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CHAPTER 7

Summarizing Discussion

Introduction

Antigens that enter the body continuously challenge the immune system. Especially at mucosal sites, which are in direct contact with the outer environment, exposure to foreign antigens occurs at high frequency. To protect against harmful antigens, specific antigen recognition receptors allow for the induction of effective immune responses. However, not all antigens are harmful and the immune system tolerates their presence by inhibiting inflammatory responses. The induction of T regulatory cells (Treg cells) that are capable of suppressing T effector cell (Te cell) responses is of major importance in this process. Since dendritic cells (DCs) are important instructors of T cells, we set out to identify regulatory molecules expressed by DCs that play a role in immune tolerance induction. In this thesis, we have shown the necessity of several molecules for the induction of mucosal tolerance. Furthermore, the influence of the microenvironment in regulation of immune responses was investigated. The results of these studies will be summarized and discussed in this chapter.

Attenuation of mucosal immune responses

Foreign antigens are abundant at mucosal sites, but many of them are non-harmful. DCs that take up antigen at these sites are able to efficiently induce immune tolerance to non-harmful antigens. This is striking, as the microenvironment in which this tolerance induction takes place is loaded with factors that are known to induce inflammatory responses. In order to recognize specific antigens for the induction of appropriate immune responses, DCs express pattern recognition receptors (PRRs) such as toll-like receptors (TLRs). Interestingly, these TLRs are able to recognize both harmful and non-harmful antigens. To further distinguish between different antigens and to induce an appropriate immune reaction, it is likely that DCs have regulatory mechanisms in order to control activation by pathogen-associated molecular patterns (PAMPs) and prevent inflammatory reactions to harmless antigens. An interesting molecule to execute this task is serine leukoprotease inhibitor (SLPI), an enzyme predominantly expressed at mucosal surfaces. SLPI inhibits lipopolysaccharide (LPS)-induced endotoxic shock by preventing CD14 – TLR-4 association and subsequent downstream signalling for activation of DCs [1-4]. We hypothesized that DCs at mucosal surfaces may express this enzyme in order to attenuate the continuous activation signals provided by the presence of large amounts of microbes at this site. To study mucosal tolerance induction, we used our model based on nasal antigen administration as previously described [5]. The first indication for a role of SLPI in DC-mediated mucosal tolerance was our finding that SLPI is only expressed by DCs isolated from lymph nodes (LNs) draining mucosal surfaces (chapter 2). The fact that

SLPI is mainly found in fluids lining mucosal surfaces further supports a mucosa-associated expression pattern. The inhibitory role of SLPI in DCs was shown by in vitro studies with SLPI$^{-/-}$ bone marrow (BM)-DCs, which showed enhanced activation when compared to wild type (WT) BM-DCs after stimulation with LPS, resulting in increased proliferation of T cells. Furthermore, when DCs were isolated from the nose draining cervical lymph nodes (CLNs) after intranasal (i.n.) antigen administration, DCs from SLPI$^{-/-}$ mice showed enhanced expression of costimulatory molecules and inflammatory cytokines when compared to WT mice. However, this elevated expression did not abrogate tolerance induction in SLPI$^{-/-}$ mice when challenged for a delayed-type hypersensitivity (DTH) response after i.n. OVA antigen administration. The difference in DTH response between SLPI$^{-/-}$ mice and WT mice only became clear after adding small amounts of LPS to the i.n. OVA adminstration. The presence of low levels of LPS resulted in impaired tolerance induction in SLPI$^{-/-}$ mice, while tolerance induction in WT mice could still occur. Thus, SLPI is produced upon antigenic stimulation to attenuate inflammatory responses simultaneously induced by LPS, hereby allowing for tolerance induction to the specific antigen. It remains to be investigated how SLPI production by DCs is induced. It is not unlikely that also stromal elements within the CLNs could play an instructive role for the induction of SLPI in DCs present within mucosa draining LNs. Whether the endogenous production of SLPI is enough for the inhibition of DC activation or additional production by for instance mucosal secretory cells is required is not yet clear. Secreted SLPI has been shown to bind LPS in vitro, preventing LPS-CD14 ligation [6]. Also blockade of CD14-TLR4 association, resulting in inhibition of downstream signalling through IRAK, IκBα, and IκBβ has been described [1-4]. Cytosolic SLPI may prevent degradation of the inhibitory factor IkBa upon TLR2 and TLR4 signalling, reducing the amount of NF-κB entering the nucleus for transcriptional activation [7]. In addition, blockade of NF-κB binding sites by SLPI, resulting in inhibition of NF-κB function has also been reported [3]. In our study, we show that SLPI is necessary for the inhibition of endotoxin-induced activation signals in DCs at mucosal sites which are continuously loaded with LPS. This attenuation minimizes LPS interference with the induction of tolerance to harmless antigen. High doses of LPS indicative of a serious pathogen challenge will abrogate the inhibition by SLPI, allowing for effective immune responses.

