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MHC class II antigen presentation by B cells in health and disease

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MHC class II antigen presentation
by B cells
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Yuri Souwer

Cover: Onze eerste humane B cel die fluorescerent gekoppeld MHC klasse II tot expressie brengt, gevisualiseerd met een confocaal microscoop.

MHC class II antigen presentation by B cells in health and disease

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Chapter 1

General introduction

The immune system protects us from harmful microbial infections. Such protection results from the interplay between innate and adaptive (acquired) immunity, both of which involve differential recognition of self from infectious non-self. While innate immunity relies upon antigen-nonspecific pattern-recognition receptors to broadly sense offensive signals, adaptive immunity utilizes far more specific antigen receptors. These antigen receptors are expressed by B and T cells, and function to better discriminate various antigenic epitopes in order to achieve specific immunity and immunological memory (1, 2).

Bone marrow

Hematopoiesis is the process of production of mature blood cells, which primarily takes place in the bone marrow (3). Here, pluripotent stem cells reside that give rise to the various types of blood cells. Pluripotent stem cells maintain, in contrast to differentiated blood cells, their proliferative ability throughout the life of an individual. These pluripotent stem cells divide to produce two types of stem cells. A common myeloid progenitor that gives rise to the myeloid lineage, which comprises erythrocytes, platelets and most of the cells of the innate immune system, such as granulocytes, macrophages, mast cells, monocytes, and dendritic cells. A common lymphoid progenitor gives rise to the lymphoid lineage, which comprises the natural killer cells of the innate immunity and lymphocytes of the adaptive immune system. There are two types of lymphocytes, B lymphocytes (B cells) and T lymphocytes (T cells) (4).

Normal B cell development

In the body, the humoral immune response is mediated primarily by B cells and requires enormous variability in immunoglobulins to deal with all possible antigens. Immunoglobulins, or antibody molecules, consist of a constant region and a variable region. The constant region takes one of only five distinguishable forms which determine the effector function. The variable region is the actual antigen binding site and can be composed of an apparently endless variety of different amino acid sequences, forming subtle differences that allow antibodies to bind specifically to an equally infinite variety of antigens. In addition, multiple regulatory mechanisms are required to discriminate self from non-self and to yield long-lasting immunological memory.

B cells arise in the bone marrow from stem cells and differentiate through a complicated and highly regulated process into cells that can produce antibodies capable of recognizing specific foreign antigens. The development of B cells in the bone marrow is initiated by the assembly of genes for the variable regions of the heavy and light chains of antibodies in B cell progenitors which is obligatory for B cell antigen receptor (BCR) expression (5). The BCR is a membrane bound immunoglobulin and consists of two heavy and two light chains. First, the heavy chains of the BCR are produced, thereafter the light chains (6).

The variable regions of heavy and light chains are formed by DNA rearrangement of multiple different segments on the heavy and light chain loci (Fig. 1). This process is called V(D)J recombination, in which the gene rearrangements start at the heavy chain locus in pro-B cells with first random combinatorial joining of the D (diversity) and J (joining) gene segments (7). Subsequently, rearrangements of V (variable) gene segments to DJ-rearranged segments are induced and if successful at one allele, the other allele is turned off by allelic exclusion (8). Light chains consist of and rearrange V and J gene segments and are combined with the heavy chain. The recombination process generates an enormous antigen receptor diversity, which is further increased by random nucleotide introduction in the joining regions during recombination. An intact pre-BCR is expressed at the cell surface of the dividing pre-B cell.

B cell selection

During B cell proliferation, positive and negative selection checkpoints are set to test the competence of the (pre-)BCR (Fig. 2). In the bone marrow, positive selection occurs in cells expressing a transmembrane pre-BCR. This selection means that signaling from the pre-BCR is required to suppress V(D)J recombination and an appropriate pre-BCR signals for proliferative expansion (9). Whether pre-BCR cross-linking by an unknown ligand or simple surface expression is sufficient is still debated. Pre-B cells that fail to fulfill proper receptor requirements are developmentally arrested or forced into apoptosis (10). Thus, B cells expressing an appropriate pre-BCR will continue to develop. These immature B cells undergo negative selection at later stages of development, since most of the generated BCRs are self-reactive (11). This negative selection occurs before the immature B cells leave the bone marrow; if a BCR reacts with self-antigen in the bone marrow, receptor editing is induced. The process of receptor editing rescues the B cells when

secondary V(D)J rearrangements induce the replacement of the autoreactive antigen receptors to non-self reactive antigen receptors (12, 13). Immature B cells progress from a receptor-editing competent, apoptosis-resistant stage into a receptor-editing incompetent, apoptosis-sensitive stage (14). A B cell that passes

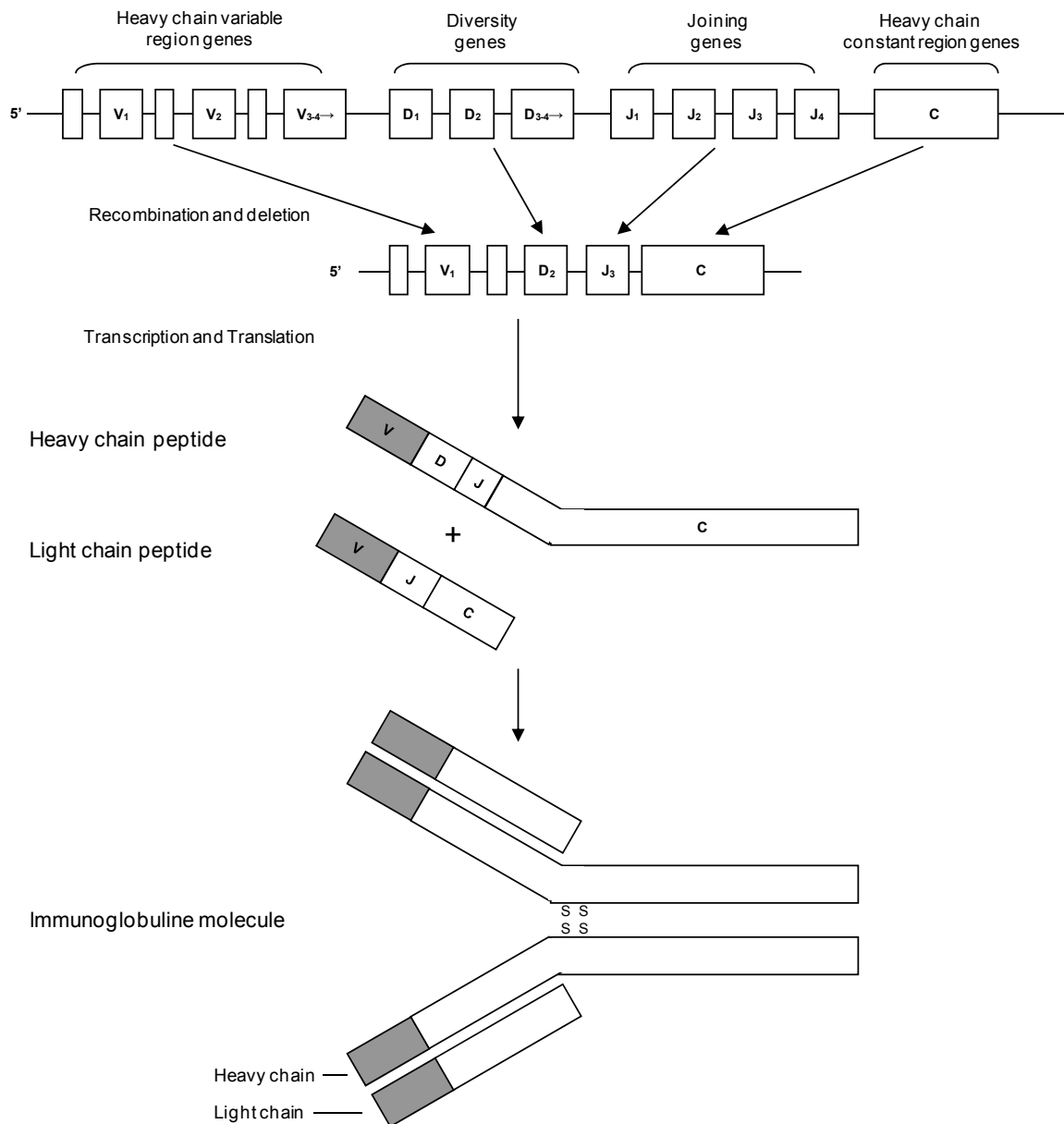


Figure 1. The heavy chain genes have no complete exon encoding the variable region domain, instead this is split into arrays of gene segments. Light chain genes are similarly organized on different chromosomes but they have no diversity gene segments. Immunoglobulin genes rearrange segments with the looping out of intervening DNA. This is done in a precise order: first the heavy chain rearranges, then if a functional heavy chain (always IgM initially) results (many joins are out of frame), the light chains rearrange also in order, first kappa then if kappa is unproductive (or cannot pair with the heavy chain) lambda.

both positive and negative selection checkpoints expresses a functional, non-self reactive BCR that is able to encounter foreign antigen as it leaves the bone marrow. The rearranged genes code for both the BCR at the cell surface and for secreted immunoglobulins after the B cell has differentiated into plasma cell. Thus, the BCR and the produced antibodies of one B cell have the same specificity for a particular antigen.

Germinal center reaction

After leaving the bone marrow, the immature B cells pass from the blood into secondary lymphoid organs: lymph nodes, spleen and mucosa-associated lymphoid tissue (MALT). Immature B cells that migrate from the bone marrow to the periphery are referred to as transitional B cells. Transitional B cells can be distinguished from mature B cells by the absence of the ATP-binding cassette (ABC)B1 transporter (15) and because they express low levels of recombination activating gene (RAG) mRNA (16). Transitional B cells are short-lived and only 10–30% of these cells become long-lived mature, naïve B cells and are localized together with follicular dendritic cells (FDCs) in discrete clusters called primary follicles. These FDCs are thought to provide signals essential for the survival and continued recirculation of the naïve B cells. FDCs have taken up foreign antigen, which is presented on their membrane. B cells monitor the antigens present on FDCs and upon recognition of the antigen by their BCR the germinal center reaction is initiated (17). In addition to FDCs recently identified 'antigen transport cells' and marginal zone B cells in the spleen have been implicated in the process of antigen presentation to B cells (18).

During the germinal center reaction the activated B cells start to divide forming a secondary follicle, the germinal center. Here their genomic DNA may undergo modifications, a process called somatic hypermutation (SHM). During SHM, small changes, mainly single nucleotide exchanges but also deletions and duplication, are introduced at a high rate into the variable-region genes of the BCR. Because of these changes a wide variety of B cells is made, all recognizing the same antigenic epitope but with a different affinity. B cells with a BCR with the highest affinity for the antigen are positively selected for survival (19). Depending on the antigen, the BCR can acquire another constant region in a process called class switch recombination (CSR) (20) by switching from IgM expression to heavy chain expression of other immunoglobulin classes: IgG, IgA or IgE, leaving the antigen

specificity unaltered (21). The subclass of the constant region confers functional specialization on the antibody: e.g. IgG1-3 and IgM can activate the classical pathway of complement activation, IgA1 the alternative pathway of complement activation, IgG1-3 can be transferred across the placenta, IgE has high-affinity binding to mast cells and basophils and, except for IgG2 and IgM, all (sub)classes can bind Fc receptors on macrophages and phagocytes. IgD is coexpressed with IgM on the surface of almost all mature B cells, although this isotype is secreted in only small amounts of plasma cells and its function is unknown. Although germinal center formation strongly facilitates CSR and SHM, other environments can also support CSR and SHM. B cells from patients of which the CD40 ligand gene is mutated (X-linked hyper-IgM syndrome type I), have a certain level of hypermutation, although there are no germinal centers (22). Also, T-cell-independent antigens initiate IgA class switching by linking B cells with multiple innate immune pathways. Whereas some T-cell-independent antigens activate B cells through Toll-like receptors (TLRs) (e.g. LPS), others activate B cells through their BCR (e.g. polysaccharides). T-cell-independent antigens can also provide additional B-cell-stimulating signals through DCs, which release soluble class-switch-inducing factors related to CD40L, including B-cell activating factor (BAFF; also known as BLyS) and a proliferation-inducing ligand (APRIL) (reviewed in (23)).

Terminal B cell differentiation

B cells that have successfully bound antigen and survived selection leave the germinal center and migrate into the periphery, where they become either memory B cells or plasma cells (24, 25). IL-10 has been put forward as the key cytokine that terminates the expansion of memory B cells by inducing differentiation into plasma cells (26). Plasma cells predominantly migrate to the bone marrow and have different live spans. Productive signaling events lead to the generation of short-lived antibody-secreting cells that survive only for a few weeks (27). Memory B cells reside in secondary lymphoid organs and rapidly proliferate and differentiate upon exposure to the same antigen without further involvement of germinal centers, generating high amounts of long-lived immunoglobulin secreting plasma cells that account for the persistence of the humoral immune response (28).

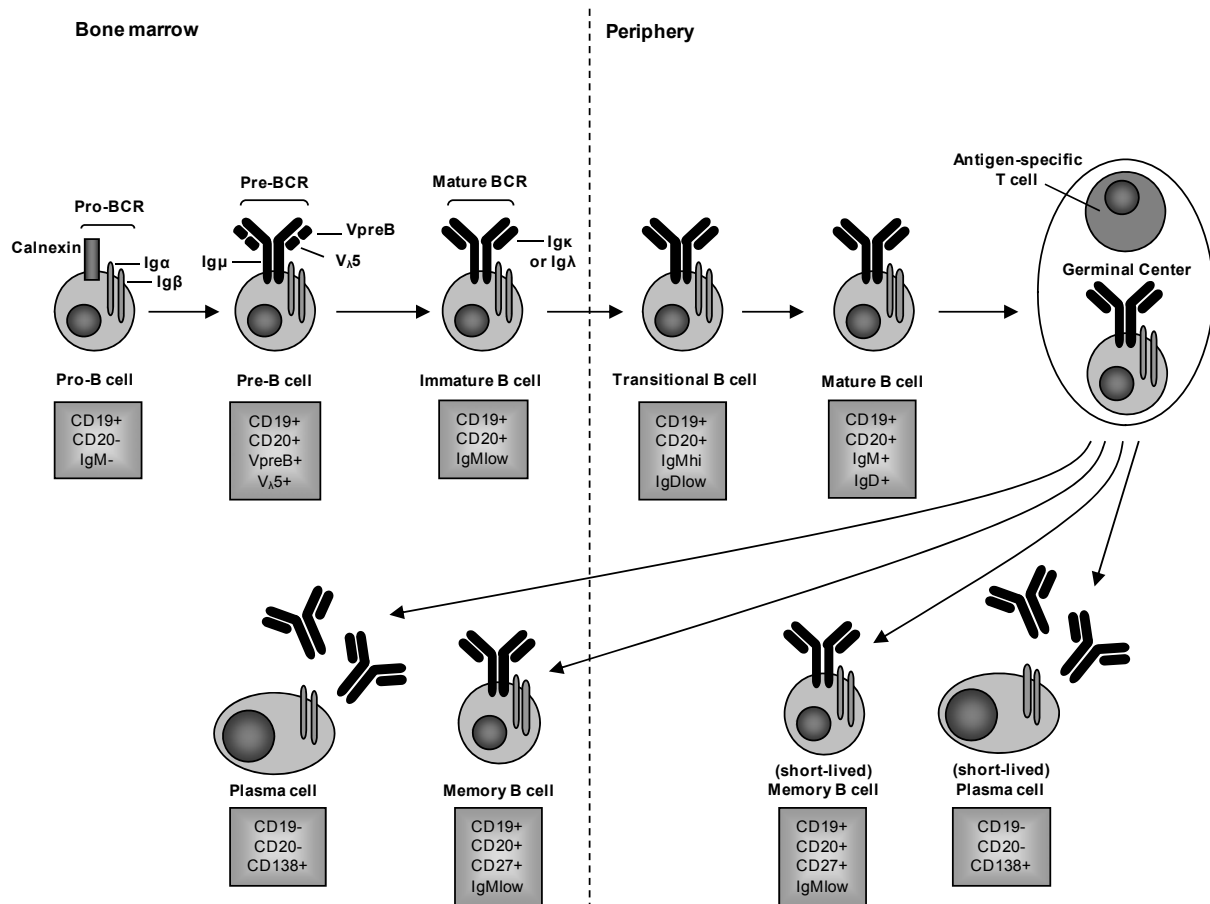


Figure 2. B-cell development occurs in both the bone marrow and peripheral lymphoid tissues such as the spleen. In the bone marrow, development progresses through the pro-B-cell, pre-B-cell and immature-B-cell stages. During this differentiation, rearrangements at the immunoglobulin locus ultimately result in the expression of a mature BCR that is capable of binding antigen. At this immature stage of development, B cells undergo a selection process to prevent any further development of self-reactive cells. Both receptor editing and clonal deletion have a role at this stage. Cells successfully completing this checkpoint leave the bone marrow as transitional B cell, eventually maturing into mature follicular B cell (or marginal-zone B cell). Following an immune response, antigen-specific B cells develop into either plasma cell or memory B cell.

Salmonella infection

Antibodies produced by antigen-specific B cells are important in the clearance of bacterial infections. On the one hand they opsonize bacteria to activate the complement system that will ultimately lead to lysis of the bacteria. On the other hand, antibodies opsonize bacteria to be recognized by phagocytic cells and subsequent destruction of the bacteria by phagocytes. *Salmonella enterica* is a Gram-negative, enteric pathogen responsible for disease syndromes of significant morbidity and mortality (29). After oral uptake, the bacterium crosses the intestinal

epithelium via M cells (or microfold cells). M cells are found in the follicle-associated epithelium of the Peyer's patch and differ from normal enterocytes in that they lack microvilli on their apical surface. Instead, M cells possess broader microfolds and the filamentous brush border glycocalyx, an extracellular polysaccharide layer found throughout the intestine attached to enterocytes, is much thinner or absent on M cells. This allows M cells to sample antigen/bacteria from the lumen and deliver it via transcytosis to the Peyer's patches (30). Another way of bacterial invasion in the intestinal mucosa is via dendritic cells (DCs). DCs express tight-junction proteins, open the tight-junctions between epithelial cells and send dendrites outside the epithelium into the lumen to directly sample microorganisms. In this way, DCs can transport bacteria to the basolateral side while preserving the integrity of the epithelial barrier (31). The bacteria are ultimately internalized by macrophages, dendritic cells, and neutrophils (32, 33). Entry into these cells is actively induced by the bacterium through an impressive array of effector proteins that orchestrate uptake by manipulating the host cellular machinery (34). *Salmonellae* manipulate host cells upon infection in order to alter the actin cytoskeleton allowing phagosomal cup formation and entry of the relatively large pathogen into the host cell. Bacterial effector proteins are therefore introduced into the host cytosol through the *Salmonella* Type III Secretion System (TTSS). After invasion, *Salmonella* forms an intracellular vacuole called the *Salmonella*-containing vacuole (SCV). Here another set of effectors is secreted into the host cytosol for vacuole maintenance and interference with the endosomal system to obtain nutrients and to prevent maturation and fusion with lysosomes (35, 36). *Salmonella* replicates in an expanding SCV (37, 38) and escapes detection by the immune system (39, 40). This feature of *Salmonella* is considered crucial for their survival and pathogenicity (41). Although *Salmonella* replicates in the phagosome, it remains unclear how they are released from the infected cell. This may follow from apoptosis or necrosis of the infected cell, but this is not established.

When *Salmonella* has passed the intestinal epithelium, it spreads via mesenteric lymph nodes to liver, bone marrow and spleen where replication continues (42). How *Salmonella* reaches these organs is unclear. So far, especially neutrophils, CD18-expressing phagocytes and DCs have been implicated (43, 44). Being facultative intracellular pathogens, immunity to *Salmonella* requires adequate humoral and cell-mediated immune responses (45, 46).

Antigen presentation by MHC class II molecules after BCR recognition

Antigen presentation by B lymphocytes is needed to generate high-affinity Abs (5, 47). Development of an effective humoral immune response is mediated by two actions of the BCR: transmembrane signaling through BCR-complexes to induce B cell differentiation and antigen internalization for processing followed by MHC class II-mediated presentation to acquire T cell help. The proper execution of both actions requires binding of a polyvalent antigen to multiple BCR molecules on one B cell.

Since primary B cells are not classical phagocytic cells, it is unclear how they acquire antigens from bacteria for antigen presentation. B cells can present particulate antigens in the context of MHC class II (48-51) and are able to extract antigen from a non-internalizable surface (52). Indeed, many B cell antigens are polyvalent as they are present in multiple copies to the particulate surfaces of microbes or cells (53). B cells use their BCR to concentrate specific antigen to the antigen loading compartments (termed MIIC for MHC class II containing compartment, see later) for loading of antigenic fragments onto newly synthesized MHC class II molecules (53). Besides internalization of antigen, the BCR drives intracellular targeting by accelerating the delivery of antigen to MIICs (54). Furthermore, BCR signaling ignited by antigen induces acidification of the MIICs which favors antigen loading onto newly synthesized MHC class II molecules (55). Together, these cellular adaptations enable B cells to preferentially present specific antigens that have been internalized via the BCR to CD4⁺ T cells. However, the exact regulation of MHC class II antigen presentation after BCR-specific recognition of particulate antigens is not clear.

The regulation of MHC class II antigen presentation

Regulation of the MHC class II antigen presentation pathway may affect the efficacy of MHC class II antigen presentation. MHC class II molecules are heterodimeric cell surface glycoproteins that present antigens to CD4⁺ T cells. After synthesis, the α and β subunits of the MHC class II molecule associate in the endoplasmic reticulum (ER) together with the invariant chain (Ii or CD74) (Fig. 3). The Ii folds in part through the antigen binding groove of the MHC class II molecule, stabilizing the $\alpha\beta$ heterodimer and preventing the binding of ER polypeptides. Another function of Ii is to direct MHC class II molecules to the lysosomal-like MIIC compartments (56), where the majority of MHC class II antigen loading occurs, as it contains two short

leucine-based sequences in the cytoplasmic tail that are responsible for trafficking through the endocytic pathway (57). During transport to the MHC compartments, the Ii is progressively degraded by various proteinases depending on the cell type (58), leaving only a small fragment (class II-associated invariant chain peptides (CLIP)) in the class II peptide binding groove (59). The dissociation of the CLIP peptide and subsequent loading of antigenic peptide is an essential step in antigen presentation. Release of CLIP is facilitated by the specialized chaperone HLA-DM (DM) (60), a nonclassical MHC molecule composed of an α and β subunit. In addition to displacing CLIP, DM catalyses the natural process of peptide dissociation (61) by associating to DR to generate a peptide-receptive conformation by opening the peptide-binding groove. DM dissociates the peptide-MHC class II complex that it recognizes by perturbing a critical hydrogen bond between a conserved histidine residue on the β -chain of the MHC class II molecule and the peptide backbone (62). DM thus releases both CLIP and other nonstable binding peptides. Consequently, DM acts as a peptide editor, favoring presentation of high affinity peptides (63-66). HLA-DO (DO), another nonclassical $\alpha\beta$ heterodimer, is expressed in human B cells (also in thymic medullary epithelium (67) and in subsets of DCs (68)). Association with DM is necessary for efficient exit of DO from the ER and for accumulation in lysosomal vesicles (69). DO acts as a negative modulator of antigen loading by inhibiting the catalytic action of DM on class II peptide loading (70, 71). As a result, cells expressing DO show elevated cell surface levels of CLIP on HLA-DR3 molecules, paralleled by a reduced, but not abolished, presentation of antigenic peptides (70). DO does not act as a simple inhibitor of DM, but modulates in a pH-dependent manner. The activity of DM itself is optimal at pH 5.0, but it still catalyzes class II-peptide loading at pH 6.0. Association of DO with DM still allows a respectable amount of DM function at lysosomal-like pH, but abolishes it completely at pH 6.0, the pH of early endosomes. Thus, DO acts as a sort of pH sensor to control the activity of DM (72, 73). Thus, DO reduces MHC class II-mediated presentation of antigenic peptides in general and modulates the antigenic peptide repertoire by facilitating presentation of particular antigens, while suppressing others. DO therefore both limits and skews the class II-associated antigenic peptide repertoire in B cells (70, 72, 74).

The relative levels of DR, DM and DO determine the efficacy of MHC class II antigen presentation together with cellular processes that determine antigen processing and

intracellular trafficking of antigens, DR, DM and DO and eventually trafficking of MHC class II molecules loaded with antigenic peptides to the cell surface.

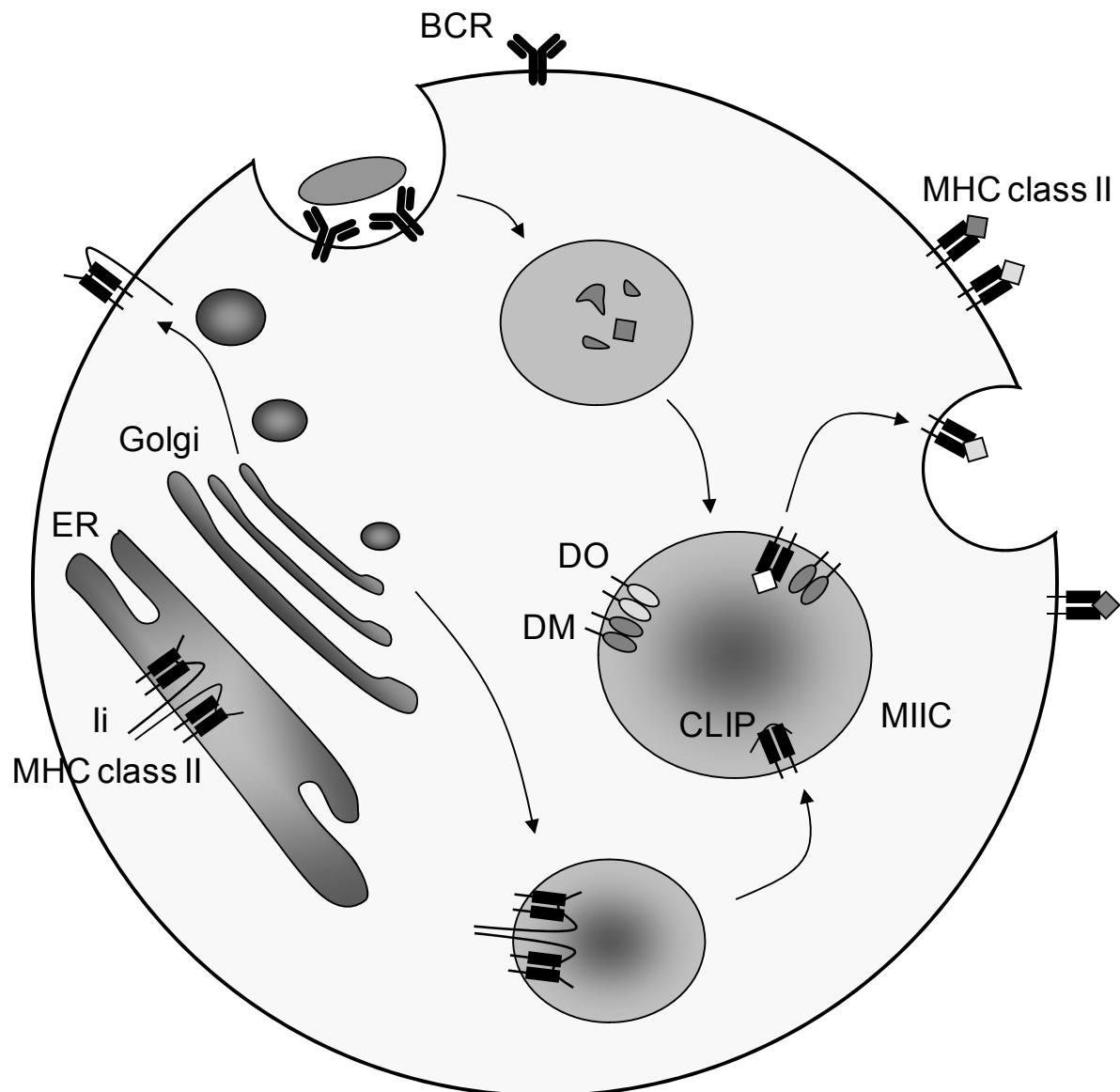


Figure 3. MHC class II $\alpha\beta$ heterodimers assemble in the endoplasmic reticulum (ER) with the assistance of invariant chain (Ii). The cytoplasmic tail of Ii contains a motif that targets the Ii-MHC class II complex to the endosomal/lysosomal pathway. During transport to the MIIC, Ii is degraded by a series of endosomal proteases with the CLIP fragment remaining, which prevents premature peptide loading. HLA-DM assists exchange of CLIP for relevant exogenous antigenic fragments in the MIICs prior to transport of stable MHC class II-antigen complexes to the plasma membrane. HLA-DO can inhibit DM-mediated peptide loading.

Regulation of MHC class II, DM and DO expression.

The relative expression of DO and DM (or the number of DM molecules in association with DO) related to the level of MHC class II expression control antigen presentation in B cells. Aberrant expression of DM, DO and/or DR could lead to an altered MHC class II peptide repertoire. Not surprisingly, DO and DM expression are very consistent and tightly regulated at different cellular levels in healthy B cells (75) and during B cell differentiation (76, 77). Expression of DR, DM and DO is regulated at the transcriptional as well as the post-translational level.

Transcription of MHC class II, DM and DO is regulated by a master regulator termed the class II transactivator (CIITA) (78, 79). CIITA is transcriptionally controlled by four distinct promoters, each transcribing a unique first exon and yielding a unique CIITA transcript (80). The promoters I, III and IV are differentially used in different cell types and in response to inflammatory stimuli. A physiological role for CIITA-PII is questioned as transcripts originating from this promoter are rare. CIITA-PI is constitutively active in myeloid dendritic cells (DCs) and CIITA-PIII constitutively in B cells, plasmacytoid DCs, monocytes and activated T cells (81). CIITA-PIV has been shown to be the promoter predominantly involved in IFN- γ -inducible CIITA expression (82, 83). In healthy B cells transcription of the MHC class II genes is tightly regulated by CIITA, but dysregulation has been observed in tumors (84, 85).

MHC-mediated antigen presentation and cancer

A successful anti-tumor T cell response is achieved by the two effector arms of the immune system. On the one hand, CD8⁺ CTLs ensure specific elimination of tumor cells upon recognition of MHC class I-antigen complexes. On the other hand CD4⁺ T helper cells generate the required T cell help upon activation by MHC class II-antigen complexes (86). The absence of T cell help can lead to abortive induction of CTL responses and subsequent tolerance (87). Moreover, T cell help is required for the maintenance of CTL responses, which is essential in diseases like cancer (88, 89). It has been shown that CD4⁺ T cell inclusion in adoptive T cell transfer studies improved tumor clearance by the CD8⁺ T cells (90, 91). MHC class II molecules play a pivotal role in the induction and regulation of an antigen-specific immune response. MHC class II antigen presentation activates antigen-specific CD4⁺ T cells. Over the last years it has become clear that the establishment of an effective CD4⁺ T cell response is required for both the induction and maintenance of anti-tumor CD8⁺ cytotoxic T lymphocytes (CTL) responses (92). Indeed, loss of MHC class II

expression has been observed in diffuse large B cell lymphomas with fewer tumor-infiltrating CD8⁺ T cells in MHC class II-negative tumors (93, 94).

B-cell chronic lymphocytic leukemia

During B cell differentiation, uncontrolled growth of B cells can occur, resulting in the formation of lymphomas or leukemia.

B-cell chronic lymphocytic leukemia (B-CLL) is the most common form of leukemia in adults in the Western world. Typical for the disease is the highly variable clinical course, with survival rates varying between a few months and two decades (95). B-CLL is characterized by a progressive accumulation of a malignant B cell population that fails to undergo apoptosis. Apparently the immune system is unable to deal with this abnormal cell population. Indeed, B-CLL is characterized by striking immune incompetence in which not only the number but also the function of the B and T cells is impaired (96). Prognostic factors for survival of B-CLL patients are the mutational status of the immunoglobulin *IGHV* genes, cytogenetic aberrancies, CD38 and ZAP-70 expression (97). Today, the strongest predictor for survival in B-CLL is the mutational status of the *IGHV* genes.

MHC-mediated antigen presentation and B-CLL

In B-CLL patients, malignant B cells accumulate in the bone marrow and periphery. Apparently, these cells are not recognized by the immune system in such a way that the malignant cells are effectively cleared. This may be due to immune escape by the malignant cells by preventing CD4⁺ T cell activation, since B-CLL cells lack significant expression of CD80 and CD86 costimulatory molecules (98). However, the absolute numbers of T cells are increased in B-CLL patients. Whether this expansion is a result of (altered) MHC class II antigen presentation by malignant B cells has not been established.

A shift in MHC class II antigen presentation by B-CLL cells may lead to altered T helper cell activation and subsequent help to CD8⁺ CTLs. It is unclear whether the T cell expansion in B-CLL is indicative for attempted but unsuccessful tumor clearance or contributes in another way to the disease, for instance by creating an environment that supports survival of neoplastic cells (99). Antigen-independent mechanisms have been implicated in the T cell expansion in B-CLL (reviewed in (100)). The TCR-dependent oligoclonal/monoclonal expansion of CD4⁺ T cells in B-CLL however, points to an antigen-driven process. How malignant B cells present

antigens via MHC class II molecules to CD4⁺ T cells and whether this may be an explanation for observed T cell expansion in B-CLL is unclear. If so, modulation of these MHC class II responses would be an interesting approach to control these tumors.

Acute myeloid leukemia

Acute myeloid leukemia (AML) is a myeloproliferative disorder, characterized by an arrest in differentiation of hematopoietic stem cells due to acquired mutations. This results in accumulation of immature non-lymphoid cells in the bone marrow and peripheral blood, often accompanied by suppression of the normal blood cells. AML is a heterogeneous disease in which subgroups can be defined with different stages of immature blasts present and each group is characterized genetic lesions, clinical behavior, prognosis and therapy (101). With chemotherapy and stem cell transplantation approximately 70 % of patients achieve complete remission, but half of these patients relapse (102). Although AML blasts generally express MHC class II molecules (103), they have escaped the initial immune response in acute disease status. How efficient AML blasts present antigens via MHC class II molecules is unclear.

Scope of this thesis

In this thesis, the role of MHC class II antigen presentation in the immune response against bacterial infections and tumors was investigated.

MHC class II antigen presentation is indispensable for activation of CD4⁺ T helper cells, which give help to B cells for antibody production. So far it is unclear how particulate antigens induce MHC class II antigen presentation and CD4⁺ T helper activation, as B cells are considered to be non-phagocytic. In **Chapter 2** we investigate how B cells deal with particulate antigens, using beads coated with antibodies specific for the BCR or the bacterium *Salmonella typhimurium* as model systems. We demonstrate that B cells phagocytose the beads and *Salmonella* upon antigen-recognition by the BCR and that these B cells subsequently activate CD4⁺ T cells. Since *Salmonella* is a facultative intracellular bacterium, we investigated the fate of *Salmonella* inside the B cell in **Chapter 3**. For elimination of infected cells, an effective induction of antigen-specific CD8⁺ T cells, which can kill infected target cells, is desirable. In **Chapter 4** we demonstrate that B cells are able to cross-present bacterial antigens via MHC class I to induce an effective CD8⁺ T cell response. We also show that the ability of B cells to activate *Salmonella*-specific CD8⁺ T cells requires help of CD4⁺ T cells.

Induction of CD4⁺ T cells is indispensable for the generation of an effective and long-lasting immune response. Some types of cancer escape immune surveillance by interfering with antigen presentation. Most AML blasts express MHC class II molecules on their cell surface which could induce CD4⁺ T cells activation. In **Chapter 5** we analyzed cell surface expression of HLA-DR and the efficacy of MHC class II antigen presentation by analyzing the amount of the self-peptide CLIP inside the peptide binding groove of HLA-DR. **Chapter 6** describes the efficacy of MHC class II antigen presentation in B-CLL and we correlated this to the *ex vivo* analysis of the T cell compartment in B-CLL patients. Evaluation of the MHC class II antigen presentation components at the mRNA level in **Chapter 7** revealed that all components are significantly higher transcribed in B-CLL patients. Survival analysis of patients showed that the level of *DOA* mRNA at the time of sample acquisition has prognostic power. **Chapter 8** contains a general discussion and a summary of the results.

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Chapter 2

BCR-mediated internalization of *Salmonella*: a novel pathway for autonomous B cell activation and antibody production

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Abstract

The present paradigm is that primary B cells are non-phagocytosing cells. In this study, we demonstrate that human primary B cells are able to internalize bacteria when the bacteria are recognized by the B cell receptor (BCR). BCR-mediated internalization of *Salmonella typhimurium* results in B cell differentiation and secretion of anti-*Salmonella* Ab by the *Salmonella*-specific B cells. In addition, BCR-mediated internalization leads to efficient Ag delivery to the MHC class II Ag-loading compartments, even though *Salmonella* remains vital intracellularly in primary B cells. Consequently, BCR-mediated bacterial uptake induces efficient CD4⁺ T cell help, which boosts *Salmonella*-specific Ab production. BCR-mediated internalization of *Salmonella* by B cells is superior over extracellular Ag extraction to induce rapid and specific humoral immune responses and efficiently combat infection.

Introduction

Defense against pathogens is essential for survival and is controlled by the innate and acquired arms of the immune system. Ag presentation by B lymphocytes is needed to generate high-affinity Abs (1, 2). Development of an effective humoral immune response is mediated by two actions of the BCR: transmembrane signaling through BCR complexes to induce B cell differentiation and Ag internalization for processing followed by MHC class II-mediated presentation to acquire T cell help. The proper execution of both actions requires binding of a polyvalent Ag to multiple BCR molecules. Indeed, many B cell Ags are polyvalent as they are bound in multiple copies to the particulate surfaces of microbes or cells (3).

The role of CD4⁺ T cells in the induction of protective immunity against pathogens is well established (4, 5). CD4⁺ T cell activation requires MHC class II Ag presentation after Ag processing in the endocytic pathway and subsequent binding of antigenic peptides to MHC class II molecules, a process controlled by the MHC class II chaperones HLA-DM and HLA-DO (6-8). B cells use their BCR to concentrate specific Ag to the Ag-loading compartments (termed MIIC for MHC class II containing compartment) for loading of Ag onto newly synthesized MHC class II molecules (3). Besides internalization of Ag, the BCR drives intracellular targeting by accelerating the delivery of Ag to MIICs (9). Furthermore, BCR signaling ignited by Ag induces acidification of the MIICs, which favors Ag loading onto newly synthesized MHC class II molecules (10). Together, these cellular adaptations enable B cells to preferentially present specific Ags that have been internalized via the BCR to CD4⁺ T cells.

Since primary B cells are considered to be not phagocytic, it is unclear how they acquire Ags from bacteria for Ag presentation. B cells can present particulate Ags in the context of MHC class II (11-14) and are able to extract Ag from a non-internalizable surface (15). Studies on MHC-mediated presentation of BCR-specific Ags are mainly performed with soluble Ags or with pre-cross-linked anti-BCR Abs. We used *Salmonella typhimurium* as a model system to study MHC class II Ag presentation of particulate, polyvalent Ags, and B cell activation. Being facultative intracellular pathogens, immunity to *Salmonella* requires adequate humoral and cell-mediated immune responses (16, 17). *Salmonella* invades host macrophages, but also many other cells and establishes an intracellular niche inside discrete vacuoles, known as *Salmonella*-containing vacuoles or SCV (18). This feature of *Salmonella* is considered crucial for their survival and pathogenicity (19, 20).

