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CHAPTER 4

CD200 expression is essential for nasal tolerance induction through IDO activity#

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Abstract

Entry of antigen via mucosal pathways will initiate the induction of immunological tolerance in the draining lymph nodes (LNs). For the induction of tolerance to intranasally administered ovalbumin (OVA), the microenvironment of the nose draining cervical lymph nodes (CLNs) is essential. Furthermore, it has been demonstrated that for intranasal tolerance induction the enzyme indoleamine 2,3-dioxygenase (IDO) is needed and that this enzyme is induced in dendritic cells (DCs) in vitro by the immune regulatory molecule CD200. Here we show that DCs in mice deficient for CD200 do not express IDO, coinciding with a failure of these mice to induce immunological tolerance to intranasally administered antigen. CD200 is expressed on stromal fibroblastic reticular cells (FRCs) in resting LNs and is also strongly induced on T cells upon their activation. By bone marrow transplantation studies, we could demonstrate that CD200 expression is required on both FRCs and hematopoietic cells for the induction of intranasal tolerance, thus pointing to a crucial role of CD200 in this process.

Introduction

CD200 (OX2) is a membrane type 1 glycoprotein, belonging to the immunoglobulin superfamily and expressed by a broad variety of cells. Expression can be found on B cells, T cells, and follicular dendritic cells (FDCs), but also on neurons from the central nervous system (CNS) and vascular endothelium [1, 2]. Expression of the receptor for CD200 (CD200R) is more restricted to hematopoietic cells, particularly myeloid cells [2-5]. Both the receptor and ligand contain two extracellular Ig-like domains and their interactions play an important role in the regulation of immune responses, as blocking resulted in expansion of the myeloid lineage and enhanced auto-immune diseases [1, 3]. Further evidence for a regulatory function of CD200R triggering comes from the observed presence of activated CD200R-expressing microglia, which are in direct contact with CD200 expressing neuronal cells. In $CD200^{-/-}$ mice, this lack of interaction resulted in increased levels of nitric oxide synthase and macrophage activation [6]. CD200R engagement on myeloid cells was shown to have a suppressive effect on IFN-γ- or IL-17-, but not LPS-induced cytokine production. In these studies no influence of CD200R on monocyte derived DCs could be observed [7]. Additionally, it has been shown that CD200R ligation prevents allograft rejection [8, 9]. An effect of CD200R signaling on DCs comes from in vitro studies in which CD200-Ig treatment of plasmacytoid DCs (pDCs) resulted in the induction of tolerogenic DCs which were capable of transferring tolerance upon in vivo transfer [10]. In these experiments, CD200R ligation together with type 1 IFN-R signaling resulted in the induction of indoleamine 2,3-dioxygenase (IDO) in pDCs. IDO is an intracellular enzyme that degrades the indole moiety of tryptophan, serotonin and melatonin [10-16]. Expression of IDO is associated with prevention of auto-immune diseases, rejection of the fetus during pregnancy and transplant rejection [14, 17-19]. In the presence of IDO expressing cells, T cell proliferation is inhibited and apoptosis is induced [19-22]. Also in tumors IDO expression in antigen presenting cells (APCs) has been observed, suggesting involvement in the suppression of anti-tumor responses by T cells [17, 23]. The depletion of tryptophan from the microenvironment in which immune cells reside can account for the regulatory effect of IDO, but also the formed metabolites, kynurenines, have immunoregulatory properties. Kynurenines may be able to induce apoptosis by processes modulating oxygen free radicals [24]. We have shown that the immunomodulatory properties of IDO expression and tryptophan degradation play a determining role in the induction of tolerance to intranasally administered antigens and suppression of T cell responses [25]. Upon intranasal exposure of harmless antigens [26-33], antigen is taken up by DCs, processed and presented in the draining LNs by non-plasmacytoid interstitial DCs, where T regulatory cells (Treg cells) are

