

MHC class II antigen presentation by B cells in health and disease $\mathsf{Souwer},\,\mathsf{Y}$

Citation

Souwer, Y. (2009, December 1). MHC class II antigen presentation by B cells in health and disease. Retrieved from https://hdl.handle.net/1887/14474

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Chapter 8

Summarizing discussion

MHC class II antigen presentation plays a pivotal role in human health and disease. One the one hand antigen presentation via MHC class II molecules activates CD4+ T cells to produce cytokines and express CD40L to stimulate B cells to produce antibodies. In this thesis we studied the importance of MHC class II antigen presentation by B cells and the antibody response in bacterial infection. On the other hand, after MHC class II antigen presentation the activated CD4+ T cells give help to CD8+ T cells. The activated CD8+ T cells can now exercise their cytotoxic activities to eliminate defective/transformed or infected cells. Disruption of MHC class II antigen presentation could play a role in the immune evasion of cancer cells; therefore we investigated the MHC class II antigen presentation pathway in leukemia. The importance of MHC class II antigen presentation becomes clear in patients that have a deficiency in MHC class II antigen presentation. This rare primary immunodeficiency disease, called Bare Lymphocyte Syndrome type II (BLSII), is characterized by the absence of expression of MHC class II proteins (1). The MHC class II genes themselves are unaltered in these patients, but their expression is abolished by mutations in transcription factor genes that initiate transcription of MHC class II genes. The result is that patients have a severe defect in both cellular and humoral immunity and exhibit an extreme vulnerability to infections. Infections start within the first year of life, there is a dramatic progression of various types of infectious complications and patients generally die before the age of 10. This demonstrates that a defect in MHC class II antigen presentation can only poorly be compensated for by the other players of the innate and acquired immune system.

Phagocytosis of bacteria by B cells

Classically, professional phagocytes include neutrophils, monocytes, macrophages, dendritic cells (DCs), and mast cells. Professional phagocytes have receptors on their surfaces that can detect harmful objects that are not normally found in the body, such as pathogenic bacteria. Phagocytes are therefore crucial in fighting infections, as well as in maintenance of health in tissues by removing dead and dying cells that have reached the end of their life-span. Hallmarks for phagocytosis are the internalization of large particles (typically >500 nm in diameter), with reorganization of the actin cytoskeleton and pseudopodia extension (the formation of a phagocytic cup). The dogma is that B cells lack phagocytic capacities, but recently it was shown that B cells from early vertebrae (teleost fish and

amphibians) are potent phagocytes (2). The authors suggested that the phagocytic capacity of B cells was already present in a common ancestor at the time of the phylogenetic split of teleosts from amphibians, but that mammalian B cells seemed to have lost that innate immune capacity. Indeed, although human B cell lines had been described to present particulate Ags in the context of MHC class II (3, 4) and to extract Ag from a non-internalizable surface (5), human primary B cells were thought not to be able of phagocytosing large particles because they have little space in the cytoplasm and a relatively large nucleus.

Since human primary B cells are not considered as phagocytic cells, how do they acquire antigens from bacteria? The dogma is that B cells capture antigen from follicular dendritic cells (FDCs) in lymphoid follicles of the spleen, lymph nodes (LNs) and mucosal lymphoid tissues (6). Another way could be via normal DCs, which have been shown to recycle internalized antigens to their cell surface and present these in an unprocessed form to B cells (7). Recently, subcapsular sinus macrophages have been identified in LNs as an important site of B cell encounter with particulate antigen (8-10). Since B cells have been shown to extract antigens from a non-internalizable surface, antigen extraction from the surface of other cells could be a way to internalize bacterial antigens. Alternatively, bacteria may translocate to regional LNs (11) or to B cell areas in the spleen and mucosaassociated lymphoid tissue (MALT), where B cells may directly extract antigens from the bacteria themselves. In Chapter 2 the human B cell line Ramos is used in combination with anti-IqM coated beads to show that human B cell lines are indeed very capable phagocytic cells when triggered via the B cell receptor (BCR). Ramos cells completely internalize anti-IgM coated beads but irrelevant coated beads are not internalized. As a more physiological model system we used the bacterium Salmonella. In contrast to the current dogma, we demonstrated that also naïve and memory primary B cells are able to phagocytose whole, living Salmonella. Further analysis showed that this occurs via the BCR and that phagocytosis via the BCR results in activation of the B cell and secretion of immunoglobulins. The antibodies produced by B cells that have internalized Salmonella are reactive to Salmonella, again showing involvement of the BCR. The relatively high percentage of circulating B cells that recognize Salmonella via their BCR can be explained by the expression of a polyreactive BCR (also reactive to other bacteria) by CD27⁺ circulating marginal zone B cells (12). As for IgM⁺ memory B cells, also a subset of mature naïve B cells in peripheral blood express a BCR of polyreactive nature (13). Next to