In this report we show that B cells are highly efficient phagocytes of inert particles, like beads, when these particles are recognized by the BCR. B cells are thus ligand-selective phagocytic cells. BCR-mediated internalization of *Salmonella* generates autonomous B cell activation and rapid anti-*Salmonella* Ab secretion. Immediate intimate contact and fusion occurs between MIICs and SCVs. Consequently, Ag presentation and proliferation of *Salmonella*-specific CD4⁺ T cells is induced. Although BCR-mediated internalization suffices to drive Ab production, T cell help further improves the response. The observation that B cells can proliferate and differentiate autonomously after *Salmonella* uptake is important in light of the remaining Ab responses to pathogens when CD4⁺ T cell help fails, as is the case in HIV patients.

Materials and methods

Antibodies, beads and fluorophores

Goat anti-mouse IgG-conjugated Dynabeads M-450 (DynaL Biotech) were coated with mAb anti-human IgM (MH15; Sanquin). The anti-human IgM antibody (MH15) was mixed with mAb anti-*S. typhimurium* LPS (1E6; Biodesign International) and rat anti-mouse IgG1 (RM161.1; Sanquin) to generate BCR-LPS tetrameric Ab complexes. The mAb anti-human HLA-DR (L243) (21) was used to block MHC class II-TCR interaction in T cell proliferation assays. For immunoelectron microscopy (EM), mAb anti-human CD63 (435; Sanquin), rabbit anti mouse (Nordic) and gold (10 nm)-conjugated protein-A (EM Laboratory, Utrecht University, The Netherlands) were used. F(ab')₂ fragments of MH15 were generated by standard pepsin digestion.

PE-conjugated anti-IgM was obtained from Sanquin (MH15-PE), anti-CD27-PE and IgG1-PE isotype control from BD Biosciences. Fluorescent secondary Ab goat anti-mouse Alexa Fluor 633 and Texas Red-phalloidin were obtained from Molecular Probes and 4',-diamidino-2-phenylindolyne (DAPI) from Sigma-Aldrich.

Transfectant cell lines

The pcDNA3 DOβGFP (22) and DR1βGFP (23) constructs have been described before. DOβGFP and DR1βGFP were demonstrated to form complexes with their respective endogenous α-chain. Transfections were performed by electroporation using a Gene Pulser II with Capacitance Extender (Bio-Rad). Stable transfectants of the human B cell line Ramos were selected and maintained in RPMI 1640

supplemented with 5% FCS (Bodinco), 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-Glutamine, 50 µM 2-ME in the presence of 2000 µg/ml G418 (Life Technologies). Stable expression of the GFP-tagged proteins was verified by Western blotting and ensured by regular selection of positive cells by FACS sorting. NIH3T3 fibroblasts expressing human CD40L (3T3-CD40L) (24) were cultured in IMDM supplemented with 5% FCS (Bodinco), 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-Glutamine, 50 µM 2-ME and 500 µg/ml G418 (Life Technologies). 3T3-CD40L cells were harvested, irradiated with 30 Gy (Gammator M38-1, MDS Nordion) and seeded without antibiotics in 96 wells flat bottom plates (2×10^4 cells per well) to form a confluent monolayer overnight.

Bacterial Strains

S. typhimurium SL1344 (*Salmonella*) (25), GFP-*Salmonella* (26) and mRFP-*Salmonella* (27) were described before. GFP-*Salmonella* defective in SPI-1 (*invA* mutant) or SPI-2 (*ssrA* mutant) were a kind gift from M. Rescigno. *Staphylococcus aureus* expressing GFP (RN4220 with pWVW189GFP) was a kind gift from S.A.J. Zaat. Bacteria were grown in Luria-Bertani (LB) broth with antibiotics overnight at 37°C while shaking, subcultured at a dilution of 1/33 in fresh LB media, and incubated at 37°C while shaking for 3.5 hours. Bacteria were washed twice with PBS, incubated 1/25 with the BCR-LPS tetrameric Ab complexes for 30 min at room temperature, and washed twice to remove unbound Abs. Dead *Salmonellae* were bacteria fixed with paraformaldehyde (3.7% in PBS).

Lymphocyte isolation and proliferation assay

Human PBMCs were isolated by centrifugation on a Ficoll-Hypaque gradient (Axis-Shield PoC AS) from a buffycoat obtained from healthy donors after informed consent (Sanquin). B and T cells were subsequently purified using anti-CD19 and anti-CD4, anti-CD8 Dynabeads and DETACHaBEAD (DynaL Biotech), according to the manufacturer's instructions.

B lymphocytes were incubated for 40 min at 37°C with *Salmonella* without antibiotics. Next, cells were washed four times and cultured for 1 h in medium containing 100 µg/ml gentamycin (Invitrogen) to eliminate non-internalized bacteria. Cells were washed and cultured in RPMI 1640 medium without phenol red, supplemented with 5% FCS (Bodinco), 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-Glutamine, 50 µM 2-ME, 20 µg/ml human apo-transferrin (Sigma-Aldrich);

depleted for human IgG with protein G sepharose (Amersham Biosciences)), and 10 µg/ml gentamycin. One $\times 10^5$ B cells and 5×10^4 T cells were cultured in 200 µl at 37°C in the presence of 5% CO₂ in 96 wells round bottom plates (Greiner Bio-One). The maximum proliferation capacity of T lymphocytes (varying between 35 to 60 $\times 10^3$ cpm) was established by stimulation with anti-CD3 (CLB.T3/4.E, Sanquin) and anti-CD28 (CLB.CD28/1, Sanquin) which were both used at 1 µg/ml. After 5 and 12 days, 150 µl of supernatant was collected for Ab measurement and fresh medium was added. To study the kinetics of Ag presentation, B cells incubated with *Salmonellae* were irradiated with 60 Gy at indicated time points before incubation with T cells. For B/T cell proliferation assays, after 5 days of culturing [³H]thymidine (GE Healthcare) was added at a final concentration of 1 µCi/ml (37 kBq/ml) for 16 h. Cells were harvested on glass fiber filters (Wallac) and radioactivity was measured with a 1205 Betaplate liquid scintillation counter (Wallac). For blocking experiments, B cells were preincubated with 5 µg/ml anti-HLA-DR (L243) for 30 minutes before T cells were added. For the Ag-specificity assay, CD4⁺ T cells were CFSE labeled and cocultured with B cells that had taken up *Salmonellae*. The dividing T cells were sorted after 6 days and cultured with 10 IU/ml IL-2 (Chiron) for 6 more days. PBMCs were labeled with CFSE and incubated with tetanus toxoid (7,5 µg/ml; Statens Serum Institut, Copenhagen, Denmark) for 11 days, with 10 IU/ml IL-2 added on day 6, and proliferating CD4⁺ T cells were sorted. B cells from the same donor were incubated with *Salmonellae* and PBMCs from the same donor with Tetanus Toxoid, irradiated after 18 h and then the sorted T cells were added for 2 days before [³H]thymidine was added for 16 h.

FACS analyses

Freshly isolated primary B cells were incubated with *Salmonella*, washed four times, and cultured for 1 h in medium containing 100 µg/ml gentamycin. Cells were incubated with directly labeled Abs and for LPS staining cells were incubated with anti-LPS Ab and subsequently with Alexa Fluor 633-conjugated goat-anti-mouse Ab. Cells were washed with PBS containing 0.1% BSA. Lymphocytes were gated by forward and side scatter and dead cells were excluded by staining with DAPI. Two hundred thousand events were acquired on an LSR II (BD Biosciences) and analyzed using FACSDiva software (BD Biosciences).

FACS sorting of B cells that had internalized uncoated living bacteria was performed on a MoFlo Sorter (Dakocytomation), populations were >75% purified.

Live cell imaging and EM analyses

For CLSM analysis, coverslips were coated with 1 mg/ml Poly-L-lysine (Sigma-Aldrich) for 1 h and washed thoroughly with Aquadest and dried on air. Cells were allowed to attach on the coated coverslips for 15 min and subsequently beads or *Salmonellae* were added. For visualization of the actin cytoskeleton, cells were fixed with 3.7% paraformaldehyde and stained with Texas Red-phalloidin and DAPI. Confocal analysis was performed at 37°C using a Leica TCS SP confocal laser scanning microscope equipped with an argon/krypton laser with x63 oil objective and 1.4 aperture (Leica Microsystems). Green fluorescence was detected at $\lambda > 515$ nm after excitation at 488 nm. For dual analyses, green fluorescence was detected at 520–560 nm. Red fluorochromes were excited at 568 nm and detected at $\lambda > 585$ nm. All experiments presented were repeated several times on different days and results were consistent and reproducible. Further image processing was performed using the ImageJ software package.

For EM, cells were allowed to take up beads or bacteria for 40 min or 4 h (to study MIIC-SCV fusion) and fixed in a mixture of paraformaldehyde (4%) and glutaraldehyde (0.5%). After embedding in a mixture of methyl cellulose and uranyl acetate, ultrathin sections were stained and analyzed with a Philips CM10 electron microscope.

Plating Assay

For enumeration of intracellular surviving bacteria, freshly isolated primary B cells were incubated with anti-IgM coated *Salmonellae* and Ramos cells with uncoated *Salmonellae* as a control, washed, and cultured in medium with 10 µg/ml gentamycin as described above. At the indicated time points, cells were washed with PBS and lysed in 0.1% Triton X-100 (Merck) for 10 min on ice, washed with PBS, and a dilution series was plated onto LB agar plates. Plates were incubated overnight at 37°C and colonies were counted.

ELISA assays

To determine IgM levels in culture supernatants, flat-bottom Maxisorb plates (Nunc) were coated with polyclonal anti-IgM (SH15; Sanquin) in 100 µl of PBS (pH 7.4; NPBI International) overnight at room temperature. Plates were washed with PBS/0.02%Tween 20 (Mallinckrodt Baker) and samples were incubated for 2 h in

high-performance ELISA buffer (Sanquin). As a standard, pooled human serum was used. Plates were washed and incubated for 1 h with 1 µg/ml mAb anti-human IgM-HRP (MH15-HRP; Sanquin).

Whole-cell *Salmonella* ELISA was performed by coating overnight at 37°C of *Salmonella* to Maxisorb plates in 100 µl 0.1 M sodium bicarbonate at pH 9.6 supplemented with 10 µg/ml gentamycin. Plates were washed extensively with PBS/0.02%Tween-20 and supernatants were incubated in high-performance ELISA buffer. Plates were washed and incubated for 1 h with 1 µg/ml mAb anti-human IgM-HRP (MH15-HRP, Sanquin).

After washing, peroxidase activity was visualized by incubation with 100 µl 3,5,3',5'-tetramethylbenzidine (Merck), 100 µg/ml in 0.11 M sodium acetate (pH 5.5), containing 0.003% H₂O₂ (Merck). The reaction was stopped by addition of an equal volume of 2 M H₂SO₄ (Merck) and the absorbance at 450 and 540 nm was measured immediately in a Titertek plate reader. Results were calculated with LOGIT software (<http://www.xs4all.nl/~ednieuw/Logit/logit.htm>).

Statistical analysis

Statistical significance was determined using the Mann-Whitney *U* test.

Results

Efficient BCR-mediated phagocytosis of large particulate Ags by B cells

Unlike other professional Ag presenting cells, primary B cells show very limited phagocytic capacity. Ag uptake by B cells is critically dependent on the selectivity of the B cell receptor (BCR) (10, 28). The current view on BCR-mediated Ag uptake by B cells mainly centers on soluble Ags like small foreign proteins or shedded bacterial coat products (29). Accordingly, most B cell activation studies involve the global triggering of BCR using soluble cross-linking Ags rather than pathogen-associated Ags. We opted to study BCR-mediated recognition of particulate Ags in B cells by inducing localized clustering of the BCR using beads decorated with mAb against the BCR. When anti-IgM coated beads contacted a Ramos B cell stably expressing MHC class II HLA-DR1 tagged with GFP (DR-GFP, which localizes to the plasma membrane and lysosomal MIIC vesicles), rapid and efficient internalization of the bead ensued (Fig. 1A and supplemental movie 1). Ramos cells polarized themselves toward the contact site during uptake of the beads such that the nucleus moved to the side opposite of the bead, analogous to the situation

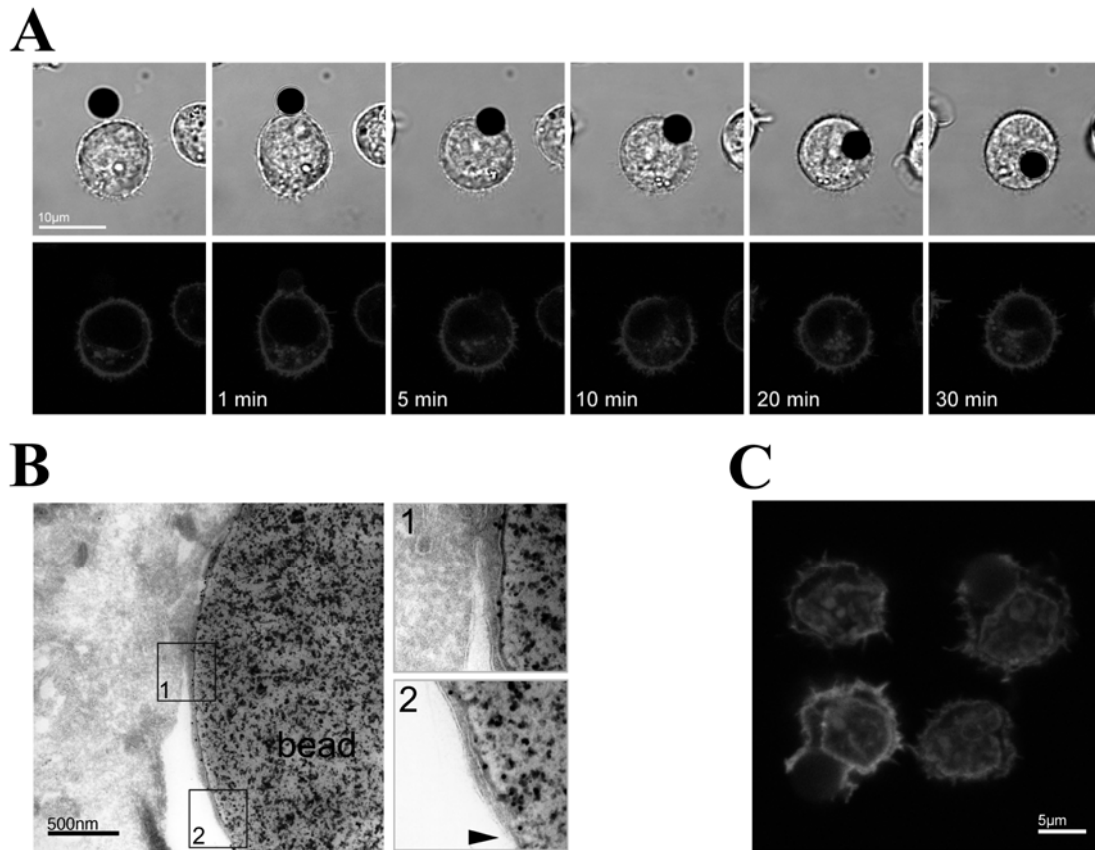


Figure 1. Efficient BCR-mediated phagocytosis of large particulate Ags.

(A) Living Ramos B cells expressing DR-GFP and anti-IgM-coated beads were imaged every 30 s. Depicted are time points after initial contact, top panel: transmission image, bottom panel: GFP signal. Scale bar = 10 μm. Images are frames from supplemental movie 1. (B) Ramos cells were fixed 10 min after addition of anti-IgM coated beads and analyzed by cryoelectron microscopy. Scalebar = 500nm. Zoom-ins of the thin membrane extrusions surrounding the bead are shown for indicated regions. The tip of the protrusion is indicated with an arrowhead in inset 2. (C) Cells were fixed 10 min after addition of anti-IgM coated beads and processed for immunofluorescence confocal microscopy. Depicted is the overlay of the signals from DAPI nuclear staining (blue), phalloidin-stained actin-cytoskeleton (red), and DR-GFP (lower left cell only). Scalebar = 10 μm. Figure represents one section from a Z-stack. A three-dimensional reconstruction is provided as supplemental movie 4.

following Th cell contact (30) or following cytotoxic CTL-target cell interactions (31). Internalization reached completion within 10-20 min and required an intact cytoskeleton (as the microtubule-disruptive agent nocodazole prevented phagocytosis, supplemental movie 2). In addition, uptake was BCR dependent because beads coated with an irrelevant Ab were not internalized (supplemental movie 3). Ramos cells do not express FcγRs, which excludes their involvement in bead uptake. A detailed analysis by cryoelectron microscopy revealed some of the

impressive cellular events underlying uptake of large particulate Ags. During the initial phase of contact, Ramos cells surrounded the bead with a surprisingly thin double membrane originating from the cell surface (Fig. 1B). Staining with phalloidin of Ramos cells in the process of bead phagocytosis revealed extensive actin fibers in the membrane protrusions surrounding the bead (Fig. 1C and supplemental movie 4). Together, these data show that B cells are able to internalize inert particles in a process that fulfills the criteria of phagocytosis. Thus, different from the general concept that primary B cells are essentially nonphagocytic cells, B cells are very efficient phagocytes when particle recognition is facilitated by the BCR.

B cell lines can internalize Salmonella via their BCR

We generated a stable transfectant of the Ramos B cell line with the MHC class II Ag presentation chaperone HLA-DO tagged with GFP (DO-GFP). DO-GFP localizes to the MIICs in living cells. As the Ag specificity of the BCR of Ramos cells is unknown, we coated the bacteria with anti-IgM-anti-LPS tetrameric Ab complexes. Within 1 mi after first contact, Ramos cells efficiently internalized GFP-*Salmonella* coated with tetrameric Ab complexes (Fig. 2A and supplemental movie 5). Uncoated *Salmonella* were ignored by Ramos B cells (supplemental movie 6), showing that *Salmonella* by itself is not able to invade the Ramos B cell line. To investigate the internalization process in more detail, we used cryoelectron microscopy on Ramos cells shortly after encounter of the coated bacteria. This showed the formation of a phagocytic cup and the extension of pseudopodia around the bacteria, demonstrating that B cells actually seem to phagocytose the *Salmonella* bacteria (Fig. 2B). To study the interaction between the green MIICs in Ramos DO-GFP with the SCVs, we used a red mRFP-*Salmonella*. Ramos DO-GFP cells incubated with coated mRFP-*Salmonella* showed bacterial uptake and rapid translocation of the MIICs to the SCVs (Fig. 2C and supplemental movie 7). Multiple intimate contact events were observed between the membrane of the SCVs and the DO-GFP positive MIICs, suggesting fusion events of the MIICs with the SCV immediately after bacterial uptake.

To study the acquisition of MHC class II molecules on the SCV membrane, we used Ramos cells expressing DR-GFP. MHC class II molecules already localized to the membrane of the SCV during the actual process of BCR-mediated uptake of coated mRFP-*Salmonella* (Fig. 2D and supplemental movie 8). Similar to the DO⁺ vesicles,

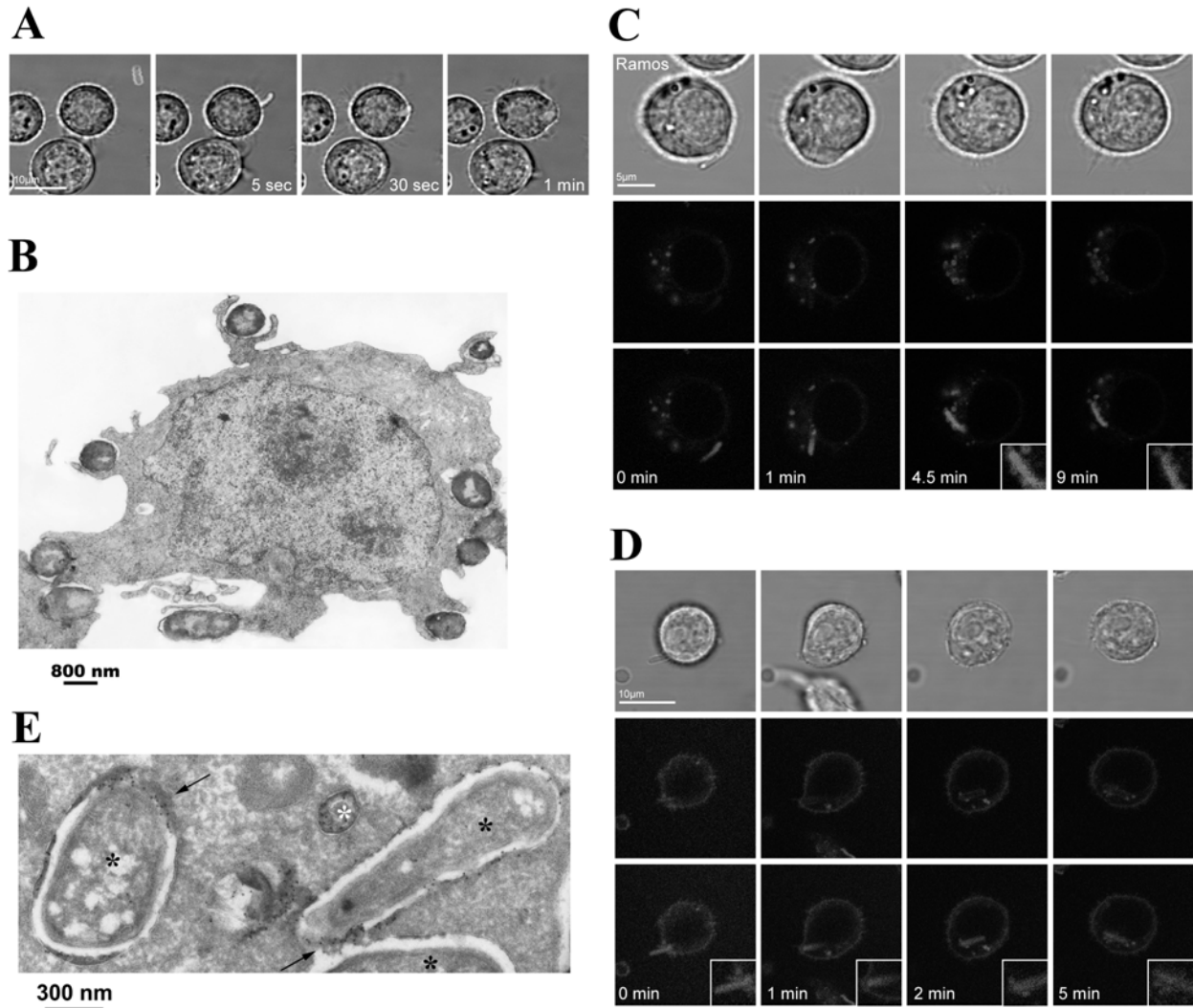


Figure 2. Efficient BCR-mediated internalization of *Salmonella*.

(A) Living Ramos cells, expressing DO-GFP, and GFP-expressing *Salmonella* were imaged every 3 s. Depicted are time points after initial contact. GFP signal is projected on top of the transmission image. Scale bar = 10 μ m. Images are frames from supplemental movie 5. (B) Electron microscopic analysis of Ramos cells in the process of phagocytosing anti-BCR-coated *Salmonella*. Scale bar = 800nm. Note the cup-shaped pseudopodia of the B cell at contact places with the bacteria. (C) Living Ramos cells expressing DO-GFP and mRFP-*Salmonella* were imaged every 10 s. Depicted are time points after initial contact, top panel: transmission image, middle panel: GFP signal, bottom panel: overlay of GFP and mRFP signal. Scale bar = 5 μ m. Inset shows zoom-in on bacterium. Images are frames from supplemental movie 7. (D) Living Ramos cells expressing DR-GFP and mRFP-*Salmonella* were imaged every 10 s. Depicted are indicated time points after initial contact, top panel: transmission image, middle panel: GFP signal, bottom panel: overlay of GFP and mRFP signal. Scale bar = 5 μ m. Inset shows zoom-in on bacterium. Images are frames from supplemental movie 8. (E) Immunoelectron microscopic analysis of primary B cells with internalized anti-BCR coated *Salmonella* and CD63 (10-nm gold particles). Black asterisks mark bacteria, the white asterisk marks an MIIC; and arrows indicate fusion events with MIICs.

we observed extensive docking of DR-GFP⁺ vesicles with the SCV membrane within minutes after entry. Because molecular exchange between the MIIC vesicles and the SCVs is critical for generation of MHC class II molecules complexed with *Salmonella* Ags, we visualized MIIC-SCV fusion by EM. Indeed, fusion between the characteristic multivesicular MIICs and the SCVs was frequently observed. Immunostaining showed that, next to the MIICs, the SCV membrane stained positive for CD63 (Fig. 2E). Quantification of the fusion events between MIICs and 100 SCVs showed that in 10% of the SCVs the actual process of MIIC-SCV fusion was captured in the time frame of cell fixation. Thus, the combined acquisition of MHC class II on the SCVs and the frequent fusion events between SCVs and MIICs generates all conditions required for Ag presentation of *Salmonella* Ags.

BCR-mediated internalization of *Salmonella* by primary human B cells

Since Ramos cells are at least 1.5 times larger than primary B cells, we tested whether primary B cells could internalize *Salmonella* as well. To distinguish between binding of bacteria to the BCR and actual uptake, we used a mAb against *Salmonella*-LPS. Completely internalized GFP-positive bacteria will not be stained, while extracellular and partially engulfed bacteria will be accessible to the anti-LPS antibody. Incubation of primary human B cells with uncoated GFP-*Salmonella* consistently revealed a small but significant population of B cells (4.3%, SD = 1.1, n = 6) that recognized and internalized the native bacterium via direct recognition of *Salmonella* Ags by the B cell's BCR (Fig. 3A). A similar proportion of primary B cells recognized and captured dead uncoated GFP-*Salmonella* via their BCR (4.1%, SD = 1.5, n = 4), but failed to internalize dead *Salmonellae* since these B cells stained all positive for LPS (Fig. 3A). Analysis by confocal microscopy confirmed that internalized viable *Salmonellae* are completely taken up by primary B cells, resulting in one to three intracellular bacteria per B cell (Fig. 3B, left panel). Incubation with fixed bacteria only showed binding but no uptake of *Salmonella* (Fig. 3B, right panel). To address the possible involvement of FcγRs on primary B cells, we preincubated primary B cells with F(ab)₂ fragments of the anti-IgM antibody MH15 to block the internalization of anti-IgM coated *Salmonella* by primary B cells. This resulted in inhibition of 80%, illustrating that internalization is indeed BCR-mediated (supplemental Fig. 1). Ideally, we would also like to block BCR-mediated internalization of *Salmonella* via direct recognition of *Salmonella* Ags by the BCR. However, since blocking of the Ag binding site of the BCR is impossible

due to lack of anti-Id Abs, we studied the effect of BCR internalization before addition of the bacteria on the efficiency of bacterial uptake. We combined Abs against the H chain of IgM with cross-linking goat-anti-mouse antibodies. This resulted in a partial internalization of the BCR (mean fluorescence intensity for membrane-bound IgM dropped from 3767 to 2275) and a concomitant reduction in GFP⁺/LPS⁻ B cells that had completely internalized *Salmonella* (supplemental Fig. 2). In addition, Ramos cells efficiently internalized GFP-*Salmonella* in a BCR dependent manner only when *Salmonella* was coated with anti-IgM (Fig. 3C). No GFP-positive Ramos cells were found without the anti-IgM coat, showing that *Salmonella* is unable to infect Ramos cells autonomously. As a control for IgM-type BCR-mediated uptake of Ramos, *Salmonella* coated with anti-IgG were incubated with Ramos cells, and (similar to uncoated bacteria) these were not internalized. Incubation of the IgG-type BCR-expressing B cell line Cess with anti-IgG-coated bacteria showed that the anti-IgG coated-bacteria were efficiently taken up by Cess (data not shown). Up to 90% of the Ramos cells acquired one or more anti-IgM coated bacteria. However, ~ 25% of Ramos cells contained internalized GFP-*Salmonella* only, while >60% of Ramos cells were also positive for LPS staining. Confocal microscopy of cells labeled by anti-LPS antibodies showed that these represented Ramos cells that had internalized some but not all bound bacteria (data not shown).

Since dead bacteria were not internalized, *Salmonella* may be requiring both recognition by BCR and bacterial-mediated processes to enter primary human B cells. *Salmonella* can invade host cells by expressing type III secretion systems encoded either by *Salmonella* pathogenicity island (SPI)-1 to translocate effector proteins into host cell cytoplasm that trigger internalization of the bacteria or by SPI-2 to modulate intracellular trafficking and replication of *Salmonella* within a modified vacuolar compartment. Recent studies however have modified this concept to some extent as they show a partial overlap in SPI-1 and SPI-2 functions (32). To address involvement of these type III secretion systems, we used *Salmonella* defective in SPI-1 (*invA* mutant) or SPI-2 (*ssrA* mutant). Incubation of primary B cells with SPI-1 defective *Salmonella* showed a reduction in internalized bacteria and no bacteria adhering to primary B cells. Incubation with SPI-2-defective *Salmonella* showed a milder reduction in internalized bacteria and a minimal reduction in adhering bacteria (Fig. 3D). This indicates that SPI-1 is involved in attachment to primary B cells in conjunction with the BCR. Components

of SPI-1, and to a lesser extent SPI-2, are involved in BCR-mediated uptake of *Salmonella* by primary B cells.

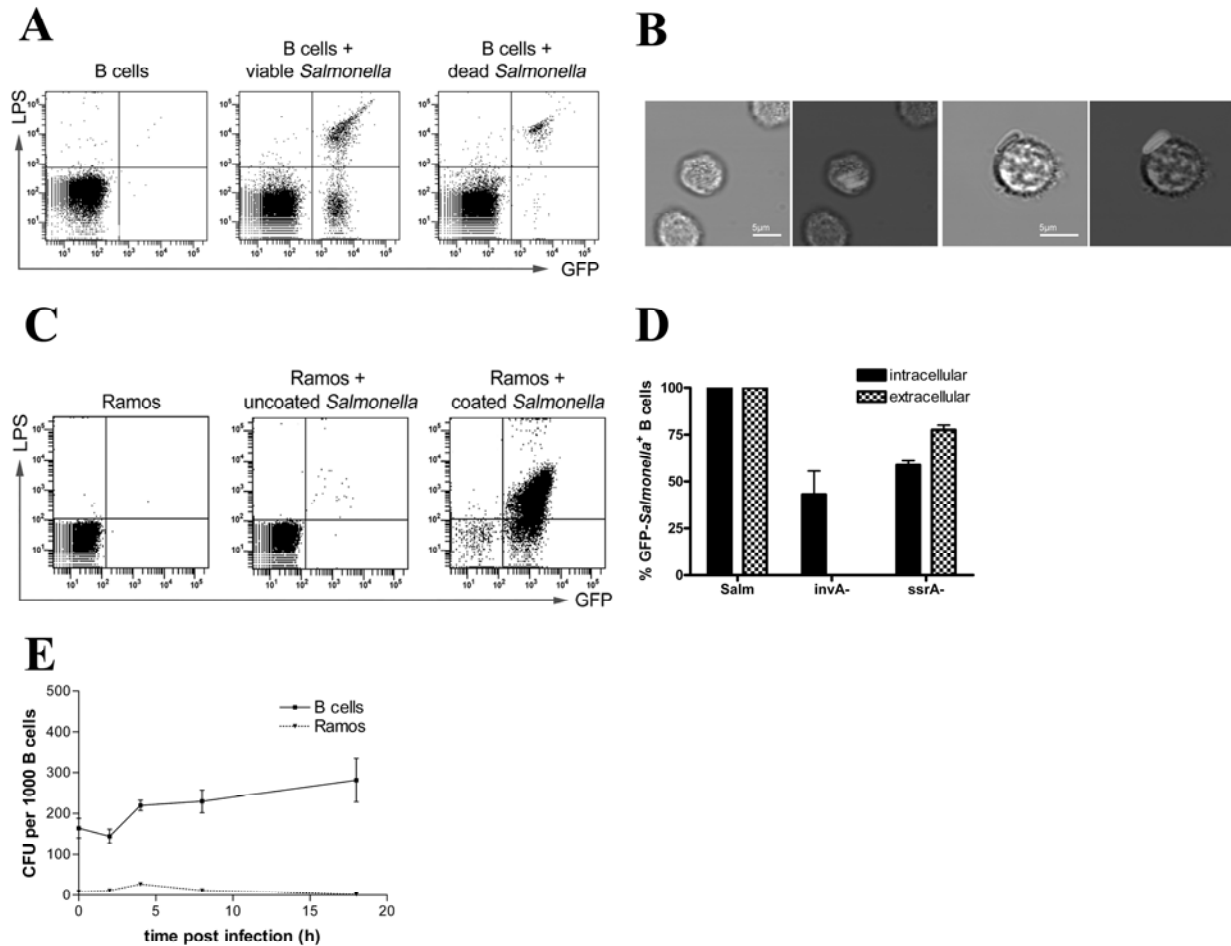


Figure 3. BCR-mediated internalization and survival of *Salmonella* in primary B cells.

(A) CD19⁺ B cells were incubated with viable GFP-*Salmonella* for 40 minutes and FACS analyzed. Anti-LPS-APC versus GFP scatter plots are depicted. (B) Living primary human B cells were incubated with viable (left) or dead (right) GFP-*Salmonella* for 40 min and imaged using confocal microscopy. Left panel: transmission image, right panel: GFP signal projected on transmission image. Scalebar = 5µm. (C) Ramos B cells were incubated with viable GFP-*Salmonella* for 40 min, stained with an antibody against *Salmonella* LPS and analyzed by FACS. Depicted are anti-LPS-APC vs. GFP scatter plots of 50.000 events. (D) CD19⁺ B cells were incubated with viable wild-type GFP-*Salmonella* or mutant for SPI-1 (invA⁻) or SPI-2 (ssrA⁻) for 40 minutes and analyzed by FACS. Intracellular indicates B cells that are GFP⁺LPS⁻ and extracellular indicates GFP⁺LPS⁺ B cells. Data are from two independent experiments with cells from four different donors, error bars indicate SEM. (E) B cells were incubated with anti-IgM-coated *Salmonella* and Ramos cells with uncoated *Salmonellae*, lysed and plated onto LB-agar plates. Data are from duplicates of experiments performed with B cells from two individual donors, error bars indicate SEM.

Phagocytosed *Salmonella* grow in many cell types but can efficiently be destroyed in specialized cells like macrophages and neutrophils (33). To examine survival of internalized *Salmonella* in primary B cells, we performed plating assays. B cells were incubated with anti-IgM coated *Salmonella* and washed and cells with internalized bacteria were followed in time. At different time points, cells were lysed and intracellular bacteria were plated onto agar. Internalized *Salmonella* remained vital for the 18 h that we tested (Fig. 3E). Ramos cells incubated with uncoated *Salmonella* were used as a control, since uncoated *Salmonella* are not taken up by Ramos cells (supplemental movie 6). Indeed, hardly any *Salmonellae* were recovered after incubation with Ramos (Fig. 3E). Thus, *Salmonella* survives intracellularly after BCR-mediated internalization by primary B cells.

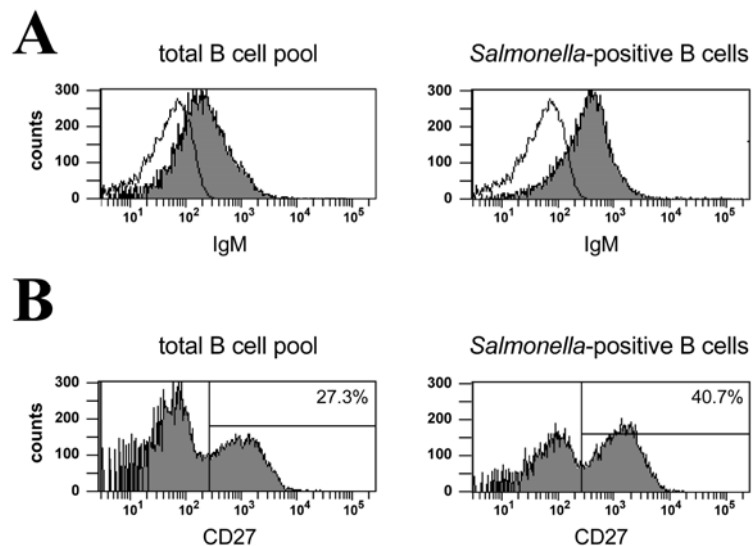


Figure 4. Both naive and memory B cells internalize *Salmonella*.

(A) Primary B cells were incubated with GFP-*Salmonella* and stained for IgM before and after FACS sorting. The open histogram represents the IgG1 isotype control. (B) The cells from 3A were analyzed for expression of CD27. Percentages of CD27⁺ B cells are given. For IgG1 isotype control, see Fig. 3A.

Which peripheral B cell type is able to internalize *Salmonella*? Most peripheral B cells express the IgM surface Ig receptor (34). To confirm that internalization of *Salmonella* occurs via the BCR, we analyzed IgM expression on *Salmonella*-containing B cells. This showed that B cells that have internalized *Salmonella* expressed surface IgM (Fig. 4A). The mean fluorescence intensity of IgM for the total B cell pool is 478 and for the *Salmonella*-containing B cells 825. Two major subsets of B cells can be identified in adult peripheral blood according to the expression of CD27. CD27 expressing B cells comprise memory B cells while CD27-

negative B cells represent naive and transitional B cells (35). FACS analysis showed that IgM⁺ memory B cells (CD27⁺) internalized *Salmonella* more efficiently than IgM⁺CD27⁻ naïve B cells (Fig. 4B). Although a proportion of the naïve IgM⁺ B cells are able to take up *Salmonella*, *Salmonella* is preferentially internalized by the circulating IgM⁺ memory B cells.

Presentation of BCR-internalized Ags by B cells to T cells

When *Salmonella* survives within the phagosome (see Fig. 3E) following BCR-mediated internalization, does this result in MHC class II mediated presentation of *Salmonella* Ags? To test this, primary human B cells were incubated with anti-IgM-coated *Salmonellae* to achieve BCR-mediated uptake by all IgM⁺ B cells and maximize the number of Ag-presenting B cells. These cells were subsequently cultured in the presence or absence of autologous primary T cells. After 5 days, [³H]thymidine was added and cells were harvested after 18 h. Incubation of B cells with anti-BCR-coated *Salmonella* induces proliferation of the B cells (green line, Fig. 5A), demonstrating that BCR ligation and BCR-mediated internalization of *Salmonella* effectively activated B cells. B cells incubated with coated *Salmonella* and cultured with autologous T cells results in an Ag-specific proliferation of T cells (red line) (Fig. 5A).

To study whether primary B cells with a BCR directed against *Salmonella* also induce T cell proliferation, we incubated uncoated *Salmonella* with primary B cells (Fig. 5B, left panel). Addition of autologous T cells yielded a *Salmonella*-specific T cell proliferation response (blue vs pink line). B cells incubated with uncoated dead *Salmonella* that could not be internalized were able to induce T cell proliferation (Fig. 5B, right panel). However, T cell proliferation is optimal when viable *Salmonellae* have been internalized by B cells (Fig. 5B: left vs right panel). Incubation of only T cells with uncoated or coated bacteria did not result in B cell-independent proliferation of the T cells (data not shown).