induced [34-36]. The exact mechanisms of the induction of immunomodulatory properties in DCs are unknown, although we have shown that non-plasmacytoid interstitial DCs in LNs draining the nasal mucosa exhibit higher expression of IDO than DCs in peripheral lymph nodes (PLNs) and that upon IDO blockade no Treg cells and no immune tolerance can be induced [25]. By LN transplantation, it was shown that the microenvironment of these organs is instrumental for the induction of tolerance to harmless antigen that enters through the mucosa. In these studies, PLNs were transplanted to the nasal mucosa draining site and these LNs failed to induce tolerance to an intranasally administered antigen, while transplanted native, nose draining cervical LNs (CLNs) were able to do so [33]. Further analysis of the transplanted LNs showed that rapidly after transplantation, virtually all donor hematopoietic cells in the transplanted LN are replaced by host cells, whereas the stromal cells (including FRCs, vascular and lymphatic endothelium and FDCs), making up for the basic structure of the LN, remain of donor origin (manuscript submitted for publication). This is clearly supporting the idea that stromal elements in the LNs are instrumental for the type of immune responses generated and also supports the suggestion that interaction between local stromal cells of the CLNs and e.g. DCs is crucial for intranasal tolerance induction [37]. Here we show an enhanced expression of CD200 on stromal cells of CLNs when compared to stromal cells of PLNs. Corresponding to the enhanced CD200 expression on stromal cells, interstitial DCs from CLNs show a higher expression of IDO when compared to interstitial DCs from PLNs. In CD200-deficient mice, IDO production in these DCs is significantly inhibited and the absence of CD200 results in impaired tolerance induction to intranasally administered OVA. In addition, activation of T cells leads to the induction of CD200 expression, and by BM transfer studies we show that both stromal and T cells are required for the induction of immune tolerance. These results emphasize the importance of the microenvironment in the regulation of immune reactivity and identify CD200 as one of the key molecules in tolerance induction.

Materials and methods

Mice

Female BALB/c and C57Bl/6 mice aged 8 to 12 weeks were purchased from Charles River (Sulzfeld, Germany) and kept under standard animal housing conditions. The D011.10 and OT-II transgenic mice were used at 8 to 12 weeks of age and were bred at our own facilities.

CD200$^{-/-}$ mice were bred and obtained from the animal facility at the Amsterdam Medical Center, The Netherlands. The Animal Experiments Committee of the VU Medical Center and the Amsterdam Medical Center approved all experiments involving animals.

Tissue isolation and stainings

Cervical (internal jugular and superficial) and peripheral (inguinal) LNs were isolated and either frozen in TissueTek for immunofluorescence or used as single cell suspensions for FACS analysis or FACS sorting. Single cell suspensions were made by cutting LNs with scissors, followed by digestion at 37ºC for 30 min, using Blenzyme 2 (Roche, Penzberg, Germany) and 100 U/ml DNAse I (Roche, Penzberg, Germany) in PBS. Cell clumps were removed by pipetting the cells over a nylon mesh. The LN cells were washed and resuspended in PBS with 2% New Born Calf Serum (PBS-NBCS). For sorting of DCs, cells were stained with biotin conjugated anti-MHCII (clone M5/114), PE-conjugated anti-CD11c (clone N418, eBioscience) and 7AAD (Invitrogen-Molecular Probes, Leiden, The Netherlands) to discriminate live vs dead cells. Stromal cells were sorted as all cells that were negative for the Alexa-488 conjugated anti-CD45 (clone MP33), while 7AAD^{+} dead cells were excluded. For FACS and immunofluorescence, anti-gp38 (anti-podoplanin, clone 8.1.1 obtained from the Developmental Studies Hybridoma Bank (DSHB) at University of Iowa, Iowa City, IA) and anti-CD200 (clone OX90) were used. Additionally, PE-Cy7 conjugated anti-CD4 (clone GK1.5, eBioscience) and Sytox Blue (Molecular Probes) were used to stain for OVA-specific T cells and live cells, respectively, which were analyzed on a Cyan ADP High-Performance Research Flow Cytometer (DakoCytomation, Glostrup, Denmark). Secondary antibodies were Alexa conjugated goat-anti-rat-Fab or goat-anti-hamster (Molecular Probes, Leiden, The Netherlands).