antibody production, we showed in **Chapter 2** that phagocytosis of *Salmonella* also leads to rapid antigen presentation via MHC class II molecules to CD4+ T cells. In turn, activated CD4⁺ T cells give help to B cells, as antibody secretion is enhanced after incubation B cells that have internalized *Salmonella* and CD4⁺ T cells. The activation of CD4⁺ T cells is bacteria-specific, as we showed that T cells primed against *Staphylococcus* do not respond upon restimulation with B cells that have phagocytosed *Salmonella*.

The B cell as transport vehicle for Salmonella

Since Salmonella is a facultative intracellular bacterium, the question arises what the fate is of Salmonella once inside the B cell. **Chapter 3** describes the possible role of B cells in the dissemination of Salmonella after oral ingestion. We showed that (unlike macrophages, neutrophils and to a lesser extent DCs (14, 15)) B cells are not able to kill Salmonella after uptake via the BCR. However, replication of Salmonella is repressed in living B cells, but in apoptotic B cells Salmonella starts to multiplicate again. We noticed release of viable bacteria from B cells hours after phagocytosis and these excreted bacteria could reinfect other cells in vitro. To evaluate the role of B cells as transporters of Salmonella in vivo, we performed experiments in mice. These experiments showed that adoptive transfer of Salmonella-specific B cells in wild-type mice enhanced mortality after oral administration of a sub-lethal dose of Salmonella. Moreover, Salmonella were found in the spleen of mice that had received Salmonella-specific B cells and not in the spleen of mice that had not received Salmonella-specific B cells.

Cross-presentation of Salmonella antigens by B cells

B cells belong to the group of "professional antigen presenting cells" mainly because of their very efficient way of internalizing antigen. Other members are DCs and macrophages, which like B cells, display fragments of antigens via MHC class II molecules on their cell surface. Next to presentation via MHC class II molecules, DCs are able to cross-present exogenous antigens via MHC class I molecules to CD8+ T cells (16). In **Chapter 4** we showed that B cells are also able to cross-present *Salmonella* antigens and activate CD8+ T cells. Not surprisingly, this activation of CD8+ T cells is dependent on CD4+ T cell help, as culturing of B cells that had phagocytosed *Salmonella* with only CD8+ T cells did not result in activation of the CD8+ T cells. Upon activation, CD4+ T cells produce IL-2 and adding IL-2

together with CD8+ T cells restored the activation of CD8+ T cells by B cells. Activation and proliferation alone is not sufficient to eliminate infected cells, CD8⁺ T cells need to degranulate upon encountering an infected cell. We performed cytotoxicity assays to show that *Salmonella*-primed CD8⁺ T cells degranulate (measured by CD107a expression on the surface of CD8⁺ T cells) and are truly killing infected cells (measured by release of ⁵¹Chromium from the cytoplasm of infected B cells). In addition we showed in **Chapter 4** that the CD8⁺ T cell response induced by infected B cells is a memory response, since naïve CD8⁺ T cells could not be primed by B cells. This in contrast to the CD4⁺ T cell response, in which both naïve and memory CD4⁺ T cells were activated by B cells that had phagocytosed *Salmonella* (unpublished results).