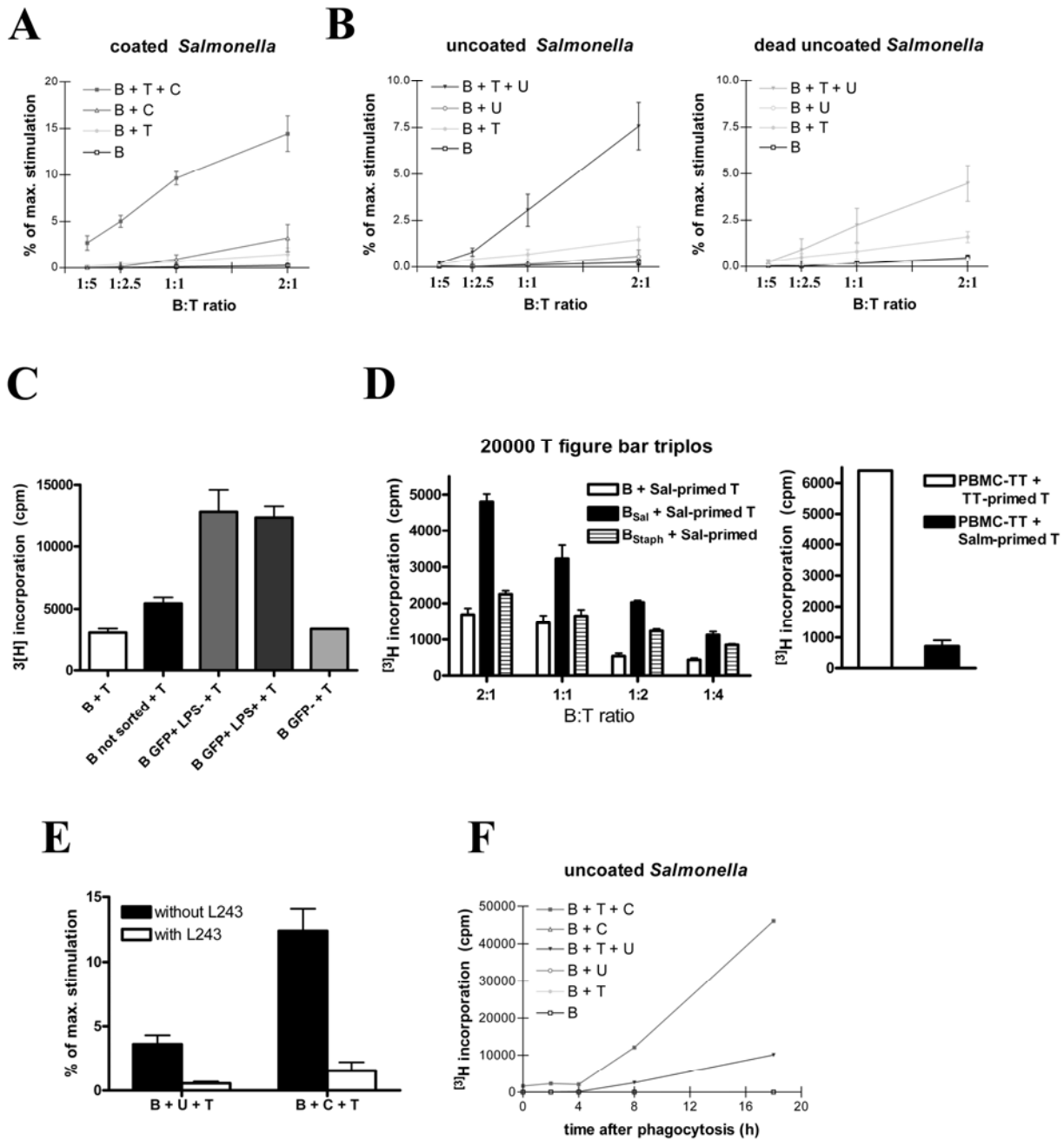
To demonstrate that the T cell proliferation in Fig. 5B was indeed induced by the fraction of B cells that had captured *Salmonella*, we FACS sorted the GFP-*Salmonella*-positive B cells in fractions positive and negative for anti-LPS staining and cultured these with autologous T cells. T cells only proliferated when cultured with B cells that had captured GFP-*Salmonella* (Fig. 5C). It may be that complete internalization is not required for Ag presentation but that only capturing of the bacteria by the BCR suffices.

We can however not exclude that GFP-*Salmonella*⁺LPS⁺ B cells also contain completely internalized bacteria as we have shown that B cells are able to take up more than one *Salmonella*. Moreover, even though *Salmonella* survives in the vacuole and suppresses MHC class II Ag presentation in phagosomes (27), *Salmonella* Ags are still efficiently presented by B cells. This probably reflects Ag degradation and loading on MHC class II molecules in normal MIICs after content exchange between phagosome and MIICs due to the observed intimate contact and extensive fusion events.

To demonstrate the Ag-specificity of the proliferating T cells, we performed restimulation assays in which we sorted the T cells that proliferated in response to B cells that had taken up *Salmonella*, and restimulated these *Salmonella*-primed T cells with autologous B cells that had taken up *Salmonella* or *Staphylococcus*. This showed that the *Salmonella*-primed T cells are indeed for the large part Ag specific, as they proliferate better in response to B cells that had internalized *Salmonella* than B cells that had internalized *Staphylococcus* or control B cells without bacteria. As a control, T cells primed against *Staphylococcus* did proliferate in response to B cells that had internalized *Staphylococcus*, demonstrating that these B cells did present *Staphylococcus* Ags to CD4⁺ T cells (Fig. 5D, left panel). In addition, we restimulated *Salmonella*-primed T cells with PBMCs presenting tetanus toxoid Ags. This showed no response of the *Salmonella*-primed T cells, while tetanus toxoid-primed T cells from the same donor proliferated after restimulation (Fig. 5D, right panel).

Furthermore, we performed blocking assays to show that the T cell response depends on the MHC class II Ag presentation pathway with L243, an Ab that blocks the MHC class II-TCR interaction (Fig. 5E). The induction of T cell activation depended on presentation of *Salmonella* Ags via MHC class II (HLA-DR), as the T cells failed to respond after blocking of MHC class II with L243.

The observation that fusion of MIICs with the phagosome occurs swiftly prompted us to examine whether this had consequences for Ag presentation. When we irradiated B cells immediately after incubation with anti-IgM coated *Salmonella*, no proliferation of B or T cells was observed after 6 days. B cells apparently need to be viable to process and present *Salmonella* Ags to T cells. To study the kinetics of Ag presentation, B cells were incubated with native or anti-IgM coated *Salmonella* and B cells were irradiated at several time points before incubation with T cells. After 5 days, [³H] thymidine was added and cells were harvested after 18 h. Noninfected B



cells did not induce T cell proliferation after irradiation. Anti-BCR coated *Salmonella* internalized by IgM⁺ B cells start to induce proliferation of T cells immediately (red line) and uncoated *Salmonella* internalized by BCR-reactive B cells 4 h after uptake of the *Salmonella* (blue line) (Fig. 5F). Ag presentation thus starts at times corresponding to the earliest phases of BCR-induced internalization and rapid fusion with the MIICs. Primary B cells rapidly present Ags of internalized *Salmonella*, even if the bacterium survives inside a B cell.

Figure 5. BCR-mediated uptake of *Salmonella* induces Ag presentation by B cells.

(A) BCR-induced internalization results in proliferation of B cells and Ag-specific T cells. B cells (B) were either or not incubated with viable anti-BCR-coated (C) *Salmonella* in the presence or absence of autologous T cells (T), as indicated. Results are shown as percentage of maximal stimulation of T cells with anti-CD3 and -CD28 Abs. (B) The same experimental setup as in Fig. 4A was performed with uncoated (U) viable and dead *Salmonellae*. Data are from four independent experiments of different donors, error bars indicate SEM. B:T represents the ratio of different amounts of B cells added to a fixed amount of T cells. Experiments with uncoated and coated *Salmonella* were performed in parallel using the same donor. (C) B cells incubated with viable uncoated GFP-*Salmonella* were FACS-sorted as indicated and incubated for 6 days with T cells. White bar: B cells without bacteria, black bar: B cells before sorting, green bar: GFP-*Salmonella*⁺LPS⁻-sorted B cells, blue bar: GFP-*Salmonella*⁺LPS⁺-sorted B cells and grey bar: GFP-*Salmonella*⁻-sorted B cells. Data are from two independent experiments with cells from different donors, error bars indicate SEM. (D) *Salmonella*-primed or *Staphylococcus*-primed T cells were restimulated for 2 days with autologous B cells that were incubated with viable *Salmonella* or *Staphylococcus* or restimulated with PBMCs incubated with tetanus toxoid. Data are representative for three independent experiments with different donors, error bars indicate SEM. (E) T cells (T) were cultured with B cells (B) that had taken up uncoated (U) or anti-BCR-coated (C) *Salmonella* either or not in the presence of the MHC class II Ag presentation blocking Ab L243. (F) Ag presentation by B cells starts immediately after internalization of the *Salmonella*. B cells (B) were either or not incubated with uncoated (U) or anti-BCR-coated (C) *Salmonella* and irradiated with 60 Gy at different time points before T cells (T) were added. Data are representative for four independent experiments of different donors.

BCR-mediated internalization induces IgM secretion

To test if BCR-mediated internalization of *Salmonella* leads to differentiation of B cells into Ab-secreting cells, supernatants of B cells that internalized bacteria were tested for the presence of human IgM after culture. After 5 days incubation with viable uncoated bacteria, no strong induction of IgM secretion following BCR-mediated internalization was detectable (Fig. 6A, *left panel*). When the *Salmonella* were coated with anti-IgM Abs, B cells produced four times more IgM than uncoated bacteria. Addition of T cells did not increase IgM production in the first 5 days, indicating that IgM production resulted from a T cell-independent activation of B cells (Fig. 6A, *left panel*). T cell help did occur within 12 days, leading to a strong increase in IgM production (Fig. 6A, *right panel*). Thus, BCR-mediated internalization of *Salmonella* induces autonomous IgM secretion by B cells, whereas T cell help is required during the late stage of Ig secretion by B cells. IgG production of B cells incubated with *Salmonella* did not significantly surpass IgG production levels from B and T cells that were not incubated with *Salmonella* (data

not shown). This is in line with the observation that the B cells that take up *Salmonella* are IgM⁺ memory B cells. It also indicates that BCR-mediated internalization of *Salmonella* by the naive IgM⁺ B cell pool does not induce Ig class switching under our culture conditions.

When B cells that internalize *Salmonella* through the BCR are activated, they might produce *Salmonella*-specific Abs. We incubated uncoated, viable GFP-*Salmonella* with primary B cells and FACS-sorted the GFP⁺ B cells. We cultured the sorted B cells on a monolayer of fibroblasts expressing human CD40L to provide costimulation. After 12 days, total human IgM as well as *Salmonella*-reactive Abs were quantified. B cells that internalized *Salmonella* produced more total IgM than B cells that did not take up *Salmonella* (Fig. 6B, upper left panel). The production of *Salmonella*-reactive Abs was measured using a whole-cell *Salmonella* ELISA.

Strikingly, the sorted *Salmonella*-containing B cells produced significant amounts of anti-*Salmonella* IgM (Fig. 6B, lower left panel), unlike control B cells from the same donor. Correction of the anti-*Salmonella* reactive IgM for total IgM production by the B cells revealed that the sorted B cells produced significantly higher levels of anti-*Salmonella* IgM compared to control B cells (Fig. 6B, right panel). The production of significantly higher levels of anti-*Salmonella* IgM clearly shows the involvement of the BCR in internalization of *Salmonellae*. If the BCR would not be involved, but bacterial internalization and subsequent B cell activation would solely depend on TLR stimulation and/or co-stimulation, the B cells that had taken up *Salmonella* would not be able to produce *Salmonella*-specific IgM. Thus, BCR-mediated internalization of *Salmonella* forms an efficient pathway to induce differentiation of *Salmonella*-specific B cells and production of *Salmonella*-reactive IgM Abs.

Discussion

B cells may encounter Ags as free Ag or delivered by Dendritic cells (DCs) (36). DCs are equipped with both nondegradative and degradative Ag uptake pathways to facilitate Ag presentation to both B and T cells. Blood DCs can capture and transport particulate Ags such as invading bacteria to the spleen, where they promote differentiation of marginal zone B cells into IgM secreting plasma cells (37). We here show a pathway independent of DCs and macrophages. In contrast to the dogma that mammalian B cells lack the ability to ingest pathogens and are only involved in the adaptive phase of the immune response (38) or that entry of

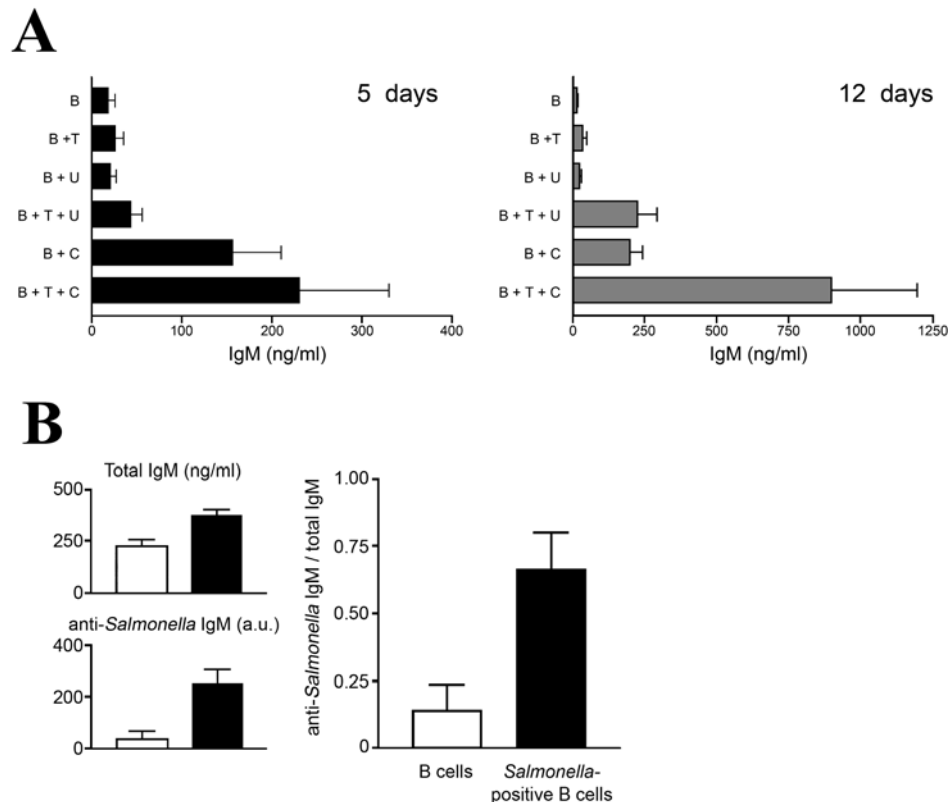


Figure 6. BCR-mediated internalization induces IgM production and B cells with a BCR reactive for *Salmonella* Ags produce anti-*Salmonella* IgM.

(A) B cells (B) were either or not incubated with uncoated (U) or anti-BCR coated (C) *Salmonella* in the presence or absence of autologous T cells (T). After 5 (black bars, *left panel*) and 12 days (grey bars, *right panel*) total secreted human IgM was determined. (B) B cells either or not incubated with viable uncoated GFP-*Salmonella* were FACS sorted and cultured on a monolayer of irradiated, CD40L-expressing fibroblasts for 12 days and supernatant was analyzed for total IgM production and *Salmonella*-reactive IgM production. White bars represent B cells and black bars represent *Salmonella*-positive B cells. Anti-*Salmonella* IgM was divided on the total amount of IgM measured in the supernatants (*right panel*, $P = 0.006$). Data are representative for three independent experiments of different donors.

Salmonella in B cells is a random process (39), primary B cells can internalize *Salmonella* via their specific BCR. So far, the general concept for Ag presentation of bacterial peptides by B cells was that B cells extract proteins from the surface of DCs or bacteria or bind shedded bacterial proteins (15). Indeed, this may occur for dead or lysed bacteria killed by Abs and complement or after antibiotic treatment. Our observation that recognition via the BCR of dead bacteria without internalization induces Ag presentation to T cells is in line with this concept. However, internalization of viable bacteria leads to superior CD4⁺ T cell activation and instantaneous generation of anti-*Salmonella* Abs by autonomous activation of the *Salmonella*-reactive B cells.

It has been proposed that Abs made by IgM memory B cells are the first-line defense mechanism against all infections and that Abs produced by IgM memory B cells are the only B cell defense against T-independent Ags (40). IgM⁺ memory B cells in peripheral blood represent circulating splenic marginal zone B cells in charge of T-independent responses (41). Since marginal zone B cells express a BCR of polyreactive nature (42), this could explain the relatively high numbers of CD27⁺ B cells that take up *Salmonella*. As for IgM⁺ memory B cells, a subset of mature naive B cells in peripheral blood are polyreactive (43). Combined, the primary B cells that we found to internalize *Salmonella* seem to represent naive and IgM⁺ memory B cells with a polyreactive BCR.

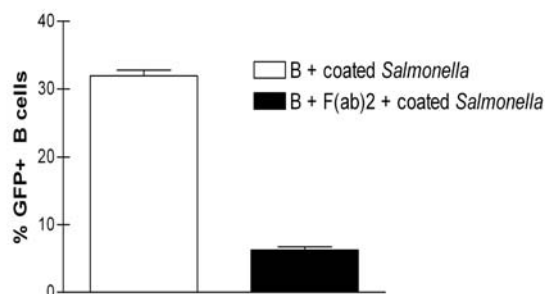
How do these findings relate in the involvement of B cells in *Salmonella* infection? Studies in B cell-deficient mice show that B cells are necessary for efficient protection against both primary and secondary infection with *Salmonella* (44). Passive transfer of *Salmonella*-immune serum could not restore resistance of mice to *Salmonella* (45), demonstrating that high-affinity Ab production alone is not the only function of B cells in salmonellosis. Moreover, at the early stage of primary infection class-switched high-affinity Abs against *Salmonella* are not yet available and cannot explain the importance of B cells at this stage. Therefore polyreactive, IgM⁺ memory B cells may well be involved in protection against primary infection via BCR-mediated internalization of *Salmonella* and rapid generation of protecting *Salmonella*-reactive IgM Abs. B cell deficient *Igh-6*^{-/-} 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 as well (46). The importance of B cells in this line of immune defense may relate to their property to present Ags to T cells. It remained unclear how Ag presentation was achieved since processing and presentation of Ags by naive B cells was not observed. In this study, we provide a missing link in these observations by showing that the IgM⁺ B cells can internalize viable bacteria and very efficiently induce Th activation. However, IgM secretion can also be induced by BCR-mediated *Salmonella* uptake and activation alone, albeit less efficient than observed with additional CD4⁺ T cell help. The rapid secretion of IgM before B cells encounter CD4⁺ T cells represents a first line of specific immune responses to pathogens and may represent the remaining humoral response when CD4⁺ T cell help fails, as is the case in HIV patients. In conclusion, we demonstrate for the first time that bacterial uptake via the BCR by B

cells forms a highly efficient pathway to generate an immediate antimicrobial humoral immune response.

Acknowledgements

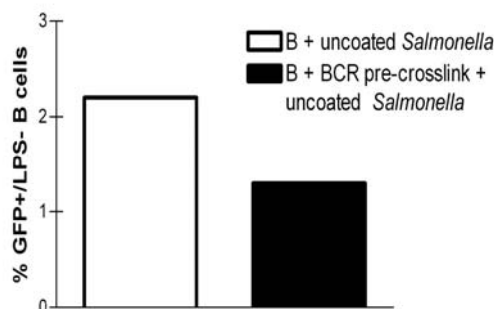
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Supplemental figure S1. Blocking of the BCR with F(ab)₂ fragments.

Primary B cells were pre-incubated with F(ab)₂ fragments of the anti-IgM antibody before incubation with anti-IgM coated *Salmonellae*. Data are from five different donors, error bars represent SEM.



Supplemental figure S2. BCR internalization before incubation with bacteria.

Primary B cells were pre-incubated with an anti-IgM antibody followed by incubation with a goat-anti-mouse antibody to achieve partial BCR internalization before incubation with uncoated *Salmonellae*. Data are one representative set of experiments from four different donors.

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Chapter 2

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Chapter 3

Immune escape and dissemination of *Salmonella* by antigen-specific B lymphocytes

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Submitted for publication

Abstract

The bacterial pathogen *Salmonella* causes worldwide morbidity and mortality. A major route of host entry in the intestinal mucosa is via M cells, directly entering the B cell-rich Peyer's Patches. *Salmonella* can be taken up by antigen-specific B cells in a B cell receptor (BCR)-dependent fashion. We studied the contribution of B cells to the pathogenicity of *Salmonella typhimurium*. Upon phagocytosis of *Salmonella* by primary human B cells, *Salmonella* survives intracellularly in a dormant state that is actively maintained by the B cell. Subsequently, *Salmonella* is excreted by B cells, allowing infection of other cell types and reinitiation of replication. B cells may thus function as a transport vehicle for *Salmonella*. Indeed, adoptive transfer of *Salmonella*-specific B cells before oral infection of mice with *Salmonella* was required to disseminate *Salmonella* to the spleen. *Salmonella*-specific B cells thus function as survival niche and reservoir to contribute to systemic dissemination of *Salmonella* and represent the first example of a pathogen that uses cells of the adaptive immune system for spreading of infection.

Introduction

Salmonella enterica is a Gram-negative, enteric pathogen responsible for disease syndromes of significant morbidity and mortality (1). After oral uptake, the bacterium crosses the intestinal epithelium and enters the Peyer's patches via specialized antigen-sampling M cells (2) or via luminal capture by dendritic cells (3, 4). They are ultimately internalized by macrophages, dendritic cells, and neutrophils (5, 6). Entry into these cells is actively induced by the bacterium through an impressive array of effector proteins that orchestrate uptake by manipulating the host cellular machinery (7). *Salmonella* manipulate host cells upon infection in order to alter the actin cytoskeleton allowing phagosomal cup formation and entry of the relatively large pathogen into the host cell. Bacterial effector proteins are therefore introduced into the host cytosol through the *Salmonella* Type III Secretion System (TTSS). *Salmonella* can thus enter most cell types to form an intracellular vacuole called the *Salmonella*-containing vacuole (SCV). Here, another set of effectors is secreted into the host cytosol for vacuole maintenance and interference with the endosomal system to obtain nutrients and to prevent maturation and fusion with lysosomes (8, 9) by manipulating the Akt-AS160-Rab14 cascade and PAK4 (10). *Salmonella* replicates in an expanding SCV (11, 12) and escapes detection by the immune system (13, 14). Although *Salmonella* replicates in the phagosome, it remains unclear how the bacteria are released from the infected cell. Obvious mechanisms would involve apoptosis or necrosis of the infected cell, but such is not established.

When *Salmonella* has passed the intestinal epithelium, it spreads via mesenteric lymph nodes to liver, bone marrow and spleen where replication continues (15). How *Salmonella* reaches these organs is unclear. So far, dendritic cells, macrophages, neutrophils and CD18-expressing phagocytes have been implicated to be the target cells of *Salmonella* infection (4, 16). Similar to HIV (reviewed in (17)), dendritic cells may act as pathogen carriers for more systemic spreading of the infection. Macrophages, dendritic cells and neutrophils however, exhibit efficient bactericidal mechanisms (6, 18) that render these cells less favorable as vehicles for bacterial dissemination. *Salmonella* is able to enter and survive within human B cell lines (19, 20). In mice, infected B cells are able to present *Salmonella* antigens to CD4⁺ T cells (21). We recently showed that primary human B cells are able to internalize *Salmonella* after recognition by the B cell receptor (BCR) and subsequently present bacterial antigens to *Salmonella*-specific CD4⁺ T cells (22).

Here we show how primary antigen-specific B cells are used as transport vehicle for spreading within the host. We show that *Salmonella* utilizes the specificity of the immune system after uptake by antigen-specific B cells to survive intracellularly in a dormant state that is actively maintained by the B cell. Ultimately, *Salmonella* is excreted by the B cell followed by reinfection and replication in other cell types. Adoptive transfer of B cells with transgenic BCRs that specifically recognize hen egg lysozyme (HEL)-expressing *Salmonella* showed that *Salmonella*-specific B cells contribute to the *in vivo* dissemination of *Salmonella* in mice after oral administration of the bacteria. The antigen-specific B cells thus act as antigen-specific reservoirs or transport vehicles to release *Salmonella* at distant sites for further infection. These data provide the first example of the use of the adaptive immune system by bacterial pathogens for spreading infection in a situation analogous to the involvement of dendritic cells in the spreading of HIV.

Materials en methods

Mice

C57BL/6 mice (6–8 weeks old) were purchased from Harlan (Udine, Italy). BCR-HEL VDJ ki mice (a kind gift of Dr. J. Cyster, University of California, San Francisco) were bred under specific pathogen-free conditions at Charles River Laboratories. All experiments were performed in accordance with the guidelines established in the Principles of Laboratory Animal Care (directive 86/609/EEC).

Evaluation of Salmonella spreading in vivo.

CD43- naïve B cells were purified from spleens of BCR-HEL VDJ ki mice with CD43 (Ly-48) Microbeads (Miltenyi Biotec, Bologna, Italy) according to the manufacturer's instructions. 10^6 - 2×10^5 cells were injected intravenously into C57BL/6 mice (14 mice per group) one day before oral infections. For survival experiments, mice received 10^5 CFUs of HEL surface-expressing *S. typhimurium* SL1344, and survival was monitored daily. For evaluation of splenic colonization, mice received 10^5 CFUs of bacteria. Sixteen hours after infection, spleens were collected and processed; a fixed number of cells was lysed with 0.5% sodium-deoxycholate and plated onto TB-agar plates for CFU counting 12 hr later.

Antibodies and bacterial strains

mAb anti-human IgM (MH15, Sanquin, Amsterdam, The Netherlands) was mixed with rat anti-mouse IgG1 antibody (RM161.1, Sanquin) and mAb anti-*S. typhimurium* LPS (1E6, Biodesign International, Kennebunk, ME) to generate BCR-LPS tetrameric antibody complexes. Fluorescent secondary antibodies and Texas Red-phalloidin were from Molecular Probes (Leiden, The Netherlands). GFP-*S. typhimurium* SL1344 has been described (23). The *S. typhimurium* strain 14028 containing the lux operon of *P. luminescens* (luxCDABE) was a kind gift from S. Vesterlund (24) and K. Nealson. Exponentially grown bacteria were washed with PBS, incubated with BCR-LPS tetrameric antibody complexes for 30 min at RT, and washed twice to remove unbound antibodies. Surface HEL-expressing *S. typhimurium* SL1344 was generated by electroporating bacteria with a pVUB4 vector (kindly provided by P. Cornelis, Flanders Institute for Biotechnology, Brussels, Belgium (25)) in which inactive HEL-encoding gene was cloned in frame with the one encoding for OprI protein from *P. aeruginosa* under the control of LacZ promoter. HEL expression was induced by the addition of 1 mmol/L isopropyl-L-thio-B-D-galactopyranoside to exponentially growing bacteria (26).

Lymphocyte isolation, infections and cell lines

Isolation of human B cells from peripheral blood and culturing of the Ramos B cell line and NIH3T3 fibroblasts expressing human CD40L (3T3-CD40L) have been described (22). B lymphocytes with viable uncoated bacteria and Ramos cells with viable anti-IgM coated bacteria were incubated for 40 min at 37°C w/o antibiotics while tumbling. Next, cells were washed four times and cultured for 1h in media containing 100 µg/ml gentamycin (Invitrogen) to eliminate non-phagocytosed bacteria. Cells were cultured in RPMI 1640 medium with 5% FCS, p/s, 2 mM L-Glutamine, 50 µM 2-mercaptoethanol, 20 µg/ml human apo-transferrin ((Sigma-Aldrich) depleted for human IgG with prot-G sepharose) and 10 µg/ml Gentamycin.

Live cell imaging

Wide field microscopy was performed at 37°C using 6-well plates (coated with Poly-L Lysine) and a Zeiss Axiovert 200 M microscope equipped with a FluorArc fluorescence lamp, motorized scanning stage, 63x LD Achromplan objective; NA 0.75 and climate chamber. Images were acquired using a Zeiss AxioCam MRm Rev.2 CCD in combination with the manufacturer's AxioVision software. All experiments

presented were repeated several times on different days, and results were consistent and reproducible. Further image processing was performed using the ImageJ software package.

Intracellular survival and growth assays

Human primary B cells were incubated in parallel experiments with either GFP- or Lux-expressing *Salmonella* with 2 bacteria per cell. The percentage of living cells and GFP levels were determined using a FACS Calibur (Becton Dickinson). Bioluminescence was measured for 5s in a luminometer (Berthold). Bacterial growth was determined by dividing the relative bioluminescence signal by the relative number of GFP⁺ living B cells, resulting in the amount of light produced per bacteria containing B cell. For induction of apoptosis, cells were treated with 0.1 μ M Edelfosine (Biomol) (27).

Bacterial excretion assay

To visualize bacterial excretion, human primary B cells were incubated with uncoated GFP-*Salmonella* at 20 bacteria per cell, and followed using wide field microscopy in medium containing anti-LPS antibodies coupled to TexasRed. To quantify excretion, cells were stained with DAPI (Sigma-Aldrich) to exclude dead cells and anti-LPS coupled to APC and fixed with 3.7% formaldehyde before analysis using a LSR II (Becton Dickinson). For the increase in LPS levels, the initial level at time point 0 was set to 1. The percentage of excreted bacteria was calculated as the loss of GFP⁺/LPS⁻ B cells compared to time point 0. To discriminate between bacterium and B cell-induced excretion, cells were cultured in medium containing 10 μ g/ml tetracycline to arrest intracellular bacteria (bacteriostatic capacity was verified using lux-*Salmonella* in Ramos cells).

Statistical analysis

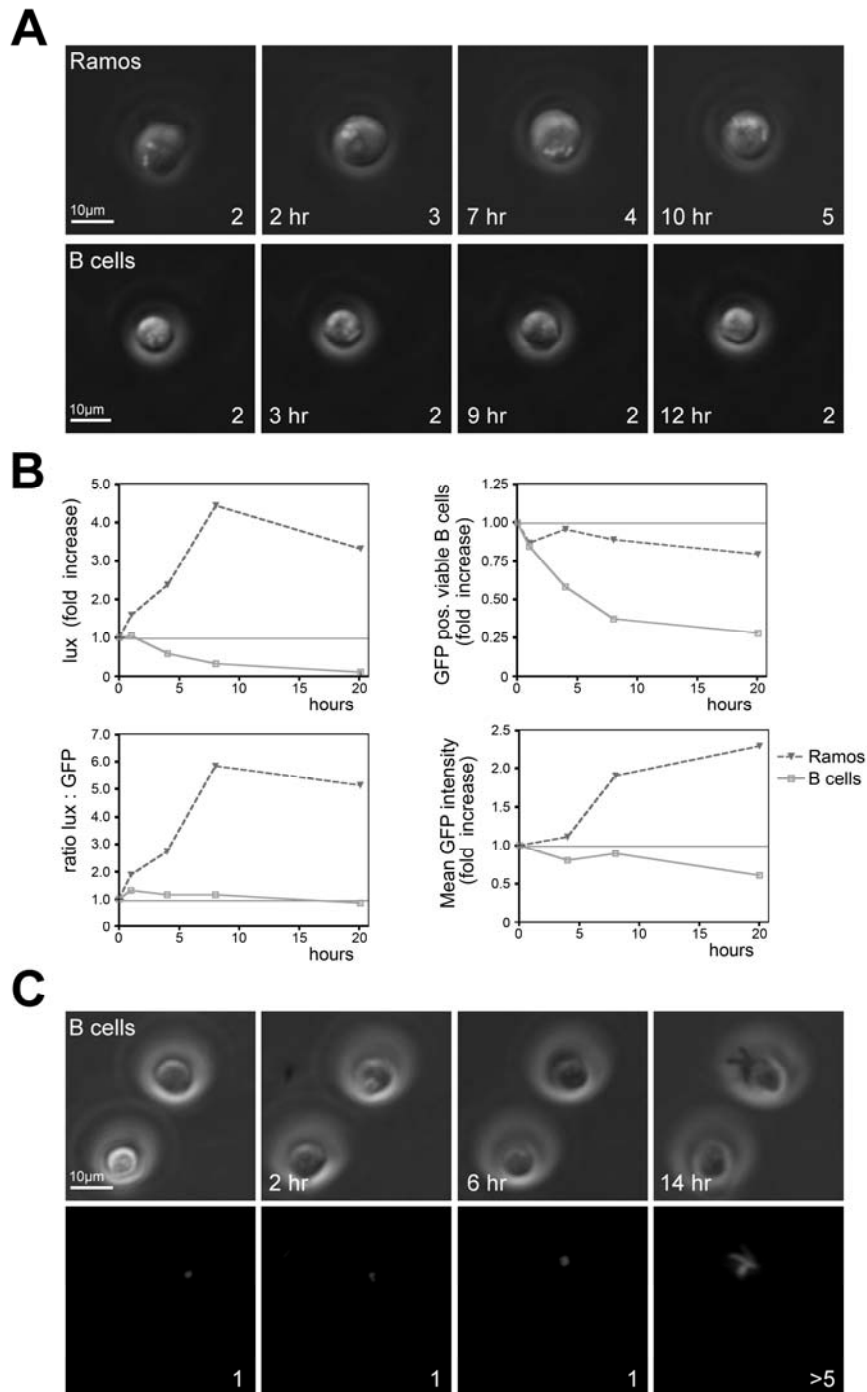
Kaplan-Meier plots and long-rank tests were used to assess survival differences of adoptively transferred mice after virulent *S. typhimurium* infection. Statistic calculations were performed by JMP 5.1 software (SAS, Cary, NC).

Results and Discussion

Primary human B cells form a survival niche for intracellular Salmonella

Recent data indicated that B cells from early vertebrates act as efficient phagocytes in contrast to mammalian B cells that do not show phagocytic behavior (28). We demonstrated however, that human B cells have not lost this phagocytic capacity but will only phagocytose particles or pathogens when first recognized by the BCR (22). The BCR acts as an antigen-specific receptor for *Salmonella*, and BCR crosslinking likely cooperates with bacterial effector proteins to facilitate uptake in B cells. Other mammalian cells are infected by *Salmonella* without any further involvement of such specific receptors. Phagocytosed *Salmonella* grows in many cell types, and can only be efficiently destroyed in specialized cells like macrophages and neutrophils in a process requiring the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system (5). We previously noticed survival of *Salmonella* in B cells after BCR-mediated internalization and now studied the fate of these phagocytosed *Salmonella* in more detail.

To study the fate of *Salmonella typhimurium* in B cells, time-lapse imaging of GFP-*Salmonella* containing B cells was performed with wide-field microscopy to limit phototoxicity. After incubation with bacteria and extensive washing, we observed replication of GFP-*Salmonella* in the Ramos B cells (Fig. 1A, *top panel* and supplemental movie 1), whereas *Salmonella* did not multiply in primary B cells (Fig. 1A, *bottom panel* and supplemental movie 2). To quantify these observations, we performed parallel experiments to compare GFP-*Salmonella* (detected by FACS analysis) with light producing lux-*Salmonella* (detected by luminometry). Light production by lux-*Salmonella* requires ATP and is thus a marker for bacterial viability (24). Figure 1B shows that anti-IgM coated GFP-*Salmonella* that have been phagocytosed by Ramos cells expanded intracellularly. Over a time course of 10h we observed a strong increase in lux activity (Fig. 1B, *top left panel*), while the number of GFP-*Salmonella* positive viable Ramos cells remained nearly constant (Fig. 1B, *top right panel*). The amount of light produced in GFP-*Salmonella* positive Ramos cells increased considerably (Fig. 1B, *bottom left panel*), indicating that the number of bacteria per Ramos cell increased over time. In accordance, the GFP signal per Ramos cell increased (Fig. 1B, *bottom right panel*). In contrast, lux activity sharply dropped over time when *Salmonella* was phagocytosed by primary human B cells (Fig. 1B, *top left panel*). This was not due to elimination of



Salmonella as the fraction of GFP-*Salmonella* containing B cells declined equally fast (Fig. 1B, top right panel). In fact, the amount of light produced per living GFP-*Salmonella* positive B cell remained constant during the course of the experiment (Fig. 1B, bottom left panel), showing that *Salmonella* remained viable in primary human B cells, albeit under conditions of inhibited proliferation. This confirms the wide-field microscopy data that showed that *Salmonella* does not replicate in primary B cells. Unlike specialized phagocytic immune cells like macrophages,

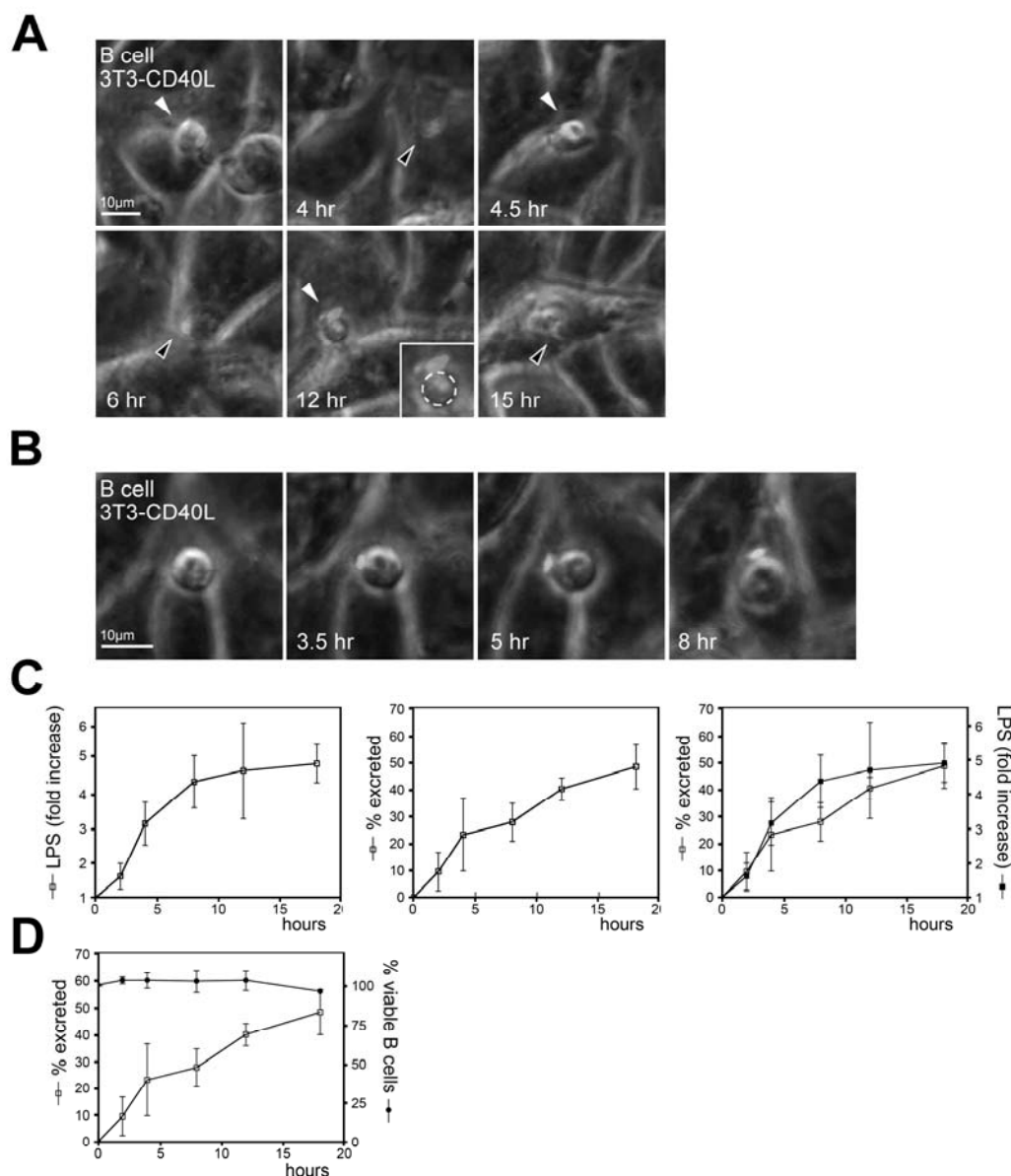
Figure 1. Primary human B cells form a survival niche for intracellular *Salmonella*.