Molecular mechanisms for the induction of antigen-specific tolerance

Although SLPI is needed for efficient tolerance induction, it seems that SLPI is not directly involved in the generation of immune tolerance. Instead, SLPI allows for the ability of tolerance induction at sites with a continuous triggering

of PPRs by attenuating LPS-induced activation signals. Therefore, the ability of mucosa-draining LNs to induce antigen-specific tolerance must be established through other factors. Together with the enhanced expression of SLPI in DCs from CLNs compared to DCs from PLNs, we also found a similar enhanced expression of indoleamine 2,3-dioxygenase (IDO), as shown in chapter 3. IDO has been described as an immune regulatory enzyme due to its ability to modulate tryptophan metabolism. By degrading tryptophan, IDO depletes proliferating cells of this essential amino acid, but also the formed metabolites called kynurenines may have immune-regulatory properties [8-11]. We set out to study the role of IDO in tolerance induction by inhibiting IDO during i.n. antigen administration. Upon IDO blockade, the induction of antigen-specific tolerance was impaired, either because Treg cells were completely absent or dysfunctional. The requirement of IDO for mucosal tolerance induction correlates with data from several other studies that reported the necessity for IDO in tolerance induction [12-15]. We did not find a differential expression of the transcription factor forkhead box P3 (FoxP3) when nasal tolerance induction was blocked, suggesting that the mechanism of Treg cell induction differs from that of naturally occurring Treg cell (nTreg cell) induction where FoxP3 expression is upregulated [16]. It has been shown that naive $CD4^+CD25^-$ cells can be converted to $CD4^+CD25^+$ Treg cells by expression of FoxP3 [16, 17], but in our model T cells from tolerant and non-tolerant mice showed equal expression of FoxP3 and also IDO inhibition did not alter FoxP3 expression in these cells. We have recently reported that for antigen-specific tolerance induction, Treg cells are induced within the $CD4^+CD25^-$ population [18]. Therefore, FoxP3 may be less important for the induction of antigen-specific mucosal Treg cells than for the development of naturally occurring Treg cells. Interestingly, IDO did have an effect on the survival of T cells. After 48 hours of i.n. antigen administration, a reduction of dead antigen-specific T cells could be seen in CLNs of mice with IDO inhibitor, suggesting that T cells are selectively deleted through the activity of IDO. However, we could not find significant differences in apoptosis or proliferation of T cells and the mechanism by which IDO induces tolerance remains to be further elucidated. By deletion of Te cells, IDO may shift the balance between Te and Treg cells towards the Treg cells, hereby allowing for tolerance induction. Also the mechanism of induction of IDO expression remains to be clarified. It has been shown that IFN-γ and CD80/CD86 ligation by CTLA-4 expressed on Treg cells can induce production of IDO in DCs [19-22]. In earlier studies, we have described the rapid upregulation of CTLA-4 on stimulated T cells in CLNs, with CTLA-4 expression already present on the early proliferating cells [23]. In PLNs, this upregulation does not show until the fourth cell division. In addition, IFN-γ production is prolonged in antigen-specific T cells in CLNs but not in PLNs. These circumstances could allow for rapid induction of IDO expression in

DCs from CLNs when compared to DCs from PLNs and this enhanced response may shift the balance towards the generation of sufficient amounts of Treg cells to suppress Te cell responses.

