12) and can not bind CD200 [11;40;41]. Inhibition of different activation signals might require different inhibitory signals. Furthermore, Zhang et al study mCD200R, whereas we have used rCD200R. Both molecules may have different requirements for Dok-1 association and inhibitory function.

Our data clearly show that all three tyrosines in CD200R are required for full inhibitory function. Our association data in yeast suggest that all three tyrosines of CD200R are required for Dok-1 association. Together, this could indicate that Dok-1 association is needed for full inhibitory function of CD200R. However, it is critical to determine which tyrosines are essential for Dok-1 association in mammalian cells.

Furthermore, we show that inhibitory function is reduced, but not abolished if one tyrosine residue is mutated. These single mutants FYY, YFY and FYY, may be unable to bind Dok-1, but still have inhibitory capacity. Therefore, other molecules could mediate this inhibitory function.

The first candidate is Shc, although, despite numerous attempts by different groups, association of Shc with CD200R has not been found in mammalian cells. The role of Shc in CD200R function is unclear and it is likely that yet other molecules associate to the intracellular CD200R domain. Performing a yeast tri hybrid screen with a CD200R construct in which the tyrosine(s) to which Dok-1 associates is mutated into a phenylalanine could allow identification of these other associating molecules.

In summary, we have shown that CD200R needs all tyrosines for its full inhibitory function, and at least two for partial inhibition. Furthermore, we have identified Shc and Dok-1 as molecules associating with the phosphorylated intracellular CD200R tail. However, we have also shown that these are probably not the sole molecules involved in exerting the inhibitory effect of CD200R and that other yet unidentified molecules could still be involved.

**Acknowledgements**

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# 4

# Ligation of CD200R by CD200 is not required for normal murine myelopoiesis

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**Abstract**

CD200R is an inhibitory receptor involved in the regulation of myeloid cells. It recruits Dok-1 and Dok-2, which are potent inhibitors of the Ras signalling pathway used by colony-stimulating factor (CSF) receptors. Dok-1/Dok-2 double knockout (DKO) mice develop leukaemia at 10-12 months of age. We investigated whether disturbed CD200R signalling could be responsible for this phenotype. Therefore, we studied whether $CD200^{-/-}$ mice have altered myelopoiesis and develop leukaemia. We report that CD200R is expressed on hematopoietic progenitor cells. However, $CD200^{-/-}$ mice have normal numbers of myeloid progenitors in the bone marrow and these cells have normal proliferative capacity. These results indicate that the development of leukaemia in Dok-1/Dok-2 DKO mice is not solely due to an absence of CD200R signalling. In addition, we show that the previously reported enhanced numbers of myeloid cells do not occur in all $CD200^{-/-}$ mice. We determined whether variations in the numbers of peripheral myeloid cells were due to an enhanced response to G-CSF or an inflammatory stimulus. Mobilisation of immature neutrophils via G-CSF and infiltration of mature neutrophils and macrophages upon thioglycolate injection were not altered in $CD200^{-/-}$ mice. We conclude that $CD200^{-/-}$ mice exhibit normal myelopoiesis and that development of leukaemia in Dok-1/Dok-2 DKO mice is not caused by a lack of CD200-mediated CD200R signalling.

## Introduction

The development of a functional immune cell pool is greatly dependent on the colony-stimulating factor (CSF) group of cytokines. Most CSFs are specific for a certain lineage (such as granulocyte-CSF (G-CSF) and macrophage-CSF or M-CSF). IL-3 however, stimulates production of for example macrophages, neutrophils, megakaryocytes and erythroid cells (reviewed in (1)). Since they mediate survival, proliferation and functional differentiation of all hematopoietic lineages, CSFs are also important for the maintenance of homeostasis and immune competence (1). Signals from many CSF receptors are mediated by the Ras signalling pathway (2-5), for example during the development of immature cells (5). To balance these activating signals, inhibitory molecules such as SOCS-3 act to prevent overproduction of immune cells (6). In addition, the adaptor molecules Dok-1 and Dok-2 are reported to be involved in myelopoiesis (7, 8). Dok-1 and Dok-2, upon phosphorylation, inhibit the Ras signalling pathway. Although the exact mechanism of inhibition still remains to be clarified (9), Dok-1 and Dok-2 most likely function by recruiting RasGAP, which converts the active RasGTP to the inactive RasGDP (10). Mice deficient for both Dok-1 and Dok-2 exhibit increased numbers of myeloid progenitors starting at 4 months of age (7, 8). Furthermore, these mice develop chronic myelogenous leukaemia (CML)-like myeloproliferative disease with predominant granulocyte and monocyte production at 10-12 months of age. At onset of leukaemia, Dok-1/Dok-2 double knockout (DKO) mice showed clear splenomegaly and peripheral blood and bone marrow hypercellularity (7, 8). However, it is unclear which signal recruits Dok-1/ Dok-2 in normal myelopoiesis.

Recently, it has been shown that both Dok-1 and Dok-2 associate to CD200R and mediate its inhibitory function (11, 12). CD200R is an inhibitory immune receptor reported to be mainly expressed on myeloid cells (13, 14). It has been shown to be able to inhibit cellular responses of different myeloid cell types in vitro after ligation by its widely expressed ligand, CD200 (15-17). In vivo, CD200$^{-/-}$ mice have been shown to be more susceptible to auto-immune diseases (18). In addition, CD200$^{-/-}$ mice were shown to contain twice the normal number of myeloid cells, and macrophages of these mice were more activated than macrophages from wild type mice (18, 19).

Since Dok-1 and Dok-2 both are mediators of CD200R signalling and are known to be suppressors of myeloid leukaemia, we investigated whether myelopoiesis in CD200$^{-/-}$ mice would be perturbed. We examined the CD200$^{-/-}$ myeloid progenitor compartment and report that, although CD200R is expressed at a high level on hematopoietic progenitor cells, there are no differences in progenitor cell numbers and types between wild type and CD200$^{-/-}$ mice. In addition, we found no differences between wild type and CD200$^{-/-}$ mice in the response to G-CSF induced mobilisation of immature neutrophils and thioglycolate-induced recruitment of mature neutrophils and macrophages to a site of inflammation.

In conclusion, we show that leukaemia development of Dok-1/Dok-2 DKO mice is not

caused by a defect in CD200R signalling.

## Materials and methods

*Mice*

Wild type C57BL/6J mice were obtained from Charles River (France). $CD200^{-/-}$ mice were bred at the Specified Pathogen Free (SPF) unit in the Central Animal Laboratory of the Utrecht University. These mice were offspring of the previously published $CD200^{-/-}$ mice on full C57BL/6J background (18). Blood was obtained by puncture of the vena saphena. Splenocytes were obtained by straining spleens through nylon sieves, after which the erythrocytes were lysed. Bone marrow was obtained by aseptically flushing the cells from femora. All animal experiments were approved by the Utrecht University animal ethics committee.

*G-CSF and thioglycolate treatment*

For mobilisation of immature neutrophils from the bone marrow to the blood wild type and $CD200^{-/-}$ mice were injected subcutaneously (s.c.) with 20 µg of pegylated hG-CSF (Amgen, Thousand Oaks, CA, United States) (20) in 150 µl PBS or with PBS only. A single injection of peg-G-CSF results in increased mobilisation of immature neutrophils from the bone marrow to the blood, peaking at 4 days after injection. The percentages and total amounts of neutrophils in peripheral blood were monitored by venal puncture and analysis by flow cytometry.

Alternatively, sterile peritonitis was induced in wild type and $CD200^{-/-}$ mice by intraperitoneal (i.p.) injection of 1 ml 4% aged thioglycolate broth (Sigma) or with PBS as a control. At 4 hours or 2 days after injection, infiltration of cells into the peritoneum was analysed by performing peritoneal lavage. Peritoneal cells were counted and analysed for cellular composition by flow cytometry.

*Flow cytometry*

Antibodies against murine Gr-1, CD11b and CD117 (c-Kit) were from Beckton Dickinson Biosciences (San Diego, CA, United States). Antibodies against mouse F4/80, CD200 and CD200R were from AbD Serotec.

Blood cells were analysed by staining the cells with antibodies, after which erythrocytes were lysed with FACS lysing solution (Becton Dickinson, San Jose, CA). Spleen cells were stained after the erythrocytes were lysed. A FACSCalibur with CellQuest software was used for acquisition and analysis.

*Colony-forming unit assay*

Bone marrow cells (10,000 or 15,000) were plated in pre-made methylcellulose medium in IMDM supplemented with foetal calf serum and cytokines (50 ng/ml hSCF, 50 ng/ml hFlt-3, 10 ng/ml mIL-3, 0.2 nM hIL-5, 5 ng/ml TPO, 0.5U/ml EPO, 60 ng/ml hG-CSF and 10 ng/ml mGM-CSF). Experiments were all performed in duplo. 10 to 12 days after plating, colonies were counted manually and the colony type was determined by visual inspection of the colony. To validate the visual inspection, several colonies from each experiment were isolated and May-Grünwald-Giemsa stained cytospins were used for microscopic evaluation of cellular morphology.

*Statistical analyses*

Nonparametric statistical tests were used since the data were not normally distributed. Differences between the groups were analysed using Mann-Whitney tests. A p-value less than 0.05 was considered statistically significant. All statistical analyses were performed using SPSS 12.0 (SPSS Inc, Chicago, IL, United States).

**Results**

*CD200R signalling does not influence formation of myeloid progenitors*

We first measured CD200R expression on c-Kit$^{+}$ hematopoietic stem and progenitor cells in the bone marrow and observed high expression levels (Figure 1A). There was no difference between CD200R expression on c-Kit$^{+}$ bone marrow cells of wild type and CD200$^{-/-}$ mice (data not shown). To investigate whether mice in which cellular inhibition by CD200R is impaired (as is the case in CD200$^{-/-}$ mice) develop leukaemia, we measured myeloid cell numbers at 14 months of age. There were no differences between absolute myeloid cell numbers (data not shown) and relative numbers of myeloid cells of wild type and CD200$^{-/-}$ mice in blood (Figure 1B) and spleen (data not shown). This indicates that CD200$^{-/-}$ mice do not develop leukaemia. Strikingly, CD200$^{-/-}$ mice 14 months old did not show increased levels of myeloid cells as previously reported for 8 to 12 week old CD200$^{-/-}$ mice (18). Rather, they contained normal neutrophil and macrophage (data not shown) numbers, which could indicate that the reported increase in myeloid cell numbers in CD200$^{-/-}$ mice is transient.

To investigate the influence of CD200 on hematopoiesis we next performed colony-forming assays with bone marrow cells from wild type and CD200$^{-/-}$ mice of different ages. Bone marrow cells were cultured in methylcellulose medium supplemented with serum and a cytokine mix enabling development of all myeloid lineages. After 12 days, established colonies were counted. We did not observe differences in total numbers, types and sizes of the colonies

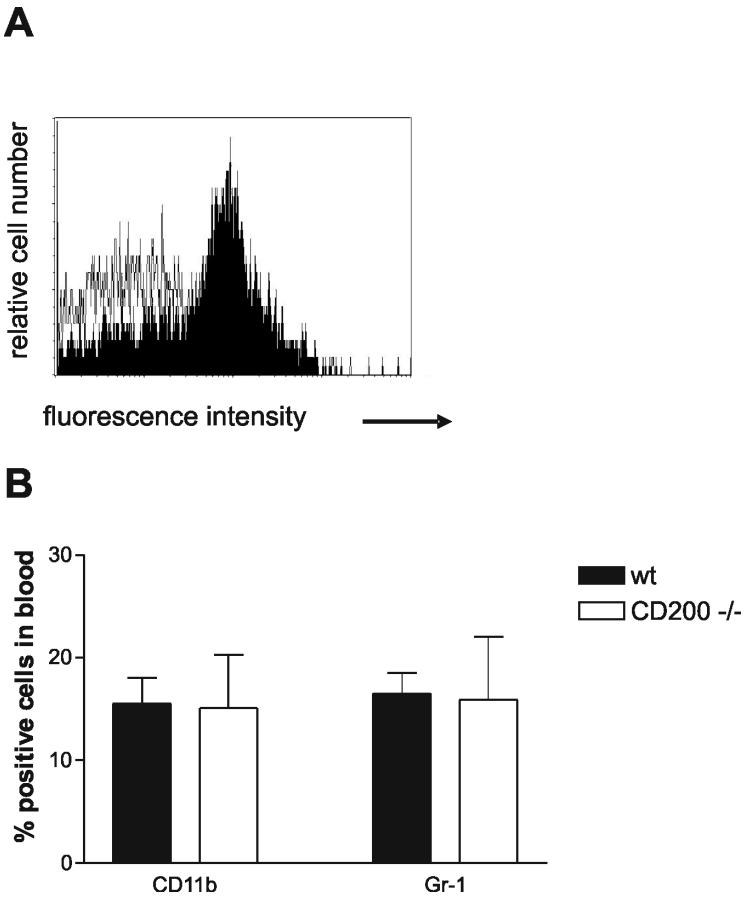


**Figure 1. Lack of CD200 expression does not induce leukaemia**
A. Bone marrow cells were harvested from femora of wild type mice. Cells were labelled with antibodies against c-Kit and CD200R and analysed by FACS. Open histogram represents isotype control staining, closed histogram represents CD200R staining. Data shown are representative of 4 independent experiments.
B. Blood from wild type mice and CD200$^{-/-}$ mice was stained with Gr-1 and CD11b antibodies. After erythrocyte lysis, cells were analysed by flow cytometry. Black bars represent wild type mice, open bars represent CD200$^{-/-}$ mice. Error bars indicate standard error of the mean (SEM) of 4 mice from 2 independent experiments.

(Figure 2A and data not shown). Although there seemed to be a small difference between wild type and CD200$^{-/-}$ mice in total colony numbers at the age of 18 weeks, this difference was statistically not significant (p=0.11). Therefore, we conclude that the bone marrow of CD200$^{-/-}$ mice does not contain different numbers of myeloid progenitors. Also, there are no differences in proliferative capacity or type of progenitors between cells from wild type and CD200$^{-/-}$ mice. In agreement with a previous report (21), wild type C57Bl/6 mice show no change in myeloid progenitor cell numbers during ageing. Surprisingly, CD200$^{-/-}$ mice do show a decrease in myeloid progenitor numbers during ageing (Figure 2B). Studying bone marrow of mice 14 months of age did not yield differences between wild type and CD200$^{-/-}$ mice, confirming that CD200$^{-/-}$ mice do not develop leukaemia (Figure 2B).

*Not all young CD200$^{-/-}$ mice have more myeloid cells*

Performing these and other experiments, we noticed that several young (8 to 12 weeks) CD200$^{-/-}$ mice did not exhibit increased numbers of neutrophils compared to wild type. This was surprising, since previously it was clearly shown that 6 to 12-week-old CD200$^{-/-}$ mice have increased levels of myeloid cells, and that these cells display an activated phenotype (18, 19).

In our hands, only a small proportion (about 25%) of CD200$^{-/-}$ mice contained more

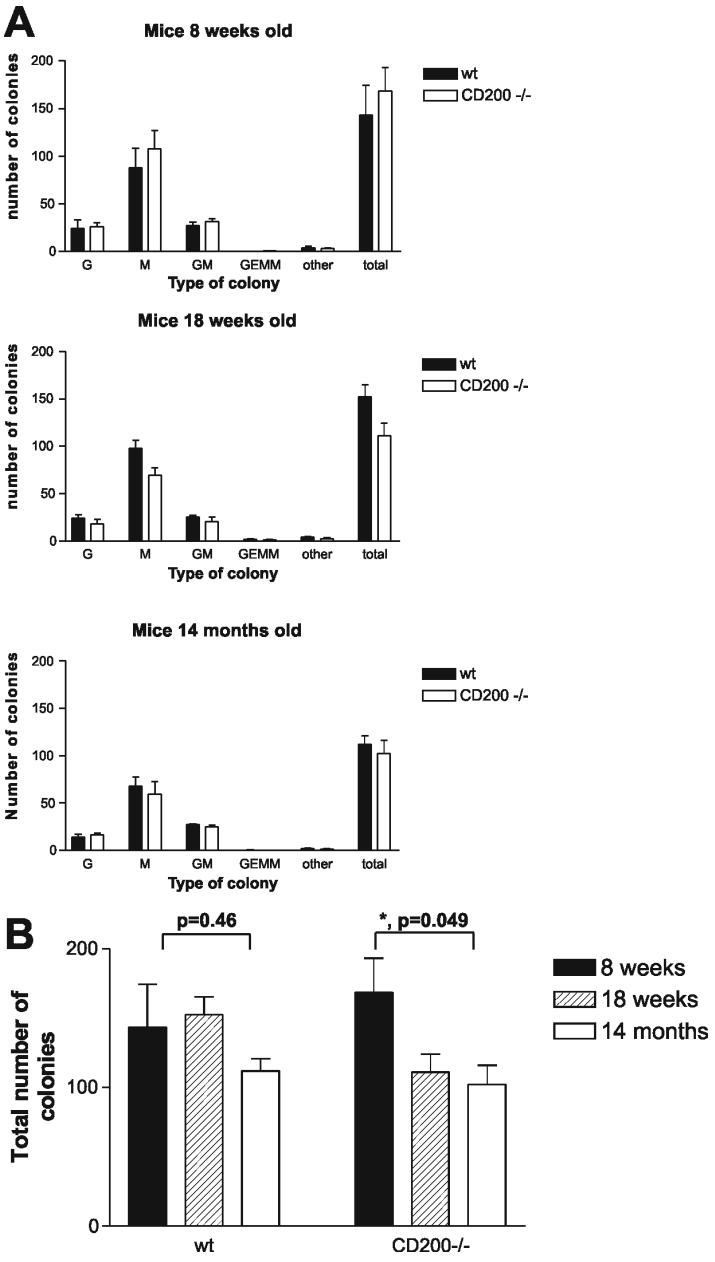


**Figure 2. CD200 expression does not influence the formation of myeloid progenitors**
Bone marrow cells from wild type and CD200$^{-/-}$ mice were cultured in methylcellulose medium containing SCF, Flt-3, G-CSF, GM-CSF, EPO, TPO, IL-5, and IL-3. After 12 days, colonies were counted per colony type. To validate visual colony typing, several colonies from each well were isolated from the medium, and cell morphology was microscopically examined after May-Grünwald-Giemsa staining of cytospins. Results are grouped per colony type observed. G: granulocyte colony, M: monocyte/ macrophage colony, GM: granulocyte/ monocyte colony, GEMM: granulocyte/erythrocyte/ monocyte/ megakaryocyte colony, other: not identifiable colony morphology, total: total number of colonies.
A. Black bars represent wild type mice, open bars represent CD200$^{-/-}$ mice. Error bars indicate SEM of 4 mice (2 independent experiments/ age group). The upper panel represents data from mice 8 weeks old, the middle panel represents data from mice 18 weeks old, and the lower panel shows data from mice 14 months old.
B. Only the total amount of colonies is plotted. Black bars indicate mice 8 weeks old, hatched bars indicate mice 18 weeks old, open bars indicate mice 14 months old. Error bars indicate SEM of 4 mice (2 independent experiments/ age group).

myeloid cells in their peripheral blood (Figure 3) and spleen (data not shown). To study whether this discrepancy could be explained by differences in exposure to environmental stimuli, this analysis included wild type and CD200$^{-/-}$ mice from both a specified pathogen free (SPF) unit and an open animal unit, as well as wild type and CD200$^{-/-}$ mice housed in a different animal facility (AMC, Amsterdam, The Netherlands). However, the variation in myeloid cell numbers in CD200$^{-/-}$ mice could not be traced to a litter, breeding pair or animal facility, and was unrelated to the age of the mice.