Real time PCR

MHCII^{high}CD11c^{high} interstitial DCs were sorted from pooled LN cells from 6 mice using a Cytomation MoFlo sorter and lysed in Trizol (Gibco BRL, Breda, The Netherlands). RNA was isolated by precipitation with isopropanol and cDNA was synthesized from total RNA using RevertAid First Strand cDNA Synthesis Kit (Fermentas Life Sciences, Burlington, Canada) according to the

manufacturer's protocol. IDO specific primers and primers for housekeeping genes β-actin and 18S RNA were designed across exon-intron boundaries using Primer Express software (PE Applied Biosystems, Foster City, CA).
Real time PCR was performed on an ABI Prism 7900HT Sequence Detection System (PE Applied Biosystems). Total volume of reaction mixture was 20 μl, containing cDNA, 300 nM of each primer and SYBR Green Mastermix (PE Applied Biosystems). To correct for primer efficiency, a standard curve was generated for each primer set with cDNA from a pool of non-activated CLNs, PLNs and mesenteric LNs (MLNs) and expression of transcripts was related to β-actin and 18S RNA.

Tolerance induction, sensitization and measurement of DTH responses
The antigen OVA (Albumin Chicken Egg 5x Crystalline, Calbiochem) or HEL (Hen Egg Lysozyme, Sigma) was administered intranasally by 3 applications of 100 μg antigen in 10 μl saline on three consecutive days as described earlier [36]. One day after the last intranasal antigen administration or one day after transfer of $CD4^+$ T cells, mice received 100 μg antigen in 25 μl saline, mixed with 25 μl IFA (Difco Laboratories, Detroit, USA) subcutaneously in the tail base. Five days later, ear thickness was measured with an engineer's micrometer (Mitutoyo, Tokyo, Japan) and a challenge of 10 μg antigen in 10 μl saline was given in the auricle of each ear. After 24 hours, the mean increase in ear thickness of both ears was expressed as the DTH response.

T cell enrichment, CFSE labelling, transfer and antigenic stimulation
Spleens and lymph nodes from DO11.10 or OT-II mice were minced through a 100-μm gauze to obtain single cell suspensions. To deplete erythrocytes from spleen cell suspension, cells were incubated for 2 minutes on ice in lysis buffer (150 mM NH_4, 1 mM $NaHCO_3$, pH 7.4). Cells were washed and resuspended in PBS-NBCS and $CD4^+$ T cells were enriched using the CD4 negative selection kit (Dynal, Oslo, Norway). The enriched cell suspension consisted of at least 60% $CD4^+$ T cells, as determined by flow cytometry (FACS Calibur, Becton Dickinson and FACS Cyan, DakoCytomation, Glostrup, Denmark). These cells were labeled with 5 μM of CFSE at $3x10^7$/ml for 10 min and washed with ice-cold PBS. BALB/c or C57BL/6 mice were injected with approximately 10^7 OVA-specific T cells and were subsequently stimulated by intranasal (i.n) or intramuscular (i.m.) administration of 400 μg OVA in 10 μl saline. Draining lymph nodes were analyzed at 72 hrs after antigen administration by either immunofluorescence or FACS.

Transfer experiments
To check for the presence of Treg cells in sensitized and tolerized mice, tolerized mice were resensitized by subcutaneous injection of 100 μg OVA in 25

μl saline mixed with 25 μl IFA at 2 weeks after ear thickness measurement to maintain Treg cells. One week after resensitization, spleens were collected and minced through a 100 μm gauze to obtain single cell suspensions. To deplete erythrocytes from this cell suspension, cells were incubated for 2 min on ice in lysis buffer (150 mM NH_4, 1 mM $NaHCO_3$, pH 7.4). Cells were washed and resuspended in PBS 2% NBCS and $CD4^+$ T cells were enriched using the CD4 negative selection kit (Dynal, Oslo, Norway). The enriched cell suspension consisted of 50-60% $CD4^+$ T cells, as determined by flow cytometry (FACS Calibur, Becton Dickinson). 2-5x10^5 $CD4^+$ T cells were injected i.v. via the lateral tail vein in acceptor mice, which were subsequently sensitized and challenged with OVA (as described above).