Environmental factors in tolerance induction

The mucosa-specific production of IDO and SLPI in DCs were obvious reasons to further investigate a role for tissue-specific factors in steering immune responses. Our finding that lymph node stromal cells express a variety of receptors and ligands allowing interactions with immune cells already implicated a role for stromal components in immunological processes (chapter 5). Additional indications that environmental factors can influence immune responses comes from transplantation studies in which CLNs were removed and replaced by PLNs. Normally, tolerance induction occurs in nose-draining CLNs, but this was not possible in fully functional nose-draining PLNs [24]. Apparently, intrinsic factors within the CLNs create a specific microenvironment that favors the induction of tolerogenic immune responses. Therefore, we decided to investigate the LN stromal cells of the fibroblastic reticular system (FRS) that create the microenvironment within the LN by providing a cellular framework through which hematopoietic cells can move. Interestingly, a study by Fallarino et al. reported induction of IDO in plasmacytoid DCs (pDCs) by ligation of CD200R. The ligand CD200 is expressed by B cells and T cells, but also by non-hematopoietic cells as follicular dendritic cells (FDCs), neurons from the central nervous system (CNS) and vascular endothelium [25, 26]. CD200 has been described as an immunoregulatory molecule, since blocking resulted in expansion of myeloid cells and enhanced auto-immune diseases [25, 27]. The enhanced IDO expression in DCs from CLNs compared to DCs from PLNs and the indications of a specific tolerogenic microenvironment in the CLNs encouraged us to compare CD200 expression on stromal cells from CLNs and PLNs. Indeed, we found enhanced expression of CD200 on stromal cells from CLNs when compared to stromal cells from PLNs (chapter 4). Immunofluorescent analysis revealed differential expression of CD200 on the gp38^{+} follicular reticular cells (FRCs) from CLNs and PLNs. In addition, T cells rapidly upregulated CD200 expression after activation. To further investigate the role of CD200 in nasal tolerance induction, we compared groups of CD200$^{-/-}$ and WT mice in our i.n. tolerance induction model. Strikingly, CD200$^{-/-}$ mice failed to become tolerant to the administered OVA as was shown by the increased DTH response when compared to WT mice. Furthermore, purified CD4^{+} cells from CD200$^{-/-}$ mice that received OVA i.n. could not induce tolerance in naive mice, indicative of impaired or dysfunctional Treg cell induction, similar to the effects of IDO blockade. To further establish an in vivo role for CD200 in IDO induction in DCs, we showed impaired IDO expression in

CLN-DCs from CD200$^{-/-}$ mice. Although the enhanced expression of CD200 on FRCs from CLNs suggests that CD200 is the discriminating factor for nasal tolerance induction, we observed that hematopoietic cells contribute to CD200-mediated tolerance induction as well. By creating bone marrow (BM)-chimeras and using them in our nasal tolerance induction model, we could show that CD200 expression on stromal cells alone is not enough for efficient tolerance induction, but also expression of CD200 on hematopoietic cells is needed. The phenotype of the hematopoietic cells responsible for this process needs further study. Interestingly, examination of LN sections from CD200$^{-/-}$ mice that had received WT BM revealed CD200 expression by some gp38^{+} stromal cells. Thus, stromal precursors may be present within the BM transplant, as also suggested in other studies [28, 29]. The higher expression of CD200 on stromal cells within the CLNs may create a tolerogenic microenvironment, enhancing IDO production in CLN-DCs.