The role of B cells in Salmonella infection

After oral uptake, Salmonella crosses the intestinal epithelium and enters the Peyer's patches via specialized antigen-sampling M cells (17) or luminal capture by DCs (18, 19). After entry in the Peyer's patches, Salmonella immediately meets with B cells that can phagocytose the bacteria and activate CD4⁺ T helper cells and secrete anti-Salmonella antibodies. B cells are necessary for efficient protection against both primary and secondary infection with Salmonella (20) and generation of high-affinity antibodies is not the only function of B cells in salmonellosis, since passive transfer of Salmonella-immune serum could not restore immunity of mice to Salmonella (21). B cell deficient mice have impaired Th1 T-cell responses from the early stage of Salmonella infection, showing that B cells play an essential role in the initiation of T-cell-mediated protection (22). Human B cells are able to prime naïve CD4⁺ T cell and also activate memory CD4⁺ cells, but are not able to kill Salmonella and disseminate the bacterium through the body. However, B cells that have taken up Salmonella are able to activate memory CD8+ T cells that subsequently kill infected cells. During primary infection, other antigen presenting cells (e.g. DCs) are still needed for priming of naïve CD8⁺ T cells. Salmonella thus uses the immune system of the host for survival and dissemination through the body via antigen-specific B cells, but the evolving immune system generates anti-Salmonella antibodies and activated CD4⁺ T helper and CD8⁺ CTLs. This is an example of interaction between bacteria and host, which seems to be difficult to balance for the human host and might explain the pathogenicity of Salmonella.

Dead bacteria are also recognized by the BCR, but not phagocytosed. In Chapter 2 we showed that although not completely taken up, antigens from dead bacteria are still presented by B cells to CD4⁺ T cells and activate the latter. In this way, B cells can be activated by CD4⁺ T cells to differentiate into antibody secreting plasma cells yielding an effective immune response for subsequent infections. Chapter 4 shows that B cells do not cross-present antigens from dead bacteria to CD8⁺ T cells, indicating that Salmonella itself contributes to cross-presentation of its own antigens. Living Salmonella injects proteins into the cytosol of the host cell via its TTSS to ensure its intracellular survival. Inevitably, these proteins will be degraded by the proteasome. Peptides can then be shuffled into the ER, loaded onto newly synthesized MHC class I molecules and presented to CD8⁺ T cells. Dead Salmonella have no functional TTSS anymore which may explain the lack of cross-presentation of dead bacteria. The finding that B cells that harbor dead Salmonella do not activate Salmonella-specific CTLs may fit with the concept that this is also immunologically unneeded; in this situation the cells do not form a niche for Salmonella survival and spreading, thus their immediate clearance may be less important for survival of the host.

MHC class II antigen presentation in leukemia

Professional APCs take up apoptotic cells and debris to initiate a CD4+ T cell response. Dying tumor cells can also be taken up, but sometimes the APC does not succeed in generating an effective immune response against the tumor cells, since the cancer is not always cleared. However, many tumors of the hematological system express MHC class II molecules on the cell surface. Apparently, MHC class II antigen presentation by these tumor cells did not succeed in the generation of an effective immune response which might be the result of aberrancies in the MHC class II antigen presentation pathway. We have studied the MHC class II antigen presentation pathway in the myeloid leukemia AML and in more detail in the lymphoid leukemia B-CLL.