Bone marrow chimeras

C57Bl/6 CD45.1 congenic and $CD200^{-/-}$ (CD45.2) mice were subjected to lethal total body irradiation (TBI) by exposure to 2 doses of 4.5 Gy gamma irradiation, and housed in individually ventilated cages (IVC). The day following TBI, bone marrow cells from four CD45.1 congenic and $CD200^{-/-}$ donor mice were prepared aseptically from both tibiae and femurs. Irradiated recipients were given 7x10^6 bone marrow cells i.v. by lateral tail vein injection, and continued to be housed in IVC for 6 weeks, at which stage the animals were immune-competent again. During this period the mice received acidified neomycin-containing drinking water (0.16% neomycin-sulphate (Sigma-Aldrich) in autoclaved tap water containing 0.037% HCl). Eight weeks following BM transplantation successful chimerism was confirmed by FACS analysis of PBMCs stained for the CD45.1 and CD45.2 congenic markers. These mice were used in a DTH experiment 19 weeks after bone marrow transfer.

Statistics

Groups of mice in the DTH experiments with ear swelling responses were compared using an one-way ANOVA followed by Bonferroni's multiple comparison test or Tukey-Kramer multiple comparison test for unequal group sizes. P values of < 0.05 were considered significant.

Results

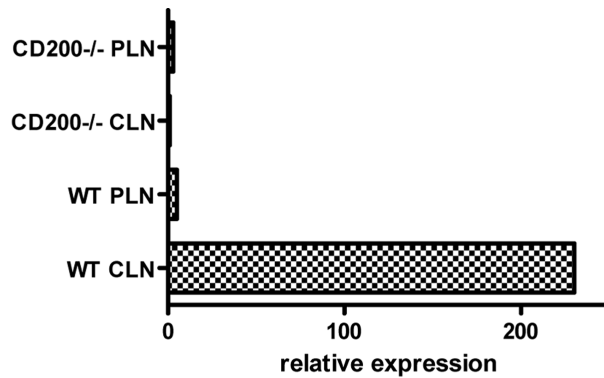

Figure 1: IDO expression is strongly reduced in CD11c^{high}MHCII^{high} DCs from CD200$^{-/-}$ CLNs when compared to WT CLNs. CLNs and PLNs (inguinal LNs) from 6 C57Bl/6 were isolated and digested using Blenzyme 2. DCs were stained for CD11c and MHC class II and MHCII^{high}CD11c^{high} cells were sorted to obtain a purity of >90% non-plasmacytoid interstitial DCs. Sorted cells were lysed in Trizol reagent, RNA was isolated and cDNA was synthesized by reverse transcription. Subsequently, expression levels of IDO were determined using real time PCR. Results normalized to internal reference genes 18S and β-actin were similar. Shown are relative expression levels in CLNs and PLNs of CD200$^{-/-}$ and WT mice.

DCs from CD200$^{-/-}$ mice exhibit a reduced expression of IDO

We previously demonstrated that IDO is differentially expressed in DCs from cervical LNs (CLN-DCs) when compared to peripheral LN DCs (PLN-DCs), and that IDO expression is instrumental for the induction of immune tolerance upon intranasal administration of antigen [25]. Since CD200R ligation on DCs has been shown to induce IDO [10], we investigated whether CD200 is involved in the differential expression of IDO in CLN-DCs. Hereto we determined the levels of IDO expression in sorted MHCII^{high}CD11c^{high} non-plasmacytoid interstitial DCs from CD200$^{-/-}$ CLNs and WT CLNs by real time PCR. Analysis of DCs from CD200$^{-/-}$ showed that IDO was virtually absent in CLN-DCs, while it could be normally detected in CLN-DCs from WT mice, indicating the involvement of CD200-CD200R interaction in the induction of IDO. IDO expression in DCs from PLNs was low in WT as well as CD200-/- mice (fig. 1).

Failure of tolerance induction upon nasal antigen administration in CD200$^{-/-}$ mice

To examine whether the strongly reduced expression of IDO in CLN-DCs from CD200$^{-/-}$ indeed resulted in a failure to induce immune tolerance, CD200$^{-/-}$ and WT mice received OVA intranasally and were subsequently sensitized in the tail