Stromal cell subsets

The differential expression of CD200 on gp38^{+} cells from CLNs led us to further investigate LN stromal cells by FACS analysis. By staining for gp38 and CD200 together with markers for endothelial cells (ECs) and follicular dendritic cells (FDCs), we could identify 5 subsets of stromal cells within the LNs. ECs positive for MECA32 and CD31 were identified as gp38^{-}CD200^{hi} cells, together with cells expressing CD35 and FDC-M2, indicative of FDCs (chapter 5). Interestingly, FDCs are known to express high levels of CD200 and these gp38^{-}CD200^{int} cells may be the progenitors of FDCs while the mature FDC population is comprised of gp38^{hi}CD200^{hi} cells. The gp38^{-}CD200^{int} and gp38^{int-hi}CD200^{int} cell populations were mostly negative for EC and FDC markers and contained the FRCs. Next to CD200, we could show expression of several other molecules by real time PCR analysis of LN stromal cells. For gp38, a role in tissue development and repair, as well as carcinogenesis has been suggested [30, 31]. Gp38 also plays a role in lymphatic vessel formation [32]. The expression of CD40 on a subset of stromal cells allows them to interact with activated T cells and this may facilitate remodeling of the extracellular matrix to accommodate the proliferation of T cells. Stromal cells also express the DC proliferation factor Flt3L, but its role here remains to be clarified. Furthermore, angiopoietins ANG-1 and ANG-2 and the VEGF molecules expressed by stromal cells may allow for rapid vasculature remodeling in LN during immune responses [33, 34]. Next to these ligands for hematopoietic cells, stromal cells also express receptors that can provide stimulatory signals upon ligation. By immunofluorescence, PDGF-Rβ expression could be seen. Signaling through PDGF-Rβ is necessary for pericyte recruitment during blood vessel differentiation [35]. Signaling through TNF-R1 and LTβ-R is crucial for the structural organization within the LNs by

inducing CXCL13, CCL19, and CCL21 chemokine production [36]. LTβR is expressed on HEVs and may interact with $LT\alpha_1\beta_2$ on transmigrating leukocytes. This interaction could induce the expression of adhesion molecules on HEVs, as has been suggested from a study showing reduced expression levels of peripheral lymph node addressin (PNAd) and mucosal addressin cell adhesion molecule (MAdCAM) on HEVs upon LTβR-blockade [37]. It is likely that ligation of stimulatory receptors such as TNF-R1 and CD40 results in remodeling of the FRS while it may also enhance chemokine production [38]. Chemokine gradients may be established by the presence of chemokine-binding glycosaminoglycans on FRCs and components of extracellular matrix, allowing for targeting of immune cells to specific sites in the LN for the efficient induction of an immune response [39]. Indeed, such a role has been described for FRCs, as it was shown that T cells migrate along FRCs towards T cell areas [40]. Thus, it seems that stromal cells are more than just passive building blocks of the LNs.

Induction of homing molecules on recirculating T lymphocytes

Since our studies showed that LNs draining mucosal contain a unique microenvironment that contributes to the induction of tolerance, it is logical to assume that also entry of immune cells to these specific sites is controlled by the microenvironment. The mechanism of mucosal T cell homing involves the induction of $\alpha_4\beta_7$ integrin and CCR9 expression on activated T cells [41-45]. To further investigate the role of stromal cells in this matter, we decided to use LN transplantation as a method to study the role of LN microenvironments in the induction of tissue tropic molecules on T cells. By transplantation of GFP-expressing LNs, we could show by immunofluorescence that only stromal cells were GFP-positive after 12 weeks of transplantation (chapter 6). Furthermore, transplantation of LNs from CD45.1 mice into CD45.2 congenic hosts showed that virtually no CD45.1 hematopoietic cells remain in the transplanted LNs. These conditions allowed us to study the effect of specific MLN stromal factors on hematopoietic cells in a peripheral location, without intestinal factors draining into the LNs. We proposed that the microenvironment in mucosa-draining LNs facilitates the induction of $\alpha_4\beta_7$ integrin and CCR9 expression in T cells. Stimulation of antigen-specific transgenic CD4 T cells in these transplanted MLNs by subcutaneous injection of antigen showed that activated T cells within the LNs upregulate $\alpha_4\beta_7$, giving them gut-homing capabilities. In addition, stimulated antigen-specific transgenic CD8 T cells showed similar results for induction of $\alpha_4\beta_7$, but failed to show a significant difference in the expression of CCR9. Other investigators have reported that induction of CCR9 on T cells is specific for $CD103^+$ DCs while induction of $\alpha_4\beta_7$ can be established by both $CD103^+$ and $CD103^-$ DCs [41, 43]. It has been suggested that $CD103^+$