(A) Widefield fluorescence microscopy of living Ramos and primary human B cells with phagocytosed GFP-expressing *Salmonella*. Bacteria were coated with anti-IgM mAbs to force BCR-specific recognition of the bacteria by the Ramos B cell line and to enhance the number of primary B cells that can phagocytose bacteria via their BCR. Depicted are GFP signals projected on the transmission image. Scalebar = 10µm. Number of bacteria in the visualized cell is given in the lower right corner. Lower left corner: time after *Salmonella* infection. Images are frames from supplemental movie 1 and 2. (B) Analysis of Ramos and primary human B cells incubated with living anti-IgM coated lux-expressing (*top left panel*) or GFP-expressing (*top right panel*) *Salmonella*. The ratio of lux over GFP shows the amount of light produced per GFP-*Salmonella* positive B cell (*bottom left panel*), indicating intracellular *Salmonella* viability. The mean fluorescence of the GFP positive population, set arbitrarily at 1 at the beginning of the experiment, shows that the GFP signal increases in Ramos B cells, whereas it decreased in primary human B cells (*bottom right panel*). A representative example of three independent experiments is shown. (C) B cells were infected with anti-BCR coated GFP-expressing *Salmonella* before exposure to Edelfosine to induce apoptosis. Cells were imaged over a 14h period. *Top panel*: transmission image, *bottom panel*: GFP-signal. Scalebar = 10µm. Images are frames from supplemental movie 3.

neutrophils, or B cells from early vertebrates (28), human B cells are apparently inefficient in producing the microbicidal conditions that are required to eliminate *Salmonella*. We next investigated whether primary B cells actively suppress *Salmonella* growth. We selectively induced apoptosis of B cells (without affecting *Salmonella*) and measured *Salmonella* replication. Intracellular replication of *Salmonella* was no longer suppressed 2h after induction of apoptosis in primary B cells with the alkyl-lysophospholipid Edelfosine (27) (Fig. 1C and supplemental movie 3), demonstrating that growth arrest of *Salmonella* requires viable primary B cells. These data suggest that primary human B cells, unlike human B cell lines, actively suppress multiplication of intracellular *Salmonella* within the SCV.

Salmonella is excreted by B cells and infects secondary host cells

The number of primary B cells that had initially phagocytosed *Salmonella* dropped over time (Fig. 1B, *top right panel*), suggesting that *Salmonella* may be released. To visualize the fate of phagocytosed *Salmonella* in B cells, primary human B cells infected with GFP-*Salmonella* were co-cultured on a monolayer of CD40L-expressing 3T3 cells and analyzed by time-lapse wide-field microscopy. In addition, CD40L will provide a pro-survival signal (29) enabling long term cell culture and imaging of primary B cells. Primary B cells that had phagocytosed GFP-*Salmonella* showed extensive invasive behavior by continuously moving under and over the



3T3-CD40L monolayer (Fig. 2A and supplemental movie 4). At later time points, some GFP-*Salmonella* appeared to be exocytosed from the B cell. To visualize this, GFP-*Salmonella* infected primary B cells were cultured in the presence of a low concentration of Texas-Red labeled anti-LPS mAb in the medium. GFP-*Salmonella* will attract and concentrate this antibody upon exposure to the medium. Figure 2B shows a B cell with phagocytosed GFP-*Salmonella* that becomes accessible for anti-LPS antibodies in the medium after 8 hr of culture (supplemental movie 5). *Salmonella* excretion from primary B cells was quantified using FACS by detecting GFP-*Salmonella* and LPS-positive B cells. Strong increase in cell surface exposed LPS on cells that were initially GFP-*Salmonella* positive and LPS negative was observed (Fig. 2C, left panel). This suggests that a large fraction of the phagocytosed *Salmonella* were exocytosed as in the example shown in Fig. 2B.

Figure 2. *Salmonella* is actively excreted by B cells and capable of infecting secondary host cells.

(A) Primary B cells having phagocytosed anti-BCR coated GFP-*Salmonella* on a monolayer of 3T3-CD40L fibroblasts were imaged using widefield fluorescence microscopy. Depicted is the GFP signal projected on the transmission image with images taken every 30 min. Scalebar = 10µm. Arrows indicate the B cell, white arrow: B cells moves op top of the monolayer, black arrow: B cells moves below the monolayer. Images are frames from supplemental movie 4. (B) Primary B cells having phagocytosed anti-BCR coated GFP-*Salmonella* on a monolayer of 3T3-CD40L fibroblasts were imaged using widefield fluorescence microscopy in the presence of TexasRed labeled anti-LPS mAbs. Depicted are GFP and Texas-Red signals projected on the transmission image. Scalebar = 10µm. Images are frames from supplemental movie 5. (C) Quantification of *Salmonella* secretion from B cells. Primary B cells were incubated with live uncoated GFP-*Salmonella*. Cells were stained with antibodies against LPS, fixed and analyzed using FACS. *Left panel:* increase in cell surface exposed LPS from bacteria exposed at the cell surface after initial uptake by B cells. *Middle panel:* percentage of B cells having excreted *Salmonella* as calculated from the percentage of B cells containing GFP-*Salmonella* followed in time. *Right panel:* left and middle panels are projected to illustrate that both processes show similar kinetics. Error bars represent SD from three independent experiments. (D) Primary B cells were incubated with live uncoated GFP-expressing *Salmonella* and followed for the time points indicated. The fraction of living B cells is plotted to demonstrate that loss of GFP-*Salmonella* positive B cells is not correlated with cell death.

Accordingly, the population of GFP-*Salmonella* positive/LPS negative B cells declined over time inferring increase in excretion (Fig. 2C, *middle panel*) with kinetics that were identical to the acquired LPS signal over time (Fig. 2C, *right panel*). GFP-*Salmonella* infection of primary B cells did not affect B cell viability or induced apoptosis (Fig. 2D). Note that during the first phase of excretion *Salmonella* was released, but remained associated to the B cells, hence the increased staining with the anti-LPS antibodies in the first 10h. The bacterium was later released from the B cell, leveling off further LPS labeling. Loss of the GFP-*Salmonella* signal from infected primary B cells increased over an 18h period in our experiments at which point more than 50% of the bacteria were released from B cells. Thus, a large proportion of phagocytosed *Salmonella* are secreted within 24 hrs of human primary B cell infection.

Excretion of Salmonella is a cell autonomous process

Does *Salmonella* actively participate to the process of excretion from B cells? So far, we used the antibiotic gentamycin in our experiments to kill extracellular *Salmonella* and prevent their overgrowth, as gentamycin does not affect intracellular *Salmonella* replication (as seen in Fig. 1A). Unlike gentamycin, the antibiotics tetracycline and erythromycin kill *Salmonella* intracellularly (30).

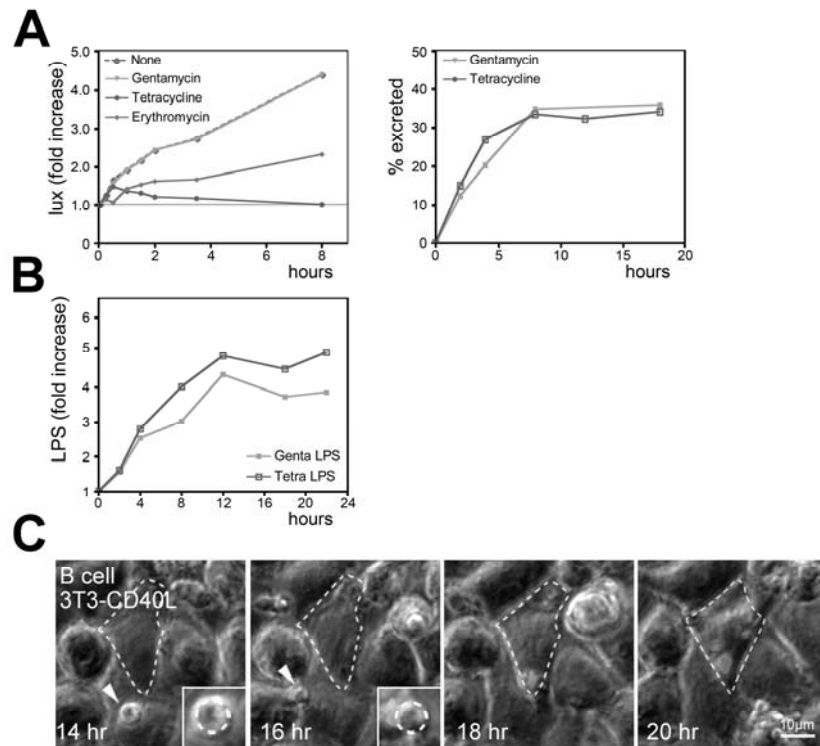


Figure 3. *Salmonella* is actively excreted by B cells and capable of infecting secondary host cells (A) *Left panel:* the effect of antibiotics on the growth of lux-*Salmonella* in Ramos B cells. *Right panel:* the same FACS analysis as in 2C was performed in presence of either Gentamycin or Tetracycline to discriminate between host and bacteria mediated excretion. (B) The same FACS analysis as in Fig. 2C was performed in presence of either Gentamycin or Tetracycline to discriminate between host versus bacterial-mediated excretion. Increase in cell surface LPS levels is similar in the presence of Gentamycin and Tetracycline, indicating that viable *Salmonella* are not required for excretion. (C) Primary B cells having phagocytosed anti-IgM coated GFP-*Salmonella* on a monolayer of 3T3-CD40L fibroblasts were imaged using widefield fluorescence microscopy for the times indicated. Imaging conditions are similar as in 2A. GFP-*Salmonella* is excreted from a primary B cell (white arrowhead), followed by infection of the 3T3-CD40L monolayer (outline of infected cell marked by a dashed line). Inset shows zoom-in on primary B cell excreting GFP-*Salmonella*. Images are frames from supplemental movie 6.

These antibiotics inhibited lux-*Salmonella* growth in Ramos cells (Fig. 3A, *left panel*) and no viable *Salmonella* bacteria were recovered in plating assays of infected B cells, in contrast to exposure to gentamycin (data not shown). *Salmonella* secretion by primary B cells was measured in the presence of either gentamycin or tetracycline. Tetracycline did not affect excretion of GFP-*Salmonella* from primary B cells, which occurred equally efficient as in the presence of gentamycin (Fig. 3A, *right panel*), indicating that *Salmonella* viability is not required for excretion. Likewise, a similar increase in cell surface LPS levels was observed in the presence

of tetracycline as with gentamycin (Fig. 3B). While *Salmonella* participates in uptake after capture by the BCR (22), excretion does not require viable *Salmonella*. Can *Salmonella* then infect other cells following transport by B cells to distant sites? We co-cultured primary human B cells containing phagocytosed GFP-*Salmonella* on a monolayer of 3T3-CD40L and followed post-excretion events using time-lapse wide-field microscopy. Figure 3C shows an example of a phagocytosed GFP-*Salmonella* that subsequently extruded from the primary B cell followed by infection of the fibroblast monolayer (supplemental movie 6). The released bacteria infected and replicated in the fibroblast monolayer, demonstrating that passage through primary B cell had not suppressed bacterial replication in an irreversible manner. These data suggest that *Salmonella* can use primary B cells as a reservoir or transport vehicle allowing it to escape immune attack and hitch-hike to distant locations.

Salmonella-specific B cells mediate spreading in acute in vivo infection.

Our observations imply that the availability of B cells with pathogen-specific BCRs would be advantageous for the spreading of infection as they may be used as *Salmonella* carriers. How *Salmonella* reaches the spleen is not clear. Possibly, *Salmonella* encounters specific B cells as they cross the intestinal epithelium via M cells directly located to gut-associated lymphoid tissue (GALT) sites where many B cells reside in Peyer's patches. Among preferred distant sites of persistent infection are the spleen and lymph nodes. *Salmonella* has been isolated from splenic macrophages and splenic B cells of orogastrically infected mice (20). *Salmonella* was thought to reach these locations after transport by neutrophils (16), a notion incompatible with the efficient bactericidal capacity of neutrophils (5). Also macrophages and DCs have been implicated, but also these cells have bactericidal capacities, like neutrophils. The possibility that *Salmonella* uses antigen-specific primary B cells as transport vehicle to distant sites in the host has not been considered. To test this option *in vivo*, we adoptively transferred C57BL/6 mice with increasing amounts of CD43- naïve B cells carrying a BCR specific for the HEL antigen. One day after transfer, mice were infected with a sublethal dose of surface HEL-expressing *Salmonella*, and survival was monitored daily. Transfer of 2×10^5 HEL-specific B cells increased C57BL/6 mortality after HEL-expressing *Salmonella* infection whereas 10^6 B cells protected (Fig. 4A). These results may be due to two opposite effects of the antigen-specific B cells; spreading of *Salmonella* and

production of antibodies against *Salmonella*, in which the balance of these activities determines survival of mice. To directly establish if HEL-specific B cells mediated systemic dissemination of HEL-expressing *Salmonella*, we investigated bacterial recovery from the spleen in the different experimental settings. *Salmonella* spread to the spleen only after adoptive transfer of antigen-specific B cells (Fig. 4B). Thus, antigen-specific B cells were required for *in vivo* dissemination of *Salmonella* to the spleen.

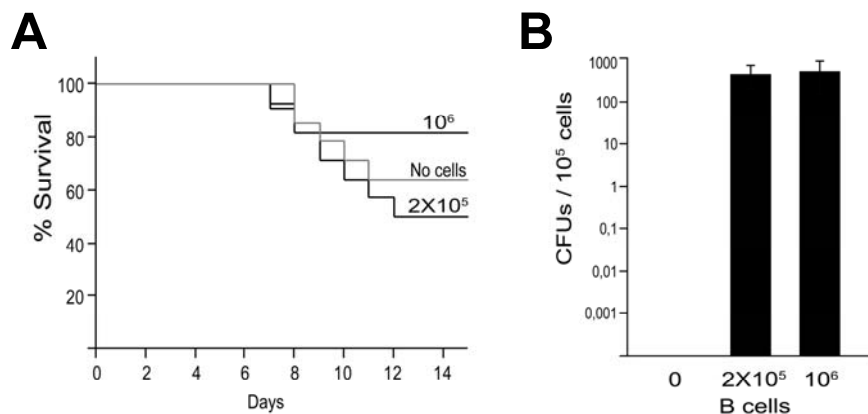


Figure 4. *Salmonella*-specific B cells form a survival niche and help *Salmonella* spreading through the body

(A) Kaplan-Meier survival curves of C57BL/6 mice left untreated or adoptively transferred with 2×10^5 , or 10^6 HEL-specific CD43⁻ naïve B cells. Mice ($n=14/\text{group}$) were orally infected with surface HEL-expressing *Salmonella* one day after transfer. Survival was monitored daily. (B) C57BL/6 mice were left untreated or adoptively transferred with 2×10^5 or 10^6 HEL-specific CD43⁻ naïve B cells, as indicated. Mice were orally infected with surface HEL-expressing *Salmonella* one day after B cell transfer. Culturable splenic bacteria (CFU/ 10^5 cells) 16 hours after infection are shown ($n=3/\text{group}$). Shown is the mean + SEM. One representative example of two independent experiments is shown.

Our data show that *Salmonella* misuses the specificity of the adaptive immune system. *Salmonella* may hide from the early innate immune defenses in antigen-specific B cells and may even create a sustained reservoir of infection by going through multiple rounds of uptake and excretion in B cell-rich areas like the Peyer's patches and spleen. In addition, *Salmonella* may hitch-hike in B cells to facilitate systemic spreading of the infection. Previously, it was shown that cells of the innate system and erythrocytes can be used by pathogens for systemic spreading of infection within immunocompetent hosts, like HIV (DCs) (31) and *P. falciparum* (erythrocytes) (32). The adaptive immune system has evolved to protect against infection, while simultaneously generating immunological memory to ensure a rapid

immune response against reinfection. We now show that *Salmonella* has adapted to this by using antigen-specific B cells for immune evasion and spreading to distant sites within the host. This is the first example of the use of cells from the adaptive immune system by bacteria to enable their dissemination.

Acknowledgements

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Chapter 3

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Chapter 4

Phagocytosis of *Salmonella* by B cells generates an effective cytotoxic T cell response via cross-presentation of *Salmonella*-antigens

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Jacques Neefjes and S. Marieke van Ham

Submitted for publication

Abstract

The eradication of facultative intracellular bacterial pathogens, like *Salmonella typhi*, requires the concerted action of both the humoral immune response and the cytotoxic CD8⁺ T cell response. Dendritic cells (DCs) are considered to orchestrate the cytotoxic CD8⁺ T cell response via cross-presentation of bacterial antigens onto MHC class I molecules. Cross-presentation of *Salmonella* by DCs however, is accompanied by the induction of apoptosis in the DCs. Besides antibody production, B cells are required to clear *Salmonella* infection for other unknown reasons. Here we show that *Salmonella*-specific B cells that phagocytose *Salmonella* upon BCR-ligation are able to activate human CD8⁺ memory T cells via cross-presentation yielding a *Salmonella*-specific cytotoxic T cell response. Unlike the DCs, B cell-mediated cross-presentation of *Salmonella* does not coincide with apoptosis. Thus, B cells are a viable alternative for DC in the activation of the cytotoxic effector arm and the generation of effective adaptive immunity against *Salmonella* reinfection.

Introduction

Salmonella is a pathogenic bacterium that causes severe disease in mice and man. *Salmonella typhi* (*Salmonella enterica* serovar Typhi) causes invasive diseases in human, which has many features in common with *Salmonella typhimurium* in mice. The gastrointestinal tract is the major site of primary infection of the host and has to be passed before systemic infection can occur. One way to infect the host cells is via sampling of bacteria by DCs in the intestine. *In vitro* studies showed that DCs located in the lamina propria under the gut epithelium of the small bowel extend processes across the tight junctions between the epithelial cells and capture bacteria from the luminal side of the gut (1,2). The major route of infection however, is via microfold cells or M cells (3,4). The specialized antigen-sampling M cells are located in the dome region of the Peyer's Patches and are efficient in transportation of macromolecules and microorganisms to the underlying immune cells (2,5). Like other Gram-negative bacteria, *Salmonella* uses specific virulence factors to invade other cell types, called the Type III Secretion System (TTSS). Many *Salmonella* virulence genes are clustered in *Salmonella* pathogenicity islands (SPIs). SPI-1 and SPI-2 encode TTSSs that mediate the injection of effector proteins into the host cell cytoplasm via sophisticated secretion devices (6). SPI-1 is associated with invasion of intestinal epithelia and enhanced intestinal inflammation in the infected host (7,8). SPI-2 modulates intracellular trafficking and enables replication within a modified vacuolar compartment, called the *Salmonella*-containing vacuole (SCV) (9-11) and enhances inflammation during enteric phase (12,13). *Salmonella* activates the PKB/Akt1 pathway to prevent maturation of SCV into destructive phagolysosomes, thus manipulating the host for its own survival (14).

After transcytosis by M cells, *Salmonella* reaches the subepithelial dome of the Peyer's patches and encounters an extensive network of resident macrophages, DCs and great numbers of B cells (15,16). Instead of being immediately destroyed by these cells, *Salmonella* have evolved several mechanisms to survive in the harsh milieu of phagosomal compartments (17) and can be cytotoxic to macrophages by inducing apoptosis *in vitro* (18,19).

Recently, we showed that recognition of *Salmonella* via the specific B cell receptor (BCR) on B cells results in internalization of *Salmonella*. *Salmonella* is able to survive intracellularly in primary B cells in a non-replicative state (20). Following uptake of *Salmonella*, B cells do not go into apoptosis, but differentiate and start to

produce *Salmonella*-specific antibodies. In addition, BCR-mediated phagocytosis of *Salmonella* by B cells leads to antigen presentation via MHC class II and subsequent CD4⁺ T cell activation, which in turn boosts antibody production by the infected B cell.

Antibody transfer studies have shown that the requirement for B cells in the clearance of *Salmonella* does not solely depend on antibody formation (21). Which additional immune responses need B cell involvement remains unclear. For clearance of *Salmonella*, not only the humoral immune response is required, but also the activation of cytotoxic CD8⁺ T cells is needed to eliminate *Salmonella*-infected cells. Recently, DCs have been shown to prime *Salmonella*-specific CD8⁺ memory T cells after direct uptake of bacteria or via suicide cross-presentation after uptake of *S. typhi*-infected human cells (22). As the generation of *Salmonella* antigens for MHC class II molecules is an efficient process in infected B cells, we tested whether BCR-mediated phagocytosis also leads to cross-presentation of *Salmonella* antigens via the MHC class I pathway of B cells and whether this elicits a cytotoxic T cell response against *Salmonella*-infected cells.

Here we show that *Salmonella*-specific primary B cells that have internalized *Salmonella* do cross-present *Salmonella* antigens via MHC class I molecules in a proteasome-dependent manner. Subsequently, we demonstrate that cross-presentation of *Salmonella* antigens by B cells activates *Salmonella*-specific CD8⁺ memory cells that acquire a cytotoxic phenotype and are efficient in the killing of *Salmonella*-infected cells. Thus, B cells are an unappreciated source of cells cross-presenting bacterial antigens for T cell stimulation.

Materials and methods

Antibodies and fluorophores

mAb anti-human IgM (MH15, Sanquin, Amsterdam, The Netherlands) was mixed with rat anti-mouse IgG1 antibody (RM161.1, Sanquin) and mAb anti-*S. typhimurium* LPS (1E6, Biodesign International, Kennebunk, ME) to generate BCR-LPS tetrameric antibody complexes, used to coat bacteria as previously described (20).

The following labeled anti-human mAbs were obtained from BD Biosciences (San Jose, CA): anti-IFN- γ -FITC, anti-CD27-PE, anti-CD107a-PE, anti-CD8-PerCP-Cy5.5, anti-CD4-APC, anti-CD45RO-PE, AnnexinV-APC and IgG1-PerCP-Cy5.5 isotype control. FITC-conjugated antibody IgG1, IgG2a and IgG2b, IgG1-PE and IgG-APC

isotype controls were obtained from DAKO (Glostrup, Denmark). Anti-CD45RA-FITC and anti-CD45RO-FITC were obtained from Sanquin and DAPI from Sigma-Aldrich (Steinheim, Germany). CFSE (Invitrogen, Paisley, UK) labeling was used in proliferation assays.

Bacterial growth conditions

GFP expressing-*S. typhimurium* SL1344 was described before (23). GFP-*Salmonella* defective in SPI-1 (*invA* mutant) or SPI-2 (*ssrA* mutant) were a kind gift of M. Rescigno (European Institute of Oncology, Milan, Italy). *Staphylococcus aureus* expressing GFP (RN4220 with pWVW189GFP) was kindly provided by S. A. J. Zaat (Academic Medical Center, Amsterdam, The Netherlands). All bacteria strains were grown overnight at 37°C in Luria-Bertani (LB) broth with carbenicillin or chloramphenicol (Sigma-Aldrich, St Louis, MO) to maintain GFP expression while shaking, subcultured at a dilution of 1:33 in fresh LB medium and incubated while shaking at 37°C for 3 to 4 hours to obtain exponentially growing bacteria. For coating, bacteria were washed twice with PBS and incubated with BCR-LPS tetrameric antibody complexes for 30 minutes at room temperature and washed twice with PBS to remove unbound antibodies. For experiments with dead *Salmonella*, bacteria were heat killed by incubation at 65°C for 15 minutes.

Lymphocyte isolation and B lymphocyte infection with Salmonella

Human PBMCs were isolated by centrifugation on a Ficoll-Hypaque gradient (Axis-Shield PoC AS, Oslo, Norway) from a buffycoat obtained from healthy donors after informed consent (Sanquin). B and T cells were subsequently purified using anti-CD19 and anti-CD4, anti-CD8 Dynabeads and DETACHaBEAD (Invitrogen), according to the manufacturer's instructions.

B lymphocytes were incubated for 45 minutes at 37°C with *Salmonella* without antibiotics. Next, cells were washed to remove unbound bacteria four times and cultured for 1 hour in medium containing 100 µg/ml gentamycin (Invitrogen) to eliminate non-phagocytosed bacteria. Cells were washed and cultured in RPMI 1640 medium w/o phenol red (Lonza, Basel, Switzerland), supplemented with 5% FCS (Bodinco, Alkmaar, the Netherlands), 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-Glutamine, 50 µM 2-ME, 20 µg/ml human apo-transferrin ((Sigma-Aldrich), depleted for human IgG with protein G sepharose (Amersham, Uppsala, Sweden)) and 10 µg/ml gentamycin.

Flow cytometry

1×10^5 B cells, or *Salmonella*-infected B cells were cultured with 5×10^4 CFSE-labeled T cells ($CD8^+$ T cells alone or in 1:1 ratio with $CD4^+$ T cells) for 6 days to activate and expand *Salmonella*-specific T cells. 50,000 events were acquired on a LSR II (BD) and analyzed with FACSDiva software (BD). To test plasma membrane markers, 1×10^5 B cells with 5×10^4 T cells were cultured for 6 days and after addition of 10 IU/ml IL-2 (Chiron, Emeryville) for another 6 days. All plasma membrane stainings were performed for 15 minutes at room temperature and washed after each incubation with PBS containing 0.1% BSA. 50,000 events were acquired on a LSR II (BD) and analyzed with FACSDiva software (BD). Lymphocytes were gated by forward and side scatter. Dead cells were excluded based on their positive reaction to DAPI staining.

For some experiments, naïve T cells were sorted as $CD8^+CD45RA^+CD45RO^-$ (T_N) and memory T cells as $CD8^+CD45RA^-CD45RO^+$ (T_{MEM}), and $CD8^+CD45RO^+CD27^-$ (T_{EM}) and $CD8^+CD45RO^+CD27^+$ (T_{CM}) cells. Populations were >98% purified.

Intracellular cytokine staining

B cells and T cells were cultured for 12 days and cytokine production was measured by intracellular staining after restimulation with 0.1 μ g/ml PMA, 1 μ g/ml ionomycin and 10 μ g/ml brefeldin A (Sigma-Aldrich) for 5 hours. Cells were washed twice with PBS, fixed with 1% formaldehyde (Merck, Darmstadt, Germany) for 15 minutes and after washing twice with PBS, permeabilized with 0.5% saponin (Calbiochem, CA) in PBS containing 1% BSA (Sigma-Aldrich) and incubated with fluorescent antibodies for 30 minutes at room temperature. 50,000 events were acquired on a LSR II (BD) and analyzed with FACSDiva software (BD).

$CD8^+$ degranulation assay

$CD8^+$ T cells were primed by 6 day incubation of 1×10^5 *Salmonella*-infected B cells with 2.5×10^5 $CD4^+$ T cells and 2.5×10^5 $CD8^+$ T cells. The dividing T cells ($CD8^+CFSE^{low}$) were FACS sorted after 6 days on a MoFlo Sorter (Dakocytomation, Glostrup, Denmark) and cultured with 50 IU/ml IL-2 for 6 more days. Next, isolated autologous B cells were thawed and infected with *Salmonella*. For proteasome inhibition, MG-132 (Sigma-Aldrich) was added at a concentration of 20 μ M before incubation with bacteria. *Salmonella*-infected B cells were incubated in medium containing 10 μ g/ml gentamycin for 15 hours at 37°C to allow processing and

presentation of *Salmonella* antigens. Subsequently, the B cells were incubated at 37°C for 5 hours together with the primed *Salmonella*-specific CD8⁺ T cells in a ratio of 4:1, in the presence of anti-CD107a-PE labeled antibodies. Cells were washed twice with wash buffer (1 mM HEPES, 0.15 M NaCl, 5 mM KCl, 1.8 mM CaCl₂, 1 M MgCl₂, 0.1% BSA) and stained for CD8 and AnnexinV. After washing twice with washing buffer, DAPI was added and CD8⁺ T cells were analyzed for CD107a expression. 50,000 events were acquired on a LSR II (BD) and analyzed with FACSDiva software (BD).

⁵¹Cr release assay

Salmonella-specific CD8⁺ T cells were activated and FACS sorted as described and expanded with 50 U/ml IL-2. Autologous B cells were thawed and infected with *Salmonella*. After 15h, B cells were labeled with ⁵¹Cr (185MBq/ml; Perkin Elmer, Boston, MA) for 45 minutes at 37°C. After washing, the B cells were incubated in a 96-wells U-bottom plate (Costar Corning Inc., NY) with primed CD8⁺ T cells in a 1:2 ratio. Incubation in medium or in Triton X-100 (1% final concentration; Merck) was used to determine spontaneous and maximum ⁵¹Cr release, respectively. ⁵¹Cr release was measured in the supernatant using filters with a gamma counter (Cobra II, Canberra Packard, Mississauga, Canada). The percentage of specific cell lysis was calculated using the following formula:

$$\% \text{ specific lysis} = \frac{[\text{experimental release (cpm)} - \text{spontaneous release (cpm)}]}{[\text{maximal release (cpm)} - \text{spontaneous release (cpm)}]} \times 100\%$$

Statistical analysis

Statistical differences were determined by a paired Student's t test, using GraphPad Prism (version 4.03, GraphPad Software, San Diego, CA)

Results

***Salmonella*-infected B cells initiate a CD8⁺ T cell response**

To study MHC class I antigen presentation by B cells, we used *Salmonella typhimurium* as a model for cross-presentation against facultative intracellular bacteria. Previously, we showed that about 4% of the B cells recognize *Salmonella* by their BCR, and subsequently initiate a CD4⁺ T cell response (20). To study T cell

responses in detail, we enhanced the uptake of *Salmonella* by B cells by coating *Salmonella* with a tetrameric antibody complex, consisting of anti-LPS antibodies and anti-IgM-BCR antibodies. As a result, all B cells expressing an IgM-BCR, recognize *Salmonella* and are able to phagocytose the bacterium via their BCR. This resulted in an uptake of *Salmonella* by 30% to 60% of the B cells (data not shown). Next, we investigated whether *Salmonella*-infected B cells were able to initiate proliferation of CD8⁺ T cells in addition to the *Salmonella*-specific CD4⁺ T cell response. Therefore we cultured B cells that had phagocytosed *Salmonella* with CFSE-labeled CD4⁺ and CD8⁺ T cells. As observed before, B cells that had phagocytosed *Salmonella* induced proliferation of the CD4⁺ T cells (20). Interestingly, a considerable amount of CD8⁺ T cells had proliferated as well (Fig. 1A). To investigate the requirement of CD4⁺ T cell help for the proliferation of the CD8⁺ T cells *Salmonella*-infected B cells were cultured with CD8⁺ T cells in the absence of CD4⁺ T cells. This situation almost completely abolished proliferation of the CD8⁺ T cells (Fig. 1B and 1C). Thus, B cells infected by *Salmonella* act as antigen presenting cells and induce CD8⁺ T cell proliferation, but activation of CD8⁺ T cells requires the simultaneous activation of *Salmonella*-specific CD4⁺ T cells to enable T cell help.

To exclude a role for the tetrameric antibody complex in cross-presentation, we analyzed the induction of a CD8⁺ T cell response by *Salmonella*-specific primary B cells that had taken up *Salmonella* via their specific BCR. Although the percentage of *Salmonella*-infected B cells was lower compared to B cells that had phagocytosed *Salmonella* via the tetrameric antibody complex, with appropriate CD4⁺ T cell help *Salmonella*-specific B cells also induce CD8⁺ T cell activation (Fig. 1D).

***Salmonella*-infected B cells activate both the central memory and effector memory CD8⁺ compartment**

Do B cells elicit a naïve or a memory CD8⁺ cell response? To study this, we cultured the total CD8⁺ T cell population with or without *Salmonella*-infected B cells, in presence of CD4⁺ T cells for help, and determined the naïve (CD45RO⁻CD27⁺; T_N), central memory (CD45RO⁺CD27⁺; T_{CM}), effector memory (CD45RO⁺CD27⁻; T_{EM}) or terminal effector (CD45RO⁻CD27⁻; T_E) phenotype of the CD8⁺ T cells after prolonged culture. Upon 11 days of culture, purified CD8⁺ T cells show mainly a naïve phenotype. In contrast, following activation with *Salmonella*-infected B cells, the CD8⁺ population shifts towards the T_{EM} phenotype (Fig. 2A). CD8⁺ T cells that are

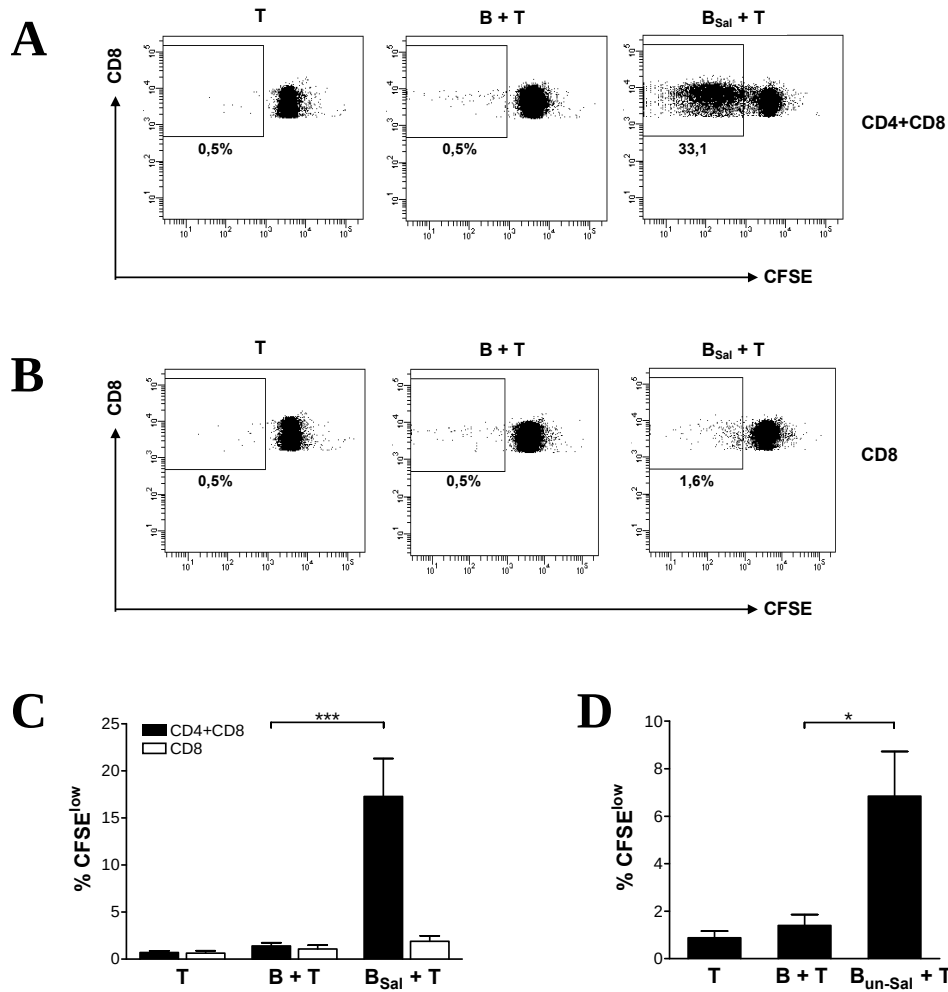


Figure 1. *Salmonella*-infected B cells induce CD8⁺ T cell proliferation with help of CD4⁺ T cells.

(A) CFSE labeled CD8⁺ T cells were cultured alone (T), together with B cells (B + T) or together with B cells that had phagocytosed *Salmonella* (B_{Sal} + T), in presence of CD4⁺ T cells. Proliferation was measured after 6 days. (B) CD8⁺ T cell proliferation was measured in absence of CD4⁺ T cells. (C) CD8⁺ proliferation (shown in A and B) of multiple independent experiments combined. Percentages are CD8⁺CFSE^{low} cells, in the presence of CD4⁺ T cells (black bars) or not (open bars). The data are expressed as mean ± SEM, of twenty-two independent experiments of different donors and *** p < 0.001. D, CD8⁺ T cell proliferation was also measured after culture with B cells that had phagocytosed uncoated *Salmonella* (B_{un-Sal}) in presence of CD4⁺ T cell help. The data are expressed as mean ± SEM, of five independent experiments of different donors, and * p < 0.05.

activated with *Salmonella*-infected B cells in the absence of help by CD4⁺ T cells, do not differentiate to a T_{EM} phenotype (Fig. 2B). As these data indicate that *Salmonella*-infected B cells mainly induce a CD8⁺ memory response, we investigated if *Salmonella*-infected B cells can prime naïve T cells at all. Therefore

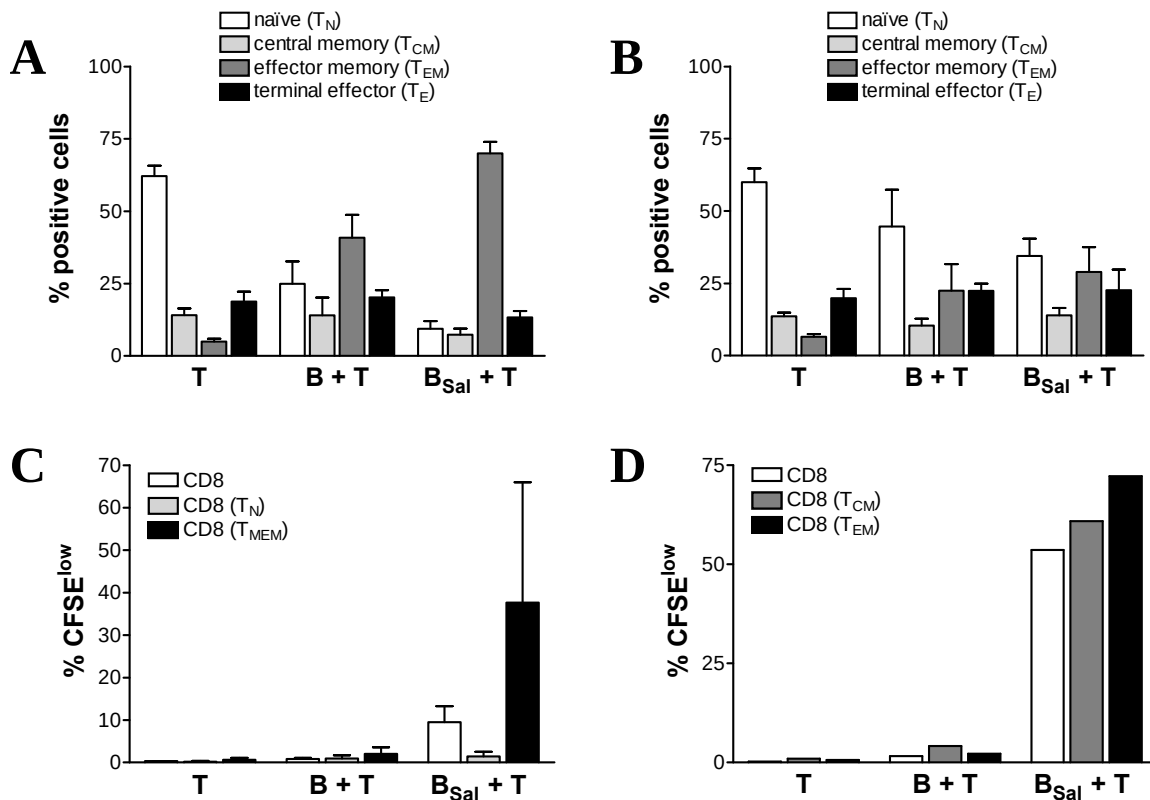


Figure 2. *Salmonella*-infected B cells elicit a CD8⁺ memory response.