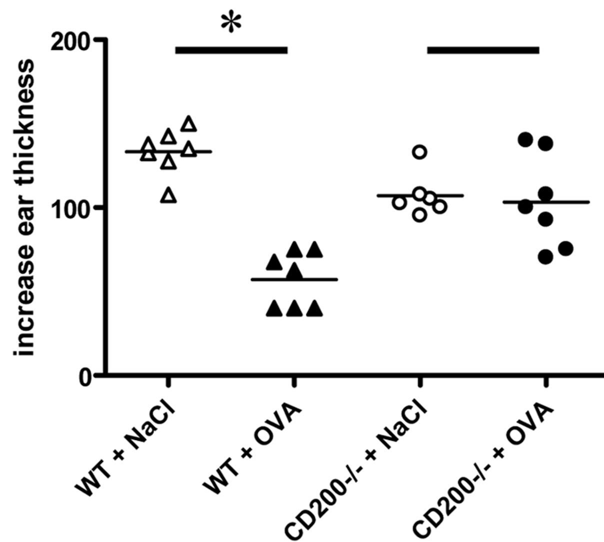

Figure 2: $CD200^{-/-}$ mice do not become tolerant to intranasal administered OVA. Both WT and $CD200^{-/-}$ mice received 100 μg OVA intranasally (i.n.) for 3 consecutive days, followed by s.c. injection of 100 μg OVA in IFA on day 4, and an injection of 10 μg OVA in the auricle of each ear on day 9. Ear thickness increase was measured as DTH response one day later. WT + NaCl: control group of WT mice that received NaCl i.n., WT + OVA: WT mice that received OVA i.n., $CD200^{-/-}$ + NaCl: $CD200^{-/-}$ mice that received NaCl i.n., $CD200^{-/-}$ + OVA: $CD200^{-/-}$ mice that received OVA i.n. As can be seen, $CD200^{-/-}$ mice failed to mount a tolerant immune response when OVA was given intranasally. Data points are means of measurements on both ears of the animals (6 or 7 mice per group). Bars indicate the mean of each group. Differences between groups are significant ($p < 0.05$) when indicated by *.

and challenged with OVA in the ear. WT animals that received OVA intranasally showed a significant lower DTH response than animals that had received NaCl intranasally (fig. 2). This tolerance induction after intranasal OVA administration could not be seen in $CD200^{-/-}$ mice, where no difference was observed in DTH response between the group that received saline or OVA intranasally (for both comparisons $p > 0.05$). From these data it can be concluded that CD200 expression plays an important role in the induction of nasal tolerance.

Induction of Treg cells is inhibited in $CD200^{-/-}$ mice

To get more insight in the mechanism behind the failure to induce tolerance in the $CD200^{-/-}$ mice, we looked for the induction of Treg cells. Upon intranasal administration of OVA, Treg cells are initially induced in the nose draining LNs from where they migrate throughout the body [33-35]. Their antigen specific regulatory capacity can be tested in a transfer system where $CD4^+$ spleen cells

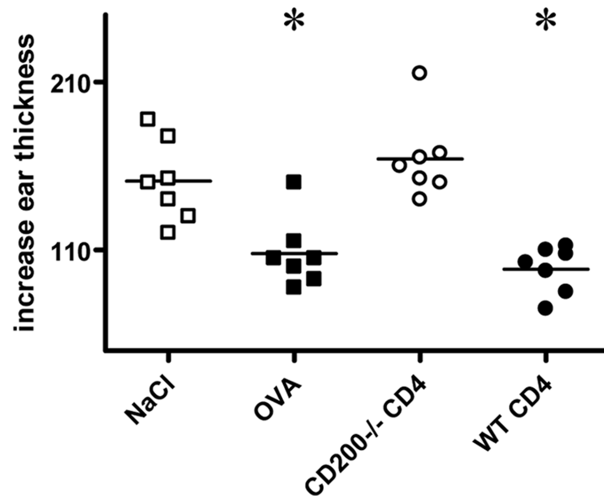

Figure 3: CD200$^{-/-}$ mice are unable to generate Treg cells upon intranasal antigen administration. Two weeks after ear thickness measurement shown in figure 2 and one week after resensitization, CD4^{+} cells from WT and CD200$^{-/-}$ mice treated i.n. with OVA were enriched by negative selection from the spleens. Enriched cells were transferred into naïve mice (group **WT CD4**; CD4 cells isolated from WT mice and group **CD200$^{-/-}$ CD4**; CD4 cells isolated from CD200$^{-/-}$ mice, respectively). These mice were sensitized by subcutaneous injection of OVA/IFA, challenged by OVA injection in the ear and ear thickness increase was measured as DTH response. As control read outs for tolerance induction and normal DTH response, two groups of naïve mice were exposed to the OVA (**OVA**) or saline (**NaCl**) i.n. before sensitization and OVA challenge. Transfer of CD4^{+} cells from CD200$^{-/-}$ mice could not transfer immune suppression to recipients, indicating the failure to generate functional Treg cells upon intranasal OVA administration in CD200$^{-/-}$ mice. Data points are the mean of both ears of individual animals (7 mice per group). Bars indicate the mean of each group. Differences between the non-tolerized WT group and other groups are significant ($p < 0.05$) when indicated by *.