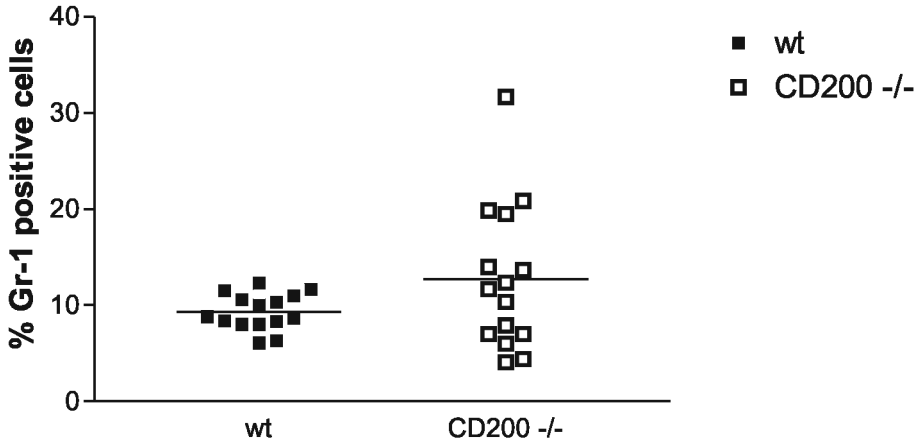


**Figure 3. Only 25% of young CD200$^{-/-}$ mice have increased numbers of myeloid cells**
Blood was drawn from wild type (n=15, closed symbols) and CD200$^{-/-}$ (n=15, open symbols) mice from different animal facilities by puncture of the vena saphena. Blood samples were stained with Gr-1 and CD11b, lysed with lysis buffer and analysed by flow cytometry. The line indicates the average value.

*Myeloid cells of CD200$^{-/-}$ mice respond normally to mobilising and inflammatory stimuli*

The observed variation of myeloid cell numbers of CD200$^{-/-}$ mice is caused by unknown circumstances. It is possible that myeloid cells of CD200$^{-/-}$ mice are hyperresponsive to a variable internal stimulus. To determine whether immature neutrophils have enhanced mobilisation kinetics when CD200 is lacking, we measured neutrophil numbers in blood of wild type and CD200$^{-/-}$ mice after injection with peg-G-CSF. There were no differences in neutrophil mobilisation of CD200$^{-/-}$ mice compared to wild type mice (Figure 4A). This indicates that immature neutrophils of CD200$^{-/-}$ mice are released with similar kinetics and in similar numbers as neutrophils of wild type mice.

To examine whether recruitment of mature cells to a site of inflammation is altered in CD200$^{-/-}$ mice compared to wild type controls, we injected 4% thioglycolate into the peritoneal cavity of wt and CD200$^{-/-}$ mice. At both 4 hours and 2 days after injection, peritoneal lavages were performed to study neutrophil and macrophage influx, respectively. Cell numbers were determined and cellular composition of the infiltrates was measured by flow cytometry. There were no differences in total cell numbers harvested from the peritoneal cavities of wt and CD200$^{-/-}$ mice. In addition, no differences were detected in absolute neutrophil or macrophage numbers (Figure 4B). Thus, we conclude that myeloid cells from CD200$^{-/-}$ mice do not migrate differently towards this inflammatory stimulus.

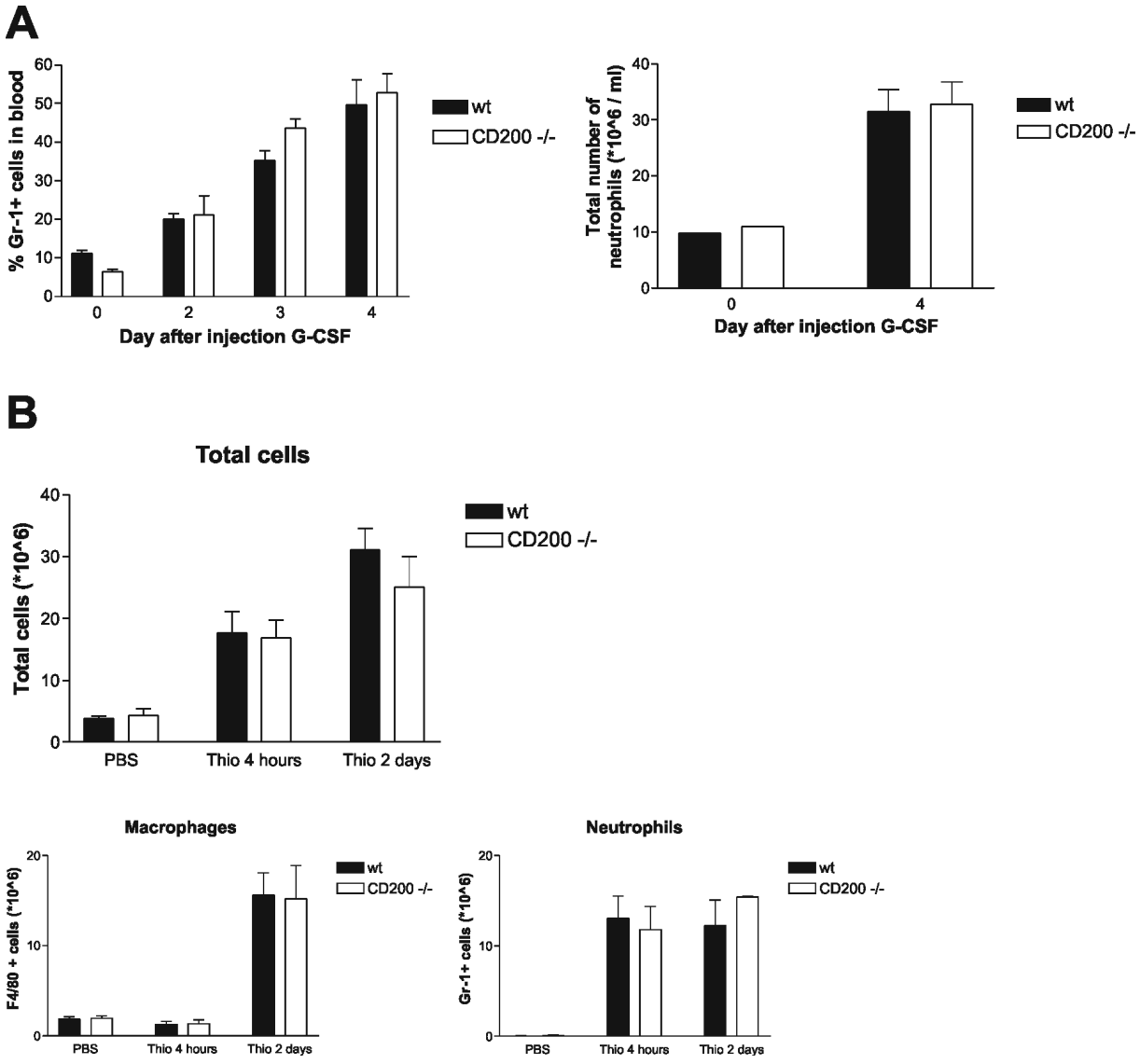


**Figure 4. Myeloid cells of CD200$^{-/-}$ mice respond normally to mobilising and inflammatory stimuli**

A. Wild type and CD200$^{-/-}$ mice were injected s.c. with 20 µg pegylated G-CSF in 150 µl PBS or with PBS only. On different days after G-CSF injection, mice were bled by puncture of the vena saphena. Total leukocyte numbers were determined and the percentage of neutrophils in peripheral blood was assessed by flow cytometry. The left panel indicates the percentage of Gr-1 positive cells in blood; the right panel indicates absolute numbers of Gr-1 positive cells per ml of blood. Black bars represent wild type mice, open bars represent CD200$^{-/-}$ mice. Error bars indicate SEM of 3 mice.

B. Wild type and CD200$^{-/-}$ mice were injected i.p. with 1 ml of 4% aged thioglycolate or with 1 ml PBS. 4 Hours or 2 days after injection, mice were sacrificed and recruited cells harvested by peritoneal lavage. Peritoneal cells were counted and assayed by flow cytometry. The upper panel indicates total amount of cells harvested from the peritoneum; the lower left panel indicates the total number of F4/80 positive cells; the lower right panel indicates the total amount of neutrophils. Black bars represent wild type mice, open bars represent CD200$^{-/-}$ mice. Error bars indicate SEM of 4 mice from 2 independent experiments.

**Discussion**

Since Dok-1 and Dok-2 are known to be suppressors of myeloid leukaemia (7, 8) and both are mediators of CD200R signalling (11, 12), we investigated whether disturbed CD200R signalling could be responsible for this phenotype and whether $CD200^{-/-}$ mice would exhibit enhanced myelopoiesis.

We report that $CD200^{-/-}$ mice have similar numbers of myeloid progenitor cells as wild type mice. In addition, we clearly show that $CD200^{-/-}$ mice do not develop a leukaemia during ageing. Therefore, the development of leukaemia in Dok-1/Dok-2 DKO mice cannot solely be explained by the involvement of Dok-1 and Dok-2 in CD200R signalling. Dok-1 and Dok-2 are adaptor molecules downstream of many other inhibitory receptors; hence it is likely that Dok-1 and Dok-2 are recruited to other receptors during tumour suppression. It should be noted that, although the only currently known ligand for CD200R is CD200, we cannot exclude that another ligand binds CD200R in $CD200^{-/-}$ mice, thereby leading to downstream signalling and, possibly, tumour suppression.

$CD200^{-/-}$ mice exhibit a decrease in myeloid progenitor numbers during ageing. This might indicate some exhaustion of the myeloid progenitor pool, possibly due to an enhanced activation of myeloid cells in $CD200^{-/-}$ mice (18). It would be interesting to investigate the renewal capacity of these cells with serial transplantations of hematopoietic stem cells. However, for these experiments, hematopoietic stem and progenitor cells lacking CD200R would be needed, which are unavailable to us.

While these experiments were being performed, we observed that not all young $CD200^{-/-}$ mice exhibited higher myeloid cell numbers. These findings could not be traced to a single breeding pair and despite identical conditions at the breeding (SPF) facility, subsequent litters from the same breeding pairs differed in neutrophil numbers (data not shown), varying between normal levels to the higher levels described previously (18). These data indicate that $CD200^{-/-}$ mice may be more sensitive than wild type mice to environmental conditions, to which they respond by producing higher numbers of myeloid cells and maintaining a higher activation status of these cells. This is likely a delicate balance and only a slight stimulation might be enough to induce myeloid proliferation. Although we show that this stimulus is not an enhanced response to G-CSF or an inflammatory stimulus, we have not been able to identify the exact nature of the stimulus.

The myeloid progenitor experiments were performed with 8 week old $CD200^{-/-}$ mice that, in hindsight, consisted of a mixed group of mice that either did or did not have an increase in peripheral myeloid cell numbers. Even when only considering the $CD200^{-/-}$ mice that did have enhanced myeloid cell numbers, we found no differences in myeloid progenitor number, subtype or proliferative capacity compared to wild type. This confirms that the increase in myeloid cell numbers observed in several $CD200^{-/-}$ mice indeed is not caused by an enlarged

population of myeloid progenitors.

Taken together, we have shown that CD200$^{-/-}$ mice do not develop leukaemia and do not exhibit altered myelopoiesis or mobilisation of myeloid cells. Thus, absence of CD200R signalling does not lead to a similar phenotype as absence of both Dok-1 and Dok-2. This indicates that development of leukaemia in Dok-1/Dok-2 DKO mice is not solely dependent on a block in CD200R signalling and that the signal that recruits Dok-1/ Dok-2 in normal myelopoiesis remains to be identified.

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# The inhibitory CD200R is differentially expressed on human and mouse T and B lymphocytes

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**Abstract**

To ensure an adequate response against pathogens and prevent unwanted self-reactivity, immune cells need to functionally express both activating and inhibitory receptors. CD200R is an inhibitory receptor mainly expressed on myeloid cells that downmodulates cellular activation both in vivo and in vitro. Although previously mainly studied as a regulator of myeloid function, we now show that CD200R is differentially expressed on human and mouse T cell subsets. In both species, $CD4^+$ T cells express higher amounts of CD200R than $CD8^+$ T cells, and memory cells express higher amounts of CD200R than naïve or effector cells. CD200R expression is upregulated on both $CD4^+$ and $CD8^+$ T cells after stimulation in vitro. Furthermore, we show CD200R expression on human and mouse B cells. In human tonsils, CD200R is differentially expressed on B cells, with high expression on memory cells and plasmablasts. Mice lacking the ligand for CD200R, $CD200^{-/-}$ mice, do not show abnormal composition of the lymphocyte compartment and have normal B cell responses to antigenic challenge. Although the functional implications remain to be elucidated, the expression of CD200R on lymphocytes suggests a much broader role for CD200R-mediated immune regulation than previously anticipated.

## Introduction

The equilibrium between inhibition and activation of the immune system is delicate. Too much inhibition may result in immune deficiency, whereas too much activation could cause damage to the host. Therefore, every immune cell expresses inhibitory receptors to balance signals from activating receptors (Ravetch and Lanier, 2000).

Most immune cells express multiple inhibitory receptors. The function of these inhibitory receptors is not redundant, as has been shown by studies using mice deficient for several B cell inhibitory receptors (Pritchard and Smith, 2003). Despite the fact that expression levels may be low, inhibitory immune receptors play an important role in maintaining immune homeostasis since absence of inhibitory immune receptors results in increased activation of immune cells, in some cases leading to spontaneous auto-immunity (Bolland and Ravetch, 2000; Takai et al., 1996).

CD200R is an inhibitory immune receptor that binds to CD200. Both molecules are members of the Ig superfamily and contain two Ig-like domains. CD200 is widely expressed and has a short intracellular tail which does not contain signalling motifs, whereas CD200R has a longer intracellular tail that contains three tyrosines, one of which is located in an NPXY motif (Wright et al., 2000). However, CD200R does not contain the common immunoreceptor tyrosine-based inhibition motifs (ITIMs) and has been described to regulate cellular activation via recruitment of the adaptor molecules Dok-1 and Dok-2 to the tyrosines in its intracellular tail (Zhang et al., 2004; Zhang and Phillips, 2005).

CD200R has been reported to be mainly expressed on myeloid cells such as monocytes, macrophages, granulocytes and dendritic cells and is an important signalling molecule in the inhibition of myeloid responses to challenge (Wright et al., 2000; Wright et al., 2003). In vitro studies have shown that ligation of CD200R results in decreased degranulation and cytokine secretion by mast cells, monocytes and macrophages (Jenmalm et al., 2006; Cherwinski et al., 2005). In addition, deletion of the CD200 gene in mice results in enhanced susceptibility to auto-immune disease and increased myeloid response to inflammation (Hoek et al., 2000). Lack of CD200R signalling in $CD200^{-/-}$ mice was found to result in enhanced Th2 switching upon induction of tolerance, implicating a role for CD200R signalling in regulating Th2 cell function (Taylor et al., 2005). Moreover, expression of CD200R on lymphocyte subsets was shown, suggesting a broader role for CD200R in the regulation of immune cells (Wright et al., 2003; Rosenblum et al., 2005). Indeed, CD3-induced proliferation and cytokine secretion by dendritic epidermal T cells can be inhibited by crosslinking of CD200R on the surface of these cells (Rosenblum et al., 2005).

Here, we study the expression of the inhibitory CD200R on different lymphocyte subsets in human and mouse peripheral blood and lymphoid organs to elucidate the potential role of CD200R expression on these cells. Most surprisingly, we report clear CD200R expression on

B cells in both species. Although the functional role of CD200R expression on lymphocyte subsets remains to be elucidated, its regulated expression pattern on lymphocytes suggests a broad role of CD200R in the regulation of immune function.

**Materials and methods**

*Mice*

Wild type C57BL/6J and CD200$^{-/-}$ mice were bred at the Amsterdam Medical Centre animal facility. For some experiments, wild type C57BL/6J mice were obtained from Charles River (France). All animals were used as approved by the Utrecht and Amsterdam University animal ethics committees.

For antigen challenge, mice received a single dose of either 10 μg TNP-Ficoll (T cell independent (TI) antigen) or 25 μg TNP-KLH (T cell dependent (TD) antigen) in 100 μl PBS by intravenous (i.v.) injection in the lateral tail vein. Serum was collected weekly, until 4 weeks after immunization, from blood obtained via the tail vein. Anti-TNP antibody titres were assessed by an isotype specific ELISA.

*Cells*

Mouse spleens and inguinal, axillary and brachial lymph nodes were strained through nylon sieves to obtain single cell suspensions, after which erythrocytes were lysed. Human peripheral blood leukocytes (PBL) were isolated from peripheral blood from healthy volunteers by density gradient centrifugation on Ficoll-Paque combined with Histopaque. Human peripheral blood mononuclear cells (PBMC) were isolated by centrifugation on Ficoll-Paque. Human tonsils were rest material from adenotomies and/ or tonsillectomies. Tonsils were minced and leukocytes were washed out to obtain single cell suspensions. All cells were resuspended in RPMI 1640 medium (Gibco BRL, Life Technologies, Paisley, United Kingdom) supplemented with 10% heat-inactivated foetal calf serum (Integro, Dieren, The Netherlands), penicillin and streptomycin (Gibco).

*T cell stimulation in vitro*

A 24-well plate was coated with 1 μg/ml anti-CD3 (OKT3, Janssen Cilag, Tilburg, The Netherlands) and/or 5 μg/ml anti-CD28 (15E8, kindly provided by Dr. R. van Lier) in a final volume of 200 μL PBS for 2 h at 37°C. $1.5 \times 10^6$ PBMC/ml were cultured for 4 days in the coated wells.

*Flow cytometry*

Antibodies against human CD3, CD4, CD8, CD25, CD27, CD45RO, CD19, CD38, IgD, CD11b, CD56 and mouse CD3, CD4, CD8, CD44, CD62L, CD25, CD69, DX5, NK1.1, B220, Gr-1, CD11b were obtained from Beckton Dickinson Biosciences (San Diego, United States). Antibodies against human IgG and IgM were obtained from DAKO (Glostrup, Denmark) and anti human IgA antibodies were obtained from Jackson Bioscience (West Grove, United States). Antibodies against human and mouse CD200 and CD200R were obtained from Serotec (Kidlington, Oxford, UK), as was anti mouse F4/80. Cells were incubated with antibodies on ice for 30 minutes. A FACSCalibur with BD CellQuest software (BD Pharmingen) was used for acquisition and analysis. For quantification of CD200R expression, MFI of isotype control stainings, although low in all cases, was subtracted from MFI values of CD200R stainings.

*Mouse antibody ELISA*

Flat-bottom 96-well MAXIsorp plates (Nunc, Roskilde, Denmark) were coated with 50 µl of 1 µg/ml isotype-specific anti-immunoglobulin antibodies in PBS and incubated overnight at 4ºC. Plates were washed with PBS and blocked with 3% BSA in PBS for 1 hour at room temperature. Sera were added in 3-fold dilutions starting at 1:150 in a total volume of 50 µl. After incubation for 2 hours at room temperature, plates were washed and 50 µl biotinylated isotype-specific antibodies (0.1 µg/ml in PBS) were added. Plates were incubated for 1 hour at room temperature and antibodies were detected by the addition of poly-streptavidin-HRP. Plates were developed with ABTS according to the manufacturer's protocol.

Anti-TNP antibody titres were assessed by an isotype-specific ELISA. MAXIsorp plates were coated with 50 µl of 1 µg/ml TNP-BSA in 0.1M carbonate buffer (pH 9.7) overnight at 4°C. Plates were blocked by incubating with 2% non-fat dry milk (NFDM) in PBS for one hour, and washed with PBS-T. Sera were applied in a 3-fold dilution series starting at 1:100 and ending at 1:24,300 and plates were incubated overnight at 4°C. After washing plates were incubated with 1µg/ml biotinylated Ig isotype-specific antibody in 2% NFDM, washed again and incubated with streptavidin-AP conjugate. Following a final wash, the plates were developed in 100 µl pNPP buffer (Sigma, St. Louis, United States) according to the manufacturer's protocol.

*Statistical analyses*

Nonparametric statistical tests were used since the data were not normally distributed. Differences between the groups were analysed using Mann-Whitney tests. A p-value smaller than 0.05 was considered statistically significant. All statistical analyses were performed using

SPSS 12.0 (SPSS Inc, Chicago, United States).

**Results**

*CD200R is expressed on human and mouse lymphocytes*

To study the distribution of CD200R, we stained human PBL with an αCD200R antibody (Figure 1A). As expected, CD200R was highly expressed on $CD11b^+$ myeloid cells. In agreement with previous results, CD200R was also expressed on $CD3^+$ T cells (Wright et al., 2003), and reproducibly at low levels on $CD3^-CD56^+$ NK cells. Unexpectedly, we also found clear expression of CD200R on peripheral $CD3^-CD19^+$ B cells. To our knowledge, this is the first time that a clear expression of CD200R on B cells is reported.