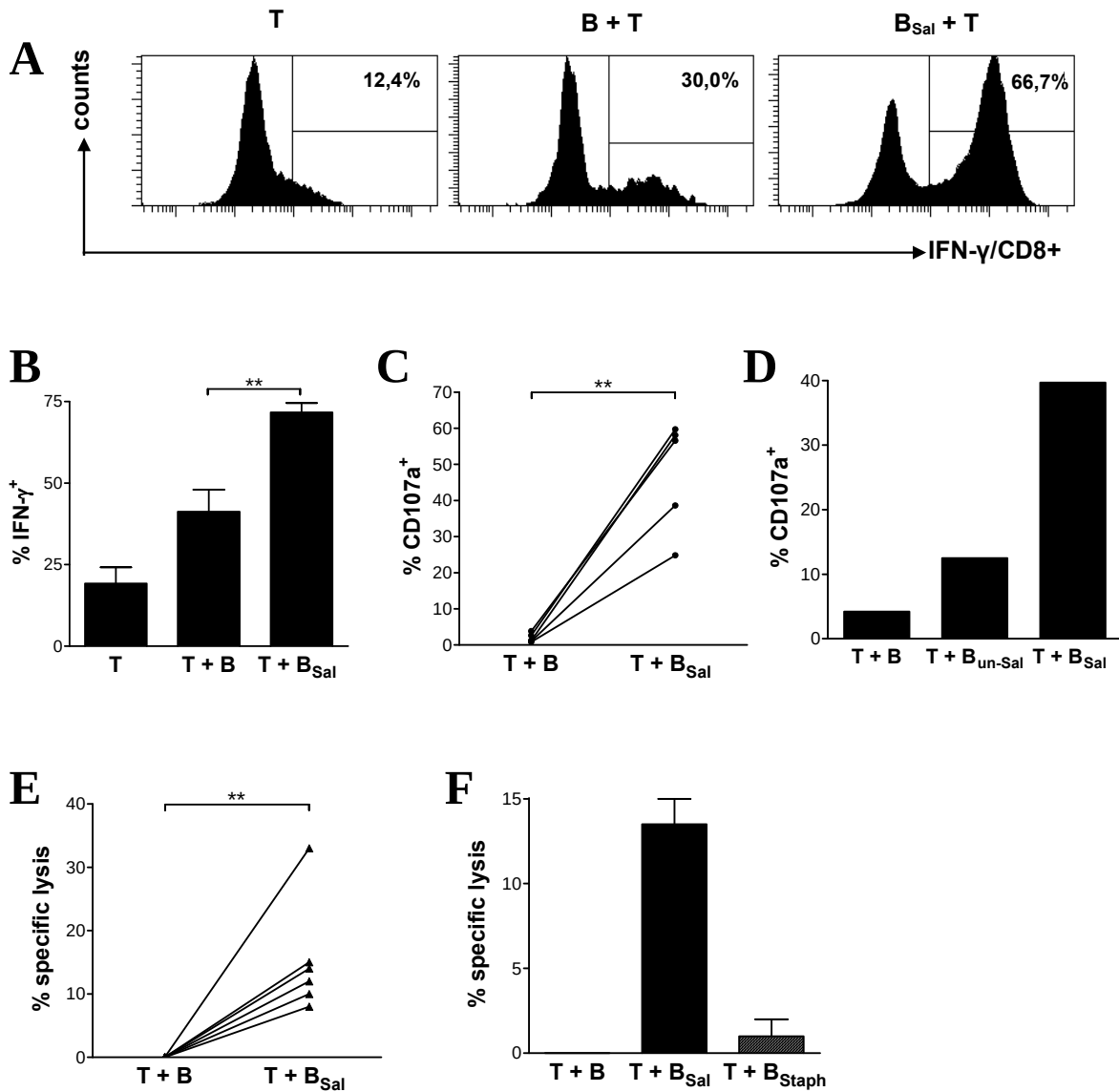
(A) CD4⁺ and CD8⁺ T cells were cultured alone (T), together with B cells (B + T) or with *Salmonella*-infected B cells (B_{Sal} + T). After 11 days, cells were stained for CD45RO and CD27 to discriminate between the different T cell populations: naïve (T_N ; CD45RA⁺CD27⁺), central memory (T_{CM} ; CD45RO⁺CD27⁺), effector memory (T_{EM} ; CD45RO⁺CD27⁻) and terminal effector (T_E ; CD45RA⁺CD27⁻) T cells. Gated CD8⁺ cells were analyzed. (B) Without CD4⁺ T cells, the differentiation of CD8⁺ T cells to effector memory is attenuated. Data are mean $\pm$ SEM from five independent experiments. (C) *Salmonella*-infected B cells activate proliferation of sorted CD8⁺CD45RO⁺ cells (T_{MEM}), but not of purified CD8⁺CD45RA⁺ cells (T_N) in the presence of CD4⁺ help. Data are mean $\pm$ SEM from three independent experiments. D, *Salmonella*-infected B cells activate proliferation of sorted CD8⁺CD45RO⁺CD27⁻ (T_{EM}) and CD8⁺CD45RO⁺CD27⁺ (T_{CM}) cells. Data are of one experiment.

we studied proliferation of sorted naïve (CD45RA⁺CD45RO⁻; T_N) or memory (CD45RA⁻CD45RO⁺; T_{MEM}) CD8⁺ T cells in response to *Salmonella*-infected B cells. Figure 2C shows that the naïve CD8⁺ population does not proliferate upon stimulation with *Salmonella*-infected B cells, whereas the memory CD8⁺ T cells proliferated vigorously. Sorting of T_{CM} (CD45RO⁺CD27⁺) and T_{EM} (CD45RO⁺CD27⁻) CD8⁺ T cells before coculture with *Salmonella*-infected B cells, showed that both T_{CM} and T_{EM} can be activated by *Salmonella*-infected B cells. (Fig. 2D). Recent experiments in mice show that both T_{CM} and T_{EM} cells can arise from activation and proliferation of the T_{CM} compartment, whereas T_{EM} cells are more terminally

differentiated and therefore proliferate poorly and only give rise to T_{EM} progeny (24). Indeed, expansion of the sorted T_{CM} by *Salmonella*-infected B cells yielded offspring with both a T_{CM} and a T_{EM} phenotype, whereas T_{EM} activation yielded mainly T_{EM} progeny. Together, these data show that *Salmonella*-infected B cells activate a recall response of memory CD8⁺ T cells, yielding expansion of both the "memory stem cell" containing T_{CM} compartment and the memory effector cell containing T_{EM} compartments.

Activated *Salmonella*-specific CTLs are able to kill *Salmonella*-infected cells

Activation of cytotoxic CD8⁺ T cells by B cells is controversial. Earlier reports showed that B cells induce tolerance or anergy in CD8⁺ T cells. In contrast, we showed proliferation of CD8⁺ T cells upon B cell activation when B cells had phagocytosed *Salmonella*. Although this proliferation is unlikely to yield tolerance, proliferation itself does not guarantee that these B cells activate functional, cytotoxic CD8⁺ T cells. CD8⁺ T cells kill intracellular pathogens either via secretion of IFN- γ or via direct killing of the infected target cell (25). To investigate if *Salmonella*-containing B cells induce a functional CD8⁺ T cell response, the IFN- γ secretion of CD8⁺ T cells was measured using an intracellular IFN- γ staining. This showed that, after culture with *Salmonella*-infected B cells, the number of CD8⁺ T cells producing IFN- γ is increased to more than 65% (Fig. 3A and 3B). Thus, cross-presentation of *Salmonella* antigens by B cells induces not only proliferation of the CD8⁺ T cells but also renders the cells functional in that it initiates IFN- γ secretion. As these data demonstrate that B cells that had taken up *Salmonella* are able to functionally activate CD8⁺ T cells, the question remained if the activated CD8⁺ T cells were *Salmonella*-specific and whether CD8⁺ T cells can acquire a cytotoxic phenotype through B cell-mediated activation. First, we investigated if the CD8⁺ degranulate their cytotoxic granules upon recognition of *Salmonella*-infected target cells. For this we analyzed expression of the marker CD107a, which is expressed at the plasma membrane of CD8⁺ T cells upon degranulation. Anti-IgM-coated *Salmonella*-infected B cells were cultured together with CFSE labeled CD4⁺ and CD8⁺ T cells and the proliferating *Salmonella*-primed CD8⁺ T cells were sorted after 6 days. After expansion with IL-2 for another 6 days, the CD8⁺ T cells were re-exposed to autologous, *Salmonella*-infected B cells and CD107a expression was measured. Upon re-exposure to *Salmonella*-infected B cells, the *Salmonella*-primed CD8⁺ T cells show an increase of CD107a expression at the plasma membrane.



Since the proliferating CD8 $^{+}$ T cells did not show degranulation when re-exposed to autologous, non-infected B cells, the CD8 $^{+}$ T cells specifically degranulated upon recognition of *Salmonella*-infected cells (Fig. 3C). To confirm our results without anti-IgM coating of the bacteria, we also primed CD8 $^{+}$ T cells with *Salmonella*-specific B cells that had phagocytosed uncoated *Salmonella* via the *Salmonella*-specific BCR. These primed CD8 $^{+}$ T cells degranulated specifically upon recognition of autologous B cells infected with uncoated *Salmonella*, of which a relative small percentage (5%, data not shown) contained *Salmonella*-infected antigen-specific B cells (Fig. 3D; middle bar). In addition, the *Salmonella*-primed CD8 $^{+}$ T cells very efficiently degranulated upon contact with autologous B cells of which a higher percentage (26%, data not shown) of cells had been infected with anti-IgM coated *Salmonella* (Fig. 3D; right bar).

Figure 3. *Salmonella* infected B cells induce *Salmonella*-specific CD8⁺ T cells to secrete IFN- γ and are cytotoxic.

(A) CD4⁺ and CD8⁺ T cells were cultured alone (T), with B cells (B + T) or with *Salmonella*-infected B cells (B_{Sal} + T). After 11 days, CD8⁺ T cells were analyzed for IFN- γ production by intracellular FACS staining. A representative experiment of five independent experiments using cells from different healthy donors is shown. (B) *Salmonella*-infected B cells induce IFN- γ expression by CD8⁺ T cells compared to non-infected B cells. Data are the mean $\pm$ SEM from five independent experiments of different donors and ** $p < 0.01$. (C) CD8⁺ T cells were primed with *Salmonella*-infected B cells and the proliferating CD8⁺ T cells were sorted after 6 days. Sorted CD8⁺ T cells were restimulated with autologous B cells that had either or not phagocytosed anti-IgM-coated *Salmonella*. *Salmonella*-specific degranulation was measured by CD107a expression at the plasma membrane CD8⁺ T cells. Data are means $\pm$ SEM of five independent experiments of different donors, and ** $p < 0.01$. (D) Proliferating CD8⁺ T cells primed with B cells that had phagocytosed uncoated *Salmonella* were sorted and CD107a expression was measured upon re-encounter of B cells (T + B), B cells infected with uncoated (T + B_{un-Sal}) or anti-IgM-coated *Salmonella* (T + B_{Sal}). Data are of one experiment. E, Sorted *Salmonella*-primed CD8⁺ T cells (see C) specifically kill *Salmonella*-infected B cells as measured by ⁵¹Cr release of autologous B cells that were either or not infected with *Salmonella*. Data are expressed as mean $\pm$ SEM, of six independent experiments of different donors, and ** $p < 0.01$. F, Sorted *Salmonella*-primed CD8⁺ T cells (see C) do not kill *Staphylococcus*-infected B cells as measured by ⁵¹Cr release of autologous B cells that were either infected with *Salmonella* or with *Staphylococcus*. The data are expressed as mean $\pm$ SEM, of two independent experiments of different donors.

Finally, we investigated if degranulation of the *Salmonella*-specific CD8⁺ T cells also leads to death of the *Salmonella*-infected cells to determine the true cytotoxic potential of the lytic granules. CFSE labeled CD8⁺ T cells were primed by incubation with *Salmonella*-infected B cells and proliferating CD8⁺ cells were sorted and expanded with IL-2 for 6 days. Re-exposure of the *Salmonella*-primed T cells to *Salmonella*-infected, ⁵¹Cr-labeled autologous B cells demonstrated that the primed CD8⁺ T cells were able to kill *Salmonella*-infected B cells but not non-infected B cells (Fig. 3E). In addition, the *Salmonella*-primed CD8⁺ T cells were not able to kill B cells that had phagocytosed *Staphylococcus* via BCR ligation (Fig. 3F), demonstrating that the CD8⁺ cells are indeed *Salmonella*-specific and do not recognize autologous B cells that are activated via BCR-mediated uptake of other bacteria. Thus, activation of CD8⁺ T cells by B cells cross-presenting *Salmonella* antigens induces a cytotoxic phenotype in the CD8⁺ T cells that specifically mediates killing of *Salmonella*-infected cells.

Cross-presentation of *Salmonella* antigens is partly proteasome dependent

We showed that after phagocytosis of *Salmonella*, B cells are able to cross-present antigens to CD8⁺ T cells and thereby initiate a *Salmonella*-specific cytotoxic T cell response. The mechanism of cross-presentation of *Salmonella* antigens by B cells is unknown. One possible mechanism is that after phagocytosis, antigens are translocated from the *Salmonella*-containing vacuole (SCV) into the cytoplasm of B cells. Next, the antigens are degraded in the cytoplasm by proteasomes into small peptides, which are presented via the classical MHC class I antigen presentation route. To investigate this mechanism of cross-presentation, we used a chemical compound that specifically blocks the proteasome (MG-132). To study the effect of cross-presentation when blocking the proteasome, we used the CD107a degranulation assay. Re-exposure of *Salmonella*-primed CD8⁺ T cell to autologous, *Salmonella*-infected B cells showed that by blocking of the proteasome, a *Salmonella*-infected B cell is much less efficient in inducing CD8⁺ T cell degranulation when proteasomes are inhibited (Fig. 4). This observation implies that the proteasome is somehow involved in cross-presentation of *Salmonella* antigens by B cells. How the proteasome is involved is unclear as proteasome inhibition has many secondary effects on the ubiquitin cycle, the modification of histones and the formation of multivesicular bodies (26).

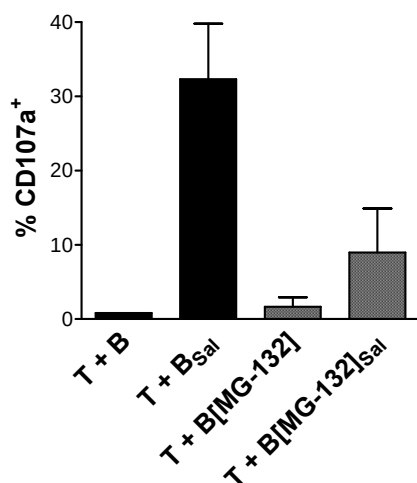


Figure 4. Proteasome inhibition of *Salmonella*-infected B cells diminishes degranulation of *Salmonella*-specific CD8⁺ T cells.

Salmonella-specific CD8⁺ T cells were sorted and co-cultured with B cells (T + B) or with *Salmonella*-infected B cells (T + B_{sal}). Treatment of B cells with proteasome inhibitor MG-132 (20 μM) leads to a decrease in degranulation of the *Salmonella*-specific CD8⁺ T cells (T + B[MG-132]_{sal}), as measured by CD107a expression.

B cells do not cross-present heat-killed *Salmonella*

Salmonella survives inside a cell via expression of the TTSS that create an intracellular environment that neutralizes the destructive forces of the host cell (27). The TTSS components SPI-1 and SPI-2 play a role in this neutralization by exporting proteins into the host cell. Because of the capacity of *Salmonella* to

invade cells and to control its maintenance inside the cell via SPI-1 and SPI-2, it is possible that *Salmonella* might directly play a role in the cross-presentation by B cells. To determine the role of SPI-1 and SPI-2 in cross-presentation of *Salmonella* by B cells, we analysed CD8⁺ T cells proliferation via co-culture with CD4⁺ T cells and B cells that had phagocytosed wild type *Salmonella*, or *Salmonella* with a mutation in SPI-1 (*invA*⁻) or SPI-2 (*ssrA*⁻). Both *Salmonella* mutants were still able to elicit a CD8⁺ T cell response, albeit at lower percentages of T cell proliferation compared to wild type *Salmonella* (Fig. 5A). Thus SPI-1 and SPI-2 each contribute to cross-presentation of *Salmonella* antigens by B cells, but are not essential.

By creating an environment in which *Salmonella* itself cannot be killed, it is possible that the intracellular survival plays a role in the efficacy of cross-presentation of *Salmonella* antigens by B cells. To study the contribution of *Salmonella* on the efficacy of cross-presentation of *Salmonella* antigens, we compared the efficiency of living and dead *Salmonella* to induce CD8⁺ T cell activation. Living and heat-killed *Salmonella* were coated with the LPS-BCR tetrameric antibody complexes to ensure similar levels of bacterial phagocytosis by B cells (data not shown). B cells that have phagocytosed dead *Salmonella* less initiate CD8⁺ T cell proliferation (Fig. 5B), indicating that *Salmonella* contributes to the process of cross-presentation in B cells.

However, another part of the response continued with dead *Salmonella*. To confirm that *Salmonella* antigens of dead bacteria are inefficiently cross-presented to CD8⁺ T cells by B cells, we primed CD8⁺ T cells with B cells infected with living or heat-killed *Salmonella*, sorted and expanded the primed T cells followed by re-exposure to autologous B cells that had not phagocytosed *Salmonella*, or had phagocytosed either living or dead *Salmonella*. Cross-presentation of *Salmonella* antigen was measured by the extent of degranulation of the primed T cells using CD107a expression.

CD8⁺ T cells that had been primed by B cells infected with living *Salmonella* efficiently degranulated upon recognition of autologous B cells that had phagocytosed living *Salmonella* (upregulation of CD107a to 60%), whereas the cells did not degranulate upon contact with B cells that had phagocytosed dead *Salmonella* (Fig. 5C; left panel). This indicates either that B cells present different *Salmonella* antigens to CD8⁺ T cells from live or dead bacteria or that B cells cannot cross-present *Salmonella* antigens when the intracellular bacterium is not alive. Furthermore, CD8⁺ T cells that had been primed with B cells that had taken up

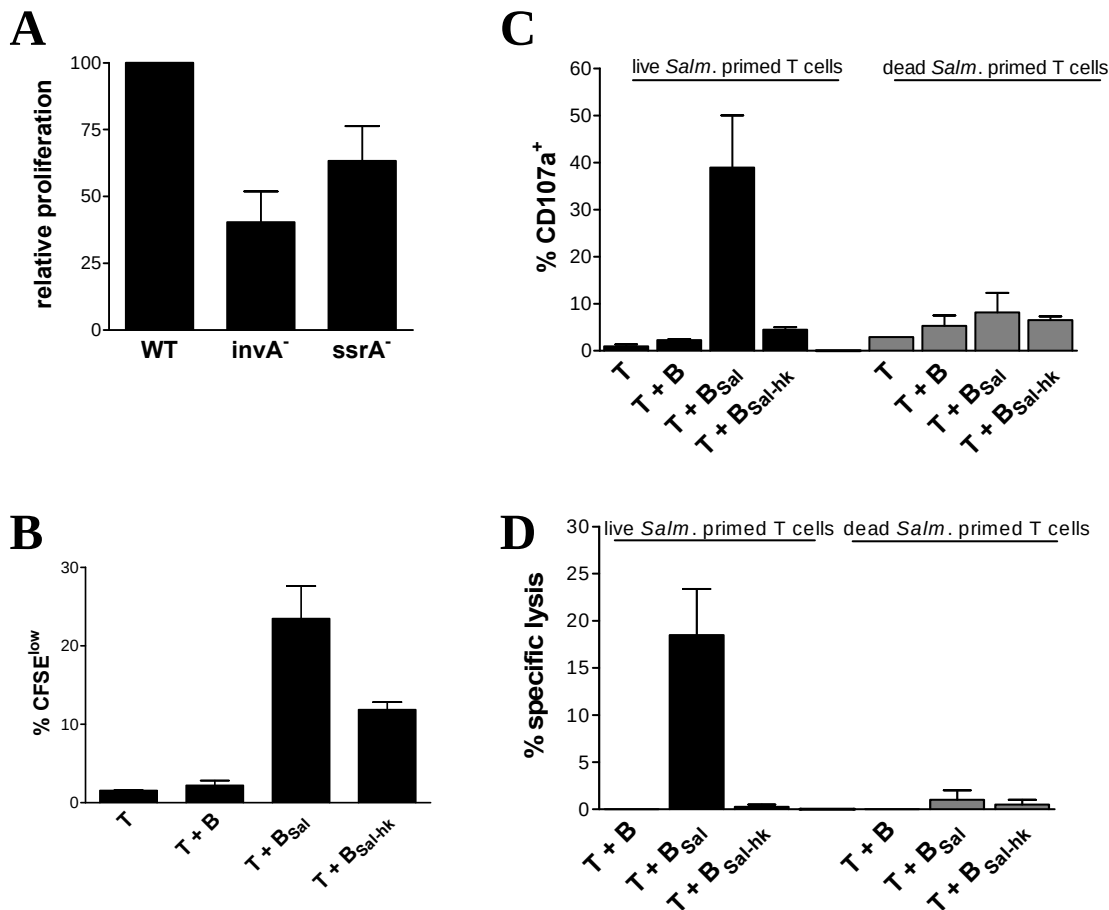


Figure 5. Induction of the cytotoxic T cell response against *Salmonella* by B cells requires uptake of living *Salmonella*.

(A) CD8⁺ T cells were labeled with CFSE and cultured with B cells that had phagocytosed either wild type *Salmonella* (WT), mutant for SPI-1 (*invA*⁻) or SPI-2 (*ssrA*⁻). Proliferation was measured after 6 days. Data shown are proliferation of CD8⁺ cells relative to wild type proliferation and are of two different donors; error bars are SEM. (B) CD8⁺ T cells were labeled with CFSE and cultured with B cells (T + B), or with B cells that have internalized either living *Salmonella* (T + B_{Sal}) or heat killed *Salmonella* (T + B_{Sal-hk}). Proliferation was measured after 6 days. C, *Salmonella*-specific T cells that were primed with B cells that had either internalized living (left panel) or dead *Salmonella* (right panel) were sorted and restimulated with B cells (T + B), B cells that had internalized living *Salmonella* (T + B_{Sal}) or dead *Salmonella* (T + B_{Sal-hk}). Degranulation was analyzed as CD107a expression by CD8⁺ T cells. D, CD8⁺ T cell mediated kill was measured as the release of ⁵¹Cr by B cells. Data are expressed as mean ± SEM, from four (live *Salmonella* primed) or two (dead *Salmonella* primed) independent experiments of using material from different healthy donors.

dead *Salmonella* showed poor degranulation to B cells that had taken up either live or heat-killed *Salmonella* (Fig. 5C; right panel), demonstrating that B cells indeed do not induce a cytotoxic CD8⁺ T cell response via cross-presentation of *Salmonella* antigens from dead intracellular bacteria. This observation was confirmed by studying elimination of *Salmonella*-infected B cells by ⁵¹Cr-release (Fig. 5D).

Together, these results show that B cells are not able to cross-present antigens of dead *Salmonella* and that *Salmonella* contributes to the activation of a *Salmonella*-specific cytotoxic CD8⁺ memory response.

Discussion

Studies in B-cell-deficient mice showed that protective immunity to *Salmonella* strongly depends on B cells (28). This dependency does not only result from antibody generation, as passive transfer of *Salmonella*-immune serum cannot transfer resistance to *Salmonella* (29). In addition, B cells are involved in the generation of a profound CD4⁺ and CD8⁺ T cell response after *Salmonella* infection (21), but the precise role of B cells remained unclear. We previously showed that human antigen-specific B cells that have internalized *Salmonella* via their BCR are able to induce a *Salmonella*-specific CD4⁺ T cell response (20). The data described here may explain the role of B cells in immune response against *Salmonella* infection other than antibody formation. In contrast to other data, in which uptake of *Salmonella* does not lead to antigen cross-presentation via MHC class I in mouse B cell lines (30), we now demonstrate that *Salmonella*-specific human B cells that have phagocytosed *Salmonella* via their BCR are capable to induce a strong recall response of cytotoxic CD8⁺ T cells after cross-presentation of *Salmonella* antigens. We previously showed that in the human Ramos B cell line, *Salmonella* is not only capable to survive, but also to replicates intracellularly. In contrast, in primary human B cells *Salmonella* survives, but is unable to replicate inside the B cell (20). The incapability to replicate in primary human B cells may play a role in cross-presentation and activation of CD8⁺ T cells. A recent report shows that the most functional CD8⁺ T cell responses are induced against *Salmonella* that do not replicate intracellularly (31). These observations may explain our finding why *Salmonella*-specific primary B cells are efficient in cross-presentation of *Salmonella* antigens from the non-replicating bacteria and activate *Salmonella*-specific CD8⁺ cells that show a functional cytotoxic T cell response. Thus, inhibition of *Salmonella* replication by primary human B cells may be beneficial for cross-presentation to CD8⁺ T cells.

After internalization by the B cell, *Salmonella* survives in the SCV. For cross-presentation, *Salmonella*-antigens should be loaded onto MHC class I. The TTSS of *Salmonella* could play a role in delivery of antigens in the cytosol by injecting proteins directly into the cytosol (32). These proteins can be degraded by the

proteasome and after transportation into the ER, loaded onto MHC class I molecules. We demonstrated that SPI-1 and SPI-2 contribute to cross-presentation, but both are not required separately. It is therefore likely that antigens excreted in the host cytosol by SPI-1 and SPI-2 are both used for antigen processing to MHC class I. In addition, we showed that B cells were not able to cross-present *Salmonella*-specific antigens of heat killed *Salmonella*. Thus, *Salmonella* itself appears to be involved in the generation of an effective cytotoxic CD8⁺ T cell response against the bacteria, a phenomenon that points to the occurrence of co-evolution of bacterial immune evasion and the generation of effective anti-bacterial immunity.

Cross-presentation of *Salmonella* antigens by B cells leads to activation of a CD8⁺ cytotoxic T cell response, but help of CD4⁺ T cells is required. The exact kind of CD4⁺ T cells help could be via cell-cell contact or cytokine mediated and is presently under investigation.

Various reports have described that B cells play a role in the expansion of *Salmonella*-specific T cells, but how specific T cell responses are induced is not understood. This has been attributed to the fact that antigen-specific B cells are probably the main B cell population with an antigen presenting function in *Salmonella* infection and that the frequency of antigen-specific B cells is elevated during a secondary infection (reviewed in (33)). Indeed, our data concur with these observations as we show that the antigen-specific B cells are the cells that are responsible for efficient cross-presentation of *Salmonella* antigens. In addition, our data provide an additional explanation why B cells are mainly involved in the recall response; *Salmonella*-infected B cells do not prime naïve CD8⁺ T cells, but are very efficient in inducing a potent recall response of cytotoxic CD8⁺ memory T cells. This implies that other APCs (e.g. DCs) are still needed to prime naïve CD8⁺ T cells. In secondary infections however, DCs can induce CD8⁺ proliferation via direct infection or via suicide cross-presentation upon ingestion of infected apoptotic cells (22), but also B cells are very efficient in generating an anti-*Salmonella* cytotoxic CD8⁺ responses. In addition, DC were shown to mainly activate the T_{EM} compartment (22), whereas *Salmonella*-infected B cells activate both the T_{CM} and T_{EM} compartment. This ensures not only the direct terminal differentiation of effector memory cells, but also the expansion of the *Salmonella*-specific CD8⁺ memory T cell compartment, which may both amplify the anti-*Salmonella* immune response

and simultaneously ensure generation of anti-*Salmonella* memory for further reinfections.

In summary, we propose a model on the role of B cells in the generation of the humoral and cellular immune response against *Salmonella*. After infection, *Salmonella* enters the body via DCs that activate CD4⁺ and CD8⁺ T cells or via M cells after which it encounters B cells, which are situated in the Peyer's Patches, directly under the M cells. *Salmonella*-specific B cells internalize *Salmonella* via their BCR and are activated. Next, internalization of *Salmonella* leads to presentation of *Salmonella* antigens MHC class II molecules and activates *Salmonella*-specific CD4⁺ T-helper cells that stimulate *Salmonella*-infected B cells to secrete *Salmonella*-specific antibodies (20), either locally or upon arrival or the infected B cells in the mesenteric lymph node. Upon re-infection, infection of *Salmonella*-specific memory B cells by *Salmonella* ensures rapid antibody production but also mediates a strong cytotoxic CD8⁺ recall response to eliminate infected cells. The *Salmonella*-specific CD4⁺ response that aided antibody production in early stages of the immune response is now also required for the reactivation of the cytotoxic T cell response against *Salmonella* in later stages of the immune response or upon reinfection. Thus, uptake of *Salmonella* by antigen-specific B cells may generate a survival niche for *Salmonella*, but at the same time strongly contributes to the generation of effective anti-*Salmonella* immunity at multiple levels of the adaptive immune response. The combined attack of pathogens by various members of the adaptive immune system will allow efficient eradication of the infection.

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Chapter 5

Class II-Associated Invariant Chain Peptide expression on myeloid leukemic blasts predicts poor clinical outcome

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Abstract

Effective antitumor responses need the activation of CD4⁺ T cells. MHC class II antigen presentation requires the release of class II-associated invariant chain peptide (CLIP) from the antigen-binding site. In antigen-presenting cells, human leukocyte antigen DM (HLA-DM; abbreviated DM in this article) catalyzes CLIP dissociation. In B cells, HLA-DO (DO) down-modulates DM function. Cell surface CLIP:HLA-DR (DR) ratio correlates to DO:DM ratio and the efficacy of antigen presentation. We examined 111 blood and bone marrow samples of patients with newly diagnosed acute myeloid leukemia (AML) for the expression of CLIP, DR, DM, and DO by flow cytometry. Patients with DR⁺/CLIP⁻ blasts had a significant longer disease-free survival than patients with DR⁺/CLIP⁺ blasts. DO, until now believed to be restricted to lymphoid cells, could be demonstrated at protein level as well as by reverse transcription-PCR. DO:DM ratio correlated to CLIP:DR ratio, suggesting that, unlike in other antigen-presenting cells of the nonlymphoid cell type, both DO and DM mediate regulation of CLIP expression in AML blasts. We hypothesize that DR⁺/CLIP⁻ AML blasts are able to present leukemia-specific antigens to CD4⁺ T helper cells initiating an effective and long-lasting antitumor response resulting in a prolonged disease-free survival.

Introduction

The role of MHC class II molecules presenting tumor antigens to CD4⁺ T helper cells in antitumor responses is well established (1). The major MHC class II molecule, DR, consists of an α and β chain that, after translocation to the endoplasmic reticulum, form a complex with the invariant chain (Ii) (2). Ii serves as a chaperone for newly synthesized class II molecules and prevents the binding of undesired antigen in the endoplasmic reticulum. The Ii is cleaved in the endosomal/lysosomal pathway until only a small remnant, called class II-associated invariant chain peptide (CLIP), remains associated with the antigen-binding groove (3). Release of CLIP is necessary for replacement with antigens, a process catalyzed by DM, a nonclassical MHC class II molecule (4). In mice lacking DM, CLIP is not released from the antigen-binding site and severely injures antigen presentation (5). In B cells and thymic epithelial cells, DO, another nonclassical MHC class II molecule, associates with DM. DO down-modulates the catalytic activity of DM in a pH-dependent manner, thus altering the repertoire of presented antigens (6). By expression of different levels of DM and DO, B cells regulate the antigen presentation capacity of their MHC class II molecules (7). High cell surface expression of CLIP correlates with a high DO:DM ratio and can be viewed as an indicator for low effectiveness of antigen presentation (8).

In MHC class II negative tumors, the activation of CD4⁺ T cells relies on presentation of tumor antigens by professional antigen-presenting cells (APCs). MHC class II transfection studies in mice have shown that, upon expression of MHC class II molecules, tumor cells can present their tumor antigens directly to CD4⁺ T cells, thus bypassing the need for professional APCs (9). Moreover, simultaneous Ii suppression by antisense therapy revealed that these cells present endogenous tumor antigens and can mount a tumor-specific immune response (10). DR⁺/Ii⁻ tumor cells, which are believed to present an optimal range of endogenous tumor antigens, are the predominant APC in vivo and have been demonstrated to be potent vaccines for tumor-bearing mice (11). Moreover, as recently described (12), human DR⁺/Ii⁻/CD80⁺-transfected tumor cell lines were demonstrated to elicit tumor-specific T-cell responses.

In AML, acquired mutations of the hematopoietic stem cells block differentiation. The result is accumulation of immature cells in the bone marrow and peripheral blood, often accompanied by suppression of the normal blood cells. With chemotherapy and stem cell transplantation, ~70% of patients achieve complete

remission, but approximately one half of these patients relapse (13). Although AML blasts generally express MHC class II molecules and costimulatory molecules (14), they must have escaped the initial immune response in acute disease status. We hypothesize that in the situation of minimal tumor burden, i.e., after achieving complete remission, AML blasts with a functional MHC class II complex could evoke effective immunosurveillance. In this study, we report that CLIP expression could be detected on AML blasts. Furthermore, we found a strong correlation between a high level of CLIP-positive AML blasts and a shortened disease-free survival. Also, the regulation of CLIP dissociation was studied. Strikingly, DO expression, until now demonstrated only in lymphoid cells (15), was demonstrated in AML blasts. This indicates that, in these myeloid cells, both DO and DM contribute to the efficacy of CLIP-antigenic peptide exchange.

Materials and methods

Patients

After informed consent, blood and bone marrow samples were collected from 111 patients with previously untreated AML between 1992 and 2003. Patients were classified according to the French-American-British (FAB) classification (13). Patients with promyelocytic leukemia (FAB-M3), whose leukemic blasts were DR negative, were excluded. Patients received remission induction and consolidation therapy according to HOVON (Dutch-Belgian Hematology-Oncology Cooperative Group) protocols. Cytogenetic risk group was defined as favorable [t(8;21), or inversion (16)], standard (neither favorable nor adverse), or adverse [complex karyotype, -5 or -7, deletion(5q), abnormality 3q or 11q] (13). Human leukocyte antigen (HLA) allotype was diagnosed with a serological microcytotoxic assay (Sanquin Research at CLB, Amsterdam). Disease-free survival was defined as the time period between achievement of complete remission and the moment of relapse or the last date of follow-up in nonrelapsed patients. Patient characteristics are shown in Table 1 and reflect a representative AML patient group.

Mononuclear Cell Isolation

Bone marrow mononuclear cells and peripheral blood mononuclear cells, withdrawn before the start of therapy, were collected through density-gradient centrifugation (Ficoll-PaquePLUS, Amersham Biosciences). Samples were analyzed immediately or cryopreserved in liquid nitrogen until analysis. For mRNA detection, cryopreserved

samples were thawed and the CD45dim/CD19-/7AAD-population (representing living myeloid leukemic, non-B cells) was isolated by sorting [FACS Vantage, Becton Dickinson (BD)]. Purity was always >99%.

Antibodies and Flow Cytometry Analysis

The following mouse antibodies were used: FITC-labeled anti-HLA-ABC (Dako), anti-DR (BD), CD86 (BD), anti-DO (BD PharMingen), CD22 (BD); phycoerythrin-labeled anti-DM (BD PharMingen), CD19 (Dako), CD20 (BD), CD14 (BD); peridinin chlorophyll protein-labeled CD45 (Coulter); allophycocyanin-labeled CD34 (BD); and 7-AAD (Via-Probe, BD PharMingen). CerCLIP.1 was kindly provided by P. Cresswell (Howard Hughes Medical Institute, Yale University School of Medicine, New Haven, CT; ref. 4). Mononuclear cell fractions were preincubated with 10% human gammaglobulin (6 mg/ml; Sanquin, Amsterdam), followed by incubation with directly labeled antibodies. For CLIP detection, cells were incubated with CerCLIP and subsequently with phycoerythrin-conjugated rabbit-antimouse immunoglobulin (Dako). A mixture of nonrelevant mouse antibodies of different isotypes was added to avoid aspecific binding of subsequently directly labeled antibodies. For intracellular staining with DO or DM antibodies, cells were, after plasma membrane staining, fixed with PBS-1% paraformaldehyde and permeabilized with PBS-0.05% saponine. All of the incubations were performed at room temperature during 15 min for extracellular and 30 min for intracellular staining. Cells were washed after every incubation step with PBS-0.1% BSA-0.05% sodiumazide and analyzed on a FACSCalibur (BD). 25,000 living cells on a forward scatter were analyzed using CellQuest software (BD). Blasts were defined as CD45dim/SSClow. Mean fluorescence intensity index (MFI) was defined by the following formula:

$$\frac{\text{Mean fluorescence (total population)} - \text{Mean fluorescence (isotype control)}}{\text{Mean fluorescence (isotype control)}}$$

The capability of eliciting effective antitumor immune responses is likely to depend on the total number of DR molecules that are not occupied by CLIP. To take both the number of DR- and CLIP-positive cells, as well as the amount of DR and CLIP molecules per cell, into consideration, we defined CLIP expression in respect to clinical data as follows:

$$\text{Relative CLIP amount} = \frac{\text{Percentage CLIP}^+ \text{ cells}}{\text{Percentage DR}^+ \text{ cells}} \times \frac{\text{MFI CLIP}}{\text{MFI DR}}$$

Real-Time Reverse Transcription-PCR

Six samples of leukemic myeloid blasts with different DO and DM protein expression were selected with the purpose of correlating protein expression with mRNA expression. Sorted cells (see above) were lysed in peqGOLD Trifast (PeQlab, Erlangen, Germany). GlycoBlue (Ambion, Austin, TX) was added as a carrier, and total RNA was extracted according to the manufacturer's instructions. First-strand cDNA was reverse transcribed using random hexamers [pd(N)6; Amersham Biosciences, Piscataway, NJ] and a SuperScript II, RNase H-reverse transcriptase kit (Invitrogen, Breda, the Netherlands). Gene expression was measured in the ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA). Selection and specificity of the primers (B&G Biotech, Freiburg, Germany) have been described previously (16). The sequences of the primers are as follows: DO α , forward 5'-GAGCCATCAACGTGCCTC-3', reverse 5'-AGTGACAGTTTGGCCGTTG-3'; DO β , forward 5'-GGAGAAAGATGCTGAGTGGC-3', reverse 5'-AGGGAGCAGAACAGCTCTTG-3'; and DM β , forward 5'-CCAGCCCAATGGAGACTG-3', reverse 5'-CAGCCCAGGTGTCCAGTC-3'. As endogenous control, primers specific for human 18S rRNA were used, forward 5'-CGGCTACCACATCCAAGGAA-3' and reverse 5'-GCTGGAATTACCGCGGCT-3'. Relative quantitation of gene expression was determined using the comparative threshold cycle method as suggested by the manufacturers. All of the results were normalized with respect to the internal control 18S rRNA and are expressed relative to CD19-positive B cells from healthy donors. We used the T2 cell line as a negative control, in this study transfected with the HLA-B27 allele (T2-B27), as described previously (17).