DCs represent the DC subset that has migrated from the intestinal lamina propria into the LNs [46]. This would explain the failure to induce CCR9 expression on T cells in transplanted MLNs, as these $CD103^+$ DCs are not present in skin. For the induction of $\alpha_4\beta_7$ and CCR9 in T cells, retinoic acid (RA)-binding to its nuclear receptor is required [42]. RA synthesis results from retinal degradation by retinal dehydrogenases (RALDH). Of the four RALDH isotypes, RALDH2 is the most prominent in MLN-DCs. By real time PCR, we showed that RALDH2 expression was enhanced in DCs from transplanted MLNs when compared to DCs from transplanted PLNs. This clearly indicates a role for stromal cells in the induction of tissue-specific homing of T cells by stimulating RA production by DCs. Additional factors from the lamina propria of the gut may also induce expression of RALDH. $CD103^+$ DCs migrating from the gut into the MLNs may express higher levels of RALDH enzymes. This, together with the RALDH enzyme expression induced by stromal cells in the MLN, may lead to higher production of RA in non-transplanted MLNs. CCR9 expression on T cells may require higher levels of RA and this would explain why we did not see CCR9 expression in transplanted MLNs.
Thus, it seems that the specific microenvironments in both gut and gut-draining secondary lymphoid tissues contribute to the induction of tissue-specific immune cells. How stromal cells in LNs are induced to distinctly differentiate in various LNs is unknown. The imprinted environmental characteristics of LNs are in agreement with a previous study that shows expression profiles becoming fixed during the development of LNs in the neonatal period [47]. In this period, it may very well be that the presence of microbes at specific sites influences the local stromal cells to enhance specific expression profiles, which subsequently become fixed during the developmental completion of the LNs. This could explain the tissue-specific expression of immune-regulatory molecules such as SLPI, IDO and CD200 and it will be interesting to see how these expression patterns emerge during LN development.

Concluding remarks

Mucosal sites are continuously challenged with large amounts of antigens. The majority of these antigens is non-harmful and therefore mucosal immune responses preferentially lead to the induction of antigen tolerance. For this tolerance induction, we have shown a role for SLPI in DCs from nasal mucosa draining LNs by reducing LPS-induced activation of DCs, resulting in decreased T cell responses. Attenuation of inflammatory signals may allow for the ability of tolerance induction by molecular mechanisms such as the expression of IDO in DCs. The expression of IDO can be regulated by CD200R ligation on DCs, as $CD200^{-/-}$ mice fail to induce tolerance and DCs from $CD200^{-/-}$ mice show

impaired IDO production. By enhanced expression of CD200, LN stromal cells from mucosa-draining LNs may influence IDO production in DCs, allowing for tolerance induction. In addition, LN stromal cells express a variety of costimulatory molecules and receptors, indicative of their capacity to closely interact with activated immune cells. This could induce remodeling of the FRS to facilitate efficient interaction of immune cells and subsequent immune responses. The induction of tissue-specific homing receptors on immune cells by microenvironmental factors allows for efficient circulation between tissues and draining LNs and may facilitate regulation of specific immune responses. Taken together, these studies indicate that LN stromal cells can no longer be seen as passive structural elements but are active cells that not only respond to- but also modulate inflammatory signals and immune responses.

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