To analyse the CD200R expression on mouse leukocytes, C57BL/6J spleen cells were harvested and flow cytometric analysis showed that highest CD200R expression was found on $CD11b^+$ myeloid cells (Figure 1B). Mouse $CD3^+$ T cells expressed low but detectable levels of CD200R. Interestingly, CD200R was expressed in significant levels on mouse $CD3^-DX5^+NK1.1^+$ NK cells. Mouse splenic $B220^+$ B cells, like human peripheral blood B cells, expressed high levels of CD200R. Furthermore, on mouse blood and lymph node cells we found similar expression patterns (data not shown).

*CD200R is differentially expressed on T-cell subsets*

To predict the potential capacity of CD200R to regulate different T cell subsets, we studied CD200R expression on these populations. In human samples, subsets were determined by staining with CD27 and CD45RO as follows: $CD27^+CD45RO^-$: naïve (N) T cells, $CD27^+CD45RO^+$: central memory (CM) T cells, $CD27^-CD45RO^+$: effector memory (EM) T cells and $CD27^-CD45RO^-$: effector (E) T cells in both $CD4^+$ and $CD8^+$ T cells (Hamann et al., 1997;Sallusto et al., 1999). In agreement with previous data (Wright et al., 2003), we found higher CD200R expression on $CD4^+$ T cells as compared to $CD8^+$ T cells (Figure 2A), and CD200R was differentially expressed on T-cell subsets (Figure 2B). Although differences were small, CD200R expression was reproducibly higher on $CD4^+$ effector memory cells as compared to naïve and central memory $CD4^+$ T cells, and CD200R expression on $CD8^+$ memory subsets was higher than on $CD8^+$ naïve and effector cells. The effector $CD4^+$ T-cell population in healthy subjects is too small to be reliably phenotyped. In mice, as in humans, CD200R was expressed higher on $CD4^+$ splenic T lymphocytes compared to $CD8^+$ cells and $CD44^+CD62L^+$ memory $CD4^+$ T cells reproducibly expressed the highest levels of CD200R (data not shown).

The regulated expression of CD200R on T cell subsets is supported by the observation

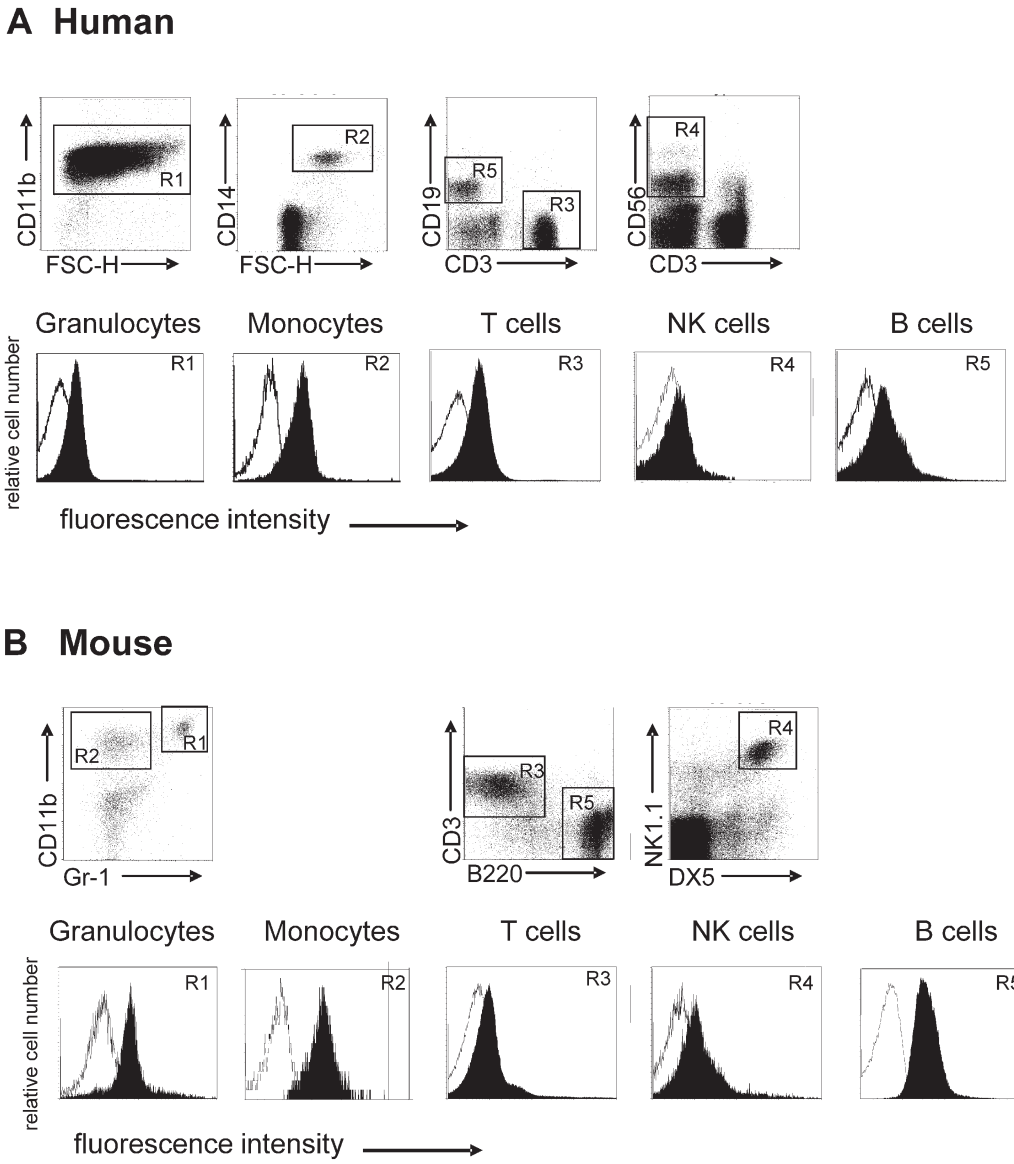


**Figure 1. CD200R is expressed on human and mouse lymphocytes**
A. Human peripheral blood leukocytes were stained using anti- CD11b, CD14, CD3, CD56 and CD19. Top panels show gating used to determine CD200R expression per cell type, as shown in the lower histogram panels. Granulocytes were identified based on forward/sideward scatter and $CD11b^+$staining, monocytes were identified based on forward/sideward scatter and $CD14^+$ staining, $CD3^+$ cells were identified as T cells, NK cells were $CD3^-CD56^+$ and B cells were $CD3^-CD19^+$. Open histograms represent isotype control staining, filled histograms represent CD200R staining. Data shown are representative for 6 different donors analysed in 3 independent experiments.
B. Spleen cells from wt C57BL/6J mice were isolated by forcing them through nylon cell strainers and were stained using anti Gr-1, CD11b, CD3, DX5, NK1.1, and B220. Top panels show gating used to determine CD200R expression per cell type, as shown in the lower histogram panels. Granulocytes were identified based on forward/ sideward scatter and $CD11b^+Gr\text{-}1^+$ staining, monocytes were $CD11b^+Gr\text{-}1^-$, T cells were $CD3^+$, NK cells were $CD3^-NK1.1^+DX5^+$ and B cells were $CD3^-B220^+$. Open histograms represent isotype control staining, filled histograms represent CD200R staining. Data shown are representative of 3 independent experiments.

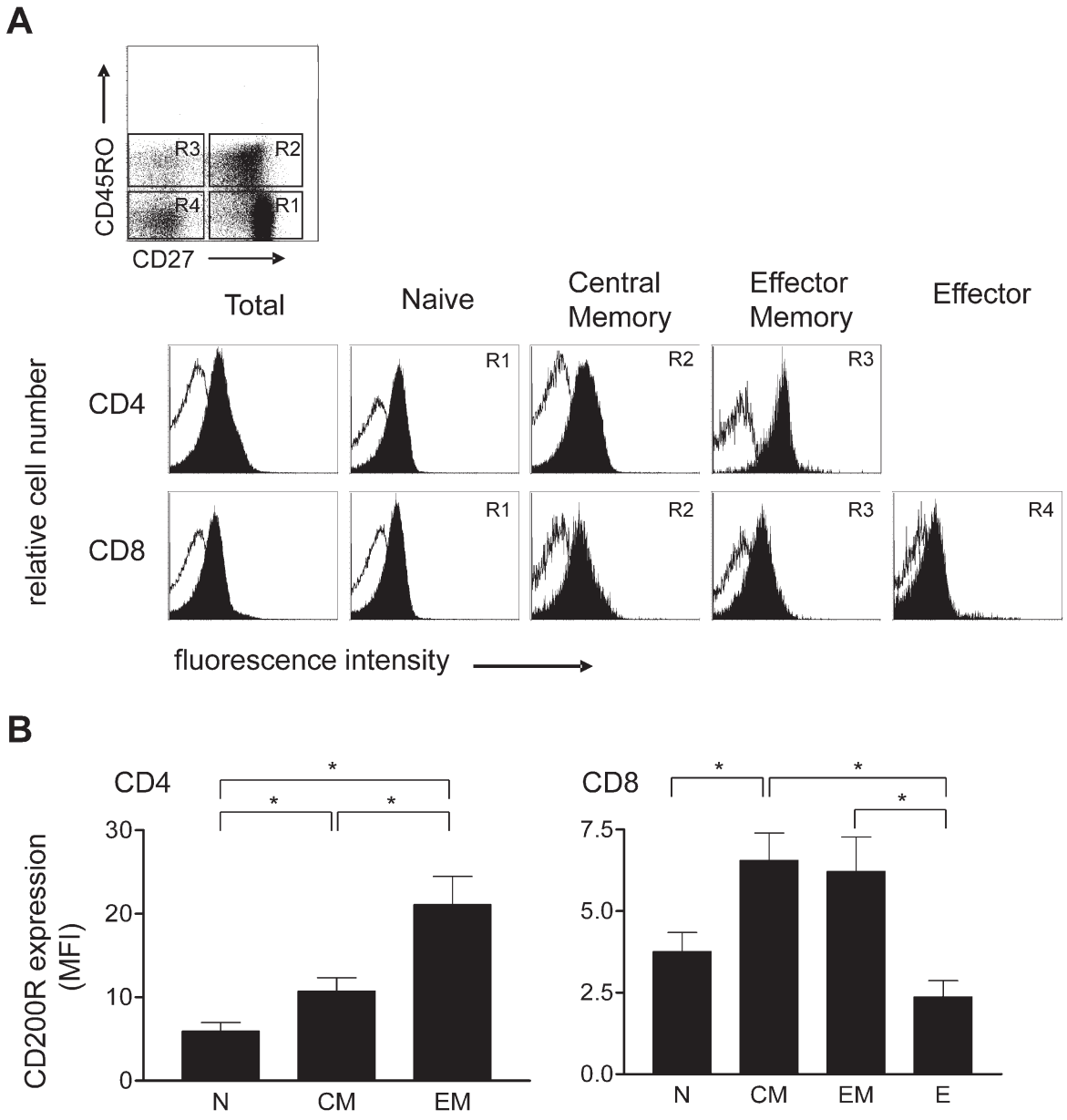


**Figure 2. CD200R is differentially expressed on T cell subsets**
A. PBMC from healthy volunteers were isolated using Ficoll. Subsets were determined by staining with CD27 and CD45RO as follows: $CD27^{+}CD45RO^{-}$: naïve (N) T cells, $CD27^{+}$ $CD45RO^{+}$: central memory (CM) T cells, $CD27^{-}CD45RO^{+}$: effector memory (EM) T cells and $CD27^{-}CD45RO^{-}$: effector (E) T cells, as is also indicated for $CD3^{+}$ $CD8^{+}$ cells in the top panel.
Histograms shown are representative of 11 donors analysed in 5 independent experiments, with open histograms representing isotype control staining and filled histograms representing CD200R staining.
B. Averages of the geometric mean fluorescence intensity of CD200R staining of 11 donors analyzed in 5 independent experiments with standard error of the mean (SEM). Left panel: CD200R on $CD4^{+}$ T-cell subsets subsets; right panel: CD200R expression on $CD8^{+}$ T-cell subsets subsets. *: significant, $p<0.05$

that both on $CD4^+$ and $CD8^+$ T cells stimulated in vitro by culture of human PBMC on plate-bound αCD3 or αCD3 together with αCD28, CD200R was upregulated after 4 days (Figure 3). In addition, stimulation of mouse splenocytes by αCD3 and αCD28 induced upregulation of CD200R on both $CD4^+$ and $CD8^+$ T cells (data not shown).

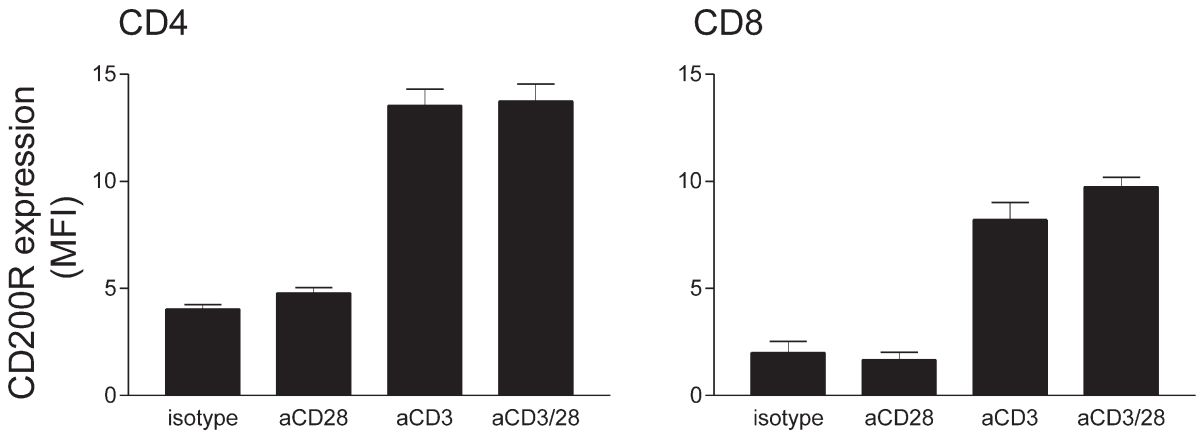


**Figure 3. CD200R expression is upregulated on T cells stimulated in vitro**
PBMC were stimulated with plate-bound αCD3 and/or αCD28 for 4 days and analysed with αCD3, αCD4 and αCD8 antibodies. Averages of the geometric mean fluorescence intensity of CD200R staining analyzed in 3 independent experiments are shown with standard error of the mean (SEM).

*CD200R is differentially expressed on peripheral and tonsillar B-cell subsets*

Once we had found expression of CD200R on peripheral B cells, we sought to determine whether CD200R is differentially expressed on B-cell subsets in peripheral blood. We co-stained $CD19^+$ cells with CD27 and CD38, to discriminate between transitional $CD19^+$ $CD27^-CD38^{++}$ B cells (immature B cells migrating from bone marrow to spleen), naïve ($CD19^+CD27^-CD38^-$) and memory ($CD19^+CD27^+CD38^-$) B cells and plasma cells ($CD19^+CD27^+CD38^{++}$) (Bhan et al., 1981; Klein et al., 1998). All subpopulations showed detectable CD200R expression, with memory B cells expressing the highest levels of CD200R (data not shown).

To further investigate differential CD200R expression on B-cell subsets in a secondary lymphoid organ, human tonsils were used. We found low but detectable expression of CD200R on total tonsillar B cells. Interestingly, the expression of CD200R on tonsillar B-cell subsets appeared to be regulated during differentiation (Figure 4A). Naïve ($CD19^+IgD^+$, R1) B cells that

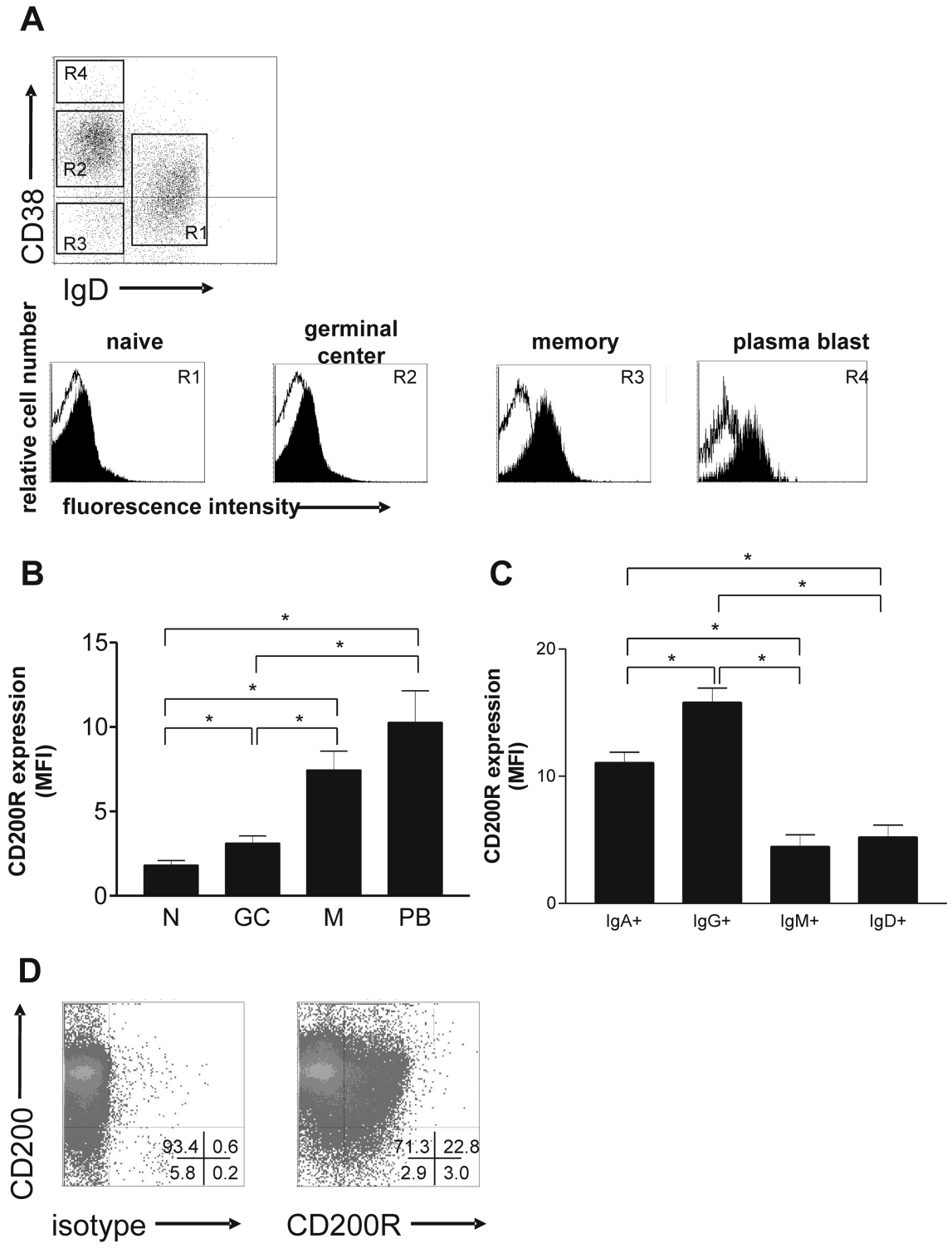
A
R4
R2
R3
R1
CD38
IgD
relative cell number
naive
germinal center
memory
plasma blast
R1
R2
R3
R4
fluorescence intensity
B
CD200R expression (MFI)
15
10
5
0
N
GC
M
PB
C
CD200R expression (MFI)
20
10
0
IgA+
IgG+
IgM+
IgD+
D
CD200
93.4
0.6
5.8
0.2
71.3
22.8
2.9
3.0
isotype
CD200R

**Figure 4. CD200R is differentially expressed on human tonsillar B cell subsets**
Human tonsils were rest material from adenotomies and/or tonsillectomies. Cells were isolated by forcing them through nylon cell strainers and then stained using the appropriate antibodies. B cell subsets were determined as follows: $CD19^+IgD^+$ naïve (N) tonsillar B cells, $CD19^+IgD^-CD38^+$ germinal center (GC) tonsillar B cells, $CD19^+IgD^-CD38^-$ memory (M) tonsillar B cells, $CD19^+$ $IgD^-CD38^{++}$ plasma blast (PB) cells.
A. Histograms shown are representative of 8 donors, with open histograms representing isotype control staining and closed histograms representing CD200R staining.
B. Shown are averages of the geometric mean fluorescence intensity of CD200R staining on tonsillar B cell subsets of 8 donors with SEM. *: significant, $p<0.05$
C. Tonsillar B cells were identified by staining with antibodies against CD19, IgA, IgG, IgM and IgD. Averages of the geometric mean fluorescence intensity of CD200R staining on tonsillar B cell subsets of 4 donors analysed in 2 independent experiments with SEM are shown. *: significant, $p<0.05$
D. Peripheral blood $CD19^+$ B cells were analysed for expression of CD200 and CD200R. Almost all B cells express CD200 and around 20% co-expresses CD200 and CD200R. Left panel indicates isotype control staining, right panel indicates CD200R staining and the percentage of cells in each quadrant is indicated. Data shown are representative for 6 donors analysed in 3 independent experiments.

have just entered the tonsils expressed low amounts of CD200R. B cells in the germinal centre reaction ($CD19^+IgD^-CD38^+$, R2) showed slightly increased levels of CD200R expression, whereas B cells that have completed the germinal centre reaction and have differentiated into memory B cells (R3, $CD19^+IgD^-CD38^-$) or plasma blasts (R4, $CD19^+IgD^-CD38^{++}$) showed higher CD200R expression (Figure 4B). Analysis of CD200R expression on B cells expressing different B-cell receptors (BCRs) revealed that CD200R expression was higher on $IgA^+$ and especially $IgG^+$ B cells compared to $IgM^+$ and $IgD^+$ B cells.