Statistical Analysis

Statistical analyses were conducted with a SPSS 9.0 software program. To analyze associations between variables, Spearman's correlation coefficient was used. Differences between patient characteristics were analyzed with the Mann-Whitney U and the χ^2 test. For survival data, Kaplan-Meier curves were constructed and compared by the log-rank test. To explore the simultaneous effect of several variables on disease-free survival, the Cox regression model was used. Relative CLIP amount was log transformed, yielding a normally distributed variable.

Results

Flow Cytometry Studies

We analyzed the cell surface expression of HLA-ABC, DR, CLIP, CD80, CD86, and CD40 expression on 111 samples of patients with AML. In line with previous observations (14), we found a consistently high percentage of cells expressing HLA-ABC, a low percentage expressing CD80, and a variable percentage expressing DR, CD86, and CD40. The percentage of cells with CLIP expression was variable (Table 1). All of the samples allowed clear analysis of these markers (Fig. 1). Double labeling of CLIP and DR was not possible because of steric hindrance of both monoclonals.

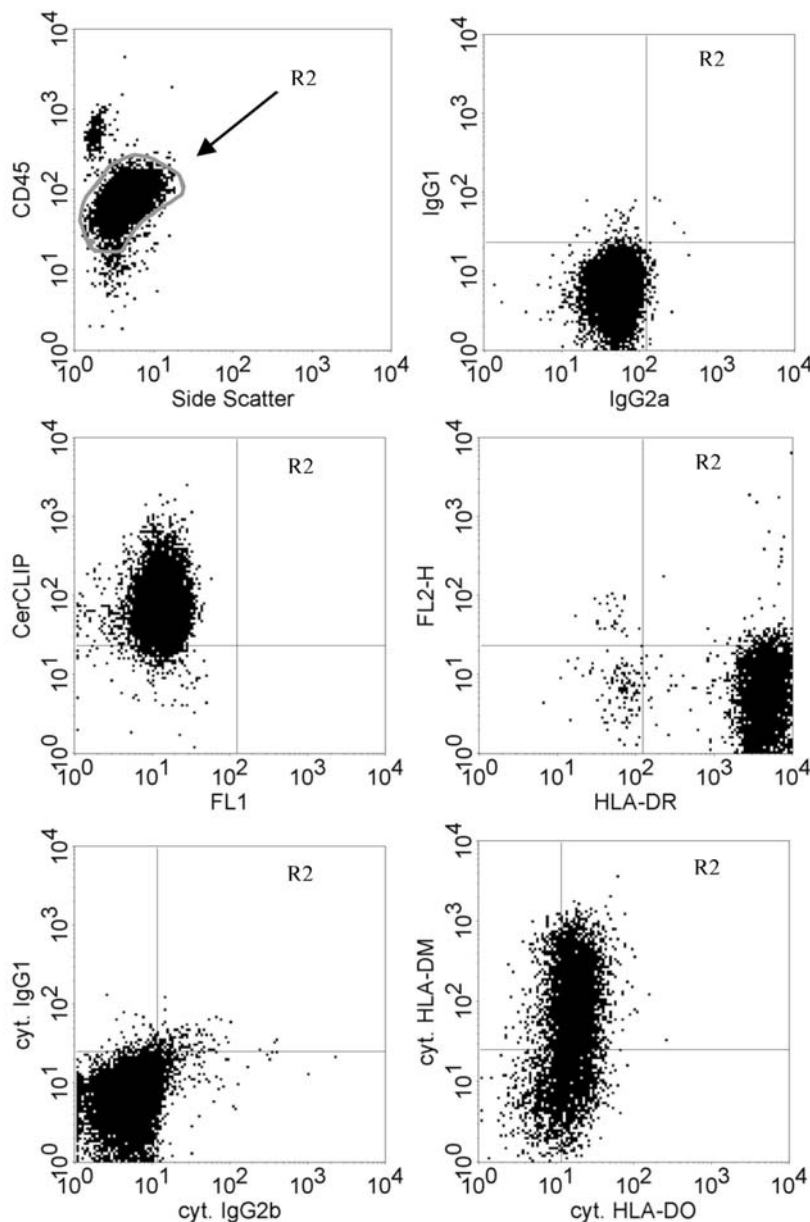


Figure 1. Example of CLIP, DR, DO and DM expression on myeloid leukemic blasts (gate R2: CD45^{dim}/SSC^{low}) with appropriate isotype controls (gated on 99% of the population).

Double labeling of CLIP and DR was not possible due to steric hindrance of both monoclonals.

(cyt., cytoplasmatic)

CLIP expression did not differ between peripheral blood and bone marrow samples, nor did it change after freezing and thawing ($n = 4$; data not shown). CLIP expression did not vary significantly among several FAB classes. Because cell surface expression of CLIP on blood and bone marrow cells was described only on mature B cells and monocytes (7), we investigated the cross-lineage expression of CD19, CD20, and CD22 (as B-cell markers) and CD14 (monocytoid marker) on AML blasts. No correlation between relative CLIP amount and these markers was observed (neither for MFI nor for percentage; $P = 0.37, 0.32, 0.56, 0.28$, and $0.53, 0.27, 0.81, 0.21$, respectively). Finally, we tested whether differences in CLIP expression were a reflection of DR allotype. We did not observe however, any relationship between DR allotype and CLIP expression ($n = 19$; data not shown) as was suggested previously (18).

Correlation of Relative CLIP Expression with Clinical Data

To test the hypothesis that a functional MHC II complex (DR+/CLIP-) would result in higher antigen-presenting capacity and, hence, a survival benefit, we excluded 11 DR- patients. We observed no differences in relative CLIP amount between patients that achieved complete remission and those that did not ($P = 0.15$). In AML patients, most relapses occur within 2 years. Therefore, we compared the relative CLIP amount between patients who survived at least 24 months after complete remission and those who suffered relapse within this period. Significantly lower relative CLIP amount was observed in patients with prolonged remission compared with that in patients who relapsed before 24 months ($P = 0.04$). In clinical practice, the percentage of positive cells of different markers is used to determine the phenotype of leukemic blasts. AML patients without relapse had a maximum level of 33% CLIP-positive AML blasts. A cutoff level of 35% resulted in strongly deviating Kaplan-Meier curves ($P = 0.015$) that clearly demonstrated the survival advantage for DR+/CLIP- patients (Fig. 2). To exclude good- and poor-risk patients based on cytogenetic risk profile, a similar Kaplan-Meier curve including only patients with an intermediate cytogenetic risk profile was constructed. The P value was slightly increased (0.08) because of the smaller group size (Fig. 2).

Because the relative CLIP amount was not related to the capability of patients to achieve complete remission, the cytogenetic risk profile, which is currently the best predictive variable for outcome for AML patients, remained a stronger predictor for

Table 1. Patients' characteristics and flow cytometric expression of different markers (percentage of positive cells) on myeloid blasts of total group and different subgroups (DR- defined as <10% of cells positive; CLIP+ defined as >35% of cells positive).

	total	patients with DR- blasts	patients with DR+ /CLIP- blasts	patients with DR+ /CLIP+ blasts
A. Clinical characteristics of patients				
Number of patients	111	11	78	22
Male/female	56/55	5/6	39/39	11/11
Age at diagnosis, y, mean (range)	52 (16-79)	46 (23-77)	52 (16-77)	52 (22-79)
WBC at diagnosis (range)	67 (1-300)	73 (1-300)	70 (1-282)	58 (1-246)
follow-up in months, mean (range)	21 (0.03-113)	21 (0.46-58)	23 (0.03-113)	14 (0.7-80)
complete remission rate, number (%)	85 (75)	9 (82)	59 (76)	15 (68)
DFS, mean (95% CI) *	41 (30-53)	32 (16-50)	48 (34-62)	11 (5-18)
Fab classification. number (%)				
AML M0	6 (5)	2 (18)	2 (3)	2 (10)
AML M1	17 (15)	5 (46)	8 (10)	4 (18)
AML M2	21 (19)	3 (27)	15 (19)	3 (14)
AML M4	29 (26)	0	25 (32)	4 (18)
AML M5	29 (26)	0	20 (26)	9 (41)
AML M6	4 (4)	1 (9)	3 (4)	0
RAEB-t	4 (4)	0	4 (5)	0
Not classified	1 (1)	0	1 (1)	0
Cytogenetic risk group, number (%)				
Favorable	9 (8)	0	9 (11.5)	0
Standard	65 (59)	8 (73)	45 (58)	12 (55)
Adverse	17 (9)	2 (18)	11 (14)	4 (18)
No metaphasis	14 (13)	0	11 (14)	3 (13.5)
Not done	6 (5)	1 (9)	2 (2.5)	3 (13.5)
B. Flow cytometric analysis of myeloid leukemic blasts				
CD40 (%) †	25.7	2	28.8	26.6
CD80 (%)	1.3	0.6	1.6	0.8
CD86 (%) ‡	22.4	4.4	24.2	25.5
MHC class I (%)	98.8	98	98.6	99.6
MHC class II (%)	66.2	1.18	70.7	83
CLIP (%)	19.8	4.1	12.3	54.5
HLA-DM (%) §	55.6	0.62	61.1	59.5
HLA-DO (%)	16.5	26.5	15.3	16.4

Abbreviations: WBC, white blood cell count; CR, complete remission; DFS, disease-free survival; CI, confidence interval, RAEB-t, refractory anemia with excess blasts in transformation.

* Significant differences were seen in DFS between DR+/CLIP+ and DR+/CLIP- patients ($P = 0.015$, log-rank). Differences in other characteristics were not significant.

† Significant differences were seen in CD40 expression between DR+ and DR- patients, ($P = 0.001$, Mann-Whitney U test). Differences in other characteristics were not significant.

‡ Significant differences were seen in CD86 expression between DR+ and DR- patients, ($P = 0.003$, Mann-Whitney U test). Differences in other characteristics were not significant.

§ Significant differences were seen in HLA-DM expression between DR+ and DR- patients, ($P = 0.001$, Mann-Whitney U test). Differences in other characteristics were not significant.

overall survival than did the relative CLIP amount ($P = 0.06$ versus $P = 0.08$, Cox regression). However, after achievement of complete remission, when the immune surveillance is supposed to control disease status, relative CLIP amount was a better predictor than cytogenetic risk profile ($P = 0.06$ versus $P = 0.36$, Cox regression) for the duration of the disease-free survival.

Antigen presentation in the absence of adequate costimulatory signals leads to anergy. DR+/CLIP- blasts do express costimulatory molecules like CD86 and CD40. DR+ cells showed significantly higher expression levels of CD86 and CD40 as compared with DR- cells ($P = 0.003$ and 0.001 , respectively), apparently above the minimal threshold required for effective costimulation.

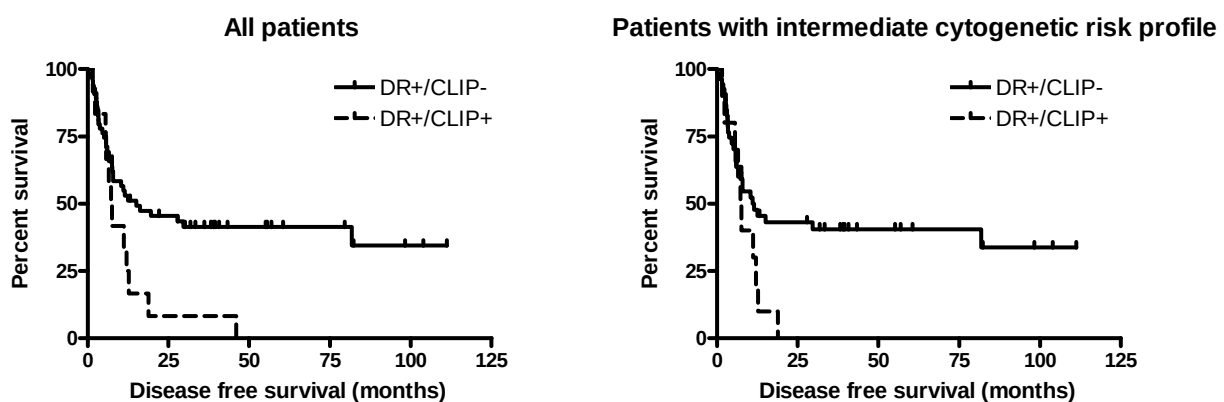


Figure 2. Kaplan-Meier analyses for disease-free survival.

Left panel, All patients, significant differences were seen between DR+/CLIP- and DR+/CLIP+ (cutoff 35% of cells CLIP positive), $P = 0.015$ (log-rank). *Right panel*, including only patients with an intermediate cytogenetic risk profile resulted in a survival curve with a similar shape

Regulation of CLIP Expression Level by HLA-DO

To elucidate the mechanism underlying the regulation of CLIP expression in AML blasts, we analyzed 89 samples for intracellular DO and DM expression by flow cytometry (Fig. 1). Surprisingly, DO was readily detectable in myeloid blasts. In B cells, the intracellular DO:DM ratio correlates with the cell surface CLIP:DR ratio. In AML blasts, we also could demonstrate a correlation [Fig. 3, *left panel* ; $P = 0.001$, correlation coefficient (R) = 0.46], indicating a functional role of DO and DM for cell surface CLIP expression in AML blasts similar to that in B cells. In B cells, reduced DO:DM ratio levels could not be explained by similar changes in transcriptional regulation (8). We assessed six samples of blasts with different DO and DM protein expression levels for DO α , DO β , and DM β that were readily detectable (Fig. 3, *right*

panel). As in B cells, however, a significant correlation between transcription level and protein level of DO and DM could not be demonstrated (data not shown).

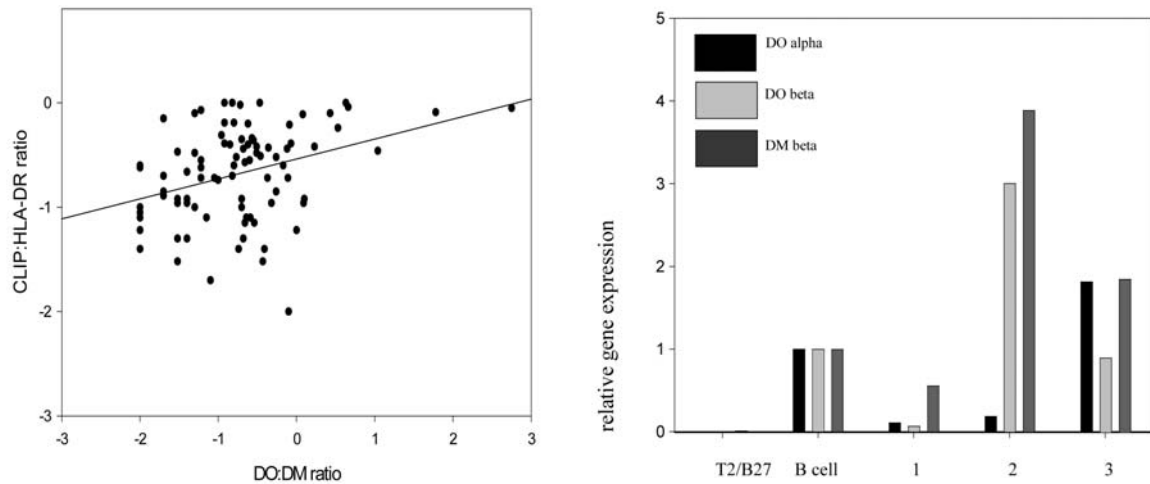


Figure 3. *Left panel:* ratio of percentage of CLIP+ to percentage of DR+ cells (CLIP:DR ratio) correlated to the ratio of DO+ to percentage of DM+ cells (DO:DM ratio) (Spearman, $P < 0.001$, $r = 0.46$, 10 log values yielding normal values). *Right panel:* relative gene expression levels of DO α , DO β and DM β of negative (T2/B27) and positive (B cells) control and 3 representative AML samples. Results are expressed relative to the positive control.

Discussion

Aberrant MHC class I antigen presentation can function as a mechanism of tumor immune escape. The importance of antigen presentation via the MHC class II pathway for establishing effective antitumor immunity via proper CD4+ helper activation is still an emerging topic of research. Here, we report that AML blasts consistently express high levels of MHC class I molecules, whereas MHC class II is variably expressed. Moreover, we show for the first time that differences in class II antigen presentation are associated with the clinical outcome of disease in humans. The immune system as surveillant in AML is not likely to play a role at the moment of diagnosis (when an enormous tumor burden exists), but rather during the period of minimal residual disease (when the patient has achieved complete remission). Indeed, we demonstrated in this study that the level of relative CLIP amount does not influence the probability of patients to achieve complete remission but that patients in complete remission with a high percentage DR+/CLIP- AML blasts have a significantly better disease-free survival rate compared with patients with DR+/CLIP+ blasts. Our finding strongly indicates that, analogous to mouse MHC class II+/Ii-tumor cells, the release of CLIP from the DR-binding site, and subsequent presentation of a broad panel of tumor antigens to CD4+ helper cells, is

a prerequisite for an effective and long-lasting antitumor response. Because of the lack of effective methods for identifying MHC class II-restricted tumor antigens in small samples of patient material, proof of this hypothesis will be difficult, but functional studies to demonstrate the higher immunogenicity of DR+/CLIP- blasts are currently being undertaken in our laboratory.

At first sight, our finding seems to contradict the fact that the patients with the FAB-M3 subtype, which is DR negative, have a better prognosis than patients with the other subtypes. However, FAB-M3 blasts present a highly immunogenic fusion protein (PML-RAR α), via their MHC class I molecule or via professional APCs, to the immune system (19).

Until now, expression of DO as a coregulator of antigen-presenting capacity was observed only in B cells that have acquired B-cell receptor expression. B cells regulate their antigen-presenting capacity during differentiation by differing levels of DO, compared with less varying DM expression (8). Loss of DO favors more efficient peptide loading on removal of CLIP. We were also able to demonstrate the presence of DO in myeloid blasts and to relate a low DO:DM ratio to a low cell surface CLIP:DR ratio. As in B cells, the absence of relationship between mRNA and protein levels of DO, which could be due to protein degradation (20), is an issue that needs further investigation.

We conclude that cell surface CLIP expression on AML blasts seems to be regulated by the balance of both DO and DM and shows a striking correlation with disease-free survival, pointing to the active involvement of the MHC class II presentation pathway. Ineffective MHC class II antigen presentation seems to be an immune escape mechanism of AML blasts that offers opportunities for developing immunotherapy for AML patients based on manipulation of the MHC class II antigen-processing pathway.

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Chapter 6

Detection of aberrant transcription of MHC class II antigen presentation genes in chronic lymphocytic leukemia identifies *HLA-DOA* mRNA as a prognostic factor for survival

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Abstract

In human B cells, effective MHC class II-antigen presentation depends not only on MHC class II, but also on the Invariant chain (CD74 or Ii), HLA-DM (DM) and HLA-DO (DO), the chaperones regulating the antigen loading process of MHC class II molecules. We analyzed immediate *ex vivo* expression of HLA-DR (DR), Ii, DM and DO in B cell chronic lymphocytic leukemia (B-CLL). Real-time RT-PCR demonstrated a highly significant upregulation of *DRA*, *CD74*, *DMB*, *DOA* and *DOB* mRNA in purified malignant cells compared to B cells from healthy donors. The increased mRNA levels were not translated into enhanced protein levels but could reflect aberrant transcriptional regulation. Indeed, upregulation of *DRA*, *DMB*, *DOA* and *DOB* mRNA correlated with enhanced expression of class II transactivator (*CIITA*). In-depth analysis of the various *CIITA* transcripts demonstrated a significant increased activity of the IFN- γ -inducible promoter *CIITA*-PIV in B-CLL. Comparison of the aberrant mRNA levels with clinical outcome identified *DOA* mRNA as a prognostic indicator for survival. Multivariate analysis revealed that the prognostic value *DOA* mRNA was independent from the mutational status of the *IGHV* genes. Thus, aberrant transcription of *DOA* forms a novel and additional prognostic indicator for survival in B-CLL.

Introduction

B-cell chronic lymphocytic leukemia (B-CLL) is the most common form of leukemia in adults in the Western world. A highly variable clinical course is typical for the disease is, with survival rates varying between a few months and two decades (1). B-CLL is characterized by a progressive accumulation of a malignant B cell population that fails to undergo apoptosis. Apparently, the immune system is unable to deal with this abnormal cell population. Indeed, B-CLL is characterized by striking immune incompetence in which not only the number but also the function of the B and T cells is impaired (2).

MHC class II molecules play a pivotal role in the induction and regulation of an antigen-specific immune response. MHC class II antigen presentation activates antigen-specific CD4⁺ T cells. The biosynthesis pathway of MHC class II is now understood in detail. MHC class II molecules bind exogenous antigens generated in the endosomal/lysosomal pathway. After synthesis, the MHC class II molecule is directed into this pathway by associating to the invariant chain (CD74 or Ii) (3). During transport to the lysosomal-like MHC class II-containing compartments (MIICs), where the majority of antigen loading occurs, CD74 is proteolytically removed, leaving only a small fragment [class II-associated invariant chain peptides (CLIP)] in the MHC class II peptide binding groove (4). Release of CLIP is facilitated by the specialized chaperone human leukocyte antigen (HLA) HLA-DM (DM), a MHC class II-like molecule. DM catalyses the natural process of peptide dissociation from MHC class II (5), thereby releasing both CLIP and other low affinity binding peptides. Consequently, DM acts as a peptide editor, favouring presentation of stable binding antigens (6-9). HLA-DO (DO), a heterodimer composed of a DO α and DO β chain, is selectively expressed in B cells and regulates the action of DM in a pH-dependent manner. We and others reported that DO reduced MHC class II-mediated presentation of antigenic peptides in general and modulated the antigenic peptide repertoire by facilitating presentation of certain antigenic peptides, while suppressing others (10-13). DO therefore both limits and skews the class II-presented antigenic peptide repertoire in B cells. The balance between DO and DM expression thus seems to be a key factor in controlling antigen presentation in B cells, which may explain why in healthy B cells DO and DM expression are tightly regulated at various levels (14).

Transcription of MHC class II, DM and DO is regulated by a master regulator, termed the class II transactivator (CIITA) (15, 16). CIITA is transcriptionally

controlled by four distinct promoters, each transcribing a unique first exon and yielding a unique CIITA transcript (17). A physiological role for CIITA-PII is questioned as transcripts originating from this promoter are rare. The promoters I, III and IV are differentially used in different cell types and in response to inflammatory stimuli. CIITA-PI is constitutively active in myeloid dendritic cells (DCs) and CIITA-PIII constitutively in B cells, plasmacytoid DCs, monocytes and activated T cells (18). CIITA-PIV has been shown to be the promoter predominantly involved in IFN- γ -inducible CIITA expression (19, 20). In healthy B cells transcription of the MHC class II genes is tightly regulated by CIITA, but dysregulation has been observed in tumors (21-23).

The purpose of this study was to perform an overall, in depth investigation of transcription and translation of the genes involved in MHC class II antigen presentation in a small, but very well-defined B-CLL cohort and to determine whether transcriptional aberrancies occurred and if so, whether they correlated to clinical outcome.

Materials and methods

Patients and healthy volunteers

Peripheral blood samples from 21 B-CLL patients were obtained after informed consent according to the declaration of Helsinki and samples were anonymized before analysis. Patients were diagnosed and staged based on standard morphologic and immunophenotypic criteria (24). Patient characteristics are shown in Table 1. Cytogenetic data are not available because this was not routinely performed at the time of sample collection. Out of the 21 patients, 15 had not received chemotherapy at the time of sample acquisition and six patients had received prior treatment. Patients that had received treatment were equally distributed between the groups with mutated and unmutated *IGHV* genes. Buffy-coats from healthy donors were obtained from the Sanquin blood supply foundation (Sanquin, Amsterdam, The Netherlands).

Purification of primary B cells

Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation on a Ficoll-Hypaque gradient (Pharmacia, Uppsala, Sweden) and B cells were purified using anti-CD19 Dynabeads and DETACHaBEAD (Dyna, Oslo, Norway), according to the manufacturer's instructions. The cell purity and viability was >99% as

determined by FACS analysis (data not shown). Purified primary B cells were pelleted, snap frozen and stored at -80°C until further use. For purification of malignant leukaemic cells from B-CLL blood samples, mononuclear cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD19 (Dakocytomation, Heverlee, Belgium) and phycoerythrin (PE)-conjugated anti-CD5 (Dakocytomation). The CD5⁺CD19⁺ population was purified by FACS sorting using a MoFlo Sorter (Dakocytomation), pelleted, snap frozen and stored at -80°C until further use. Purity was >99% as determined by FACS analysis (data not shown).

Real-time PCR

CD5⁺CD19⁺ cells were lysed in peqGOLD Trifast (PeQlab, Erlangen, Germany). GlycoBlue (Ambion, Austin, TX) was added as a carrier and total RNA was extracted according to the manufacturer's instructions. First strand cDNA was reverse transcribed using random hexamers (pd(N)₆, Amersham Biosciences, Piscataway, NJ) and a SuperScript II, RNase H-reverse transcriptase kit (Invitrogen, Breda, The Netherlands). Gene expression was measured in the ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA). Primers were selected to span exon-intron junctions to prevent amplification of genomic DNA. Primer sets (sense sequence, antisense sequence and transcript size, respectively) for the following genes were used:

HLA-DRA (5'-CATGGGCTATCAAAGAAGAAC-3', 5'-CTTGAGCCTCAAAGCTGGC-3', 180 bp)

HLA-DMB (5'-CCAGCCCAATGGAGACTG-3', 5'-CAGCCCAGGTGTCCAGTC-3', 136 bp)

HLA-DOA (5'-GAGCCATCAACGTGCCTC-3', 5'-AGTGACAGTTTGGCCGTTG-3', 146 bp)

HLA-DOB (5'-GGAGAAAGATGCTGAGTGGC-3', 5'-GCTCTTGAGACCTCATTACC-3', 133 bp)

CD74 (5'-CACCTGCTCCAGAATGCTG-3', 5'-CAGTTCCAGTGA CTCTTTCG-3', 210 bp)

Total *CIITA* (5'-AACCTCAATCTGTCCCAG-3', 5'-TG TACTGGACGTCCATCAC-3', 191 bp)

For specific *CIITA* promoter transcripts, primer pairs analogous to Hornell et al. were used (25):

CIITA-PI (5'-GGAGACCTGGATTTGGCCC-3', 5'-GAAGCTCCAGGTAGCCACCTTCTA-3', 145 bp)

CIITA-PIII (5'-GGGGAAGCTGAGGGCACG-3', 5'-GAAGCTCCAGGTAGCCACCTTCTA-3', 182 bp)

CIITA-PIV (5'-GCGGCCCCAGAGCTGG-3', 5'-GAAGCTCCAGGTAGCCACCTTCTA-3', 125 bp).

As endogenous control, primers specific for human 18S rRNA were used (5'-CGGCTACCACATCCAAGGAA-3', 5'-GCTGGAATTACCGCGGCT-3', 187 bp). Product specificity of each primer set was verified by agarose gel electrophoresis and by dissociation curve analysis. PCR products were further confirmed by sequence analysis. Transcript levels and relative gene expression of mRNA were determined

as described by Pfaffl (26). All results were normalized for starting template with respect to the internal control, and were expressed relative to the expression levels found in one of the CD19⁺ B cells from a healthy donor.

SDS-PAGE and Western blot analyses

The DR α -specific monoclonal antibody (mAb) 1B5 (27), DM α -specific mAb 5C1 (28), the previously described DO β polyclonal Ab (10) and the anti-actin mAb Ab-1 (Oncogene Research Products, Boston, MA) were used for Western blot analyses. Cells were lysed in 1% Nonidet P-40 (NP-40) lysis buffer [50 mM Tris/HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA and protease inhibitors] for 30 minutes at 4°C and centrifuged for 30 minutes at 10,000 x g at 4°C. The protein content of the cell lysates was quantified using the BCA protein assay (Pierce, Rockford, IL). For Western blot analysis, equal amounts of proteins were boiled for 5 minutes in reducing Laemmli sample buffer and separated by SDS-PAGE on 12% polyacrylamide gels and transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon P, Millipore Corp., Bedford, USA) in 25 mM Tris, 192 mM Glycine, 20% methanol, pH 8.3. Membranes were blocked in 5% skimmed milk in phosphate-buffered saline (PBS). Ab binding (in 5 % skimmed milk in PBS) was detected by incubation with secondary horseradish peroxidase-conjugated rabbit anti-mouse or swine anti-rabbit immunoglobulin Abs (Dakocytomation), followed by enhanced chemiluminescence detection (Amersham). Washes were performed using 0,1% Triton X-100 in PBS. For semi-quantitative analysis of the Western blots, subsaturated autoradiograms were scanned and the signals analyzed using TINA 2.09 software (Raytest, Staubenhardt, Germany). The relative expression levels were correlated to the cellular actin levels, measured as a reference in each sample on the blots.

Determination of mutation status

The mutational status of the immunoglobulin heavy chain variable (*IGHV*) genes of 20 patients was determined as described (29). Patient 16 had no material available for *IGHV* analysis. In brief, *IGHV* transcripts were amplified using a mixture of forward primers located in the FR1 regions of the *IGHV* gene families *IGHV1* to *IGHV6* or alternatively in the FR3 region of *IGHV1* to *IGHV6* in combination with one of the FAM-labelled reverse primers located in the C μ , C δ , C α or C γ regions.

Less than or equal to 2% difference from the most homologous germ line gene was considered to be mutated.

Statistical methods

Statistical analyses were conducted with the SPSS 15.0 software program. Differences between groups were analyzed with the 2-sided Mann-Whitney *U* test. For correlations, the Spearman non-parametric correlation test was used. Multivariate analysis was performed using the Cox's proportional-hazards model. $P < 0.05$ were considered statistically significant.

Survival time after sampling was measured from the time of sampling until death, or until the end of follow-up. Survival curves were constructed using the Kaplan-Meier method. Differences between curves were analysed using the Log-rank test. The optimal cut-off value for *DOA* transcription levels in this patient cohort was determined using two methods: (i) the Log-rank test, by testing the prognostic value for each possible cut-off point (3.1, 3.2, 3.3 etc); and (ii) Cox regression analysis, including all cut-off points as continuous variables. Both methods gave identical results.

Table 1. Characteristics of the B-CLL patients included in our analyses.

Patient	Gender	Age (years)	Months from diagnosis	Treatment	leucocyte count ($\times 10^9/l$)	Stage		Mutation status
1	F	76	217	-	20.2	0	A	MUT
2	F	61	95	-	21.4	0	A	MUT
3	F	77	6	-	22.6	0	A	UM
4	M	73	118	-	24.1	0	A	MUT
5	F	75	115	-	39.4	0	A	MUT
6	F	66	150	-	43.5	0	A	MUT
7	M	63	165	-	72.8	0	A	MUT
8	F	57	11	-	7.7	I	A	MUT
9	F	41	14	-	38.6	I	A	MUT
10	M	59	88	-	58.4	I	A	MUT
11	M	54	19	Chlo	13.4	0	A	UM
12	F	62	41	-	94.2	0	A	UM
13	M	57	79	Pred	7.2	I	A	UM
14	M	57	80	-	41.0	I	B	UM
15	M	62	98	Chlo	62.5	I	B	UM
16	M	55	33	-	17.0	III	C	ND
17	M	45	105	Chlo	84.3	III	C	MUT
18	M	59	46	Pred	7.0	IV	C	MUT
19	M	77	25	Chlo	7.1	IV	C	MUT
20	M	61	125	-	9.0	IV	C	MUT
21	M	74	108	-	52.6	IV	C	MUT

All variables indicated were assessed at time of sampling.

Chlo, chlorambucil; Pred, prednisone. Rai and Binet refer to current standard clinical staging systems. MUT indicates mutated *IGHV* genes and UM unmutated and ND indicates not determined.

Results

Transcriptional upregulation of the MHC class II components in B-CLL

To avoid patient selection biases, PBMCs were collected from a random cohort of 21 B-CLL patients, either receiving therapy or not (Table I). In purified CD5⁺CD19⁺, malignant B-CLL cells and mature, resting CD19⁺ B cells from healthy donors, we analyzed immediate *ex vivo* gene expression of *DRA*, *CD74*, *DMB* and both chains of the DO molecule, as unlike the DR and DM chains (16), *DOA* and *DOB* are not completely co-regulated (15, 30). Gene expression in eight samples of six independent healthy donors showed little variation in expression of *DRA* ($0.67 \pm [0.37]$ [SD]), *CD74* ($0.58 \pm [0.27]$), *DMB* ($0.79 \pm [0.44]$), *DOA* ($0.84 \pm [0.36]$) and *DOB* ($1.02 \pm [0.42]$) (Fig. 1), demonstrating that HLA-DR, CD74 and both class II chaperones are tightly regulated in B cells from healthy volunteers. Transcription of HLA-DR, CD74, DM and DO was markedly elevated in the malignant B-CLL cells. Overall, the patients showed a highly significant upregulation of the mRNA levels of *DRA* ($1.49 \pm [0.83]$; $P=0.022$), *CD74* ($1.11 \pm [0.39]$; $P=0.001$), *DMB* ($1.81 \pm [0.72]$; $P=0.001$), *DOA* ($3.60 \pm [1.47]$; $P<0.001$) and *DOB* ($4.57 \pm [2.64]$; $P<0.001$) (Fig. 1).

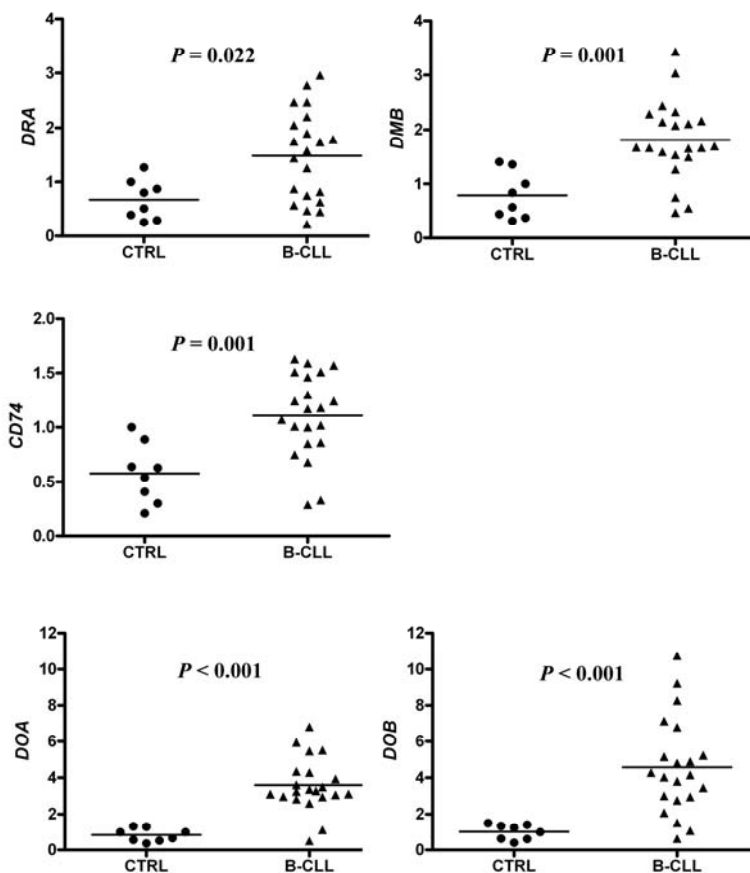


Figure 1. Transcriptional upregulation of *DRA*, *DMB*, *CD74*, *DOA* and *DOB* in B-CLL.

Comparison of relative mRNA levels between CD19⁺ B cells from healthy volunteers (= CTRL) and the CD5⁺CD19⁺ cells from B-CLL patients. Results are normalized to the internal control 18S rRNA and expressed relative to the value of CD19⁺ B cells from a healthy volunteer.

Downmodulation of DR, DM and DO protein expression in B-CLL

Expression of the components of the MHC class II pathway is not only regulated at the transcriptional level, but also at the post-transcriptional and translational level (14). Thus, the anomalous transcriptional upregulation of DR, DM and DO in B-CLL is not necessarily reflected at the protein level. We analyzed the total protein expression of DR, DM and DO using SDS-PAGE and semi-quantitative Western blotting. Unlike in healthy B cells, a highly variable expression pattern of DR, DM and DO was observed in the malignant B-CLL population (Fig. 2A). Subsequent semi-quantitative analysis demonstrated that in B cells obtained from healthy donors ($n=3$), DM and DO expression was almost invariable ($1.01\pm[0.03]$ and $1.00\pm[0.03]$ respectively), with minor DR variation ($1.13\pm[0.21]$). Surprisingly, B-CLL cells did not express more DR ($0.73\pm[0.04]$), DM ($0.75\pm[0.18]$) and DO ($0.56\pm[0.37]$) protein compared to control B cells (Fig. 2B), in contrast to the significant upregulation of mRNA. DM protein is even significantly lower in B-CLL patients as compared to healthy controls.

Increased transcription of the MHC class II genes is correlated with enhanced transcription of total CIITA in B-CLL

Since CIITA is the master regulator of MHC class II transcription, we measured total *CIITA* mRNA levels in B cells from healthy donors and B-CLL patients. B-CLL patients had significant more *CIITA* mRNA than B cells from controls ($P < 0.001$) (Fig. 3A). The enhanced mRNA levels of *DRA*, *DMB*, *DOA* and *DOB* correlated to the *CIITA* mRNA levels ($R = 0.486$, $P = 0.026$; $R = 0.530$, $P = 0.013$; $R = 0.621$, $P = 0.003$; $R = 0.614$, $P = 0.003$ respectively), suggesting that enhanced transcription of these MHC class II genes is mediated by enhanced CIITA expression. As expected, *CD74* mRNA expression did not correlate with CIITA expression since *CD74* transcription involves other factors as well (31).

CIITA-PIV expression is increased in B-CLL

CIITA is a complex regulated gene, with three promoters known to display distinct cell type- and cytokine-specific responses (32). Transcription initiated by each of the *CIITA* promoters leads to synthesis of distinct *CIITA* mRNAs containing alternative first exons spliced to a shared second exon. To identify which promoter(s) of CIITA was responsible for this upregulation of CIITA, we used primers specific for the individual CIITA-PI, -PIII and -PIV transcripts.