In **Chapter 5** we showed that the self-peptide CLIP was expressed by MHC class II positive AML blasts. Patients with HLA-DR⁺/CLIP⁻ blasts had a significant longer disease-free survival than patients with HLA-DR⁺/CLIP⁺ blasts, indicating that the expression of CLIP on the blasts could be a measure for immune escape by the leukemic cells. Exploring the MHC class II antigen presentation pathway, we found that HLA-DO and HLA-DM (the peptide editors of the MHC class II antigen

presentation pathway) were readily detectable in AML blasts both on the mRNA and protein level. The relative expression of DO and DM correlated with the efficiency of antigen loading on DR molecules: the more DO relative to DM, the more CLIP relative to DR. Together this led to the hypothesis that HLA-DR⁺/CLIP⁻ blasts are able to present leukemia-specific antigens to CD4⁺ T cells, priming an effective antitumor response that results in a prolonged disease-free survival. Strategies to down-modulate CLIP expression on leukemic blasts are being explored to provoke an effective anti-tumor response in AML. Retroviral transduction of specific Ii siRNAs in two human myeloid leukemic cell lines shows a reduced expression of CLIP relative to DR (van Luijn et al., submitted). Despite the reduced levels of DR at the cell surface (as a secondary consequence of knocking-down Ii), both cell lines strongly enhance activation of allogeneic CD4⁺ T cells. However, the induction of tumor-specific CD4⁺ T cells (and subsequent help by the induction of tumorspecific CD8⁺ T cells) by AML cells transduced with Ii siRNAs is not demonstrated yet. Other strategies could target DM and/or DO, the peptide editors. Unpublished data of our group show that when exposing B cells in vitro to dexamethasone, a strong increase in the amount of DM was observed, with no change in DR or DO protein levels. More DM relative to DO could enhance peptide loading of MHC class II molecules, without affecting the total amount of DR molecules available for antigen presentation.

The HLA-DOA mRNA expression level predicts prognosis in B-CLL

Next to the myeloid leukemia AML, we studied the MHC class II antigen presentation pathway in the lymphoid leukemia B-CLL. In **Chapter 6** we showed that expression of *DRA*, *DMB*, *DOA* and *DOB*, but also *Ii* is highly aberrant on the mRNA level. Since transcription of these genes is regulated (*DOB* and *Ii* partly) by CIITA, we analyzed mRNA levels of total CIITA and the lymphoid-specific promoters PIII and PIV. Total CIITA mRNA was significantly enhanced in comparison with mRNA levels in B cells from healthy controls. mRNA from CIITA-PIII was detected in all samples, but showed no difference with controls. The mRNA levels from the IFN-γ-inducible promoter CIITA-PIV were however significantly enhanced, indicating that transcription of CIITA in B-CLL is controlled by the coordinated activity of the B cell-lineage-specific promoter PIII and the IFN-γ-inducible promoter PIV. Increased transcription does not always lead to increased translation and enhanced protein levels, as we showed in **Chapter 6**. Analysis of protein

expression by semi-quantitative Western blotting showed no increase in protein expression by B-CLL cells compared with B cells from healthy controls. DM protein expression was even significantly lower in B-CLL patients.

We investigated the mechanism underlying the discrepancy between the observed transcriptional upregulation and the unvaried or downmodulated total protein levels of DR in the malignant B-CLL cells. We performed pulse chase analysis of DR in the B cells of three healthy donors and four B-CLL patients exhibiting various levels of transcriptional upregulation of DRA. A similar half-life was observed for DR in the malignant B-CLL cells and the healthy B cells, as in all cases approximately 90% of the newly synthesized DR pool was still present 24 hrs after biosynthesis. Moreover, the maturation of the DR complex and degradation rate of the class IIassociated Ii was comparable between healthy B cells and malignant B-CLL cells (unpublished results). Thus, unexpectedly, the malignant B-CLL cells demonstrated a comparable biosynthesis rate of DR as the healthy B cells, in spite of their up to 3 fold higher mRNA levels. Recently it was shown that mesenchymal stem cells show increased levels of CIITA-PIV in response to IFN-y, but that this is not reflected in an increase of HLA-DR protein due to cytoplasmic retention of the CIITA transcript (23). Since we also found upregulation of transcripts downstream of CIITA, it is not likely that CIITA-PIV is retained in the cytoplasm. Whether cytoplasmic retention of DRA transcripts could be an explanation for the normal or decreased levels of DR protein remains to be investigated. These data nicely illustrate that evaluating mRNA levels does not necessarily reflect expression levels of functional proteins.