from tolerized animals can confer tolerance to naïve recipients [32]. To check for the presence of Treg cells in WT and CD200$^{-/-}$ mice that had received OVA intranasally, naïve recipients were injected with CD4^{+} cells from both groups. At one day after receipt of the CD4^{+} cells, the animals were subsequently sensitized and challenged with OVA in the tail base and the ear, respectively (fig. 3). As controls for ear swelling in the tolerant versus DTH group, WT mice that had received OVA or saline intranasally were also sensitized and challenged with OVA. Measurement of the ear swelling showed that CD4^{+} cells from WT mice were able to transfer tolerance for OVA to naïve mice, while CD4^{+} cells from CD200$^{-/-}$ mice were not capable of doing so. These data show that CD200$^{-/-}$ mice are unable to generate functional OVA specific Treg cells upon intranasally administered OVA.

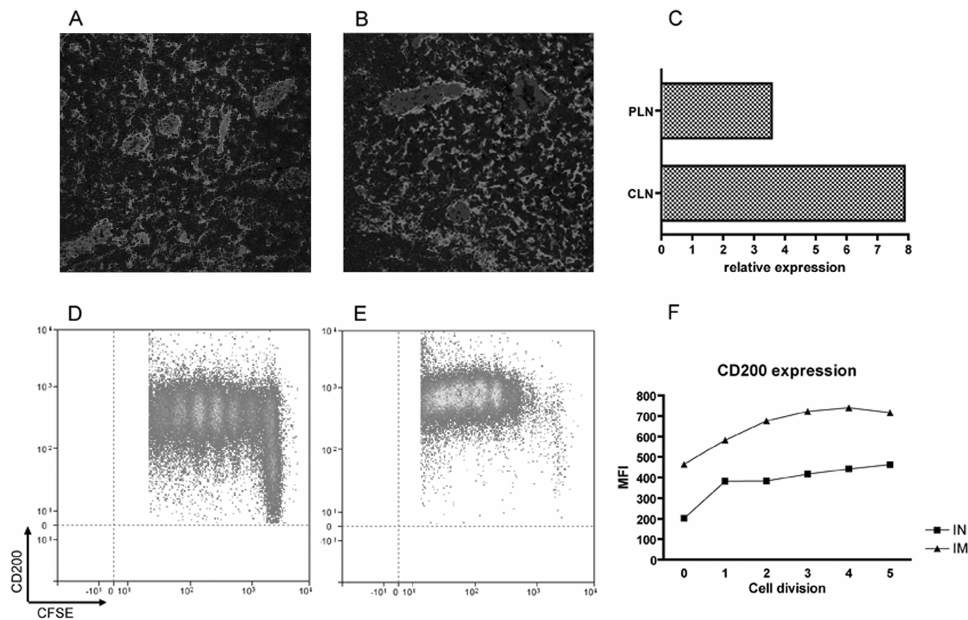

Figure 4: CD200 is expressed by LN stromal cells and activated T cells. CLNs and PLNs were isolated 72 hours after stimulation with OVA in the presence of OVA-specific transgenic T cells and frozen for immunofluorescence. Frozen tissue sections were stained for CD200 (blue) and gp38 (red) and pictures were taken of T cell areas of (A) activated CLNs and (B) activated PLNs. (C) Sorted $CD45^-$ cells from resting CLNs and PLNs of 6 mice were analyzed for CD200 mRNA expression by real time PCR. Results normalized to internal reference genes 18S RNA and β-actin were similar. Shown are the results normalized to 18S RNA. CD200 expression was studied by FACS analysis of CFSE labeled OVA-specific OTII $CD4^+$ cells in (D) CLNs and (E) PLNs at 72 hrs after i.n. and i.m. antigen administration, respectively. (F) The mean fluorescence intensity for each cell division in CLNs and PLNs (inguinal LNs) was determined for i.n. (**IN**) and i.m. (**IM**) antigen administration, respectively. Data is representative of LNs from 3 separate mice.