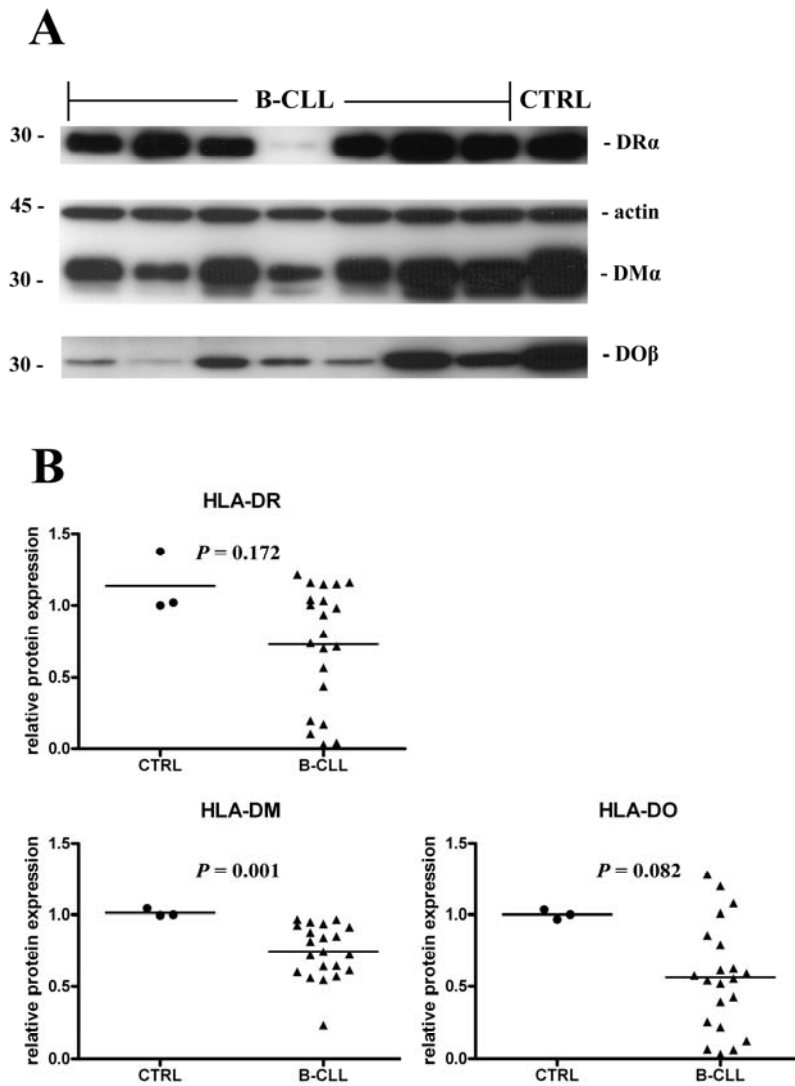


Figure 2. Variable and deviant protein expression of DR, DM and DO in B-CLL.

(A) Western blot analysis of total cell lysates of purified, malignant B-CLL cells and B cells from healthy donors immediately after isolation. A representative healthy control is shown on the right. Equal amounts of cellular proteins were analyzed (2 μ g for DR, 5 μ g for DM and 15 μ g for DO) as demonstrated by the comparable amounts of actin in each sample. Molecular marker sizes are indicated on the left (kDa). (B) Semi-quantitative Western blot analysis of the relative DR, DM and DO expression in healthy B cells *versus* malignant B cells from B-CLL patients. Subsaturated autoradiograms were scanned and signals were quantified by densitometric analysis. The values for DR, DM and DO expression were correlated to actin in each sample and subsequently expressed relative to the value obtained from a healthy control.

No messenger from CIITA-PI was found in B cells or B-CLL cells (data not shown). Transcription of *CIITA*-PIII mRNA was observed both in B cells from healthy donors and B-CLL patients with no significant difference between these two groups (Fig. 3B, *left panel*). In line with a previous report, only low levels of IFN- γ -inducible *CIITA*-PIV mRNA were found in B cells from healthy controls (33). In B-CLL

however, the activity of the *CIITA*-PIV promoter was strongly and significantly enhanced ($P < 0.001$; Fig. 3B, *right panel*). Thus, transcription of *CIITA* in B-CLL is controlled by the co-ordinated activity of the B cell-lineage-specific promoter PIII and the IFN- γ -inducible promoter PIV.

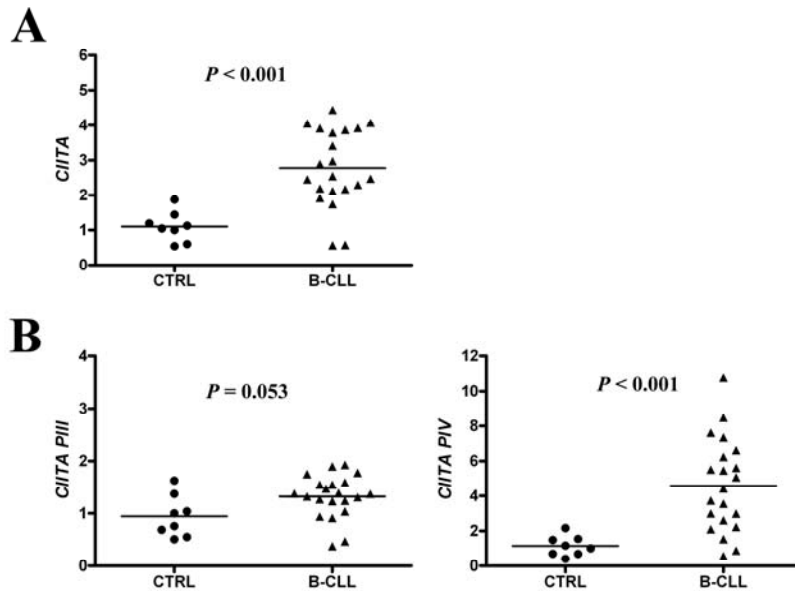


Figure 3. Transcriptional upregulation of *CIITA* in B-CLL.

Comparison of relative mRNA levels between $CD19^+$ B cells from healthy volunteers (= CTRL) and the $CD5^+CD19^+$ cells from B-CLL patients. Results are normalized to the internal control 18S rRNA and expressed relative to the value of $CD19^+$ B cells from one healthy volunteer.

Prognostic value of *DOA* mRNA expression

Now that we uncovered transcriptional aberrancies in the MHC II genes in B-CLL, we analyzed if differences in mRNA levels for the respective MHC II components showed a correlation with clinical outcome. Variables included were *DRA*, *DMB*, *CD74*, *DOA* and *DOB*, with only *DOA* mRNA levels showing a significant difference between surviving and non-surviving patients (Fig. 4A). If patients were divided into a group with *DOA* mRNA levels < 3.4 and ≥ 3.4 (the threshold with the most discriminative power in this patient cohort), *DOA* mRNA levels ≥ 3.4 defined a subgroup of patients with an unfavourable prognosis: six out of eight patients with *DOA* mRNA levels ≥ 3.4 died during follow-up as compared to four out of 12 patients with *DOA* mRNA levels < 3.4 (Log-rank test; $P = 0.031$, Fig. 4B). When entered as continuous variables using Cox regression analysis, a similar result was obtained ($P = 0.027$).

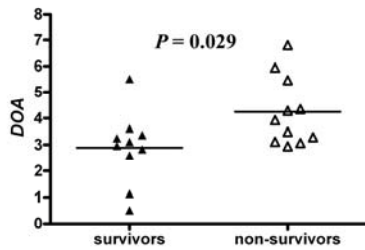
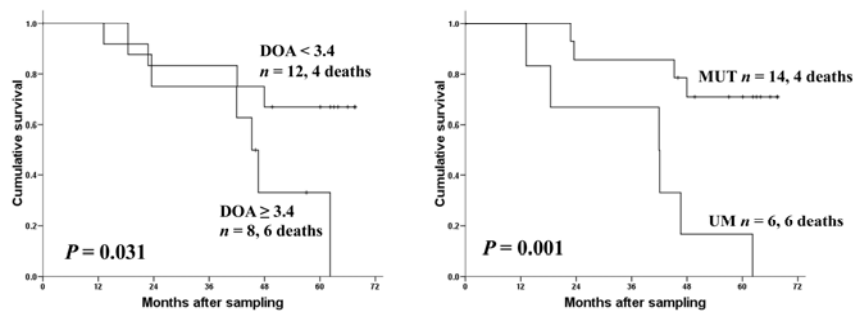
A**B**

Figure 4. DOA mRNA expression has prognostic power in B-CLL.

(A) Comparison of DOA mRNA levels between surviving and non-surviving B-CLL patients. (B) Comparison of survival time after sampling in B-CLL according to DOA mRNA expression levels and mutation status. UM, unmutated; MUT, mutated *IGHV* genes.

To date, the mutational status of the *IGHV* genes has been considered the best prognostic marker for survival in B-CLL and indeed we confirmed this in our cohort ($P = 0.001$). When DOA mRNA levels and *IGHV* mutational status were entered as categorical variable in the Cox's proportional-hazards model for multivariate analysis, both indices remained independent prognostic markers. Thus, these data indicate that aberrant DOA mRNA expression is a novel and additional prognostic indicator for survival in B-CLL, with the potential to be a risk stratifier in B-CLL patients with mutated *IGHV*.

Discussion

A plethora of factors has been identified that may play a role in tumor immune evasion. Some of these directly affect tumor recognition by CD8⁺ effector CTLs, such as deficiencies in components of the MHC class I antigen processing pathway (34-37). Also, in B-CLL, MHC class I has been suggested to play a role in tumor immune evasion (38). Over the last years it has become clear that the establishment of an effective CD4⁺ T cell response via MHC class II antigen

presentation is required for both the induction and maintenance of anti-tumor CD8⁺ CTL responses (39). Indeed, loss of MHC class II expression has been observed in diffuse large B cell lymphomas with fewer tumor-infiltrating CD8⁺ T cells in MHC class II-negative tumors (40, 41).

Here, we identified transcriptional aberrancies in the genes of the MHC class II antigen processing machinery in B-CLL. In B-CLL, the *DRA*, *CD74*, *DMB*, *DOA* and *DOB* were transcribed at higher levels than in healthy controls. We demonstrated that the transcriptional upregulation of the MHC II genes was not translated in increased protein levels. Therefore, the observed transcriptional aberrancies in B-CLL may not result in a form of immune evasion. Instead, it is more likely to point to an aberrant regulation of transcription. Indeed, upregulation of the transcriptional master regulator CIITA correlated to transcriptional upregulation of the MHC II genes. We demonstrated that CIITA transcription in leukaemic B cells is the resultant of both PIII and PIV promoter activity. Currently, it remains unclear why CIITA-PIV is active in B-CLL. CIITA-PIV is responsive to inflammatory stimuli. It has been shown in B-CLL that several serum cytokine levels are elevated in B-CLL (42-45) and that both CD4⁺ and CD8⁺ T cells express significantly more IFN- γ and IL-4 than in healthy controls (46, 47). Thus, activation of CIITA-PIV may be the resultant of the aberrant immunological environment in B-CLL.

Correlation of the identified transcriptional aberrancies with clinical outcome showed that the level of *DOA* mRNA predicts patient survival in B-CLL with high *DOA* mRNA levels correlating with poor survival time after sampling and low *DOA* mRNA correlating to good survival time after sampling. The finding that mRNA levels of *DRA*, *CD74*, *DMB* and *DOB* are not predictive indicates that transcription of *DOA* is not fully coregulated with the transcription of the other MHC class II genes. To validate the predictive power of *DOA* mRNA, we are currently expanding our cohort of B-CLL patients. The identification of *DOA* mRNA as a prognostic marker in our patient group is remarkable with potential clinical implication.

IGHV mutational status is the best stage-independent prognostic marker for B-CLL, but *IGHV* gene analysis is expensive and laborious and therefore unsuitable for standard diagnostic purposes. *DOA* mRNA analysis would be an additional diagnostic marker and can be readily applied in routine diagnostic purposes. Since novel therapies in the form of therapeutic antibodies or purine analogues can now induce complete remission, alternative prognostic markers become increasingly important. Even more so, the early identification of asymptomatic patients likely to progress is

of great clinical importance as these patients are eligible for early treatment protocols. Indeed, recently other prognostic markers have been identified, with only *CLLU1* (48) adding prognostic value independent of *IGHV* mutational status, but only in patients younger than 70 years. Here we present *DOA* mRNA as a novel, *IGHV* mutational status-independent prognostic marker for clinical outcome in B-CLL in a cohort of only 21 patients. By investigating the expression of the components of the MHC class II pathway at the transcriptional and protein level in a well defined B-CLL cohort with clinical follow-up, we defined a novel factor that is predictive for clinical outcome. Validation of this marker in a larger cohort of patients will show if *DOA* mRNA can be used for further tailoring therapy in B-CLL patients.

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Chapter 7

Aberrant MHC class II antigen presentation is linked to expansion of the activated T cell compartment in B-cell chronic lymphocytic leukemia

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Abstract

In patients with B-CLL, the immune dysfunction of T cells towards malignant B cells may contribute to the pathobiology of B-CLL. TCR-dependent oligoclonal/monoclonal expansion of the CD4⁺ T cells however points to antigen-dependent T cell activation. For this reason, we investigated the role of MHC class II antigen presentation in B-CLL. Using flow cytometry, we analyzed the expression levels of HLA-DR, CLIP, HLA-DM and HLA-DO of B cells from 17 B-cell chronic lymphocytic leukemia patients and 10 healthy donors. In addition we analyzed the T cell compartment of patients by expression of CD45RO, CD27, HLA-DR and CD38 and correlated these results to the MHC class II antigen loading pathway results. B-CLL cells showed ubiquitous expression of MHC class II that did not vary from healthy controls. B-CLL cells however showed disturbed expression of HLA-DM and HLA-DO, the editors of the MHC class II antigenic peptide repertoire. The perturbed DM/DO balance altered the peptide repertoire, as it was related to reduced expression of the self-peptide CLIP at the plasma membrane. 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. Our data show that in B-CLL a relative increase in HLA-DM and a concomitant change in the MHC class II peptide repertoire of the malignant B cells is related to ongoing T cell activation. Altered MHC class II antigen presentation may thus be a new factor in the immune dysfunction and pathobiology of B-CLL.

Introduction

A successful anti-tumor T cell response is achieved by the two effector arms of the immune system. CD8⁺ cytotoxic T lymphocytes (CTLs) ensure specific elimination of tumor cells upon recognition of MHC class I-antigen (Ag) complexes and CD4⁺ T helper cells generate the required T cell help upon activation by MHC class II-Ag complexes (1). The absence of T cell help can lead to abortive induction of CTL responses and subsequent tolerance (2). Moreover, T cell help is required for the maintenance of CTL responses, which is essential in diseases like cancer (3, 4). Indeed, CD4⁺ T cell inclusion in adoptive T cell transfer studies improved tumor clearance by the CD8⁺ T cells (5, 6) and aberrant MHC class II Ag presentation in acute myeloid leukemia was related to poor prognosis (7).

B-cell chronic lymphocytic leukemia (B-CLL) is the most common form of leukemia in adults in the Western world. Typical for the disease is the highly variable clinical course, with survival rates that vary between a few months and two decades (8). B-CLL is characterized by a progressive accumulation of malignant B cells that fail to undergo apoptosis. Notably, B-CLL is characterized by striking immune incompetence and T cell expansion combined with T cell dysfunction (9). It is unclear whether this T cell expansion is indicative for attempted but unsuccessful tumor clearance or contributes in another way to the disease, for instance by creating an environment that supports survival of neoplastic cells (10). Antigen-independent mechanisms have been implicated in the T cell expansion in B-CLL (reviewed in (11)). The TCR-dependent oligoclonal/monoclonal expansion of CD4⁺ T cells in B-CLL however, points to an antigen-driven process. How malignant B cells present antigens via MHC class II molecules to CD4⁺ T cells and whether this may be an explanation for observed T cell expansion in B-CLL is so far unclear.

MHC class II molecules bind exogenous Ags generated in the endosomal/lysosomal pathway. After synthesis, the MHC class II molecule is directed into this pathway by associating to the invariant chain (Ii) (12). During transport to the lysosomal-like MIIC compartments where the majority of Ag loading occurs, the Ii is proteolytically removed, leaving only a small fragment (class II-associated invariant chain peptides (CLIP)) in the class II peptide binding groove (13). Release of CLIP is facilitated by the specialized chaperone HLA-DM (DM). DM catalyses the natural process of peptide dissociation (14). DM thus releases both CLIP and other nonstable binding peptides. Consequently, DM acts as a peptide editor, favoring presentation of stable binding Ags (15-19). HLA-DO (DO), a heterodimer of DO α

and DO β , is expressed in B cells and regulates the action of DM in a pH dependent manner. We and others reported that DO reduces MHC class II-mediated presentation of antigenic peptides in general and modulates the antigenic peptide repertoire by facilitating presentation of particular Ags, while suppressing others. DO therefore both limits and skews the class II-associated antigenic peptide repertoire in B cells (20-22). The relative expression of DO and DM (or the number of DM molecules in association with DO) thus controls Ag presentation in B cells. Not surprisingly, DO and DM expression are very consistent and tightly regulated at different cellular levels in healthy B cells (23) and B cell differentiation status (24). Aberrant expression of DM and/or DO could lead to an altered MHC class II peptide repertoire. This shift in antigen presentation may lead to altered T helper cell activation and subsequent help to CD8⁺ CTLs.

We recently showed that increased transcription of *HLA-DOA* mRNA is correlated with shorter survival of B-CLL patients (25). To determine the role of MHC class II-mediated antigen presentation in B-CLL at the protein level, we set out to determine whether anomalies in the MHC class II Ag presentation pathway occurred in patients suffering from B-CLL and whether this is related to the observed expansion of T cell subsets in this disease. Here we show that the relative expression of DO compared to DM is affected in malignant B-CLL cells, improving dissociation of the self-peptide CLIP in MHC class II molecules. This change in the MHC class II peptide repertoire is strongly correlated to a shift from the naïve T cell compartment towards the activated effector T cell compartment.

Materials and methods

Patients and healthy volunteers

Peripheral blood samples from 17 B-CLL patients were obtained after informed consent according to the Declaration of Helsinki and samples were anonymized before analysis. Patients were diagnosed and staged based on standard morphologic and immunophenotypic criteria (26). Cytogenetic data are not available since this was not routinely performed at the time of sample collection. Out of the 17 patients, 14 had not received chemotherapy at the time of sample acquisition and 3 had received prior treatment with chlorambucil. Ten buffy-coats from healthy donors were obtained from the Sanquin Blood Supply Foundation (Sanquin, Amsterdam, The Netherlands).

Isolation of peripheral blood mononuclear cells

Heparinized blood was diluted in PBS and layered on a Ficoll-Hypaque density gradient (Axis-Shield PoC AS, Oslo, Norway). PBMCs were collected from the interphase and washed twice with RPMI 1640 medium w/o phenol red, supplemented with 5% FCS (Bodinco, Alkmaar, the Netherlands), 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-Glutamine, 50 µM 2-mercaptoethanol and 20 µg/ml human apo-transferrin ((Sigma-Aldrich, Munich, Germany), depleted for human IgG with protein G sepharose (Amersham, Uppsala, Sweden)). Cells were cryopreserved and stored in liquid nitrogen until analysis.

Antibodies and Flow Cytometry Analysis

Rapidly thawed mononuclear cell fractions were preincubated with 10% human gammaglobulin (6 mg/ml, Sanquin) and incubated with different combinations of directly labeled antibodies. The following mouse monoclonal antibodies were used: FITC-labeled anti-HLA-DR (BD Biosciences (BD), San Jose, CA, clone L243), anti-HLA-ABC (BD), anti-HLA-DO (BD), -CD8 (BD), -CD45RO (Sanquin), -CD80 (BD); Phycoerythrin-labeled anti-HLA-DM, -CD4, -CD27, -CD38, -CD86 (all BD); Peridinin chlorophyll protein-Cy5.5-labeled anti-CD8 and -CD19 (both BD) and Allophycocyanin-labeled anti-CD4 and -CD5 (both BD). For CLIP detection, cells were incubated with the Cerclip.1 mAb (kindly provided by P. Cresswell)¹⁷ and subsequently stained with PE-conjugated rabbit-anti-mouse immunoglobulin (Dakocytomation, Heverlee, Belgium). A mixture of non-relevant mouse Abs of different isotypes was added to prevent non specific binding of subsequently added directly labeled fluorescent Abs. All plasma membrane stainings were performed at room temperature for 15 minutes. For intracellular staining of HLA-DM and HLA-DO, cells were fixed and permeabilized with a Fix & Perm kit (Caltag Laboratories, Burlingame, CA) and subsequently incubated with antibodies for 20 min at room temperature. After each incubation, cells were washed twice with PBS containing 0.1% bovine serum albumin and 100,000 events were acquired by a FACS Calibur (BD). Lymphocytes were gated by forward and side scatter and analyzed using CellQuest software (BD). B-CLL cells were defined as CD5⁺CD19⁺.

Median fluorescence intensity (MFI) index was defined by the formula:

$$\frac{\text{median fluorescence intensity (total population)} - \text{median fluorescence intensity (isotype control)}}{\text{median fluorescence intensity (isotype control)}}$$

Determination of anti-CMV serology and mutation status

Anti-CMV IgM and IgG was determined by ELISA in plasma samples of 13 patients at the Department of Virus Serology at Sanquin Diagnostics Division, from 4 patients plasma was not available. Determination of the mutational status of the immunoglobulin heavy chain variable (*IGHV*) genes of 20 patients was determined as described (25).

Statistical methods

Statistical analyses were conducted with the SPSS 15.0 software program. Differences between groups were analyzed with the 2-sided Mann-Whitney *U* test. For correlations, the Spearman nonparametric correlation test was used. P values less than 0.05 were considered statistically significant.

Table 1. Patients' characteristics and expression of different markers on B-CLL cells

Clinical characteristics of patients	
Gender	9 Male / 8 Female
Age at time of sample, years, mean	64 (41-78)
Months from diagnosis, mean (range)	87 (7-217)
Rai stage, number of patients	0 - 8 I - 5 II - 0 III - 2 IV - 2
Mutational status of the <i>IGHV</i> genes	12 mutated, 4 unmutated, 1 ND
CMV serology	5 seronegative, 8 seropositive, 4 ND
Flow cytometric analysis of B-CLL	
Leucocyte count at time of sample,	42 (7-94)
CD4/CD8 ratio (% , mean (range))	1.3 (0.4-2.5)
CD40 (median FI (range))	6.4 (4.1-9.4)
CD80 (median FI (range))	3.4 (0-5.5)
CD86 (median FI (range))	13.8 (6.3-50.0)

Abbreviations: ND, not determined; FI, fluorescence intensity

Results

B-cell chronic lymphocytic leukemia cells express less CLIP in their peptide binding groove

We analyzed cell surface expression of DR and CLIP on the B cells of 17 samples of a random group of B-CLL patients (Table 1) and 10 healthy volunteers by flow cytometry. Expression of MHC class I (HLA-ABC) was not different between healthy

volunteers and B-CLL patients (data not shown). We also found no difference in DR expression on the B cells between control and B-CLL cells (Fig. 1A). B-CLL patients however, have significantly less CLIP ($P < 0.001$) associated to DR (Fig. 1B). The amount of DR molecules still associated to the self-peptide CLIP is indicative for the efficacy of the MHC class II peptide loading process. To analyze the relative occupancy of plasma membrane expressed DR with the self-peptide CLIP, the CLIP level on B cells was related to the DR level. This showed a reduced expression of CLIP associated to DR at the plasma membrane (CLIP/DR) in B-CLL patients compared with healthy controls ($P < 0.001$) (Fig. 1C). Thus, in B-CLL a relative larger proportion of the DR molecules is available for MHC class II mediated antigen presentation to CD4⁺ T cells.

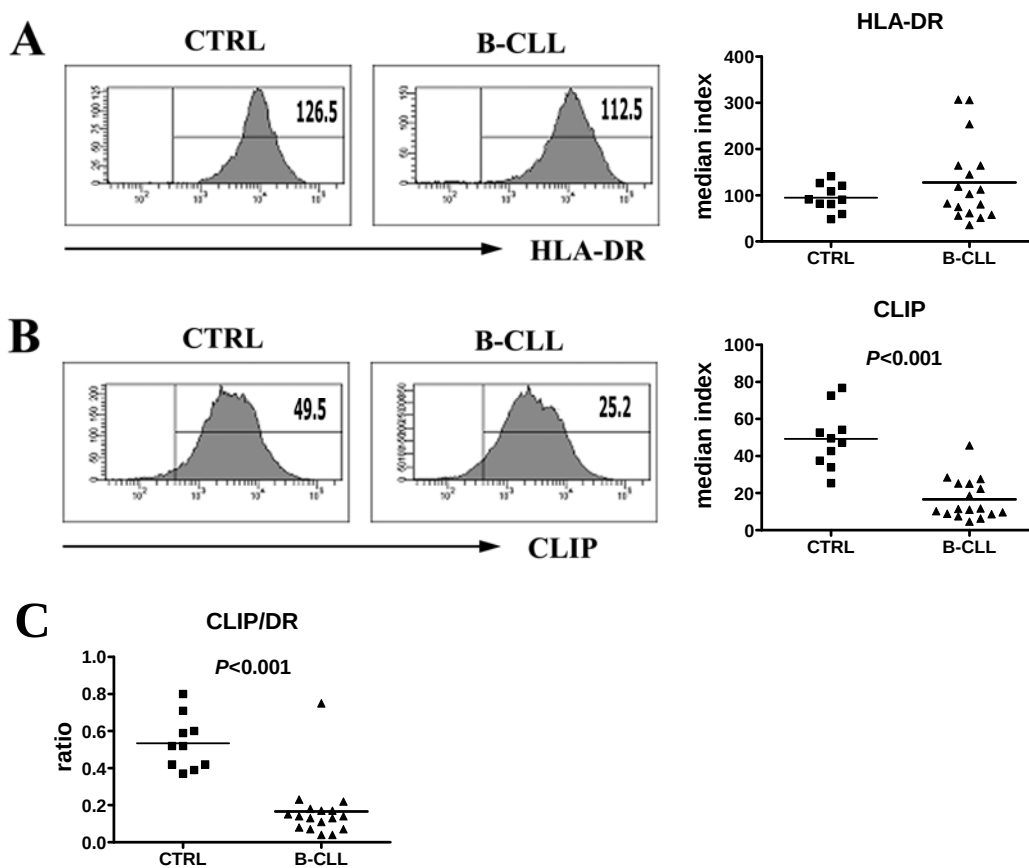


Figure 1. Decreased relative CLIP occupancy of DR in B-CLL.

(A) and (B) Representative flow-cytometric examples of HLA-DR and CLIP expression (left panels, numbers indicate the median index). DR expression is not significantly different between patients and healthy controls, CLIP expression is significantly decreased ($P < 0.001$) (right panels). (C) The relative CLIP amount in the MHC class II peptide binding groove (CLIP/DR) is significantly decreased ($P < 0.001$). Controls (CTRL) are represented by squares and B-CLL patients by triangles.

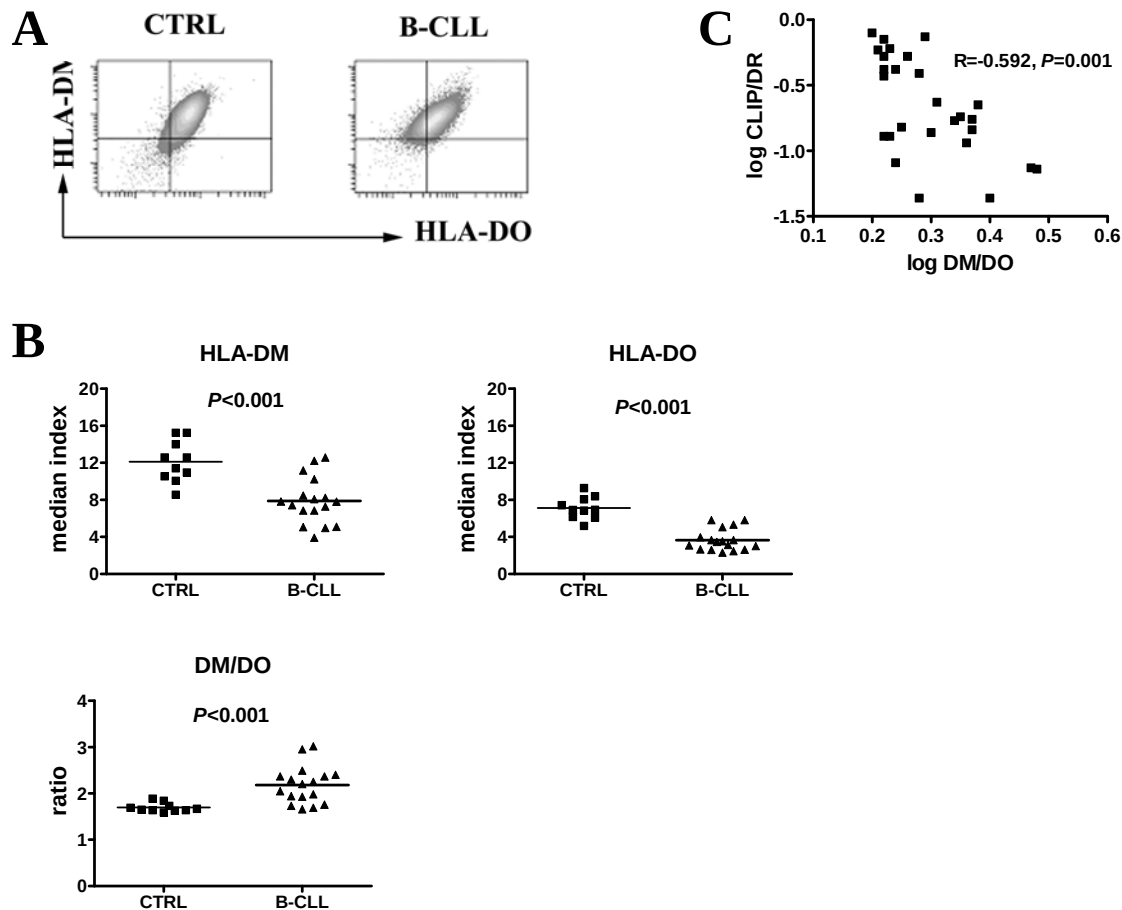


Figure 2. Aberrant expression of DM and DO in B-CLL.

(A) Representative flow-cytometric examples of intracellular HLA-DM and HLA-DO expression. (B) Both DM (upper left panel) and DO (upper right panel) are significantly decreased (both $P < 0.001$) in B-CLL patients. The relative expression of DM and DO (bottom panel) is increased ($P < 0.001$). Controls are represented by squares and B-CLL patients by triangles. (C) Relative CLIP occupancy of DR (CLIP/DR) correlated with the overexpression of DM (DM/DO) ($R = -0.592$, $P = 0.001$; 10 log values yielding normal distribution).

Reduced DM and DO expression in B-cell chronic lymphocytic leukemia cells

Antigen binding to newly synthesized MHC class II molecules is modulated by the expression of the peptide editors DM and DO. A high expression of DM compared to DO favors exchange of CLIP for antigenic peptides (20). Since DR molecules expressed reduced CLIP levels in B-CLL, we investigated the intracellular expression levels of DM and DO. Representative examples are shown in Figure 2A. The relative expression levels of DM and DO in B-CLL were markedly different from healthy controls, with both DM and DO being significantly reduced ($P < 0.001$ for both) (Fig. 2B, upper panels). When DM was compared to DO, a relative overexpression of DM

was observed in B-CLL ($P<0.001$) (Fig. 2B, *bottom panel*). This correlated with the efficiency of CLIP removal from DR (CLIP/DR) ($R = -0.592$, $P=0.001$) (Fig. 2C). Both relative overexpression of DM and the relative CLIP occupancy of DR did not correlate to B-CLL mutational status (as measured by *IGHV* gene analysis), CMV status, costimulatory markers (CD40, CD80 and CD86), Rai stage of disease or treatment regimen (data not shown). Collectively, these data show differential expression of the MHC class II peptide editors in B-CLL in combination with a reduced expression of CLIP in plasma membrane deposited DR.

Expansion of CD4⁺ and CD8⁺ effector T cell compartments in B-cell chronic lymphocytic leukemia

How does the altered CLIP expression on B-CLL cells relate to differences in CD4⁺ T cell differentiation between CLL patients and healthy controls? In line with previous observations (9), the CD4⁺/CD8⁺ ratio in B-CLL patients was lower compared to healthy controls (Table 1). No correlation was observed between the relative CLIP occupancy of DR and the CD4⁺/CD8⁺ ratio. We analyzed the peripheral T cells for the CD4⁺CD45RO⁻CD27⁺ naïve T cells, CD4⁺CD45RO⁺CD27⁺ central memory T cells and the CD4⁺CD45RO⁺CD27⁻ memory effector cells. Representative examples are shown in Figure 3A. Patients with B-CLL showed a lower percentage of naïve CD4⁺ T cells compared to healthy controls ($P=0.009$) (Fig. 3B, *upper left panel*), an unvaried central memory CD4⁺ T cell compartment (Fig. 3B, *upper right panel*) and an expansion of the memory effector CD4⁺ T cell compartment ($P=0.001$) (Fig. 3B, *bottom panel*). This points to ongoing activation of CD4⁺ T cells in B-CLL.

We analyzed the percentages of CD8⁺CD45RO⁻CD27⁺ naïve T cells, CD8⁺CD45RO⁺CD27⁺ central memory T cells, CD8⁺CD45RO⁺CD27⁻ memory effector T cells and CD8⁺CD45RO⁻CD27⁻ cytotoxic effector T cells. Representative examples are shown in Figure 3C. Patients with B-CLL showed a lower the percentage of naïve CD8⁺ T cells ($P<0.001$) (Fig. 3D, *upper left panel*) and no difference in CD8⁺ central memory T cells (Fig. 3D, *upper right panel*). The percentage of CD8⁺ memory effector T cells was increased ($P=0.001$) (Fig. 3D, *bottom left panel*), as well as the CD8⁺ cytotoxic effector T cells ($P=0.003$) (Fig. 3D, *bottom right panel*). Because the increase in cytotoxic effector T cells in B-CLL patients has been related to CMV infection (27), we tested patients for CMV infection. In our patient cohort, no significant difference in CD8⁺ T cell populations was observed between CMV seropositive and CMV seronegative B-CLL patients (data not shown).

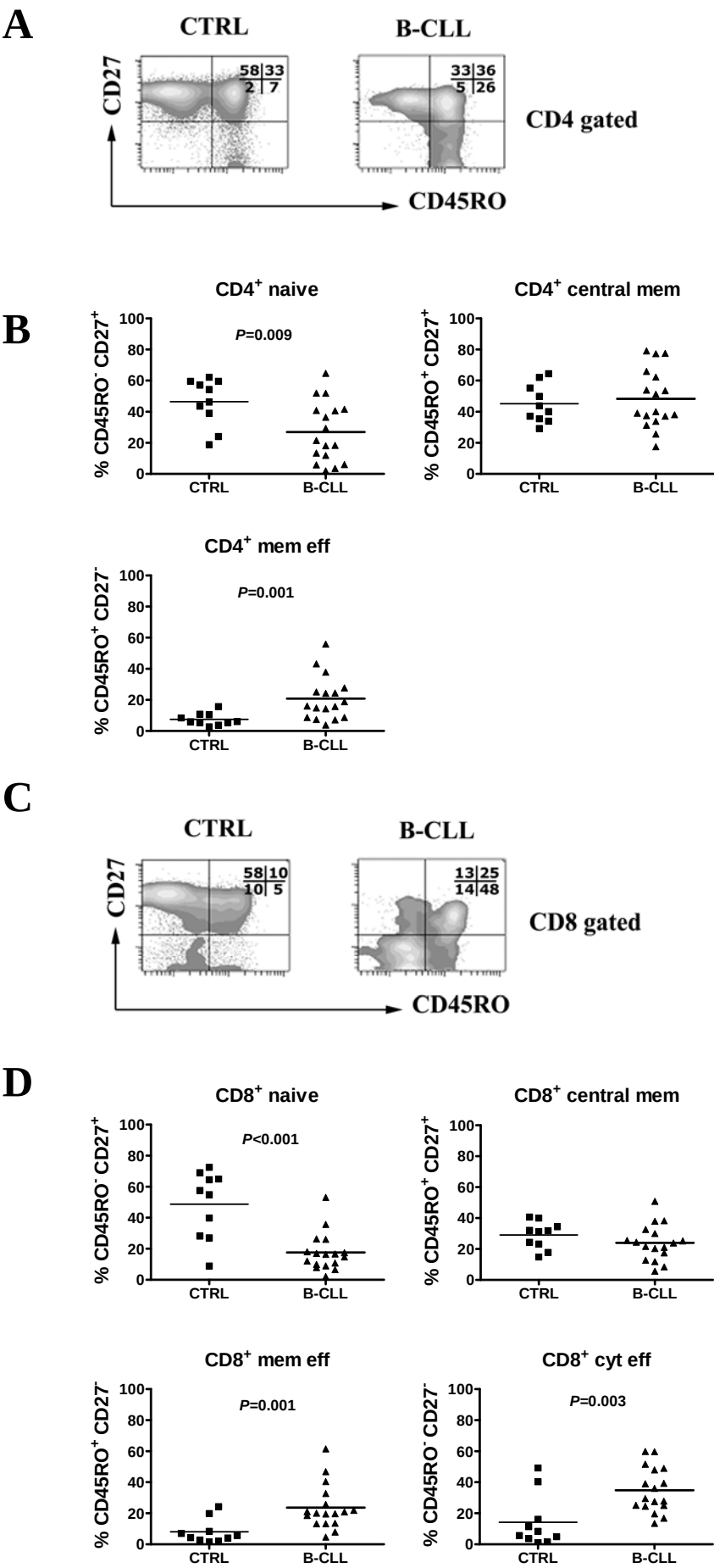


Figure 3. Expansion of effector type CD4⁺ and CD8⁺ T cells in B-CLL.

(A) Representative flow-cytometric examples of CD45RO and CD27 expression on CD4 gated cells. Percentages of cells in each quadrant are given. (B) The naïve CD4⁺ T cell compartment (upper left panel) is significantly decreased ($P = 0.009$) in B-CLL patients. The CD4⁺ central memory T cells (upper right panel) are not significantly different between patients and healthy controls and the CD4⁺ memory effector subset (bottom panel) is significantly increased ($P = 0.001$). Controls are represented by squares and B-CLL patients by triangles. (C) Representative flow-cytometric examples of CD45RO and CD27 expression on CD8 gated cells. Percentages of cells in each quadrant are given. (D) The naïve CD8⁺ T cell compartment (upper left panel) is significantly decreased ($P < 0.001$) in B-CLL patients. The CD8⁺ central memory T cells (upper right panel) are not significantly different between patients and healthy controls. The CD8⁺ memory effector subset (bottom left panel) is significantly increased ($P = 0.001$) as well as the CD8⁺ cytotoxic effector subset (bottom right panel) ($P = 0.003$). Controls are represented by squares and B-CLL patients by triangles.

CD4⁺ T cells provide help to the effector function of CD8⁺ T cells. Is there a relationship between the expanded CD4⁺ effector and CD8⁺ compartments in B-CLL? Indeed, the expansion of the CD4⁺ memory effector T cell compartment correlated with the observed expansions in CD8⁺ compartment in B-CLL, with the strongest correlation between the CD4⁺ memory effector and CD8⁺ memory effector compartments (Table 2).

Table 2. Correlations between the CD4⁺ memory effector and CD8⁺ T cell compartments

	CD4 ⁺ CD45RO ⁺ CD27 ⁻ memory
CD8 ⁺ CD45RO ⁻ CD27 ⁺ naïve	$R = -0.835, P < 0.001$
CD8 ⁺ CD45RO ⁺ CD27 ⁻ memory	$R = 0.881, P < 0.001$
CD8 ⁺ CD45RO ⁻ CD27 ⁻ cytotoxic	$R = 0.584, P = 0.001$

Increased T cell activation in B-cell chronic lymphocytic leukemia

As a marker for ongoing T lymphocyte activation, we analyzed the expression of HLA-DR and CD38 on T cells. Representative examples are shown in Figure 4A. Patients with B-CLL showed increased levels of CD4⁺HLA-DR⁺CD38⁺ T cells and CD8⁺HLA-DR⁺CD38⁺ T cells compared to healthy controls (both $P < 0.001$) (Fig. 4B). The percentage of activated CD4⁺ T cells showed a positive correlation with the percentage of activated CD8⁺ T cells ($R = 0.846, P < 0.001$) (Fig. 4C). Thus, patients with B-CLL show a higher percentage of activated CD4⁺ and CD8⁺ T cells than healthy controls.