In agreement with previous reports, we find that almost all B cells express CD200. As shown in Figure 4D, around 20% of B cells co-express CD200 and CD200R.

*Absence of CD200 – CD200R interaction does not lead to changes in the lymphoid cell compartment in vivo*

We compared the lymphoid compartment of $CD200^{-/-}$ mice with that of wild type control mice to assess whether lack of CD200R triggering on B- and T-cell subsets had direct effects on the composition of these compartments in vivo. CD200R expression in $CD200^{-/-}$ animals was similar to that of wild type controls (Figure 5A). In both young (10 weeks old) and aged (14 months old) mice, no differences were observed in the composition of the splenic lymphoid compartment, both in relative (Figure 5B) and absolute T-cell, B-cell and NK-cell numbers (data not shown). Further analysis of T-cell subsets in spleen showed that $CD200^{-/-}$ mice tended to have less naïve T cells, although the difference was not significant (Figure 5C). This difference was not found studying spleens of mice 14 months old and lymph nodes of both young and old mice (data not shown).

To determine whether $CD200^{-/-}$ mice had a higher fraction of T cells with an activated phenotype in vivo, we also measured the percentage of activated T cells in spleen and inguinal,

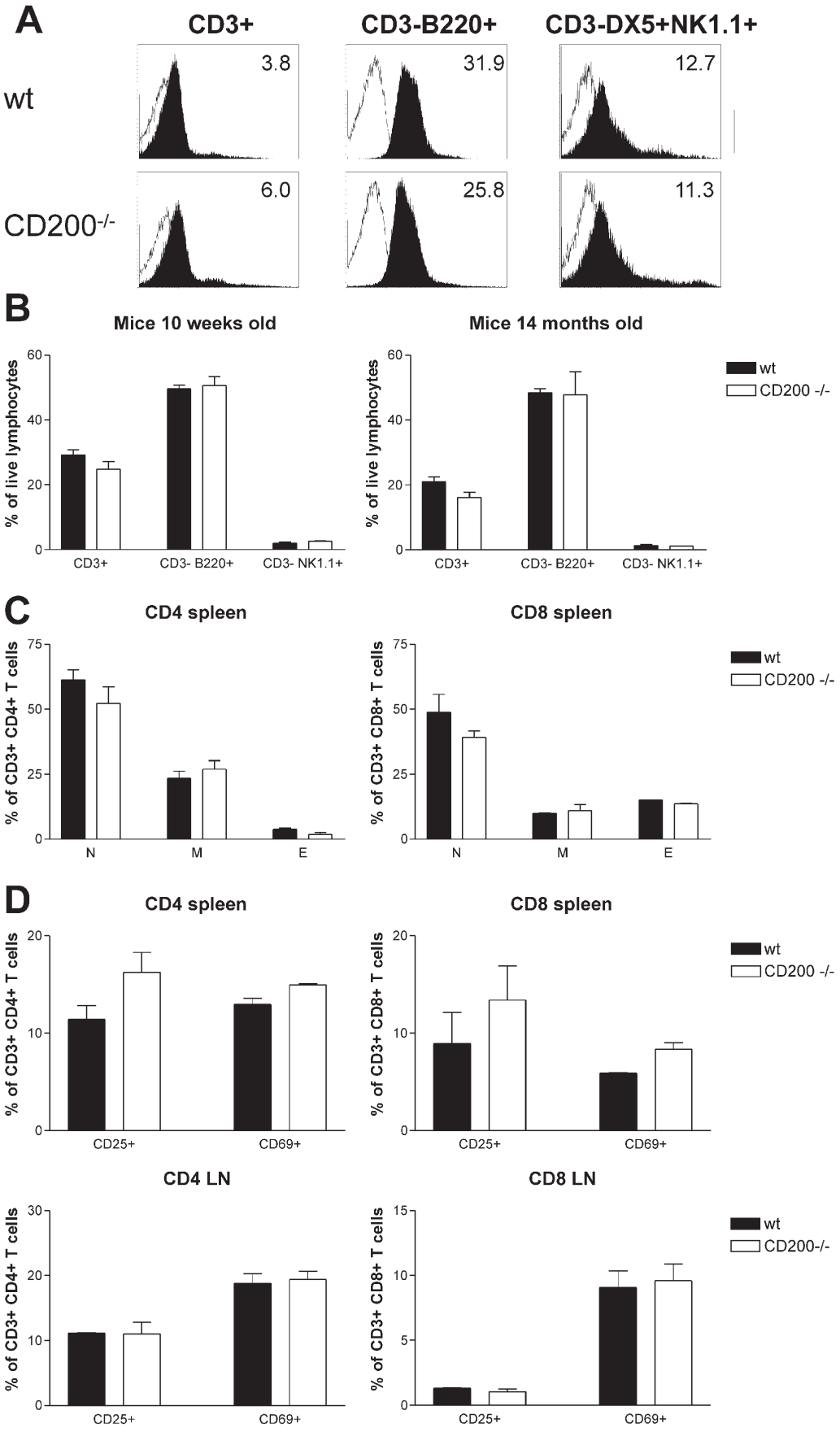
A
CD3+
CD3-B220+
CD3-DX5+NK1.1+
wt
CD200-/-
3.8
31.9
12.7
6.0
25.8
11.3
B
Mice 10 weeks old
Mice 14 months old
% of live lymphocytes
CD3+
CD3- B220+
CD3- NK1.1+
wt
CD200 -/-
C
CD4 spleen
CD8 spleen
% of CD3+ CD4+ T cells
% of CD3+ CD8+ T cells
N
M
E
D
CD4 spleen
CD8 spleen
CD4 LN
CD8 LN
CD25+
CD69+
CD200-/-

**Figure 5. CD200$^{-/-}$ mice have a normal lymphocyte compartment**
A. Mouse splenocytes (from wt and CD200$^{-/-}$ mice 10 weeks old) were stained using antibodies against CD3, B220, NK1.1, DX5 and CD200R. Open histograms represent isotype control staining, closed histograms indicate CD200R staining. Geo mean for CD200R staining is indicated.
B. Mouse splenocytes (from wt and CD200$^{-/-}$ mice 10 weeks and 14 months old) were stained using antibodies against CD3, B220, NK1.1, DX5, CD4 and CD8. T cells were CD3$^{+}$, NK cells were CD3$^{-}$ NK1.1$^{+}$DX5$^{+}$ and B cells were CD3-B220$^{+}$. Black bars represent wt mice, open bars represent CD200$^{-/-}$ mice. Averages of the geometric mean fluorescence intensity with SEM of 4 mice from 2 independent experiments are shown.
C. Mouse spleen cells (from wt and CD200$^{-/-}$ mice 10 weeks old) were stained for CD4, CD8, CD44 and CD62L. Naïve T cells were identified as CD44$^{low}$CD62L$^{+}$, memory T cells were CD44$^{+}$CD62L$^{+}$ and effector T cells were CD44$^{+}$CD62L$^{-}$. Black bars represent wt mice, open bars represent CD200$^{-/-}$ mice. Averages of the geometric mean fluorescence intensity with SEM of 4 mice from 2 independent experiments are shown.
D. Mouse spleen cells (from wt and CD200$^{-/-}$ mice 10 weeks old) were stained using CD4, CD8, CD25 and CD69. Black bars represent wt mice, open bars represent CD200$^{-/-}$ mice. Averages of the geometric mean fluorescence intensity with SEM of 4 mice from 2 independent experiments are shown.

axillary and brachial lymph nodes by CD25$^{+}$ and CD69$^{+}$ expression. Both young (Figure 5D) and aged (data not shown) CD200$^{-/-}$ mice tended to have higher numbers of activated splenic T cells, although these differences were not statistically significant. Numbers of activated T cells in lymph nodes were not different between wild type and CD200$^{-/-}$ mice (data not shown).

*Lack of CD200 – CD200R interaction has no effect on Ig production in vivo*

Next, the role of CD200R signalling on B cell function in vivo was investigated, by measurement of serum immunoglobulin levels of wild type and CD200$^{-/-}$ mice. In unchallenged mice, no differences in steady state serum immunoglobulin levels were observed (data not shown). To measure the antigen-specific immunoglobulin (Ig) production in CD200$^{-/-}$ mice, we challenged mice with T-cell dependent (TNP-KLH) and T-cell independent (TNP-Ficoll) antigens. Serum was obtained weekly up to 4 weeks after immunization. The production of specific antibodies by CD200$^{-/-}$ mice was comparable to that of wild type animals in response to both T-cell dependent (Figure 6) and T-cell independent antigens (data not shown). Thus, the role of CD200R expression on B lymphocytes remains elusive.

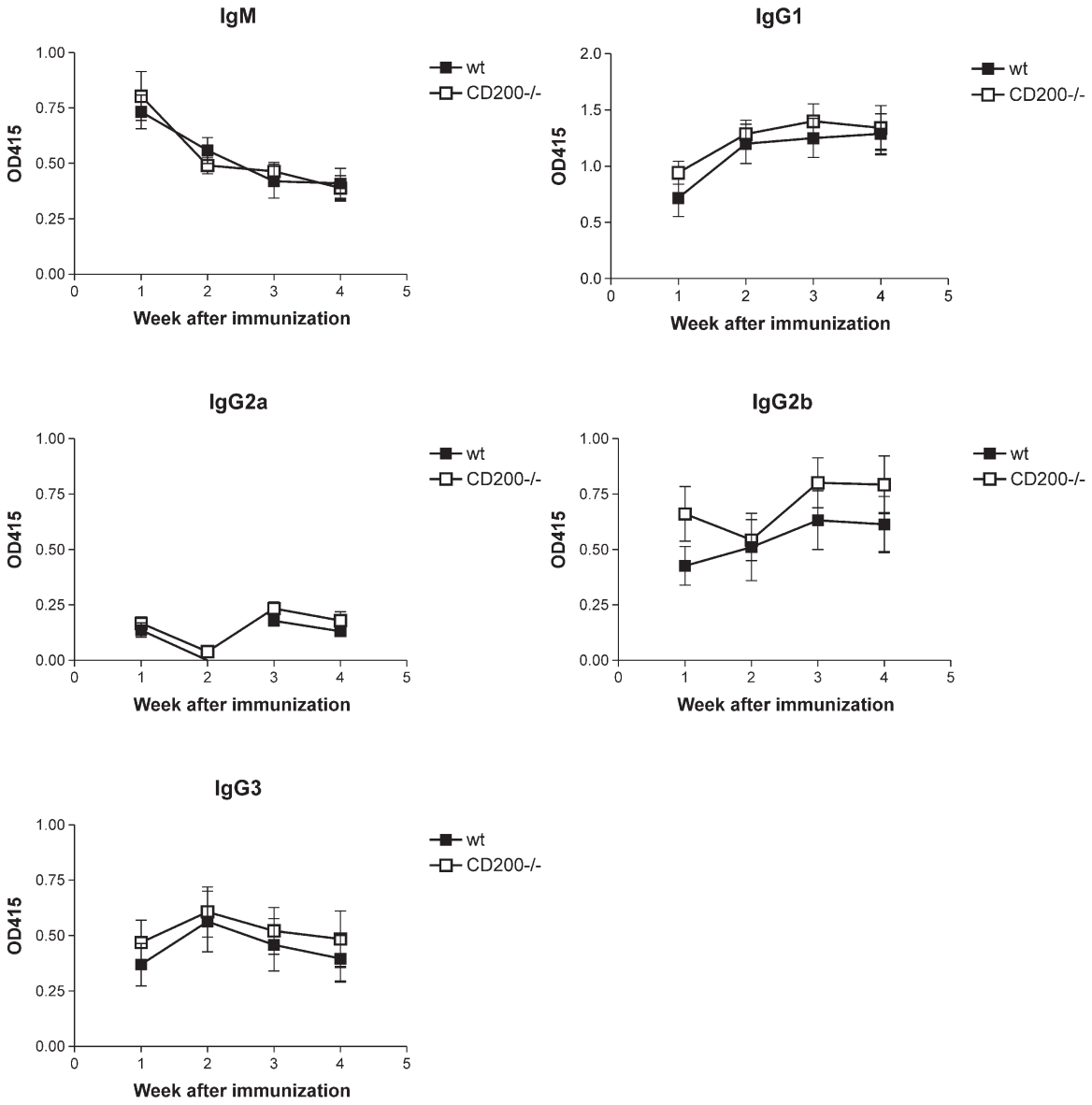


**Figure 6. CD200$^{-/-}$ mice have normal antibody responses**
Wild type and CD200$^{-/-}$ mice were injected with TNP-KLH. After immunisation, serum was collected weekly and anti-TNP antibody titer determined using isotype-specific ELISA. Depicted are the mean OD415 values of each isotype tested (indicated at the top of the panels) at each time point of 5 mice per group.

**Discussion**

Here, we show that expression of the inhibitory immune receptor CD200R is not restricted to cells of the myeloid lineage, but that CD200R is also expressed on human and mouse B and T lymphocytes and NK cells. In addition, CD200R expression is differentially regulated and it is clearly expressed on some T- and B-lymphocyte subsets. For example, CD200R expression is higher on $CD4^+$ T cells than on $CD8^+$ T cells both in mice and humans, indicating differential regulation. This could fit with the observation that $CD4^+$ T cells have a higher endogenous threshold of activation than $CD8^+$ T cells: higher concentrations of antigen, longer exposure times or more costimulatory signals are needed for activation of $CD4^+$ T cells (Kaech et al., 2002). This may be influenced by higher expression of CD200R on $CD4^+$ T cells compared to $CD8^+$ T cells, resulting in a stronger inhibitory signal in these cells and hence a higher threshold for activation.

Interestingly, different inhibitory receptors may have distinct effects on the immune system. The expression pattern of CD200R on peripheral T cell subsets differs from the expression pattern of other inhibitory receptors like LAIR-1 (Maasho et al., 2005; Jansen et al., 2007) and KLRG1 (Robbins et al., 2003). This finding highlights the non-redundant role different inhibitory immune receptors play in the regulation of immune cells and suggests that, depending on the context, cellular activation signals are modulated by specific inhibitory immune receptors. The exact mechanism of lymphoid cell regulation by different inhibitory receptors remains to be investigated.

We report clear CD200R expression on both human and mouse B cells. In addition, we show that CD200R expression is differentially regulated on human peripheral blood and tonsillar B cell subsets, suggesting a functional role for CD200R in B-cell regulation. This finding is surprising since almost all B cells express high levels of the ligand for CD200R, CD200. We now identify a subpopulation of B cells that expresses both CD200 and CD200R. This could have important implications for the regulation of the activity of these cells, especially in locations where B cells are in close proximity, for example tonsils. CD200R expression was highest on terminally differentiated B cells: memory B cells and plasma cells in peripheral blood, memory cells and plasmablasts in tonsils. This could indicate that CD200R is not involved in setting a threshold of activation for naïve B cells, but rather in preventing reactivation of memory or effector B cells. The fact that clear CD200R expression has not been reported on B cells earlier (Wright et al., 2003) might be due to variation of CD200R expression levels between different donors and mouse strains, and/or variation in the antibodies used.

The clear and differentially regulated expression of CD200R on some lymphocyte subsets, and the fact that this expression increases upon lymphocyte activation, together have the interesting implication that CD200R not only has a role as regulator of myeloid cells but is also involved in the regulation of the function of lymphocyte subsets. Earlier studies showed that

the auto-immune diseases experimental auto-immune encephalomyelitis (EAE) and collagen-induces arthritis (CIA) can be induced more easily in $CD200^{-/-}$ mice than in wild type mice (Hoek et al., 2000). Although this phenomenon has previously been attributed to myeloid cells, lymphocytes also are involved in the development of these auto-immune diseases (Corthay et al., 1999; Sospedra and Martin, 2005). The current finding that CD200R is clearly expressed on some lymphocyte subsets, and upregulated after stimulation, might indicate that lack of CD200R signalling in lymphocytes is also involved in development of autoimmunity in $CD200^{-/-}$ mice.

Previously, CD200R expression was reported on $CD4^+$ T cells and triggering of CD200R on PBMC resulted in monocyte-mediated inhibition of tetanus toxoid induced cytokine responses (Jenmalm et al., 2006). On dendritic epidermal T cells, CD200R crosslinking inhibited αCD3-induced proliferation and cytokine secretion (Rosenblum et al., 2005). Recent findings indicate that CD200 expression is upregulated on leukaemias (Moreaux et al., 2006; Tonks et al., 2007; McWhirter et al., 2006). In addition, blockade of the interaction between CD200 on leukaemic B cells and CD200R on PBMC was found to inhibit tumour growth in an in vivo model of tumour rejection (Kretz-Rommel et al., 2007). Although the cell type responsible for tumour rejection has not been identified, these data suggest an important role for CD200R signalling in regulation of mononuclear cell effector functions. Our experiments so far have not yet elucidated a prominent role for CD200R in the regulation of the lymphocyte subsets. $CD200^{-/-}$ mice do not produce different amounts of specific antibodies in response to immunization with T-cell dependent and T-cell independent antigens. However, we show that spleens of $CD200^{-/-}$ mice tend to contain less naïve and more activated $CD25^+$ and $CD69^+$ T cells, suggesting increased T-cell activation. The fact that observed differences in vivo are small may be explained by the previous observation that $CD200^{-/-}$ do not show a clear phenotype until severely challenged (Hoek et al., 2000). In addition, we cannot exclude that, in $CD200^{-/-}$ mice, CD200R might bind another ligand. To further investigate the functional role of CD200R expression on lymphocyte subsets studies with $CD200R^{-/-}$ mice are required, comparing the outcome of a range of stimuli that specifically activate lymphocytes in $CD200^{-/-}$ or $CD200R^{-/-}$ mice.

In summary, we have shown that CD200R is not only expressed on cells of the myeloid lineage, but is also expressed on human and mouse T-lymphocyte subsets. We report regulated expression of CD200R on B-lymphocyte subsets, revealing a B-cell subpopulation that expresses both CD200 and CD200R. Although CD200R signalling is not prominently involved in the development of the lymphoid compartment and B-cell responses to immunization, its regulated expression pattern could indicate a much broader role for CD200R signalling in the regulation of the immune balance than previously anticipated.

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# 6

# CD200 is a host factor in determining the course of influenza infection in mice

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**Abstract**

Both viral and host factors determine the outcome of influenza infections. Infections with highly pathogenic influenza strains are usually characterised by an over-active immune response, and loss of control of the antiviral immune response may lead to severe immune pathology. Inhibitory immune receptors may contribute to limiting and controlling such immune responses. CD200R is an inhibitory immune receptor expressed on myeloid cells and lymphocytes. We infected $CD200^{-/-}$ mice with influenza virus and found that these mice develop severe and often fatal disease, associated with prolonged presence of virus in the lungs. $CD200^{-/-}$ mice produced normal amounts of influenza-specific $CD8^{+}$ T cells, but tended to show reduced levels of influenza-specific antibodies. Depletion of T cells from $CD200^{-/-}$ mice resulted in complete absence of disease after influenza infection, indicating that pathogenic T cells possibly are the causative agents of the severe course of influenza infection in $CD200^{-/-}$ mice. This reveals the importance of CD200 as a host factor in the determination of the outcome of influenza infection in mice.