Both LN stromal cells and activated T cells express CD200

Our earlier observations regarding CLN-restricted tolerance induction strongly suggested that stromal cells are capable of instructing immune cells in such a way that immune tolerance is induced in cervical, but not in peripheral LNs [33]. Since we demonstrated that IDO is differentially expressed in CLN-DCs when compared to PLN-DCs and that this expression requires CD200, we hypothesized that stromal cells within the T cell area instruct DCs to express IDO. We analyzed the expression of CD200 on LN stromal cells in immune activated CLNs and PLNs by immunofluorescence. BALB/c mice were injected with T cells with a transgenic TCR for OVA (isolated from D011.10 mice) and PLNs and CLNs were analyzed 3 days after administration of OVA. Double

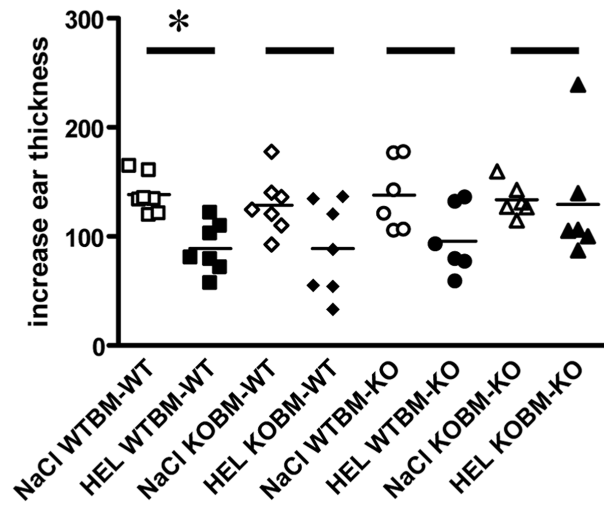

Figure 5: Both stromal and hematopoietic cells need to express CD200 for immune tolerance induction to intranasally administered antigen. Irradiated WT mice infused with WT BM (**WTBM-WT**), irradiated WT mice infused with CD200$^{-/-}$ BM (**KOBM-WT**), irradiated CD200$^{-/-}$ mice infused with WT BM (**WTBM-KO**) and irradiated KO mice infused with KO BM (**KOBM-KO**) received either HEL or NaCl intranasally. On day 4 after intranasal administration, they were challenged s.c. with 100 μg HEL in IFA. Subsequent challenge took place on day 9 by injection of 10 μg HEL in the auricle of each ear. Ear thickness increase was measured as DTH response one day later. Data are means of both ears of the animals (6 or 7 mice per group). Bars indicate the mean of each group. Differences between groups are significant ($p < 0.05$) when indicated by *.

staining for gp38 and CD200 was performed and T cell areas adjacent to the B cell follicles were analyzed (fig. 4A and 4B). Within CLNs, the FRCs were often double positive for gp38 and CD200, as can be seen by the violet color, while this was less evident in PLNs, suggesting a lower expression of CD200 on FRCs in PLNs. Expression of CD200 on FRCs could also be observed in resting LNs (data not shown). In addition, high endothelial venules (HEVs) were gp38$^-$ CD200$^+$, while all FDCs expressed both CD200 and gp38 (fig. 4A and 4B and data not shown). Although these data are suggestive for enhanced expression of CD200 in CLNs compared to PLNs, we wished to see whether there is indeed a differential level of production of CD200 by stromal cells in these LNs. Therefore, real time PCR was performed on CD45$^-$ stromal cells sorted from resting CLNs and PLNs. Results indicate that indeed CD200 mRNA expression was higher in stromal cells from CLNs when compared to PLNs (fig. 4C). To further address whether also T cells could contribute to CD200R triggering, CFSE labeled OTII cells were injected into C57BL/6 mice and analyzed at 72 hrs after intranasal administration of OVA. While resting T cells did not express CD200, activation of T cells in CLN lead to a strong induction of CD200, which

could be observed in activated non-divided as well as divided T cells (fig. 4D). After i.m. administration of OVA, CD200 expression was induced on T cells in PLN at even higher levels when compared to antigen specific T cells in CLN (fig. 4E and 4F).