In addition we analyzed whether the increased mRNA levels of the MHC II components showed a correlation with clinical outcome. Only *DOA* mRNA levels were significantly different when we compared survival after sampling between patients. If patients were divided into a group with *DOA* mRNA levels < 3.4 and ≥ 3.4 , *DOA* mRNA levels ≥ 3.4 defined a subgroup of patients with an unfavorable prognosis: 6 out of 8 patients with *DOA* mRNA levels ≥ 3.4 died during follow-up as compared to 4 out of 12 patients with *DOA* mRNA levels < 3.4.

As the change in transcription of the DO genes is not reflected by their protein levels, the question arises why DOA mRNA correlates with clinical outcome. It has been shown in B-CLL that several serum cytokine levels are elevated (24-26) and that both $CD4^+$ and $CD8^+$ T cells express significantly more IFN- γ and IL-4 than in healthy controls (27, 28). These pro-inflammatory cytokines can initiate transcription of CIITA-PIV, which is elevated in B-CLL. Thus, overexpression of DOA

mRNA might be the resultant of the aberrant immunological environment in B-CLL but can predict survival in B-CLL. This needs to be validated in a larger cohort of B-CLL patients and preferably in samples from the time of diagnosis.

Altered MHC class II antigen presentation is reflected in expansion of the activated T cell compartment in B-CLL

In Chapter 7 we quantitatively showed that actually both DM and DO are expressed lower at the protein level in B-CLL in comparison with healthy controls. Although we did not examine the biosynthesis rate of DM and DO, less expression of protein would suggest a difference in turnover of DM and DO protein in B-CLL patients compared to healthy controls. This is an interesting topic that deserves further research. However, the relative expression of DM over DO was significantly higher in B-CLL patients. Expression of DR at the plasma membrane did not differ, but CLIP levels were significantly lower in B-CLL patients. The correlation between the relative higher expression of DM and the reduced expression of the self-peptide CLIP suggests that variation in DM levels changed the peptide repertoire presented by DR molecules as it correlated to a reduced expression of the self-peptide CLIP. We next analyzed the differentiation and activation status of the T cell compartment. Chapter 7 shows that the T cell compartment in B-CLL patients was significantly decreased in naïve CD4⁺ and CD8⁺ subsets in favor of increased activated effector populations. The percentage of activated T cells inversely correlated with CLIP expression, pointing to improved antigen presentation. Altered MHC class II antigen presentation may thus be a new factor in the immune dysfunction and pathobiology of B-CLL.

New therapies against B cell malignancies comprise the use of monoclonal antibodies, especially against CD20 and CD52. The effector mechanism of anti-CD20 and anti-CD52 antibodies is mainly based on complement-dependent cellular cytotoxicity (CDCC) and antibody-dependent cellular cytotoxicity (ADCC) (29, 30). Treatment with these monoclonal antibodies seems promising, but in case of high B cell burden, exhaustion of the body's effector mechanisms may lead to substantial decreases of the immunotherapeutic efficacy.

The data in this thesis may form a new approach for therapy of B cell cancers. All chronic B cell leukemia's and B cell lymphoma's express IgM and/or IgD at the cell surface (31). Since we have shown that targeting particles to the IgM-type BCR will

lead to internalization and recently the same was demonstrated for IgD (32), targeting microparticles with anti-cancer agents to the constant region of the BCR would be an option. This approach may especially be attractive in the case of mantle cell lymphoma, which express high levels of IgM and/or IgD and in which patients have the poorest prognosis of all B cell cancers (33).

Anti-cancer agents packed in microparticles are less harmful for the host and targeted to the constant region of the BCR this will bring the drugs directly inside the cancer cells. High drug concentrations can be reached in the target cells, without harming other cells in the body. Thus, this strategy, may reduce the unwanted side effects of systemic treatment with anti-cancer drugs.

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