## Introduction

Influenza A viruses pose a major threat to global health. The most deadly influenza pandemic was the 1918 "Spanish flu", which caused an estimated 40 million deaths worldwide [1]. Influenza viruses undergo continuous antigenic change, enhancing the chance for new epidemics and pandemics [2]. Recently, the H5N1 bird influenza virus has spread globally and it is feared that this influenza strain will acquire easy transmissibility between humans resulting in the next influenza pandemic [3].

Determinants of the outcome of influenza virus infections include both viral and host factors. Viruses can use a variety of strategies to modulate and evade host immune responses induced after infection. Influenza A viruses use many mechanisms to evade both innate and adaptive immunity. For example, to evade adaptive immune responses, influenza viruses accumulate amino acid substitutions in antigenic sites and thereby create new antigenic determinants [2]. Furthermore, the NS1 protein of influenza A inactivates the host innate immune response by blocking IFNα/ß signalling, allowing viral replication in the host cell [4].

On the other hand, co-evolution of pathogens with their hosts has resulted in the shaping of the host immune system [5]. The immune response to influenza viruses has been mainly studied using mouse models. The adaptive immune response is initiated by dendritic cells (DCs) migrating from the respiratory tract to the lung-draining mediastinal lymph nodes and spleen during the early stages of infection [6], where they stimulate the development of influenza-specific cellular immunity. Clearance of influenza virus from the lungs is dependent on a functional $CD8^+$ T-cell response or a functional $CD4^+$ T-cell assisted B-cell response [7-9].

However, immune responses against influenza virus are not solely beneficial. Infection of T-cell deficient mice with influenza virus resulted in decreased lung pathology compared to wild type mice [9], indicating that T cells may also enhance pathology. Recently, development of severe disease after infection with H5N1 influenza virus was found to be due to immune-mediated lung pathology in both humans and mice [10-12]. In addition, recent studies with the reconstructed 1918 pandemic influenza virus strain showed severe immune-mediated pathology after infection of mice and non-human primates [13-15]. Therefore, adequate restriction of the immune response is critical in influenza virus infection to prevent pathology.

Host factors involved in the down regulation of immune responses may include inhibitory immune receptors. CD200R is an inhibitory immune receptor mainly described to be expressed on myeloid cells [16]. Recently, CD200R expression was also found on lymphocyte subsets [17-19], suggesting a broader role for CD200R in regulation of immune function than previously suggested. Its ligand, CD200, is widely expressed, for example on endothelial cells, neurons and lymphocytes [20]. Previously, $CD200^{-/-}$ mice were found to have enhanced responses of CD200R-expressing microglia, the resident macrophages of the brain, to neuronal damage and increased susceptibility to induction of auto-immune diseases [21]. Infection of $CD200^{-/-}$ mice

with the intracellular pathogen *Toxoplasma gondii* resulted in increased activation of microglia and decreased intracerebral parasite burden, corresponding with increased survival [22]. In addition, mice lacking a functional phagocyte NADPH oxidase, thus lacking a major source of reactive oxygen species (ROS), have higher numbers of activated macrophages in their lungs after influenza infection, coinciding with decreased CD200 expression in the lungs [23]. These data suggest that CD200R signalling regulates macrophage function during influenza infection and implicate a role for CD200R in the down modulation of cellular responses to inflammation.

In this study, we have characterised the role of CD200R in the regulation of lymphocyte function using the in vivo model of influenza infection. We infected $CD200^{-/-}$ mice with H3N2 influenza A viruses A/HK/2/68 and X-31, which cause relatively mild infections in wild type mice, and observed the development of severe disease in $CD200^{-/-}$ mice after influenza infection. These data point to a role for CD200 as an important host factor in the down modulation of the immune response after influenza infection.

## Materials and methods

*Mice and influenza infections*

Wild type C57BL/6J mice were obtained from Charles River (France). $CD200^{-/-}$ mice were bred at the Specified Pathogen Free (SPF) unit at the Utrecht University Central Animal Laboratory. All animal experiments were approved by the Utrecht University animal ethics committee.

Influenza virus strains A/HK/2/68 and X-31 were propagated in 11-day old embryonated chicken eggs. Mice were infected with influenza virus in 50 µl PBS intranasally (i.n.) under light ether anaesthesia. Mice were monitored daily at approximately the same time for symptoms of illness (weight loss, ruffled fur, hunched posture and general behaviour). Unless stated, mice were infected with A/HK/2/68.

*Depletion of cells*

Alveolar macrophages were specifically depleted by inhalation of 50 µl clodronate liposomes or PBS liposomes as a control on days -4 and -2 relative to influenza infection. $Cl_2MDP$ (dichloromethylene bisphosphonate or clodronate) was a gift of Roche Diagnostics GmbH, Mannheim, Germany. This method of depletion was tested first on mice that were not infected with influenza and alveolar macrophage numbers in BAL fluid were diminished to a sufficient extent for our purposes.

$CD4^+$ and $CD8^+$ T-cells were depleted by injection of 50 µg αCD4 antibody (clone GK 1.5) and 50 µg αCD8 antibody (clone YTS 169.4) on days -2 and +1 relative to influenza

infection. Antibodies GK 1.5 and YTS 169.4 were a kind gift of Peter van Kooten (Hybridoma Facility, Utrecht University).

*Dexamethasone treatment*

Mice were injected daily with 10 mg/kg dexamethasone (Intervet, Boxmeer, the Netherlands) intraperitoneally (i.p.). Control groups were injected with an equal volume of PBS daily.

*Flow cytometry*

Antibodies against mouse CD3, CD4, CD8, CD44 and CD62L were obtained from Beckton Dickinson Biosciences (San Diego, CA, United States). APC labeled tetramers of the mouse MHC class 1 H2-Db heavy chain, ß2 microglobulin, and the influenza virus nucleoprotein (NP)366-374 peptide ASNENMDAM were prepared as described [24] and used for immunofluorescence in combination with the indicated antibodies. A FACSCalibur with BD CellQuest software (BD Pharmingen) was used for acquisition and analysis.

Blood cells were analysed by staining the cells with antibodies, after which erythrocytes were lysed and the cells fixed with FACS lysing solution (Becton Dickinson, San Jose, CA). Spleen, lung-draining mediastinal lymph node and lung cells were analysed by straining the organs through nylon sieves, lysing the erythrocytes and staining with antibodies for flow cytometric analysis.

Bronchoalveolar lavage (BAL) fluid was obtained by flushing the lungs through a canula inserted into the trachea three times with 700 µl PBS, yielding around 1.5 ml BAL fluid. BAL fluids were kept on ice until further processing. Cells from BAL fluid were counted and cytospins were prepared and stained with Quik-Diff (Dade Behring, Switzerland). BAL fluid supernatant was stored at -80º until further analysis.

*Quantitative influenza PCR*

Lung viral load was determined by quantitative PCR essentially as described previously [25]. Briefly, lung homogenate was dissolved in TRIzol reagent. RNA was extracted in a MagnaPure LC robot (Roche applied science, Penzberg, Germany). 80 µl of the tissue homogenate and 10 µl of internal control (EMC) was isolated using the MagNa Pure LC Isolation Kit II (Tissue), exactly as described by the manufacturer. The nucleic acid was resuspended in a final volume of 200 µl. cDNA was synthezised by using the Taqman Reverse Transcription Reagents (Applied Biosystems). After incubation for 10 minutes at 25°C, RT was carried out for 30 minutes at 48°C, followed by RT inactivation for 5 minutes at 95°C. cDNA samples were assayed in a 25-µl reaction mixture containing 5 µl of cDNA, 12.5 µl of 2x Taqman Universal PCR Master Mix

(Applied Biosystems), 900 nM influenza virus A forward primer (AAG ACC AAT CCT GTC ACC TCT GA), 900 nM influenza virus A reverse primer (CAA AGC GTC TAC GCT GCA GTC C), and 200 nM influenza A probe (TTT GTG TTC ACG CTC ACC GT). Amplification and detection were performed in the ABI Prism 7700 sequence-detection system (Applied Biosystems), using the following conditions: 2 min at 50°C and 10 min at 95°C, followed by 45 cycles of 15 s at 95°C and 1 min at 60°C. The viral load present in a sample was calculated using a standard curve of influenza virus.

*Histopathology*

Organs were fixed in EAF (40% ethanol, 5% acetic acid, 10% formalin in PBS), and all organs were paraffin-embedded. Section of 5µm were prepared and stained with Hematoxylin-Eosin. All processing of organs and the HE-staining was performed at the Dept. of Pathology, UMCU. HE-stained lung slides were scored in a blinded manner by an experienced experimental-animal pathologist.

*Influenza-specific antibody ELISA*

$4*10^4$ TCID50 influenza virus in a total volume of 50µl in PBS was coated onto Maxisorp plates (Nunc, Roskilde, Denmark) and incubated overnight at 4ºC. Plates were washed with PBS and blocked with 3% BSA in PBS for 1 hour at room temperature. After washing with PBS, sera were added in 3-fold dilutions starting at 1:150 in a total volume of 50 µl. After incubation for 2 hours at room temperature, plates were washed and 50µl biotinylated isotype-specific antibodies at 0.1 µg/ ml were added. Plates were incubated for 1 hour at room temperature and influenza-specific antibodies were detected by the addition of poly-streptavidin-HRP. Plates were developed with ABTS according to the manufacturer's protocol.

*Cytokine multiplex analysis*

Cytokine levels in BAL fluid was measured by Bio-Plex Cytokine Assay (Biorad Laboratories, Veenendaal, The Netherlands) of IL-1ß, IL-6, IL-10, MIP1α, RANTES, KC, tumour necrosis factor (TNF)α, GM-CSF, IFN-γ, and MCP-1 according to the manufacturer's protocol.

*Cytokine ELISA*

IFN-γ ELISA antibody pair was from U-Cytech (Utrecht, the Netherlands), MCP-1 ELISA kit was from eBioscience (San Diego, United States). Cytokine levels in BAL fluid and sera of influenza-infected mice were determined according to the manufacturer's protocols. IL-6 levels in BAL fluid and sera of influenza-infected mice were determined using capture ELISA. Flat-

bottom 96-well MAXIsorp plates (Nunc) were coated with 100 µl of 1.5 µg/ml rat anti mouse IL-6 antibodies (BD Pharmingen) in 0.1M carbonate buffer (pH 9.5) and incubated overnight at 4º. Plates were washed with 0.02% Tween-20 in PBS (PBS-T) and blocked with 5% BSA in PBS for 1 hour at room temperature. Sera and cytokine standard (recombinant mouse IL-6, BD Pharmingen) were added in 3-fold dilutions starting at 1:150 in a total volume of 100 µl. After incubation for 2 hours at room temperature, plates were washed and biotinylated rat anti mouse IL-6 antibodies (BD Pharmingen, 2 µg/ml in PBS) and horseradish peroxidase-conjugated streptavidin (Roche, 200mU/ml) were added in a total volume of 100 µl. Plates were incubated for 30 minutes at room temperature, washed and developed with ABTS according to the manufacturer's protocol.

*Statistical analyses*

Nonparametric statistical tests were used since the data were not normally distributed. Differences between the groups were analysed using Mann-Whitney tests. A p-value smaller than 0.05 was considered statistically significant. All statistical analyses were performed using SPSS 12.0 (SPSS Inc, Chicago, IL, United States).

**Results**

*$CD200^{-/-}$ mice are critically ill after influenza infection*

To study the role of CD200R signalling during influenza infection, we infected wild type and $CD200^{-/-}$ mice with influenza virus A/HK/2/68 and monitored weight loss as a marker for disease severity. Both wild type and $CD200^{-/-}$ mice developed severe disease in response to a high dose (350 TCID50) of influenza virus (Figure 1A, upper panel). However, when lower infectious doses of influenza virus were used, clear differences were found. Wild type mice did not develop disease upon infection with 3.5 TCID50 A/HK/2/68. Strikingly, $CD200^{-/-}$ did develop severe disease, appeared dehydrated, and were sacrificed for ethical reasons when more than 20% of the original weight was lost (Figure 1A, middle panel). Similar to wild type mice, $CD200^{-/-}$ mice did not develop disease upon infection with a low dose (0.35 TCID50) of influenza virus (Figure 1A, lower panel), indicating that development of disease in $CD200^{-/-}$ mice is dependent on the initial infectious dose.

We investigated whether $CD200^{-/-}$ mice would also develop severe disease upon infection with an alternative influenza strain. For this purpose, we infected wild type and $CD200^{-/-}$ mice with X-31, a vaccine reassortant strain of A/Aichi/2/68 and A/PR/8/34. As was the case for HK/2/68, infection with a low dose of influenza virus X-31 did not result in weight loss of wild type or $CD200^{-/-}$ mice (Figure 1B, lower panel). Infection with 28.1 TCID50 X-31 virus

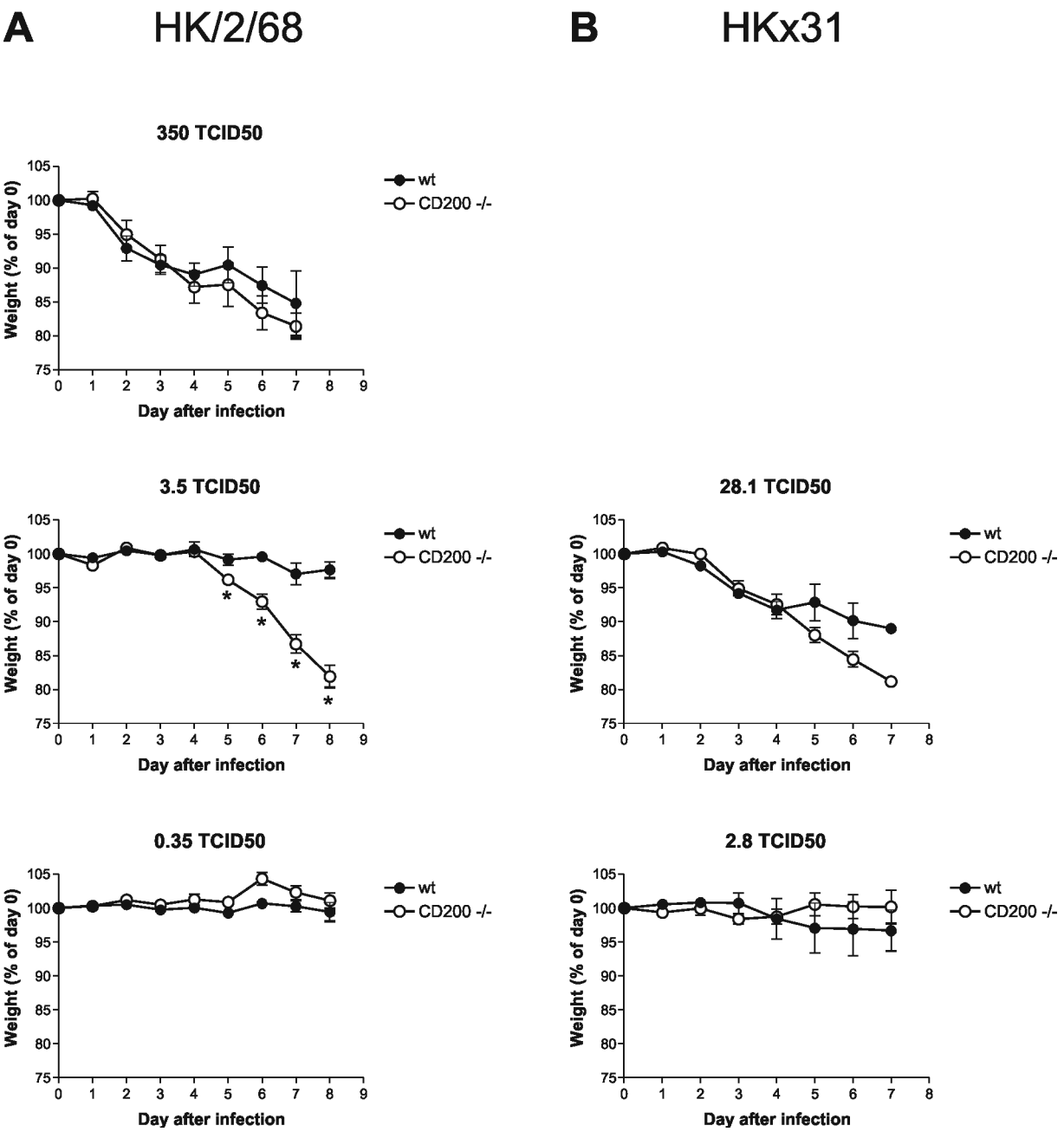


**Figure 1. CD200$^{-/-}$ mice develop severe disease after infection with influenza**
Wild type and CD200$^{-/-}$ mice were anesthetized using a humidified ether chamber and infected intranasally with 50 µl influenza virus strain A/HK/2/68 or X-31 in PBS. Weight is indicated as percentage of the original weight. Closed symbols indicate wild type mice, open symbols indicate CD200$^{-/-}$ mice. Error bars indicate standard error of the mean (SEM) of 6 mice per group. *: significant, $p<0.05$

resulted in a maximal weight loss of 10% in wild type mice. However, CD200$^{-/-}$ mice lost on average 20% of their original weight (Figure 1B, upper panel). Intriguingly, while most influenza-infected CD200$^{-/-}$ mice did not recover from the infection and were sacrificed for ethical reasons, some influenza-infected CD200$^{-/-}$ mice recovered even after a weight loss close to 20% of their original weight within 8 days after infection with both strains (data not shown). These data indicate that, depending on the infectious dose, CD200$^{-/-}$ mice develop severe disease after infection with influenza viruses A/HK/2/68 and X-31, which is in striking contrast to wild type C57BL/6J mice.

*Influenza virus is not cleared from the lungs of CD200$^{-/-}$ mice*

To determine whether the development of severe disease by CD200$^{-/-}$ mice after infection with influenza was associated with an increased lung viral load, we measured the viral load in left lung lobes of infected wild type and CD200$^{-/-}$ mice at several time points after infection (Figure 2A). The viral load during the infection tended to be higher in CD200$^{-/-}$mice, but more striking was the finding that the viral load decreased slower in these animals. This could indicate that CD200$^{-/-}$ mice have difficulty clearing influenza virus from their lungs and thus suffer from higher viral loads late in infection.

In addition, we measured influx of cells into the lungs and BAL fluid of influenza-infected wild type and CD200$^{-/-}$ mice (Figure 2B). We found no statistically significant differences in cell numbers in the lungs between influenza-infected wild type and CD200$^{-/-}$ mice. However, we observed higher numbers of cells in the BAL fluid of CD200$^{-/-}$ mice at days 6 and 8 after influenza infection, time points at which cellular influx into BAL fluid of wild type mice is declining. This decline was not found in BAL fluid of CD200$^{-/-}$ mice, suggesting sustained inflammation.

Histological analysis of lungs of wild type and CD200$^{-/-}$ mice was performed 8 days after influenza infection. Notably, at this time point, the wild type mice had lost on average 5% of their original weight, whereas the analysed CD200$^{-/-}$ mice exhibited on average weight loss of 19% (Figure 1A). Influenza-infected lungs were inflated and fixed in EAF. Hematoxylin-eosin stained sections of paraffin-embedded tissue showed clear signs of (broncho-) pneumonia in one or all lung lobes, with intra-alveolar and intra-epithelial cellular infiltrates (Figure 2C). Although in one CD200$^{-/-}$ mouse severe broncho-pneumonia was found, there were no clear differences in severity of lung pathology between wild type and CD200$^{-/-}$ mice. In addition, in most cases the observed lung pathology was not severe enough to be the probable cause of death. Furthermore, histological analysis of spleens and nasal cavities of infected CD200$^{-/-}$ mice revealed no differences compared to wild type mice (data not shown).