CD200 expression by stromal cells as well as hematopoietic cells is required for efficient tolerance induction

Since both stromal as well as T cells could contribute to CD200R triggering on DCs, we wished to determine which cell type was required for intranasal tolerance induction. Therefore, BM chimeras were generated to study the contribution of CD200 expression on stromal and hematopoietic cells in the induction of immune tolerance. Irradiated $CD200^{-/-}$ (KO) and WT mice received either KO BM or WT BM infusions. After 19 weeks, these chimeric mice received antigen intranasally and were subsequently sensitized and challenged with antigen. While we observed that WT mice that received WT BM (WTBM-WT mice) became immune tolerant as a result of intranasal antigen administration, KOBM-KO mice failed to do so, confirming our earlier observations. In the chimeric mice, intranasally administered antigen did not lead to a significant different DTH response when compared to mice that received NaCl intranasally, showing that tolerance could not be induced in WT mice with KO BM (KOBM-WT) nor in KO mice that had received WT BM (WTBM-KO) (fig. 5). Therefore, CD200 expression on both stromal cells as well as on hematopoietic cells is necessary for tolerance induction.

Discussion

Here we show that intranasal tolerance induction is dependent on the presence of CD200 and that this correlates with impaired expression of IDO in non-plasmacytoid interstitial DCs present in the nose draining CLNs. Furthermore, expression of CD200 on stromal cells was enhanced in CLNs when compared to PLNs, which could account for the differential expression of IDO in CLN-DCs versus PLN-DCs. Our results emphasize that local differences in stromal elements between mucosal and peripheral LNs may be of utter importance to direct immune responses, underlying the default ability of cervical LNs to induce tolerance to soluble antigens. This is also evident from our transplantation studies where we could show that a substantial time after transplantation, stromal elements such as FRCs and endothelial cells (ECs) are still of donor origin (manuscript submitted for publication). The persistence of donor stromal cells in the transplanted LNs suggests that these cells may proliferate locally and that their functional behavior is determined locally as well, likely by epigenetic regulation. CD200 may therefore be one of the intrinsic factors in cervical LNs with the ability of instructing DCs to upregulate IDO after CD200-CD200R interaction. However, also activated T cells may contribute to the induction of IDO, since our BM-chimeras showed that CD200 expressing hematopoietic cells are required for tolerance induction upon intranasal antigen administration.

Stromal cells may therefore predominantly contribute to the expression of IDO in resting CLN-DCs, which express higher levels of IDO than PLN-DCs, while activated T cells may contribute to further induction during an immune response. However, IDO expression in resting CLN-DCs might not solely be controlled by CD200 on stromal cells, since CD200 is also expressed on PLN stromal cells, albeit at lower levels. Additional regulatory mechanisms may exist as multiple CD200R homologues have been characterized (CD200RLa and CD200RLb), although at this point little is known about expression of these receptors on DCs [4, 38]. These receptors can pair with the adaptor protein DAP12, which is implicated in downregulation of IDO. Hypothetically, differential expression of these receptors on DC subsets may therefore add a possibility to steer IDO expression and further studies are needed to address this issue.

The induced expression of CD200 on activated T cells in both stimulated CLNs and PLNs might also be part of a mechanism to control the resolution phase of the immune response. Perhaps through the expression of CD200, T cells can induce IDO expression in DCs, thus controlling their own expansion. Timing of IDO expression may be of critical importance for modulation of the immune response. Whereas IDO is expressed at the start of the immune response in CLN-DCs, IDO expression in PLN-DCs is induced at a later time and this may

determine whether immune tolerance or immunity will occur. This is in line with recent findings showing that Tregs can induce IDO in dendritic cells by interaction of CTLA4 and CD80 [39-42]. In addition, DCs themselves can confer tolerance to other DCs in an IDO-dependent fashion. This does not necessarily need to depend on tryptophan starvation only but could also be a result of paracrine action of kynurenines, produced by enzymes downstream of IDO [43, 44].

Taken together, we show an important role for CD200 in mucosal tolerance induction, most likely through CD200-CD200R interaction between DCs and stromal cells in the LNs, leading to upregulation of IDO expression by DCs and the induction of mucosal Treg cells.

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