Histological analysis of thymi of wild type mice revealed no pathological differences,

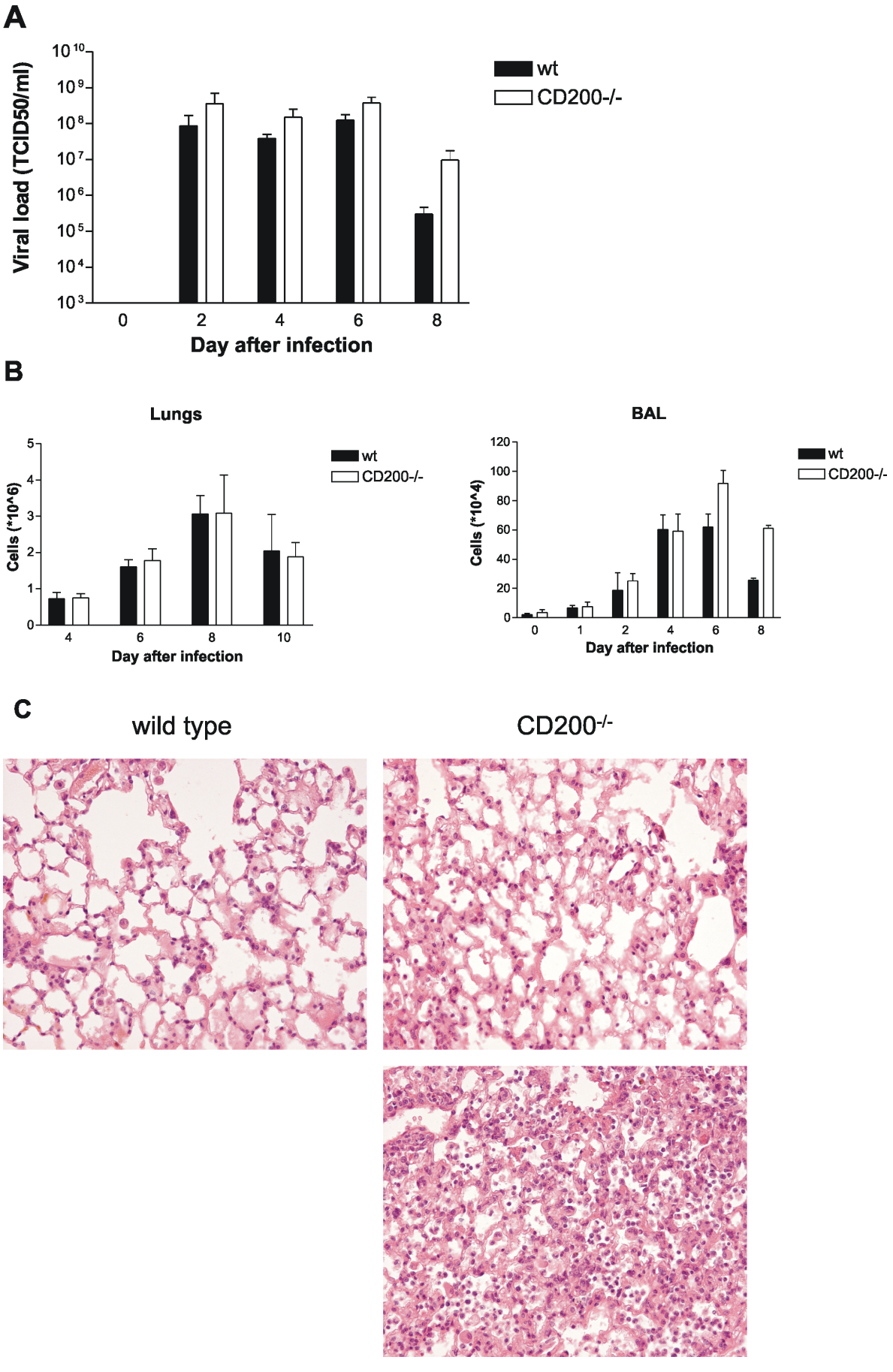
A
Viral load (TCID50/ml)
10 10
10 9
10 8
10 7
10 6
10 5
10 4
10 3
0
2
4
6
8
Day after infection
wt
CD200-/-
B
Lungs
Cells (*10^6)
5
4
3
2
1
0
4
6
8
10
Day after infection
wt
CD200-/-
BAL
Cells (*10^4)
120
100
80
60
40
20
0
0
1
2
4
6
8
Day after infection
wt
CD200-/-
C
wild type
CD200-/-

**Figure 2. CD200$^{-/-}$ mice show delayed clearance of influenza virus and increased cell numbers in BAL fluid**
A. Homogenates of the left lung lobe were assessed for viral load by quantitative PCR. Closed bars indicate wild type mice, open bars indicate CD200$^{-/-}$ mice. Error bars indicate standard error of the mean (SEM) of at least 2 mice per group.
B. Total recovered cell numbers from lungs (left panel) or BAL fluid (right panel) of wild type (closed bars) and CD200$^{-/-}$ mice (open bars) after influenza infection. Error bars indicate standard error of the mean (SEM) of 4 mice per group.
C. Tissues were fixed in EAF and the organs were processed to HE-stained slides. The left panel indicates representative lung tissue of influenza-infected wild type mice and the right panels indicate representative lung tissue of influenza-infected CD200$^{-/-}$ mice. Upper panels show intra-alveolar oedema, thickened alveolar septae and inflammatory infiltrates. The lower panel shows obstructive pneumonia as observed in a part of a lung lobe of an influenza-infected CD200$^{-/-}$ mouse. Original magnification: 20*.

whereas the thymus of a CD200$^{-/-}$ mouse showed atrophic cortex without apoptosis (data not shown).

*Influenza-specific T- and B-cell responses in CD200$^{-/-}$ mice are similar to wild type mice*

Next, we analysed the development of a functional CD8$^{+}$ T-cell response to influenza virus in CD200$^{-/-}$ versus wild type mice, using an H2-Db MHC class I tetramer loaded with NP366-374 (ASNENMDAM) peptide to measure influenza-specific T cells in the lungs of infected mice. During the course of infection, we measured an increase of influenza-specific CD8$^{+}$ T cells, indicating the development of an immune response against the virus. There were no differences between wild type and CD200$^{-/-}$ mice in the percentage of tetramer-positive CD8$^{+}$ T cells in the lungs (Figure 3A). In addition, no differences in absolute numbers of total CD8$^{+}$ T cells in the lungs of wild type and CD200$^{-/-}$ mice were found (data not shown).

We carefully analysed the T-cell compartment in lung-draining lymph nodes (Figure 3B) and spleen (data not shown) of wild type and CD200$^{-/-}$ mice during influenza infection. Interestingly, whereas CD200$^{-/-}$ mice had lower numbers of memory CD8$^{+}$ T cells before infection, they contained higher numbers of these cells at day 6 after influenza infection compared to wild type mice. We have not found any additional differences in naïve, memory and effector cell numbers of both CD4$^{+}$ and CD8$^{+}$ T cells in mediastinal lymph nodes (Figure 3B) and spleen (data not shown).

Next, we measured serum influenza-specific antibody levels per isotype to measure the influenza-specific B cell response. At day 8 after infection, CD200$^{-/-}$ mice tended to have increased levels of influenza-specific IgG3 and IgM antibodies, whereas at day 10, CD200$^{-/-}$ mice tended to have decreased influenza-specific IgG2a, IgG2b and IgG3, but not IgM levels (Figure 3C), but the differences were not significant.

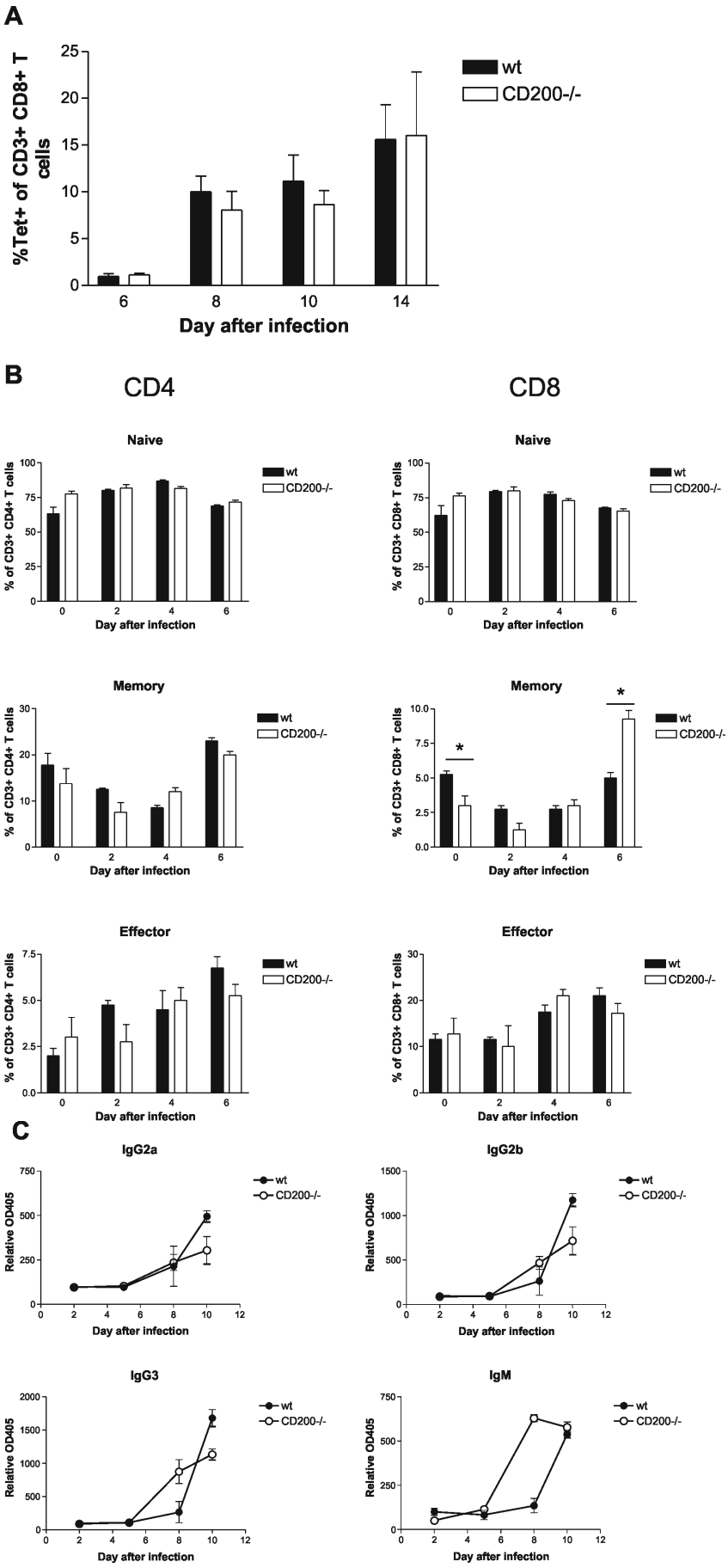


**Figure 3. CD200$^{-/-}$ mice develop normal levels of influenza-specific T- and B-cell responses**

A. Lung cells were analysed by flow cytometry with αCD3, αCD8 and MHC class I tetramers loaded with the ASNENMDAM peptide to analyse influenza-specific CD8$^{+}$ T-cell responses. Closed bars indicate wild type mice, open bars indicate CD200$^{-/-}$ mice. Error bars indicate standard error of the mean (SEM) of 4 mice per time point.

B. Lung-draining lymph nodes were analysed by flow cytometry with αCD3, αCD4, αCD8, αCD44 and αCD62L for the presence of naive, memory and effector T cells. The left panels indicate CD4$^{+}$ T-cells, the right panels indicate CD8$^{+}$ T-cells. Upper panels indicate naïve T cells (defined as CD44$^{low}$CD62L$^{+}$), middle panels indicate memory T cells (CD44$^{+}$CD62L$^{+}$), lower panels indicate effector T cells (CD44$^{+}$CD62L$^{-}$).
Results are plotted as percentage of cells within the CD4$^{+}$ or CD8$^{+}$ T-cell gate. Closed bars indicate wild type mice, open bars indicate CD200$^{-/-}$ mice. Error bars indicate standard error of the mean (SEM) of 4 mice per group. *: significant, $p<0.05$

C. Levels of influenza-specific antibodies were determined per isotype using ELISA. Data are plotted as relative to the average OD value of wild type mice at day 2. Closed symbols indicate wild type mice, open symbols indicate CD200$^{-/-}$ mice. Error bars indicate standard error of the mean (SEM) of 2 to 5 mice per group.

*Cytokine responses in BAL fluid of CD200$^{-/-}$ mice are similar to wild type mice*

To investigate whether the severe disease course of influenza infection is due to increased cytokine production, we measured cytokine levels in BAL fluid samples at different time points after infection. Multiplex analysis did not reveal significant differences in IL-10, MIP1α, RANTES, KC, TNFα, GM-CSF and IL-1ß levels (data not shown). Although initial analysis in three wild type and three CD200$^{-/-}$ mice showed that CD200$^{-/-}$ mice tended to have higher levels of IFN-γ, IL-6 and MCP-1, analysis of additional samples by ELISA did not sustain this finding (Figure 4). In addition, no differences in serum levels of these cytokines were found between influenza-infected wild type and CD200$^{-/-}$ mice (data not shown). Furthermore, levels of IFNα and IFNß in BAL fluid were assessed by ELISA, which were found to be equal in wild type and CD200$^{-/-}$ mice, with very low levels of IFNß (Figure 4, and data not shown). Thus, we conclude that CD200$^{-/-}$ mice do not produce enhanced cytokine levels compared to wild type mice after influenza infection.

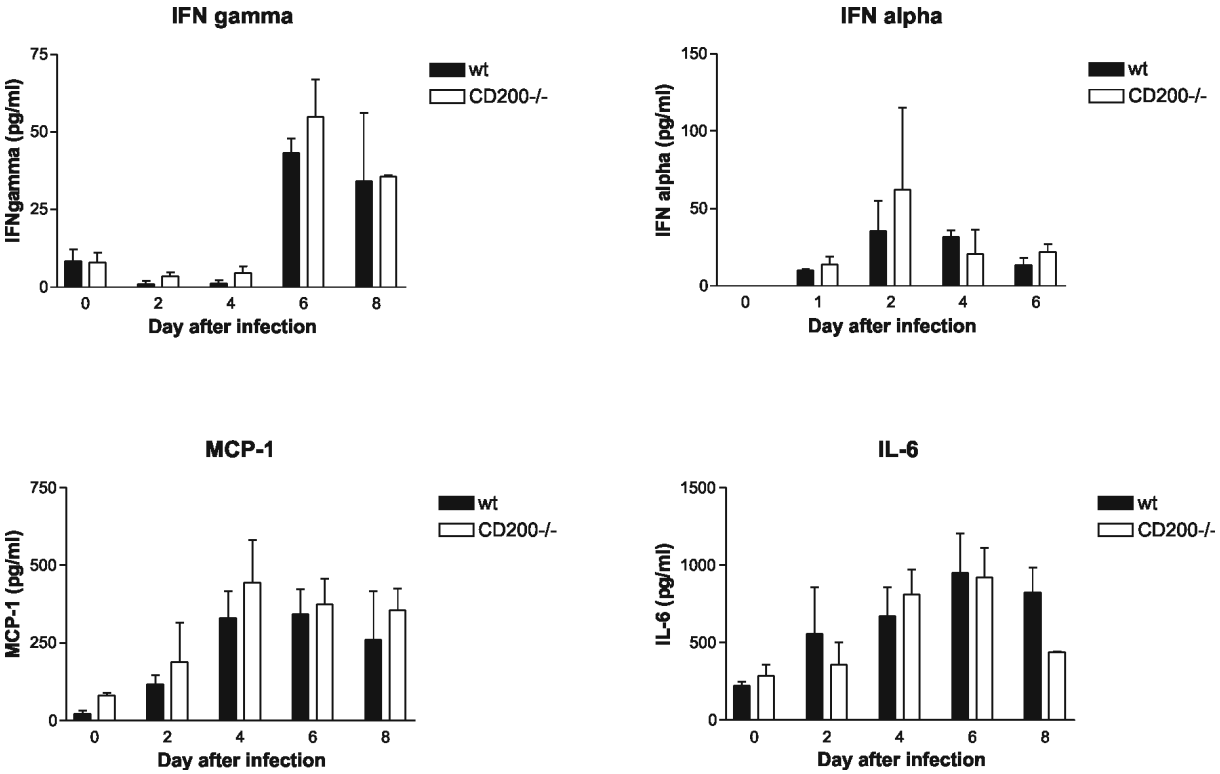


**Figure 4. CD200$^{-/-}$ mice exhibit normal cytokine levels in BAL fluid**
Cytokine levels in BAL fluid were measured using ELISA. The upper left panel indicates IFNγ levels, the upper right panel indicates IFNα, the lower left panel indicates MCP-1 and the lower right panel indicates IL-6. Closed bars indicate wild type mice, open bars indicate CD200$^{-/-}$ mice. Error bars indicate standard error of the mean (SEM) of 2 to 8 mice per group.

*Dexamethasone treatment or macrophage depletion do not ameliorate disease in CD200$^{-/-}$ mice*

Previous studies on CD200$^{-/-}$ mice have revealed an over activation of cells expressing CD200R in these mice. Therefore, we hypothesized that disease development in CD200$^{-/-}$ mice is caused by an over activation of CD200R-expressing immune cells. To investigate this hypothesis, we treated influenza-infected wild type and CD200$^{-/-}$ mice daily with 10 mg/kg/day of the immunosuppressive drug dexamethasone starting at day -1 relative to influenza infection. The control groups were injected with the similar volume of PBS daily. Treatment of CD200$^{-/-}$ mice

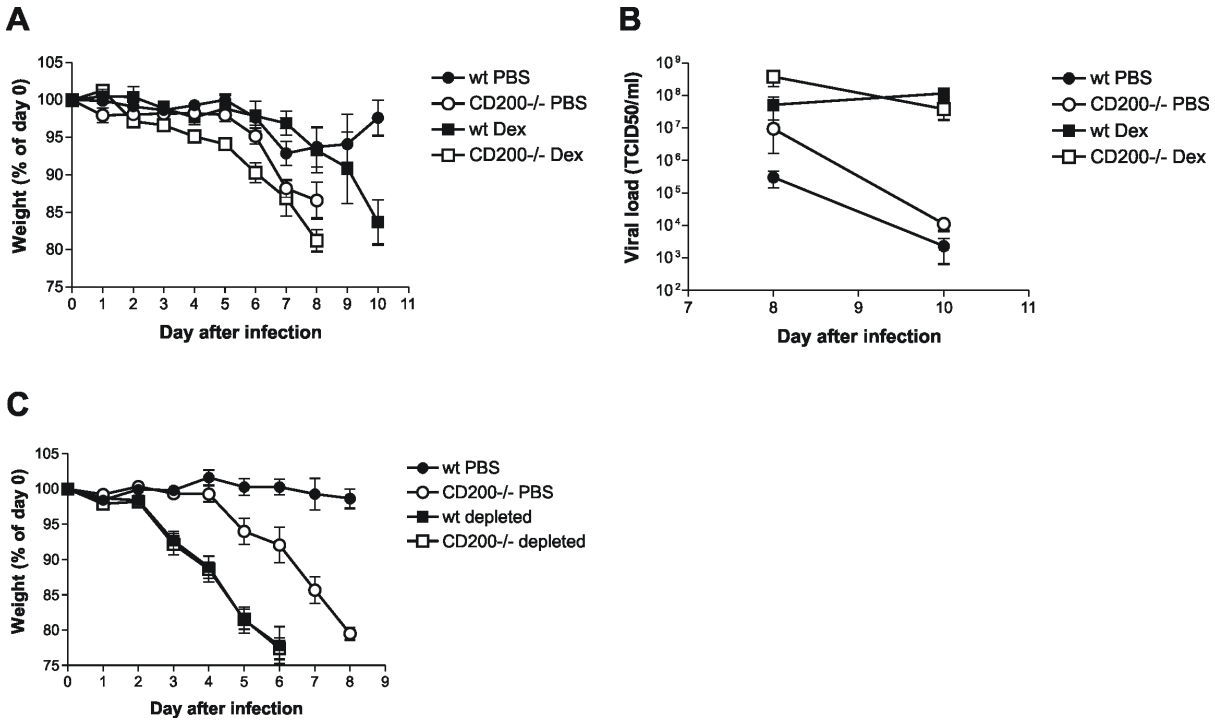


**Figure 5. Dexamethasone treatment or macrophage depletion exacerbates disease development after influenza infection in wild type and CD200$^{-/-}$ mice**
A. Starting at day -1 relative to influenza infection, mice were treated with 20 mg/ kg body weight dexamethasone (squares) or with an equal volume PBS as a control (circles) daily. Disease development was measured by determination of weight loss daily. Closed symbols indicate wild type mice, open symbols indicate CD200$^{-/-}$ mice. Error bars indicate standard error of the mean (SEM) of 6 mice per group.
B. Homogenates of the left lung lobe of dexamethasone-treated (squares) or PBS-treated (circles) wild type (closed symbols) and CD200$^{-/-}$ (open symbols) mice were assessed for viral load by quantitative PCR.
C. Alveolar macrophages were depleted by inhalation of clodronate liposomes (squares) or, as a control, PBS liposomes (circles) at days -4 and -2 relative to influenza infection. Disease development was measured by determination of weight loss daily. Closed symbols indicate wild type mice, open symbols indicate CD200$^{-/-}$ mice. Macrophage-depleted CD200$^{-/-}$ mice are not well visible since this curve exactly overlaps the macrophage-depleted wild type curve. Error bars indicate standard error of the mean (SEM) of 6 mice per group.

with dexamethasone did not ameliorate the disease. In fact, dexamethasone treated CD200$^{-/-}$ lost weight more rapidly than did PBS-treated CD200$^{-/-}$ mice (Figure 5A). Dexamethasone-treated wild type mice also developed severe disease over time, showing enhanced and sustained viral load compared to PBS-treated wild type mice (Figure 5B), indicating that this treatment regime suppressed the influenza-specific response resulting in lethal disease in both wild type and CD200$^{-/-}$ mice.

In addition, we depleted alveolar macrophages before influenza infection to determine the role of these cells in development of severe disease in CD200$^{-/-}$ mice. Alveolar macrophages express high levels of CD200R and are in close proximity to CD200-expressing epithelial cells lining the alveoli (data not shown). Depletion of alveolar macrophages was achieved by intranasal instillation of clodronate liposomes (or PBS liposomes as a control) at days -4 and -2 relative to influenza infection. Depletion of alveolar macrophages resulted in lethality of influenza infection in both wild type and CD200$^{-/-}$ mice, with similar kinetics (Figure 5C) and high viral loads (data not shown). Apparently, alveolar macrophages are critically important in the development of a protective immune response against this strain of influenza virus.

*Development of disease in CD200$^{-/-}$ mice is dependent on T-cells*

To determine the role of T-cells in CD200$^{-/-}$ disease development upon influenza infection, we depleted both CD4$^{+}$ and CD8$^{+}$ T cells by a simultaneous injection of αCD4 and αCD8 antibodies at days -2 and +1 relative to infection with influenza virus. Injection of these antibodies resulted in reduction of CD3$^{+}$ cells from 30% of living cells in peripheral blood to 5% in depleted mice (Figure 6A). In agreement with our previous results, PBS-treated wild type mice did not develop disease after influenza infection, whereas PBS-treated CD200$^{-/-}$ mice did develop severe disease. Surprisingly, T-cell depleted CD200$^{-/-}$ mice did not develop disease, indicating that T cells are an important component in the development of severe disease in CD200$^{-/-}$ mice after influenza infection (Figure 6B).

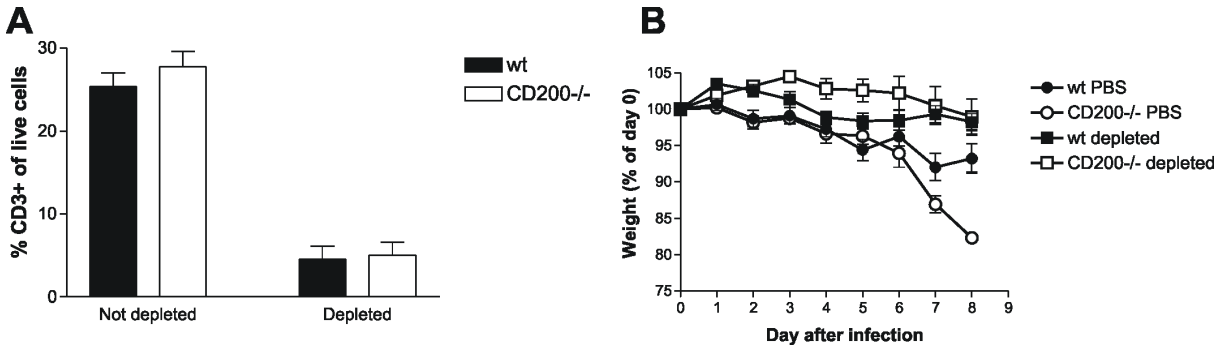


**Figure 6. T-cell depletion of CD200$^{-/-}$ mice results in absence of disease development after influenza infection**
A. T cells were depleted by injection of 50 µg αCD4 and 50 µg αCD8 antibodies in a total volume of 100 µl PBS (squares) or PBS alone as a control (circles). T-cell depletion was monitored by analysis of peripheral blood (obtained by puncture of vena saphena) just before influenza infection. Plotted is the percentage CD3$^{+}$ cells of all live lymphocytes.
B. Disease development of T-cell depleted and PBS-injected wild type and CD200$^{-/-}$ mice was measured by determination of weight loss daily. Closed symbols indicate wild type mice, open symbols indicate CD200$^{-/-}$ mice. Error bars indicate standard error of the mean (SEM) of 6 mice per group.

## Discussion

Here, we show that CD200$^{-/-}$ mice develop critical disease after influenza infection. Differences in disease progression between wild type and CD200$^{-/-}$ mice occur dose-dependently and were found after infection with two different influenza strains, which are related with regard to their envelope proteins HA and NA.

After depletion of a large proportion of both CD4$^{+}$ and CD8$^{+}$ T cells, influenza infection did not cause disease development in CD200$^{-/-}$ mice, even when mice were monitored for two weeks (data not shown), in contrast to influenza-infected CD200$^{-/-}$ mice that were treated with PBS. Although this experiment was performed only once, this clear result indicates that, either directly or indirectly, (a subset of) T cells mediate development of severe disease in CD200$^{-/-}$ mice upon influenza infection. Possible mechanisms of T-cell contribution to the severe course of influenza infection in CD200$^{-/-}$ mice include bystander T-cell activation or lack of T-cell inhibition.

Influenza-associated lung pathology is largely mediated by the immune system itself, despite regulatory mechanisms. For example, expression of OX40 on T cells is associated with immune pathology [26]. Regulatory mechanisms to restrict the T-cell pool during influenza infection include control of T-cell responses in regional lymph nodes in a Fas-dependent manner [27], and TNF receptor 2 (TNFR2)-mediated restriction of the T-cell response in the influenza-infected lung [28]. Although further research is needed to establish the cause of severe disease in influenza-infected CD200$^{-/-}$ mice, it is possible that these regulatory mechanisms are affected by the absence of CD200.

Intriguingly, wild type mice depleted of both $CD4^+$ and $CD8^+$ T cells also did not develop disease after influenza infection. This may be explained by the incomplete depletion which results in a lower, but still functional influenza-specific adaptive immune response in both wild type and $CD200^{-/-}$ mice, while the depletion of the majority of T cells in $CD200^{-/-}$ mice eradicates auto-reactive or uninhibited pathogenic T cells.

A protective immune response against influenza, resulting in clearance of the virus from the lungs, is dependent on a functional $CD8^+$ T-cell response or $CD4^+$ T-cell-assisted B cell response [7-9]. Infusion of hemaglutinnin (HA)-specific IgG2a antibodies into severe combined immune deficient (SCID) mice results in viral clearance [29]. In addition, infusion of influenza-specific $CD8^+$ effector cells into lethally infected mice resulted in clearance of influenza infection [30], mainly by Fas-mediated or by perforin/ granzyme mediated cytotoxicity [31]. In $CD200^{-/-}$ mice, influenza-specific $CD8^+$ T-cell numbers were normal, and preliminary experiments revealed that, upon restimulation with the viral peptide ASNENMDAM, $CD8^+$ T cells from lungs of infected $CD200^{-/-}$ mice were able to produce IFN-γ to a similar extent as cells from wild type mice (data not shown). In addition, although production of influenza-specific antibodies tended to occur with different kinetics and at lower levels (at day 10 after infection) in $CD200^{-/-}$ mice compared to wild type mice, there were no significant differences in production of these antibodies between wild type and $CD200^{-/-}$ mice. This indicates that disease development in $CD200^{-/-}$ mice apparently is not due to an ineffective $CD8^+$ T-cell or antibody response.

Disease development and viral load in both wild type and $CD200^{-/-}$ mice is enhanced when mice are treated with the immuno-suppressive corticosteroid dexamethasone [32;33]. In addition, depletion of alveolar macrophages before infection with influenza strain HK/2/68 resulted in enhanced disease with equal kinetics in both wild type and $CD200^{-/-}$ mice, indicating that treatment with dexamethasone or depletion of alveolar macrophages inhibits the development of a functional immune response against influenza and results in accelerated disease development in both wild type and $CD200^{-/-}$ mice.

On the basis of lung histology, we have not been able to identify the cause of the severe disease development in $CD200^{-/-}$ mice after influenza infection. This was surprising, since the tissues were taken at a time point when wild type mice had only lost around 5% of their original weight, whereas $CD200^{-/-}$ mice had lost 19% of their original weight. There were differences within lobes and between $CD200^{-/-}$ mice in severity of lung pathology. The thymic cortex of one influenza-infected $CD200^{-/-}$ mouse revealed atrophy without apoptosis, indicating that this atrophy likely is not due to a stress factor, for example dehydration, but rather migration of thymocytes to unknown destination. However, we have been able to analyse only one $CD200^{-/-}$ thymus, making conclusions difficult.

Although all influenza-infected $CD200^{-/-}$ mice developed severe disease, some $CD200^{-/-}$ mice survived the infection, which is in agreement with the observed differences in lung pathology

between CD200$^{-/-}$ mice and suggests that the development of a functional and appropriately controlled immune response against influenza is possible in these mice. Preliminary experiments with secondary influenza infections of CD200$^{-/-}$ mice revealed no disease development upon secondary infection, indicating that these mice had developed a functional adaptive immune response upon primary infection (data not shown).

Concluding, we report that CD200$^{-/-}$ mice develop severe disease after influenza infection. CD200$^{-/-}$ mice contained normal number of influenza-specific T cells in their lungs, but influenza-specific IgG2a, IgG2b and IgG3 antibody levels were reduced in CD200$^{-/-}$ mice compared to wild type mice. Depletion of both CD4$^{+}$ and CD8$^{+}$ T cells resulted in complete lack of disease development in CD200$^{-/-}$ mice, indicating that T cells are directly or indirectly responsible for the development of disease in CD200$^{-/-}$ mice. Thus, we show that CD200 is a host factor in the determination of the outcome of an influenza infection in mice.

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# 7
# Summarizing discussion

Inhibitory immune receptors play important roles in the control of immune responses and the prevention of auto-immunity. Many different inhibitory immune receptors exist, differing in expression pattern, ligand recognition and/or signal transduction pathways. Their respective roles in maintaining the immune balance are non-redundant.

In this thesis, signal transduction by the inhibitory immune receptors LAIR-1 and CD200R was investigated, as well as the role of CD200R in haematopoiesis and its expression pattern on lymphocytes. In addition, CD200 was identified as an important host factor determining the outcome of influenza infection in mice. Here, the results of the research presented in this thesis are summarized and discussed.

*Inhibitory receptor signalling is not solely mediated by recruitment of phosphatases to ITIMs*

The existence of the wide variety of inhibitory immune receptors illustrates the importance of restricting immune activation. Different inhibitory immune receptors have unique roles in maintaining immune homeostasis or restricting inflammation. Out of the many different inhibitory immune receptors, only some do not signal via ITIMs.

Is all ITIM-mediated signalling dependent on phosphatase recruitment? Unlike other ITIM-bearing receptors like KIR and PECAM-1, which require either SHP-1 or SHP-2 [1;2], we report that LAIR-1 has phosphatase-independent function and that LAIR-1, like SIRPα, ILT-2 and SIT [3-5], recruits Csk (Chapter 2). Csk is a kinase that inactivates Src family kinases and thus inhibits cellular activation [6]. For all four inhibitory immune receptors found to associate to Csk, the functional role of Csk association was not established yet. This may be due to the dual role Src family kinases play in intracellular signalling: they phosphorylate both activating receptors as well as inhibitory receptors.

Our data show that immune inhibition by LAIR-1 is not solely mediated by recruitment of phosphatases to the ITIM and illustrate other possibilities of ITIM-mediated, but phosphatase-independent, immune inhibition. Possibly, other ITIM-bearing receptors also have phosphatase-independent function and our findings raise the possibility that also other inhibitory receptors signal by recruiting Csk.

CD200R belongs to a small but important group of inhibitory immune receptors that does not signal through ITIMs [7-9]. We report that mutation of two or three intracellular tyrosines completely abrogates inhibitory function, but that mutation of any single tyrosine results in clear, although diminished, inhibitory function. In addition, using a yeast system, we identify Shc and Dok-1 as molecules associating to the intracellular domain of CD200R in a phosphorylation dependent manner. These findings were confirmed independently, although only Dok-1 and Dok-2, and not Shc, were found to associate to CD200R in mammalian cells [10]. Dok-1 was found to associate to the tyrosine located in the NPXY motif [11]. As we show that mutation of

this tyrosine results in diminished but clearly present inhibitory capacity, it is likely that Dok-1 is not the sole mediator of CD200R function. This would imply that phosphorylation of the other tyrosines leads to recruitment of alternative signal transduction molecules. However, as these tyrosines are not located in a known signalling motif, it is difficult to predict which signal transduction molecules could be recruited and this remains to be investigated.

Dok-1/Dok-2 double knockout mice (DKO) mice develop leukaemia. Although both molecules mediate CD200R signalling, $CD200^{-/-}$ mice do not develop leukaemia (Chapter 4). These data indicate that CD200R is not involved in the development of leukaemia in Dok-1/Dok-2 DKO mice. However, Dok-1 and Dok-2 are involved in the inhibition of many cellular activation signals [12]. Dok-1 mediated signalling can occur through interactions with SHIP, for example in Dok-1-mediated function of FcγRIIb and KLRG1 (or MAFA) [13;14]. Possibly, these inhibitory immune receptors play a regulatory role in the control of leukaemia development. However, it is also possible that other, yet unidentified, molecules mediate Dok-1 and Dok-2-mediated leukaemia suppression.

*Which signals are modulated by inhibitory receptors?*

The phenotypes of mice deficient for different inhibitory receptors reveal distinct roles for inhibitory receptors. Thus, each inhibitory receptor inhibits specific activating signals at distinct stages of maintaining immune homeostasis or the development of an immune response. This might not only be due to differences in ligand and receptor distribution but also to differences in intracellular signals. The current model for ITIM-bearing receptors is that they inhibit ITAM-mediated signals, but it has not been thoroughly investigated whether these signals are the only activating signals they can inhibit. In addition, it is unknown which activating signals can be modulated by ITIM-independent inhibitory signals. For example, can inhibitory immune receptors modulate signals from cytokine receptors or pattern recognition receptors such as C-type lectins or TLRs? Dok-1 and Dok-2 can inhibit LPS-induced TLR4 signalling [12]. This inhibition may be mediated by an inhibitory immune receptor, but if so, which one? In addition, recently the ITIM-bearing paired inhibitory receptor (PIR)-B was found to modulate TLR-mediated signals by functioning as a pathogen receptor [15]. Perhaps other inhibitory immune receptors can also modulate signals from this or other pattern recognition receptors.

*CD200R signalling regulates lymphocyte function*

Recently CD200 was found to be overexpressed on several leukaemias and CD200 expression was associated with unfavourable leukaemia prognosis [16-18]. In addition, blockade of the interaction between CD200 on leukaemic B cells and CD200R on PBMC was found to inhibit tumour growth in an in vivo model of tumour rejection [19]. Thus, tumours take advantage of

the inhibitory potential of the CD200-CD200R interaction. Blockade of this interaction by therapeutics may, in the future, promote tumour rejection in vivo.

Interestingly, viral homologues of CD200 have been identified [20-24]. The vCD200 produced by human herpes virus 8 down-modulates the activity of myeloid cells through an interaction with CD200R, suggesting that the ability to restrictively control host cells is an important viral immunomodulatory mechanism. The recently described activating CD200RL molecules do not bind CD200 and it was speculated that, similar to activating KIRs, these activating CD200RL molecules have evolved as a mechanism to recognize bacterial or viral components [25]. If so, it is likely that these vCD200 proteins function as ligands for the activating CD200RL molecules. However, this needs yet to be established.

Since both tumours and viruses use CD200 expression to down-modulate the immune response, it is tempting to speculate that CD200R plays a much broader role in modulating the immune system than restriction of only myeloid cells in the development of auto-immunity and inflammatory situations. Although the expression of CD200R on lymphocytes is lower than on myeloid cells, it is differentially regulated and induced upon activation in vitro (Chapter 5), suggesting a functional role for CD200R signalling in restricting lymphocyte responses. CD200R expression is highest on differentiated lymphocytes such as memory T and B cells and plasma B cells. Thus, CD200R likely is not so much involved in maintaining lymphocyte homeostasis, but rather in restricting secondary responses.

Support for a functional role of CD200R expression on lymphocytes comes from our studies on influenza infection in mice. Challenge of $CD200^{-/-}$ mice with influenza results in severe and often fatal disease (Chapter 6). Depletion of T cells from these mice before influenza infection resulted in complete absence of disease, suggesting that T cells contribute either directly or indirectly to the development of disease. Influenza-associated lung pathology is largely mediated by the immune system itself and T cells contribute greatly to immune pathology after influenza infection [26]. However, several regulatory mechanisms exist to restrict the T cell pool during influenza infection [27;28]. Is one of these regulatory mechanisms affected by the absence of CD200? Or does the absence of CD200 lead to bystander T cell activation?

Both in mice and in humans, CD200R on T cell subsets is highest on $CD4^{+}$ memory T cells. These cells might be dysregulated in $CD200^{-/-}$, thereby causing the severe disease course of influenza infection. However, further research is needed to accept or reject this hypothesis. Additional studies using challenges of the lymphocyte compartment of $CD200^{-/-}$ mice or, preferably, $CD200R^{-/-}$ mice are required to elucidate the role of CD200R signalling in lymphocyte regulation.

*CD200 is a host factor in the determination of the outcome of an influenza infection*

Host factors are important determinants in the outcome of viral infections. For example,

HLA-B27 is associated with favourable prognosis of HIV infection [29]. Genetic host factors, together with viral load and viral pathogenicity, determine the outcome of disease. During the 1918 influenza pandemic, mainly young, otherwise healthy adults succumbed to influenza infection. The case mortality rate of this severe pandemic averaged 2.5% [30], which is several times higher than the contemporary average. However, this number also indicates that 97.5% of infected individuals survived the infection, underlining the importance of host factors in the outcome of viral infection. Our data show that CD200 is a host factor in the determination of severity of influenza infection, most likely by restricting T cell mediated pathology. Of note, molecules involved in proper activation of the immune response, such as the activating NK-cell receptor Ncr1, are also important host factors determining the outcome of influenza infection [31].

To determine whether CD200 is a host factor determining the outcome of other infections as well, the immunity of $CD200^{-/-}$ or, preferably, $CD200R^{-/-}$ mice to other viruses should be studied. Preliminary studies with respiratory syncytial virus (RSV) infections in $CD200^{-/-}$ showed no differences in disease outcome and RSV-specific $CD8^{+}$ cell numbers (Michael Lukens, Eva Rijkers, Grada van Bleek et al, unpublished observation). However, since we observed differences in outcome of influenza infection only in a limited dose range and we have tested only one infectious dose of RSV, it is yet possible that, after infection with a different infectious dose, $CD200^{-/-}$ mice do develop more severe disease than wild type mice.

The identification of CD200 as a host factor in the determination of the outcome of influenza infection suggests possible intervention mechanisms during diseases in which over-activation of the immune system causes pathology. Severe clinical course of infections, for example development of severe acute respiratory distress-like syndrome (ARDS) with H5N1 bird influenza and the 1918 influenza strain is associated with immune pathology [32-34]. It is tempting to speculate that the immune pathology found in these diseases may be restricted by ligation of CD200R, for example by administration of an agonistic, soluble form of CD200. However, this hypothesis should first be investigated in a mouse model, for example by local treatment of influenza-infected mice with a soluble CD200 fusion protein.

Recognition of host factors determining the outcome of infections is important to manipulate the outcome of these infections in the future. The role of genetic factors, involved in determination of the disease course, in influenza infection should be more extensively studied, for example by infecting mice deficient in different inhibitory receptors with influenza. This may help to identify patients at high risk of developing ARDS after influenza infection and could make tailored treatment regimes for influenza-infected patients possible. Especially in the view of a possible new influenza pandemic, it is critical to identify host factors involved in the determination of the outcome of an influenza infection and genetic risk assessment may be of importance for the management of influenza-infected patients.

*Concluding remarks*

As shown by genetic analysis of human diseases and studies using knockout mice, inhibitory immune receptors play essential and non-redundant roles in the control of the immune system. The biological functions of LAIR-1 and CD200R are not yet clear. However, our studies on the signal transduction of LAIR-1 and CD200R have indicated that other mechanisms for immune inhibition exist than the commonly investigated ITIM-phosphatase pathway. Further studies should elucidate these mechanisms of immune regulation. Studies on the expression patterns of CD200R have broadened our understanding of this receptor. In addition, in vivo studies have revealed an important role for CD200R signalling in the response to a viral infection. Detailed analysis of the interaction between CD200 and CD200R may reveal even more functional implications of CD200R signalling in health and disease, for example in lymphocyte regulation, rejection of a variety of tumours, and restriction of immune responses. To assess the biological role of CD200R signalling, further analysis of the functional implications of CD200R signalling should be performed, and may lead to the development of therapeutics that exploit the CD200-CD200R interaction. In addition, analysis of host factors involved in modulation of the immune response against influenza may in the future lead to improved and tailored treatment regimes to restrict excessive immune activation in influenza-infected patients.

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Samenvatting in het Nederlands

Dankwoord

Curriculum Vitae

List of publications

**Inleiding**

Het lichaam heeft voortdurend te maken met aanvallen van bijvoorbeeld bacteriën, virussen en parasieten (verzamelnaam: pathogenen), die het goed functioneren van het lichaam bedreigen. Om het lichaam te beschermen tegen deze mogelijke pathogenen heeft het lichaam een eigen verdedigingsmechanisme, het afweersysteem. Cellen van dit afweersysteem (immuunsysteem) patrouilleren door het lichaam om afwijkingen van 'normaal' op te sporen. Als het afweersysteem een afwijking, bijvoorbeeld een infectie met een virus opspoort, komt het in actie om te proberen het virus op te ruimen. Bij deze immuunreactie zijn afhankelijk van de omstandigheden verschillende typen immuuncellen betrokken.

Tijdens een afweerreactie is een rem van cruciaal belang. Vergelijk het met een coureur in een Formule 1-race: activatie (gas geven) is nodig om een kans te maken in de wedstrijd. Maar: zonder rem ga je de finish nooit veilig halen! Zo is het in het afweersysteem ook. Het afweersysteem is een krachtig mechanisme om bijvoorbeeld bacteriën en virus-geïnfecteerde cellen op te ruimen. Als dit mechanisme niet wordt gecontroleerd, kan het schade aan het lichaam aanrichten. De activiteit van het afweersysteem wordt tijdens verschillende stadia van de afweerreactie door verschillende mechanismen gecontroleerd. Zo wordt bijvoorbeeld een soort activatiedrempel opgeworpen om te voorkomen dat het afweersysteem actief wordt op een moment dat dat niet nodig is. Ook wordt, als het infecterende pathogeen opgeruimd is, het afweersysteem teruggebracht naar de 'ruststand' om te voorkomen dat het actief blijft en cellen van het eigen lichaam gaat aanvallen.

Grof gezegd kunnen immuuncellen, op basis van hun herkomst, in twee groepen worden ingedeeld: lymfoide cellen en myeloide cellen. Lymfoide cellen omvatten T cellen, B cellen en NK cellen. Myeloide cellen zijn granulocyten (onder te verdelen in neutrofielen, basofielen en eosinofielen), dendritische cellen, monocyten en macrofagen. Iedere immuuncel heeft specifieke taken tijdens een immuunrespons.

Op de immuuncellen zitten verschillende moleculen die signalen van buiten de cel naar binnen doorgeven (receptoren). Receptoren kun je vergelijken met een soort vangnetje, die bedoeld zijn om een speciaal type molecuul (het ligand) mee te vangen. Die liganden kunnen op andere cellen zitten, maar ook bijvoorbeeld een eigen plekje (de extracellulaire matrix) hebben. Omdat cellen van het afweersysteem door het lichaam zwerven, komen ze langs allerlei andere cellen. Als een cel met een bepaalde receptor langs een ligand komt, binden deze twee moleculen, receptor en ligand, aan elkaar. De receptor kan vervolgens allerlei verschillende soorten signalen aan de cel doorgeven. Het doorgeven van signalen via receptoren naar binnenin de cel noemen we signaaltransductie. De signalen die doorgegeven worden door deze receptoren kunnen activerend zijn (ze zetten de cel aan tot een bepaalde actie) of inhiberend (remmend).

Een van de manieren om de activiteit van het afweersysteem in de hand te houden is via remmende receptoren. Er zijn veel verschillende soorten inhibitoire (remmende) receptoren.

Eerdere studies hebben laten zien dat deze receptoren op verschillende soorten immuuncellen zitten en dat ze verschillende, niet-overlappende functies hebben. De meeste inhibitoire receptoren bevatten een speciaal motief (rijtje aminozuren in het deel wat in de cel steekt) waar moleculen die de signalen binnen de cel doorgeven aan kunnen binden. Dit motief wordt ITIM genoemd.

**Dit proefschrift**

Onze onderzoeksgroep is geïnteresseerd in de inhibitoire receptoren LAIR-1 en CD200R. Van LAIR-1 was bekend dat het op bijna alle typen immuuncellen zit en dat het verschillende functies (acties) van die cellen kan remmen. LAIR-1 bindt aan verschillende soorten collageen. Collagenen komen veel in het lichaam, vooral in de extracellulaire matrix. Omdat beide moleculen veel voorkomen is de kans groot dat ze elkaar regelmatig tegen komen. Dit zou kunnen betekenen dat de binding van LAIR-1 aan collageen veel voor komt tijdens verschillende (stadia van) immuunreacties en dat LAIR-1 belangrijk is tijdens het reguleren van de activatie van immuuncellen.

CD200R is een bijzonder lid van de familie van inhibitoire immuunreceptoren omdat het, in tegenstelling tot de meeste inhibitoire immuunreceptoren, geen enkele ITIM heeft. Eerder is beschreven dat het ligand van CD200R CD200 is (daar staat de R voor: CD200 Receptor). CD200 zit op veel verschillende soorten cellen in het lichaam. Van CD200R is alleen goed beschreven dat het op myeloide immuuncellen zit. Er zijn muizen gemaakt die genetisch zo gemanipuleerd zijn dat ze geen CD200 meer hebben (CD200 knockout of $CD200^{-/-}$ muizen). In deze muizen kan CD200R niet meer aan CD200 binden. Daardoor geeft CD200R de cellen waar het op zit geen remmende signalen meer. Dit betekent dat de cellen waar CD200R op zit in deze muizen wat geactiveerd zijn. Daardoor krijgen $CD200^{-/-}$ muizen makkelijker ziektes die veroorzaakt worden door een hyperactief afweersysteem (bijvoorbeeld auto-immuunziektes). Dus is CD200R belangrijk bij het afremmen van immuuncellen.

In hoofdstuk 2 hebben we onderzocht welke signaal transductie paden gebruikt worden door LAIR-1. Eerder was al bekend dat LAIR-1 de fosfatases SHP-1 en SHP-2 gebruikt om activerende signaal transductie paden uit te zetten. Fosfatases werken remmend omdat ze een cruciale stap in de activatie van cellen kunnen omkeren. We vonden dat in cellen waarin geen SHP-1 en SHP-2 aanwezig is, LAIR-1 toch een remmende functie heeft. Er moest dus nog minimaal een ander molecuul zijn dat de remmende functie van LAIR-1 kan doorgeven. Met behulp van een screen in gistcellen hebben we gevonden dat het molecuul Csk aan LAIR-1 kan binden. Deze binding hebben we bevestigd in zoogdiercellen. Van Csk is bekend dat het, op een andere manier, verschillende signaal transductie paden in de cel kan remmen. Er is meer

onderzoek nodig om te achterhalen hoe dit precies werkt.

Vervolgens hebben we onderzocht welke moleculen binnen de cel aan CD200R kunnen binden, om uit te zoeken hoe CD200R zijn remmende functie uitoefent (hoofdstuk 3). Met dezelfde soort gistscreen vonden we dat Dok-1 en Shc aan CD200R binden. De vinding dat Shc aan CD200R bindt is vreemd, want van Shc is bekend dat het een activerend molecuul is. We hebben deze binding niet kunnen bevestigen in zoogdiercellen, dus we weten niet precies wat dat nu betekent. Van Dok-1 aan de andere kant, is bekend dat het een remmende functie heeft in de cel doordat het het activerende molecuul Ras kan uitzetten. Dok-1 is dus een logische kandidaat om CD200R signalering te door te geven. Verder laten we zien dat alle drie de tyrosines in de intracellulaire staart nodig zijn voor de volledige remmende functie van CD200R. Omdat het erop lijkt dat voor binding van Dok-1 maar één tyrosine nodig is, zou dit kunnen betekenen dat Dok-1 niet het enige molecuul is dat de functie van CD200R doorgeeft. Ook dit moet nog verder uitgezocht worden.

Van Dok-1 (en Dok-2, waarvan ook is gevonden dat het aan CD200R bindt) is bekend dat ze invloed hebben op de vorming van een bepaald type bloedcellen (myeloide cellen). Muizen waarin geen Dok-1 en Dok-2 meer aanwezig is (Dok-1/Dok-2 dubbel knockout muizen) krijgen na verloop van tijd een tumor van myeloide cellen, leukemie. Dat komt omdat Dok- en Dok-2 samen celdeling kunnen remmen. Als Dok-1 en Dok-2 er allebei niet zijn, wordt de celdeling niet meer geremd en loopt uit de hand, waardoor een tumor ontstaat. Omdat Dok-1 en Dok-2 allebei aan CD200R binden, hebben we in hoofdstuk 4 uitgezocht of de remming van tumorvorming door Dok-1 en Dok-2 via CD200R loopt. Daarvoor hebben we gebruik gemaakt van muizen die geen CD200 hebben (de $CD200^{-/-}$ muizen). In deze muizen hebben we gekeken naar de vorming van myeloide cellen uit voorlopers van bloedcellen (hematopoietische stamcellen). We vinden in $CD200^{-/-}$ muizen normale hoeveelheden hematopoietische stamcellen en dat deze cellen in normale mate uitgroeien tot volwassen bloedcellen. Verder krijgen $CD200^{-/-}$ muizen, ook op latere leeftijd, geen leukemie. We concluderen dus dat binding van CD200 aan CD200R, wat leidt tot signalering door CD200R, geen invloed heeft op de vorming van myeloide cellen en het ontstaan van leukemie.

In hoofdstuk 5 onderzoeken we op welke cellen CD200R nu precies zit. Er is eerder altijd gezegd dat CD200R vooral op myeloide cellen zit, maar wij laten nu zien dat het ook op lymfoide immuuncellen (T, B en NK cellen) voorkomt. We laten zien dat de expressie van CD200R op T en B cellen gereguleerd is, dat wil zeggen dat verschillende ondersoorten T en B cellen verschillende hoeveelheden CD200R op het celoppervlak hebben. Dit wil zeggen dat het kennelijk iets doet op die cellen, waarom zou anders de hoeveelheid verschillen? Muizen zonder CD200 hebben normale aantallen T, B en NK cellen en de afweerreactie van B cellen tegen iets wat ze niet kennen verloopt normaal, dat wil zeggen op precies dezelfde manier als in gewone muizen. We hebben dus nog niet kunnen ontdekken wat precies de functie is van

CD200R op lymfoide cellen, maar wel dat CD200R dus waarschijnlijk een bredere rol heeft in het reguleren van het afweersysteem dan eerder werd gedacht.

Als een virus het lichaam binnen komt, is een aantal factoren belangrijk voor het bepalen van de uitkomst van die infectie. Natuurlijk is het van belang hoe agressief (pathogeen) het virus is en hoe snel het kan delen. Aan de andere kant speelt het afweersysteem ook een belangrijke rol in deze infectie. Hoe beter opgeleid het afweersysteem is, hoe effectiever het de infectie kan aanpakken. Maar als een rem ontbreekt kan de reactie tegen de infectie uit de hand lopen, waardoor de afloop alsnog ongunstig kan zijn. Om te kijken hoe het afweersysteem reageert op een infectie als CD200 wel of niet aanwezig is, hebben we normale (wild type) en $CD200^{-/-}$ muizen geïnfecteerd met influenza virus (beschreven in hoofdstuk 6). Het blijkt dat, terwijl wild type muizen geen probleem hebben met het opruimen van de influenza infectie (ze niezen twee keer en dan is het klaar), $CD200^{-/-}$ muizen ernstig ziek worden van deze griep. We vinden dat het influenza virus langer in de longen van $CD200^{-/-}$ muizen blijft dan in de longen van wild type muizen. We hebben ook gekeken of T cellen belangrijk zijn bij het onstaan van ziekte in $CD200^{-/-}$ muizen na influenza infectie. Dat hebben we gedaan door er eerst voor te zorgen dat de muizen geen T cellen meer hebben (dat proces heet T cel depletie). Daarna hebben we de muizen geïnfecteerd met influenza. We vonden dat $CD200^{-/-}$ muizen die bijna geen T cellen meer hadden, niet zo ziek werden van influenza infectie als $CD200^{-/-}$ muizen die hun T cellen nog wel hebben. Het lijkt er dus op dat T cellen betrokken zijn bij het ernstige verloop van ziekte in influenza-geïnfecteerde $CD200^{-/-}$ muizen. De conclusie van dit hoofdstuk is dat CD200 een gastheerfactor is die, naast virale factoren, belangrijk is bij het bepalen van het verloop van een influenza-infectie.

**Samengevat**

Voor het afweersysteem zijn remmende receptoren belangrijk om te voorkomen dat het overactief raakt en schade aan het eigen lichaam veroorzaakt. In dit proefschrift is de precieze werking van de remmende receptoren CD200R en LAIR-1 bestudeerd. Voor het doorgeven van de signalen van deze receptoren naar binnenin de cel, zijn verrassend genoeg niet altijd fosfatases nodig. LAIR-1 kan zijn functie ook uitoefenen als er geen fosfatases in de cel aanwezig zijn. We hebben aanwijzingen dat Csk de functie van LAIR-1 kan doorgeven als er geen fosfatases zijn. CD200R bindt het inhibitoire molecuul Dok-1, dat belangrijk is bij het uitzetten Ras, een belangrijk activerend molecuul binnen de cel. Dok-1 en Dok-2 samen zijn bekende remmers van het ontstaan van leukemie, maar dit wordt niet geregeld via CD200R. In tegenstelling tot eerdere bevindingen laten we hier zien dat CD200R ook op lymfoïde immuuncellen zit. De expressie van CD200R op T en B cellen is gereguleerd, wat een aanwijzing is dat CD200R een rol speelt in de regulatie van de activiteit van deze cellen.

Verder hebben we de verrassende vinding gedaan dat als CD200 niet aanwezig is, het verloop van een influenza-infectie veel ernstiger is dan wanneer CD200 er wel is. Waarschijnlijk zijn T cellen, een bepaald type immuuncel, betrokken bij het ontstaan van deze symptomen. Voor de uitkomst van een virus infectie is een aantal factoren van zowel virus als gastheer van belang. CD200 is een belangrijke gastheerfactor in het bepalen van de uitkomst van een influenza-infectie. Dit onderzoek en het verder in kaart brengen van gastheer factoren kan van belang zijn voor de preventie en behandeling van ernstig verlopende influenza-infecties.

*Don't be afraid of what your mind conceives*
*You should make a stand*
*Stand up for what you believe*
*And tonight, we can truly say*
*Together we're invincible*

Matthew Bellamy

Het is af! Hoera! Ik vind het een raar idee dat er maar één naam voor op dit boekje staat: er zijn zoveel mensen bij het bij elkaar pipetteren/ schrijven/ maken van dit proefschrift betrokken geweest! Dit is mijn kans om jullie te bedanken, die ga ik meteen grijpen.

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*Eva*

*Birds flying high you know how I feel*
*Sun in the sky you know how I feel*
*Reeds driftin' on by you know how I feel*

*It's a new dawn*
*It's a new day*
*It's a new life*
*For me*
*And I'm feeling good*

Bricusse & Newley

Eva Suzanne Katrijn Rijkers (geboren op 15 maart 1979 te Eindhoven) heeft met dit proefschrift haar promotieonderzoek afgerond, dat zij als Assistent in Opleiding vanaf oktober 2002 heeft uitgevoerd. Dit onderzoek verrichtte zij onder begeleiding van Dr. L. Meyaard, Dr. R. Hoek en Prof. Dr. P. Coffer bij de afdeling Immunologie van het UMC Utrecht.

Haar promotieonderzoek volgde op haar opleiding Biomedische Wetenschappen aan de Universiteit Utrecht, een opleiding die zij in 1997 startte en die zij in oktober 2002 met het behalen van het doctoraal diploma afsloot.
Ter afronding van deze opleiding volgde zij een bijvakstage bij de vakgroep Immunologie, aan CD200R, onder begeleiding van Dr. L. Meyaard, Dr. R. Hoek en Prof. Dr. H. Clevers, van maart 2002 tot oktober 2002.
Daaraan voorafgaand volgde zij een hoofdvakstage aan cryptococcen, bij de vakgroep Microbiologie van het UMC Utrecht, van maart 2001 tot december 2001, onder begeleiding van Dr. F. Coenjaerts en Prof. Dr. A. Hoepelman.
Tijdens deze studie Biomedische Wetenschappen was Eva tevens gedurende een jaar praeses van de Medisch Biologen Vereniging Mebiose.
Het voorbereidend wetenschappelijk onderwijs volgde zij van 1991 tot 1997 aan het Stedelijk Gymnasium te Arnhem. In de acht jaren daaraan voorafgaand bezocht zij basisscholen in Veldhoven en Arnhem.

Eva Rijkers is vanaf juli 2007 werkzaam als junior beleidsmedewerker bij WOTRO Science for Global Development, onderdeel van de Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO) te Den Haag.

**Interference of Cryptococcus neoformans with human neutrophil migration**

Annemieke Walenkamp, Pauline Ellerbroek, Jelle Scharringa, Eva Rijkers, Andy I. Hoepelman and Frank E.J. Coenjaerts

*Advances in Experimental Medicine and Biology, 2003, 531: 315-339*

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Michiel van der Flier, Frank E.J. Coenjaerts, Pauline N. Mwinzi, Eva Rijkers, Maartje Ruyken, Jelle Scharringa, Jan L. Kimpen, Andy I. Hoepelman and Sybil P. Geelen

*Journal of Neuroimmunology, 2005, 60(1-2):170-177*

**Leukocyte-associated Ig-like receptor-1 has SH2 domain-containing phosphatase-independent function and recruits C-terminal Src kinase**

Annelies Verbrugge, Eva S.K. Rijkers, Talitha de Ruiter and Linde Meyaard

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**Ligation of CD200R by CD200 is not required for normal murine myelopoiesis**

Eva S.K. Rijkers, Talitha de Ruiter, Miranda Buitenhuis, Henrike Veninga, Robert M. Hoek and Linde Meyaard

*In press, European Journal of Haematology, 2007*

**The inhibitory CD200R is differentially expressed on human and mouse T and B cells**

Eva S.K. Rijkers, Talitha de Ruiter, Ajoeb Baridi, Henrike Veninga, Robert M. Hoek and Linde Meyaard

*In press, Molecular Immunology, 2007*