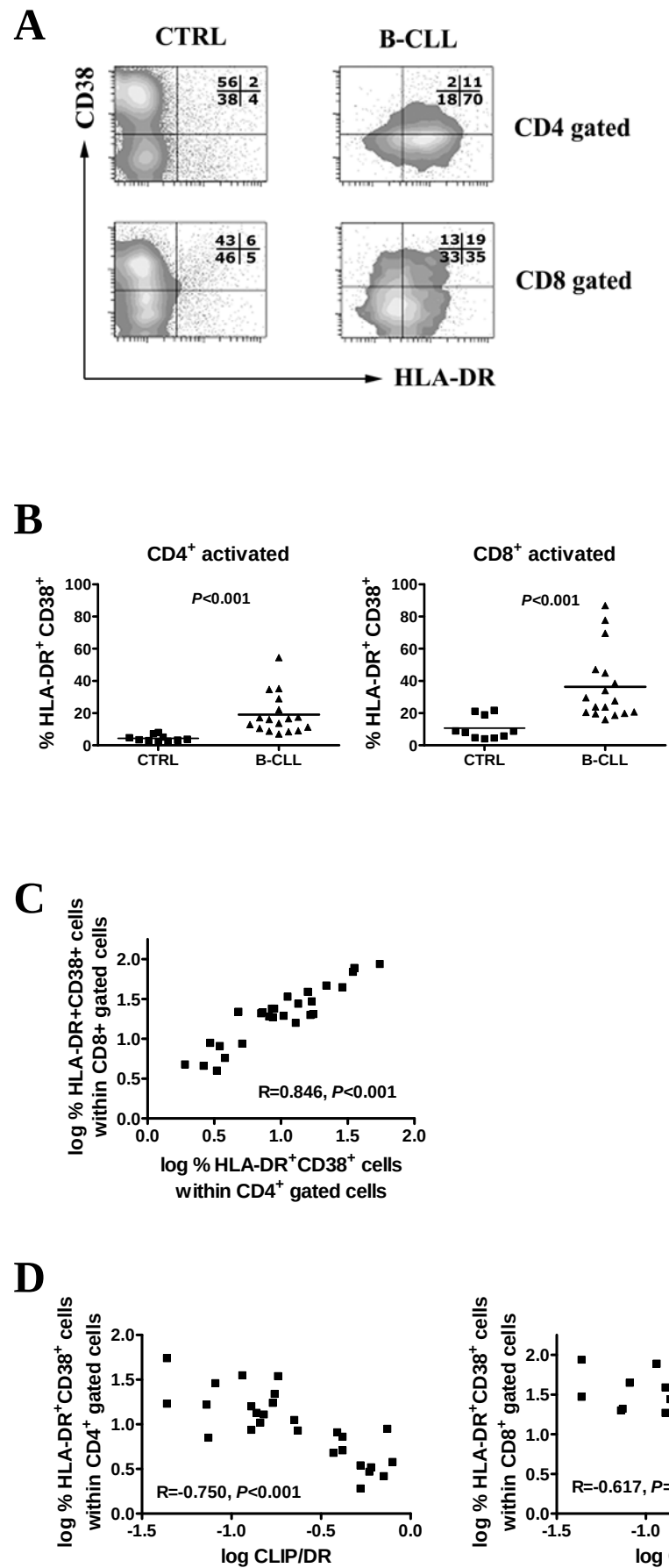


Figure 4. Increase in subsets of activated T cells correlates with the relative CLIP occupancy of DR.

(A) Representative flow-cytometric examples of HLA-DR and CD38 expression on CD4 (upper panels) and CD8 (lower panels) gated cells. Percentages of cells in each quadrant are given. (B) Both in the CD4⁺ (left panel) and CD8⁺ (right panel) T cell compartment more of the T cells have an activated phenotype (both $P < 0.001$). (C) The percentage of activated CD4⁺ T cells correlated with the percentage of activated CD8⁺ T cells ($R = 0.846, P < 0.001$). (D) Relative CLIP occupancy of DR (CLIP/DR) correlated with the percentage of CD4⁺ activated T cells ($R = -0.750, P < 0.001$) and to a lesser extent CLIP/DR correlated with the percentage of CD8⁺ activated T cells ($R = -0.617, P = 0.001$).

Correlation of the T cell parameters with the parameters involved in antigen presentation in our samples demonstrated a strong negative correlation between the relative CLIP levels associated to DR and the percentage of activated CD4⁺ T cells ($R=-0.750$, $P<0.001$) (Fig. 4D, *left panel*). To a lesser extent, the relative CLIP expression correlated to the percentage of activated CD8⁺ T cells ($R=-0.617$, $P=0.001$) (Fig. 4D, *right panel*).

Thus, in B-CLL a lower occupancy of the MHC class II peptide binding groove with CLIP strongly correlates with an increase in activated CD4⁺ and CD8⁺ T cell compartments.

Discussion

Deficiencies in components of the MHC class I Ag processing pathway have been shown in a variety of human cancers (28, 29), and some studies have correlated these deficiencies with tumor progression (30, 31). Here we identify aberrancies in the MHC class II Ag processing machinery in B-CLL and demonstrate that this is accompanied with increased T cell activation in B-CLL patients. B-CLL cells always express DR and the class II chaperones DM and DO. Thus, tumor immune escape due to genetic silencing of the MHC class II genes does not seem to occur in B-CLL. This in contrast to poor prognosis correlated to the overall loss of MHC class II expression in diffuse large B cell lymphomas (32, 33).

It is under debate whether B-CLL disease is a homogenous entity. Based on the mutational status of the immunoglobulin heavy-chain variable-region (*IGHV*) genes, B-CLL cases can be divided into two subgroups, resembling either a resting or a germinal center-experienced phenotype. DO expression is reported to vary during B cell development (24, 34), but in our cohort we could not demonstrate a difference in DO expression between patients with mutated and unmutated *IGHV* genes.

In B-CLL patients the presence of T cells with an anti-tumor specificity declines during disease progression (35). A lower CD4/CD8 ratio is observed in patients with progressive disease together with a concomitant Th1 to Th2 shift, which is detrimental for an effective anti-tumor response. These observations point to perturbed MHC class II-mediated CD4⁺ and CD8⁺ activation in B-CLL. Since the malignant B cells are poor APCs and DR cell surface expression is not altered, Dazzi and colleagues described that poor Ag presentation is due to a low B7 molecule expression (36). Although reduced expression of the costimulatory markers CD80 and CD86 is an established phenomenon in B-CLL (and confirmed in this study, see

Table 1), we now show that additional aberrancies in antigen presentation are present in the MHC class II antigen loading pathway itself. In order to get stable binding peptides in the peptide binding groove of a class II molecule, DM associates with DO which results in the release of CLIP and the preferential binding of Ags with an optimal binding motif to the class II backbone. In normal B cells about 50% of DM is associated to DO which then fails to properly support MHC class II peptide loading (24), whereas the other 50% is free for peptide editing of the class II Ag repertoire. The expression of DM in B-CLL shows that CLIP on newly synthesized class II molecules can be exchanged with antigenic peptides through the editing function of DM. The result that DM is relatively higher expressed than DO in B-CLL implies that more free DM is available for the generation of MHC class II complexes with antigens after removal of CLIP. Indeed, a decreased level of CLIP associated to DR at the plasma membrane in B-CLL patients is observed. Thus, the peptide repertoire presented by MHC class II molecules is modulated by DM and DO in healthy controls as well as in B-CLL patients. In addition, the composition of the MHC class II peptide repertoire is different in B-CLL compared to healthy controls.

The amount of CLIP associated to DR apparently varies between different types of leukemias. In acute myeloid leukemia we recently observed a relative overexpression of MHC class II complexes still containing CLIP at the plasma membrane in patients with poor prognosis (7). For acute myeloid leukemia, CLIP may constitute a form of tumor immunoediting or tumor immune escape. In B-CLL, reduced CLIP levels may serve another function. There is ample evidence that in B-CLL aggressive and non-aggressive forms arise due to the intrinsic properties of the B-CLL cells themselves and therefore the relative contribution of failed immune surveillance is under debate. The strong clinical manifestations of immune dysfunction and the expanded circulating T cell compartment have lead to the hypothesis that T cells may be involved in the pathobiology of B-CLL through the creation of a "leukemia-supportive" environment (37). Still, the mechanisms underlying the onset and sustainment of the expansion of these T cell populations in B-CLL were poorly defined. Our observations suggest that altered MHC class II antigen presentation by the malignant B cells may be involved; T cells of B-CLL patients are more differentiated towards effector and immune activated T cells and these findings correlate with parameters of improved MHC class II antigen presentation (a reduced CLIP expression and a relative overexpression of DM). In spite of the lack of strong costimulation, this correlation suggests that T cell

activation is still antigen-driven, in line with the observation that T cell expansion in B-CLL is oligoclonal or monoclonal (38). Whether initiation of T cell activation and maintenance of T cell activation are both antigen-driven remains to be established, but the observed correlations propose a contribution of MHC class II antigen presentation at certain stages in the pathobiology of B-CLL. A perturbed MHC class II antigen presentation pathway in B-CLL may thus be a new factor in the immune dysfunction and pathobiology of B-CLL.

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Chapter 8

Summarizing discussion

MHC class II antigen presentation plays a pivotal role in human health and disease. On 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 vertebrates (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 mucosa-associated 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-IgM 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 multiply 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 tumor-specific 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. DR 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 II-associated 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- γ , 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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Nederlandse samenvatting

In de mens speelt MHC klasse II antigeen presentatie een tweezijdige rol bij ziekte en gezondheid. Aan de ene kant activeert antigeen presentatie via MHC klasse II moleculen $CD4^+$ T cellen om cytokines te produceren en $CD40L$ tot expressie te brengen zodat B cellen gestimuleerd worden om antistoffen te produceren. In dit proefschrift hebben we de rol van MHC klasse II antigeen presentatie door B cellen bestudeerd in de antistof respons bij bacteriële infecties. Aan de andere kant geven na MHC klasse II antigeen presentatie de geactiveerde $CD4^+$ T cellen hulp aan $CD8^+$ T cellen. De geactiveerde $CD8^+$ T cellen kunnen nu hun cytotoxische activiteiten uitvoeren om defecte/getransformeerde of geïnfecteerde cellen te elimineren. Verstoring van MHC klasse II antigeen presentatie zou een rol kunnen spelen bij het ontduiken van het immuunsysteem door kankercellen, daarom hebben wij de MHC klasse II antigeen presentatie route bestudeerd bij leukemie. Het belang van MHC klasse II antigeen presentatie wordt duidelijk in patiënten met een defect in MHC klasse II antigeen presentatie. Deze zeldzame primaire immuundeficiëntie ziekte, genaamd Bare Lymphocyte Syndrome type II (BLSII), wordt gekenmerkt door de afwezigheid van MHC klasse II eiwit expressie. De MHC klasse II genen zelf zijn onveranderd in deze patiënten, maar hun expressie is verstoord door mutaties in transcriptiefactor genen die de transcriptie van MHC klasse II genen initiëren. Het resultaat is dat patiënten een ernstig defect hebben in zowel de cellulaire als humorale immuniteit en een extreme gevoeligheid voor infecties laten zien. Infecties beginnen in het eerste levensjaar, er is een dramatische progressie van verschillende soorten infectieuze complicaties en patiënten sterven doorgaans voor de leeftijd van 10 jaar. Dit laat zien dat een defect in MHC klasse II antigeen presentatie slecht gecompenseerd kan worden door de andere onderdelen van het aangeboren en verworven immuunsysteem.

Fagocytose van bacteriën door B cellen

Klassiek gezien omvatten professionele fagocyten de neutrofielen, monocysten, macrofagen, dendritische cellen (DCs) en mast cellen. Professionele fagocyten hebben receptoren op hun oppervlak die schadelijke objecten kunnen detecteren die normaal niet in het lichaam gevonden worden, zoals pathogene bacteriën. Fagocyten zijn daarom cruciaal bij het bestrijden van infecties en in het behouden van de gezondheid van weefsels door het verwijderen van dode en stervende cellen die het einde van hun levensduur bereikt hebben.

Kenmerken van fagocytose zijn de internalisatie van grote partikels (meestal > 500 nm in diameter) met reorganisatie van het actine cytoskelet en de extensie van pseudopodia (vorming van een fagocytische cup). Het dogma is dat B cellen geen fagocytische capaciteiten hebben, maar recent is aangetoond dat B cellen van zeer vroege gewervelden (teleost vissen en amfibieën) potente fagocyten zijn. De auteurs suggereerden dat de fagocytische capaciteit van B cellen al aanwezig was in een gezamenlijke voorouder op het moment van de fylogenetische splitsing van teleosten en amfibieën, maar dat B cellen van zoogdieren deze aangeboren immuun capaciteit verloren lijken te hebben. Inderdaad, hoewel beschreven is dat humane B cellijnen antigenen gebonden aan deeltjes presenteren in de context van MHC klasse II en antigenen van een niet-internaliseerbaar oppervlak kunnen onttrekken, werden primaire B cellen niet gedacht in staat te zijn om grote partikels te fagocyteren omdat zij weinig ruimte hebben in het cytoplasma en een relatief grote kern.

Aangezien humane primaire B cellen niet als fagocytische cel gezien worden, hoe verkrijgen zij dan antigenen van bacteriën? Het dogma is dat B cellen antigeen verkrijgen van folliculaire dendritische cellen (FDCs) in lymfoïde follikels van de milt, lymfe knopen (LNs) en mucosale lymfoïde weefsels. Een andere manier zou via normale DCs kunnen zijn, waarvan is aangetoond dat ze geïnternaliseerde antigenen recycleren naar hun cel oppervlak en deze in onaangepaste vorm aan B cellen presenteren. Recent zijn subcapsulaire macrofagen geïdentificeerd in LNs als belangrijke plaats waar B cellen aan deeltjes gebonden antigenen tegen komen. Aangezien aangetoond is dat B cellen antigenen kunnen oppikken van een niet-internaliseerbaar oppervlak, zou het vangen van antigeen van het oppervlak van andere cellen een manier kunnen zijn om bacteriële antigenen te internaliseren. Een alternatief is dat bacteriën naar regionale LNs zouden kunnen verplaatsen of naar B cel gebieden in de milt en mucosa-geassocieerd lymfoïd weefsel (MALT), waar B cellen direct antigenen van de bacteriën zelf zouden kunnen oppikken. In **Hoofdstuk 2** is de humane B cellijn Ramos gebruikt in combinatie met anti-IgM gecoate beads om aan te tonen dat humane B cellijnen inderdaad zeer capabele fagocytische cellen zijn als de beads via de B cel receptor (BCR) herkend worden. Ramos cellen internaliseren anti-IgM gecoate beads compleet, maar irrelevant gecoate beads worden niet geïnternaliseerd. Als een meer fysiologisch model hebben we de *Salmonella* bacterie gebruikt. In tegenstelling tot het huidige dogma, hebben we laten zien dat ook naïeve en memory primaire B cellen in staat zijn hele,

levende *Salmonella* te fagocyteren. Verder onderzoek liet zien dat dit via de BCR plaats vindt en dat fagocytose via de BCR resulteert in activatie van de B cel en secretie van immuunglobulinen. De antistoffen die geproduceerd worden door B cellen die *Salmonella* hebben geïnternaliseerd zijn reactief tegen *Salmonella*, dat wederom betrokkenheid van de BCR aantoont. Het relatief hoge percentage circulerende B cellen dat *Salmonella* herkent via de BCR kan verklaard worden door de expressie van een polyreactieve BCR (ook reactief tegen andere bacteriën) op CD27⁺ circulerende marginale zone B cellen. Net als IgM⁺ memory B cellen, is er ook een subset van mature naïeve B cellen in perifeer bloed die een polyreactieve BCR tot expressie brengen. Naast antistof productie, hebben we in **Hoofdstuk 2** laten zien dat fagocytose van *Salmonella* ook leidt tot snelle antigeen presentatie via MHC klasse II moleculen aan CD4⁺ T cellen. De activatie van CD4⁺ T cellen is bacterie-specifiek, aangezien we hebben aangetoond dat T cellen die opgewekt zijn tegen *Staphylococcus* niet reageren op restimulatie met B cellen die *Salmonella* hebben gefagocyteerd.

De B cel als transportmiddel voor *Salmonella*

Aangezien *Salmonella* een facultatief intracellulaire bacterie is, rijst de vraag wat het lot is van *Salmonella* eenmaal binnen in de B cel. **Hoofdstuk 3** beschrijft de mogelijke rol van B cellen in de verspreiding van *Salmonella* na orale infectie. We hebben laten zien dat (anders dan macrofagen, neutrofielen en in mindere mate DCs) B cellen niet in staat zijn om *Salmonella* te doden na opname via de BCR. De replicatie van *Salmonella* wordt echter onderdrukt in levende B cellen, terwijl in apoptotische B cellen *Salmonella* weer begint te vermenigvuldigen. We hebben gezien dat uren na fagocytose levende bacteriën uit B cellen vrij kwamen en dat deze uitgescheiden bacteriën *in vitro* opnieuw andere cellen konden infecteren. Om de rol van B cellen als verspreider van *Salmonella in vivo* te bestuderen, hebben we experimenten gedaan in muizen. Deze experimenten hebben laten zien dat adoptieve overdracht van *Salmonella*-specifieke B cellen in wild-type muizen de mortaliteit significant verhoogt na orale toediening van een sub-lethale dosis *Salmonella*. Bovendien werden verhoogde aantallen *Salmonella* bacteriën gevonden in de lever van muizen die *Salmonella*-specifieke B cellen hadden gekregen.

Cross-presentatie van *Salmonella* antigenen door B cellen

B cellen behoren vooral tot de groep van “professionele antigeen presenterende cellen” om hun zeer efficiënte manier van internaliseren van antigeen. Andere leden zijn DCs en macrofagen, die net als B cellen fragmenten van antigenen tonen via MHC klasse II moleculen op hun cel oppervlak. Naast presentatie via MHC klasse II moleculen, zijn DCs in staat om exogene antigenen te cross-presenteren via MHC klasse I moleculen aan CD8⁺ T cellen. In **Hoofdstuk 4** hebben we aangetoond dat B cellen ook in staat zijn om *Salmonella* antigenen te cross-presenteren en CD8⁺ T cellen te activeren. Niet verbazingwekkend is dat deze activatie van CD8⁺ T cellen afhankelijk is van CD4⁺ T cel hulp, aangezien het incuberen van B cellen die *Salmonella* hadden gefagocyteerd met alleen CD8⁺ T cellen niet resulteerde in activatie van de CD8⁺ T cellen. Na activatie wordt IL-2 geproduceerd door CD4⁺ T cellen en het toevoegen van IL-2 samen met CD8⁺ T cellen herstelde de activatie van CD8⁺ T cellen door B cellen. Alleen activatie en proliferatie is niet voldoende om geïnfecteerde cellen te elimineren, CD8⁺ T cellen moeten ook degranuleren na interactie met een geïnfecteerde cel. We hebben cytotoxiciteits proeven gedaan om te laten zien dat *Salmonella* geprimeerde CD8⁺ T cellen degranuleren (gemeten door CD107a expressie op het oppervlak van CD8⁺ T cellen) en werkelijk geïnfecteerde cellen doden (gemeten door het vrijkomen van ⁵¹Chroom uit het cytoplasma van geïnfecteerde B cellen). Ook hebben we in **Hoofdstuk 4** laten zien dat de CD8⁺ T cel respons geïnduceerd door geïnfecteerde B cellen een memory respons is, aangezien naïeve CD8⁺ T cellen niet geprimeerd konden worden door B cellen. Dit in tegenstelling tot de CD4⁺ T cel respons, waarin zowel naïeve als memory CD4⁺ T cellen werden geactiveerd door B cellen die *Salmonella* hadden gefagocyteerd (ongepubliceerde resultaten).

De rol van B cellen in *Salmonella* infectie

Na orale opname doorkruist *Salmonella* het darmepitheel en treedt het de Peyer's patches binnen via gespecialiseerde antigeen-sampling M cellen of opname door DCs aan de luminale zijde van de darm. Na het binnentreden in de Peyer's patches, komt *Salmonella* direct B cellen tegen die de bacteriën kunnen fagocyteren, CD4⁺ T cellen activeren en anti-*Salmonella* antistoffen uitscheiden. B cellen zijn nodig voor efficiënte bescherming tegen zowel primaire als secundaire infecties door *Salmonella* en vorming van antistoffen met hoge affiniteit is niet de enige functie van B cellen in salmonellosis, aangezien passieve overdracht van *Salmonella*-

immuun serum de immuniteit van muizen tegen *Salmonella* niet kan bewerkstelligen. B cel deficiënte muizen hebben een verstoorde Th1 T cel response in het vroege begin van een *Salmonella* infectie, wat laat zien dat B cellen een essentiële rol spelen in het initiëren van T cel gemedieerde bescherming. Humane B cellen kunnen naïeve CD4⁺ T cellen primen en ook memory CD4⁺ cellen activeren, maar zijn niet in staat om *Salmonella* te doden en dus verspreiden B cellen de bacterie door het lichaam. Desalniettemin, B cellen die *Salmonella* hebben opgenomen kunnen memory CD8⁺ T cellen activeren die vervolgens de geïnfecteerde cellen doden. Tijdens een primaire infectie zijn nog steeds andere APCs (zoals DCs) nodig voor het activeren van naïeve CD8⁺ T cellen. *Salmonella* gebruikt dus het immuunsysteem van de gastheer om te overleven en zich te verspreiden door het lichaam via antigeen-specifieke B cellen, maar het zich ontwikkelende immuunsysteem maakt anti-*Salmonella* antistoffen en activeert CD4⁺ T helper en CD8⁺ CTLs. Dit is een voorbeeld van interactie tussen bacterie en gastheer welke voor de mens moeilijk in balans te houden lijkt en de pathogeniciteit van *Salmonella* zou kunnen verklaren.

Dode bacteriën worden ook herkend via de BCR, maar niet gefagocyteerd door B cellen. In **Hoofdstuk 2** hebben we aangetoond dat, hoewel ze niet helemaal opgenomen worden, antigenen van dode bacteriën door B cellen gepresenteerd worden aan CD4⁺ T cellen en dat de CD4⁺ T cellen ook geactiveerd worden. Op deze manier kunnen B cellen door CD4⁺ T cellen geactiveerd worden om te differentiëren in antistof producerende plasma cellen, wat resulteert in een effectieve immuunrespons tegen volgende infecties. **Hoofdstuk 4** laat zien dat B cellen geen antigenen van dode bacteriën presenteren aan CD8⁺ T cellen, wat aantoont dat *Salmonella* zelf bijdraagt aan de cross-presentatie van zijn eigen antigenen. Levende *Salmonella* injecteert eiwitten in het cytosol van de gastheercel via zijn Type 3 secretie systeem (TTSS) om zijn intracellulaire overleving veilig te stellen. Deze eiwitten worden onoverkomelijk gedegradeerd door het proteasoom. Peptides kunnen dan getransporteerd worden naar het endoplasmatisch reticulum (ER), op nieuw gesynthetiseerde MHC klasse I moleculen geladen worden en gepresenteerd aan CD8⁺ T cellen. Dode *Salmonella* hebben geen functioneel TTSS meer, wat zou kunnen verklaren waarom dode bacteriën niet gecrosspresenteerd worden. De bevinding dat B cellen die dode *Salmonella* bij zich dragen geen *Salmonella*-specifieke CTLs activeren zou kunnen kloppen met het idee dat dit

immunologisch gezien ook niet nodig is; in deze situatie vormen de cellen geen niche voor *Salmonella* overleving en verspreiding, dus hun onmiddellijke opruiming zou minder belangrijk kunnen zijn voor de gastheer.

MHC klasse II antigeen presentatie in leukemie

Professionele APCs nemen apoptotische cellen en puin op om een CD4⁺ T cell respons te initiëren. Stervende tumorcellen kunnen ook opgenomen worden, maar soms slaagt de APC er niet in om een effectieve immuunrespons te genereren tegen de tumorcellen, aangezien de kanker niet altijd wordt opgeruimd. Desalniettemin brengen veel tumoren van het hematologisch systeem MHC klasse II tot expressie op hun cel oppervlak. Blijkbaar is de MHC klasse II antigeen presentatie door deze tumorcellen er niet in geslaagd om een effectieve immuunrespons te genereren wat het resultaat zou kunnen zijn van afwijkingen in de MHC klasse II antigeen presentatie route. Wij hebben de MHC klasse II antigeen presentatie route bestudeerd in de myeloïde leukemie AML en in meer detail in de lymfoïde leukemie B-CLL.

In **Hoofdstuk 5** hebben we laten zien dat het zelf-peptide CLIP tot expressie werd gebracht in AML blasten die MHC klasse II tot expressie brachten. Patiënten met HLA-DR⁺/CLIP⁻ blasten hadden een significant langere ziekte-vrije overleving dan patiënten met HLA-DR⁺/CLIP⁺ blasten. Dit laat zien dat de expressie van CLIP op de blasten een maat zou kunnen zijn voor ontsnapping aan het immuunsysteem door de leukemie cellen. Tijdens het onderzoek naar de MHC klasse II antigeen presentatie route, vonden we dat in AML blasten HLA-DO en HLA-DM (de peptide editors van de MHC klasse II antigeen presentatie route) duidelijk detecteerbaar waren op zowel mRNA als eiwit niveau. De relatieve expressie van DO en DM correleerde met de efficiëntie van antigeen belading op DR moleculen: hoe meer DO relatief aan DM, hoe meer CLIP relatief aan DR. Samen leidde dit tot de hypothese dat HLA-DR⁺/CLIP⁻ blasten in staat zijn om leukemie-specifieke antigenen aan CD4⁺ T cellen te presenteren. Dit zou een effectieve anti-tumor respons teweeg brengen die in een verlengde ziektevrije overleving resulteert. Strategieën om CLIP expressie omlaag te brengen op leukemische blasten worden onderzocht om een effectieve anti-tumor respons op te wekken in AML. Retrovirale transductie van specifiek Ii siRNAs in twee humane myeloïde leukemische cellijnen laat een gereduceerde expressie van CLIP zien relatief aan DR (van Luijn et al., submitted). Ondanks de verlaagde hoeveelheden DR op het celoppervlak (als

secondaire consequentie van de verlaging van Ii), verhogen beide cellijnen sterk de activatie van allogene CD4⁺ T cellen. Echter, de inductie van tumor specifieke CD4⁺ T cellen (en daaropvolgende hulp voor de inductie van tumor specifieke CD8⁺ T cellen) in AML blasten getransduceerd met Ii siRNAs is nog niet aangetoond. Andere strategieën zouden DM en/of DO, de peptide editors, kunnen aanpakken. Ongepubliceerde data van onze groep laten zien dat wanneer B cellen *in vitro* worden blootgesteld aan dexamethason, een sterke toename van DM eiwit wordt gezien, zonder veranderde DR of DO eiwit hoeveelheden. Meer DM relatief aan DO zou peptide belading van MHC klasse II moleculen kunnen verbeteren, zonder de totale hoeveelheid DR moleculen die beschikbaar zijn voor antigeen presentatie te beïnvloeden.

Het HLA-DOA mRNA expressie niveau voorspelt prognose in B-CLL

Naast de myeloïde leukemie AML, hebben we de MHC klasse II antigeen presentatie route bestudeerd in de lymfoïde leukemie B-CLL. In **Hoofdstuk 6** hebben we laten zien dat in B-CLL de expressie van *DRA*, *DMB*, *DOA* en *DOB* maar ook *Ii* sterk afwijkend is op mRNA niveau in vergelijking met B cellen van gezonde controles. Aangezien transcriptie van deze genen gereguleerd wordt (*DOB* en *Ii* deels) door CIITA, hebben we mRNA levels bestudeerd van totaal CIITA en de lymfoïd-specifieke promoters PIII en PIV. Totaal CIITA mRNA was significant verhoogd in vergelijking met mRNA levels in B cellen van gezonde controles. mRNA van CIITA-PIII werd in alle monsters gedetecteerd, maar liet geen verschil zien met controles. De mRNA levels van de IFN-γ-induceerbare promotor CIITA-PIV waren echter significant verhoogd, wat laat zien dat transcriptie van CIITA in B-CLL gecontroleerd wordt door de gecoördineerde activiteit van de B cel-specifieke promotor PIII en de IFN-γ-induceerbare promotor PIV. Verhoogde transcriptie leidt niet altijd tot verhoogde translatie en verhoogde eiwit levels, zoals we hebben laten zien in **Hoofdstuk 6**. Analyse van eiwitexpressie met semi-kwantitatieve Western blot liet geen toename zien in eiwit expressie door B-CLL cellen vergeleken met B cellen van gezonde controles. DM eiwit expressie was zelfs significant lager in B-CLL patiënten.

We hebben het mechanisme bestudeerd onderliggend aan het verschil tussen de geobserveerde transcriptionele opregulatie en de onveranderde of gedownreguleerde totale eiwit levels van DR in de maligne B-CLL cellen. We hebben pulse chase analyse gedaan in B cellen van drie gezonde donoren en vier B-CLL

patiënten met verschillende niveaus van transcriptionele opregulatie van *DRA*. Voor DR zagen we een vergelijkbare halfwaardetijd in de maligne B-CLL cellen en de gezonde B cellen, aangezien in alle gevallen ongeveer 90% van de nieuw gesynthetiseerde DR moleculen 24 uur na biosynthese nog steeds aanwezig was. Bovendien was de maturatie van het DR complex en de degradatie snelheid van het klasse II-geassocieerde Ii vergelijkbaar tussen gezonde B cellen en maligne B-CLL cellen (onpubliceerde resultaten). Onverwachts laten de maligne B-CLL cellen een vergelijkbare biosynthese snelheid van DR zien als de gezonde B cellen, ondanks hun tot drievoudige hogere mRNA levels. Recent is aangetoond dat mesenchymale stamcellen in respons op IFN- γ verhoogde levels van CIITA-PIV hebben, maar dat dit niet tot uiting komt in een verhoging van HLA-DR eiwit als gevolg van cytoplasmatische retentie van het CTIIA transcript. Aangezien wij ook opregulatie hebben gevonden van transcripten downstream van CIITA is het niet waarschijnlijk dat CIITA-PIV in het cytoplasma vast gehouden wordt. Of cytoplasmatische retentie van *DRA* transcripten een verklaring zou kunnen zijn voor de normale of verlaagde DR eiwit levels moet nog onderzocht worden. Deze data laten zien dat het bestuderen van mRNA levels niet noodzakelijkerwijs een afspiegeling is van de expressie levels van functionele eiwitten.

Daarnaast hebben we bestudeerd of de verhoogde mRNA levels van de MHC II componenten een correlatie vertonen met klinische uitkomst. Alleen *DOA* mRNA levels waren significant verschillend als we de overleving na monsterafname vergeleken tussen patiënten. Als patiënten werden ingedeeld in een groep met *DOA* mRNA levels < 3.4 en ≥ 3.4 , definieerde *DOA* mRNA levels ≥ 3.4 een subgroep van patiënten met een minder gunstige prognose: 6 van de 8 patiënten met *DOA* mRNA levels ≥ 3.4 stierven tijdens follow up vergeleken met 4 van de 12 patiënten met mRNA levels < 3.4 .

Aangezien de verandering in transcriptie van de DO genen niet tot uiting komt in hun eiwit levels, rijst de vraag waarom *DOA* mRNA correleert met klinische uitkomst. Het is beschreven in B-CLL dat verscheidene serum cytokine gehalten verhoogd zijn en dat zowel CD4⁺ als CD8⁺ T cellen significant meer IFN- γ en IL-4 produceren dan in gezonde controles. Deze ontstekings cytokines kunnen de transcriptie van CIITA-PIV initiëren, welke verhoogd is in B-CLL. Dus, overexpressie van *DOA* mRNA zou het resultaat kunnen zijn van een afwijkend immunologisch milieu in B-CLL maar kan overleving in B-CLL voorspellen. Dit moet gevalideerd

worden in een groter cohort van B-CLL patiënten en bij voorkeur in monsters afgenomen op het moment van diagnose.

Veranderde MHC klasse II antigeen presentatie wordt gereflecteerd door expansie van het geactiveerde T cel compartiment in B-CLL

In **Hoofdstuk 7** hebben we kwantitatief aangetoond dat eigenlijk zowel DM als DO op eiwit niveau lager tot expressie komen in B-CLL in vergelijking met gezonde controles. Hoewel we niet de biosynthese snelheid van DM en DO hebben bestudeerd, zou een lagere eiwit expressie van DM en DO eiwit in B-CLL patiënten een verschil in turnover suggereren vergeleken met gezonde controles. Dit is een interessant onderwerp dat meer onderzoek verdient. De relatieve expressie van DM ten opzichte van DO was echter hoger in B-CLL patiënten. Expressie van DR op het plasma membraan verschilde niet, maar CLIP levels waren significant lager in B-CLL patiënten. De correlatie tussen de relatief hogere expressie van DM en de verlaagde expressie van het zelf-peptide CLIP suggereert dat door variatie in DM levels het peptide repertoire dat gepresenteerd wordt door DR moleculen wordt veranderd, aangezien het gecorreleerd is aan een verlaagde expressie van het zelf-peptide CLIP. Vervolgens hebben we de differentiatie en activatie status van het T cel compartiment bestudeerd. **Hoofdstuk 7** laat zien dat het T cel compartiment in B-CLL patiënten significant verlaagd was in naïeve CD4⁺ en CD8⁺ subsets ten gunste van verhoogde geactiveerde effector populaties. Het percentage geactiveerde T cellen is omgekeerd gecorreleerd met CLIP expressie, wijzend op verbeterde antigeen presentatie. Veranderde MHC klasse II antigeen presentatie zou dus een nieuwe factor kunnen zijn in de immuun dysfunctie en pathobiologie van B-CLL.

Nieuwe therapieën tegen B cel maligniteiten omvatten het gebruik van monoklonale antistoffen, in het bijzonder tegen CD20 en CD52. Het effector mechanisme van anti-CD20 en anti-CD52 antistoffen is voornamelijk gebaseerd op complement gemedieerde cellulaire cytotoxiciteit (CDCC) en antistof gemedieerde cellulaire cytotoxiciteit (ADCC). Behandeling met deze monoklonale antistoffen lijkt veelbelovend, maar in het geval van hoge B cel belasting zou uitputting van de effector mechanismes kunnen leiden tot substantiële vermindering van de immunotherapeutische efficiëntie.

De data in dit proefschrift zouden een nieuwe benadering kunnen vormen voor therapie tegen B cel kankers. Alle chronische B cel leukemieën en B cel lymfomen brengen IgM en/of IgD tot expressie aan het cel oppervlak. Aangezien wij hebben

laten zien dat het richten van deeltjes naar de IgM-BCR leidt tot internalisatie, en recent hetzelfde is aangetoond voor IgD, zou het richten van micropartikels met anti-kanker middelen naar het constante domein van de BCR een optie kunnen zijn. Deze aanpak zou in het bijzonder aantrekkelijk kunnen zijn in het geval van mantelcel lymfomen, welke hoge levels IgM en/of IgD tot expressie brengen en waar patiënten de slechtste prognose hebben van alle B cel kankers.

Anti-kanker middelen verpakt in micropartikels zijn minder schadelijk voor de patiënt en gericht naar het constante domein van de BCR brengt dit de drugs direct binnen in de kankercellen. Zo kunnen hoge concentraties drugs bereikt worden in de target cellen, zonder andere cellen in het lichaam te schaden. Deze strategie zou dus de ongewilde bijwerkingen van systemische behandeling met anti-kanker medicijnen kunnen verminderen.

Curriculum Vitae

De auteur van dit proefschrift, Yuri Souwer, werd geboren op 25 mei 1975 te Hoorn. In 1994 behaalde hij zijn VWO diploma aan het Marcus College te Grootebroek, waarna hij anderhalf jaar Medische Biologie studeerde aan de Universiteit van Utrecht. In 1996 startte hij met zijn studie Zoölogie aan de Hogeschool van Utrecht. Na het behalen van zijn diploma in 1999 begon hij aan de verkorte opleiding Medische Biologie aan de Vrije Universiteit te Amsterdam. Na een stage van 7 maanden op de afdeling Pathologie van het VU medisch centrum en het behalen van zijn diploma in 2001, was hij vanaf november 2001 werkzaam als onderzoeker in opleiding op een gezamenlijk project van het NKI-AVL (afdeling Tumor Biologie) en het VU medisch centrum (afdeling Pathologie). Onder begeleiding van Prof.dr. J.J. Neefjes en dr. S.M. van Ham deed hij promotieonderzoek naar MHC klasse II antigeen presentatie door B cellen. In november 2003 verhuisde hij mee met dr. S.M. van Ham naar de afdeling Immunopathologie van Sanquin om daar het onderzoek voort te zetten en uit te breiden met onderzoek naar de fagocytose van bacteriën via de B-cel receptor. De resultaten van dit onderzoek staan beschreven in dit proefschrift. Vanaf november 2006 werkte hij als postdoc op de afdeling Immunopathologie en vervolgde hij het onderzoek naar de fagocytose door B cellen en antigeen presentatie aan T cellen. Sinds mei 2009 is hij werkzaam als postdoc op de afdeling Celbiologie en Histologie van het AMC in Amsterdam in de groep van Prof.dr. M.L. Kapsenberg en dr. E.C. de Jong.

Nawoord

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Het begon allemaal op het ASI-lab van de pathologie in het VUmc bij Marieke, Marion, Sander, Liesbeth, Tineke en Stephan. We hadden een gezellig kluppie in dat kleine hokje en toch alle ruimte om western blotjes te doen toen jullie klinische studie een beetje stil viel. Bedankt voor jullie geduld als ik geheel tegen de cGMP regels in weer eens vergeten was iets af te tekenen, dat is ook zo niet-research :-).

In deze tijd is ook mijn enige student begonnen; maar je bleef wel veel langer dan een gemiddelde student doordat je deelnam aan de masterclass oncologie. Jouw werk is de basis voor een stuk dat binnenkort geschreven gaat worden. Canan, heel veel succes met je promotieonderzoek bij de hematologie van het VUmc.

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Appendix I

Color figures

Figure 1A and C from Chapter 2, page 37

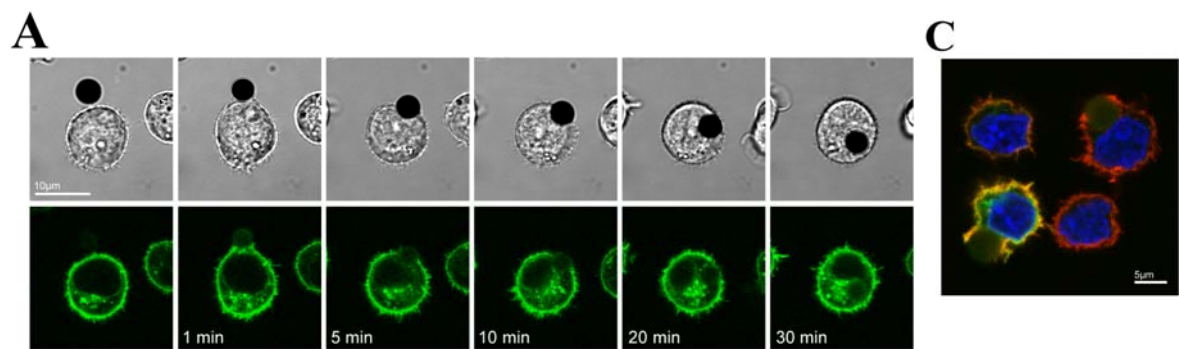


Figure 2A, C and D from Chapter 2, page 39

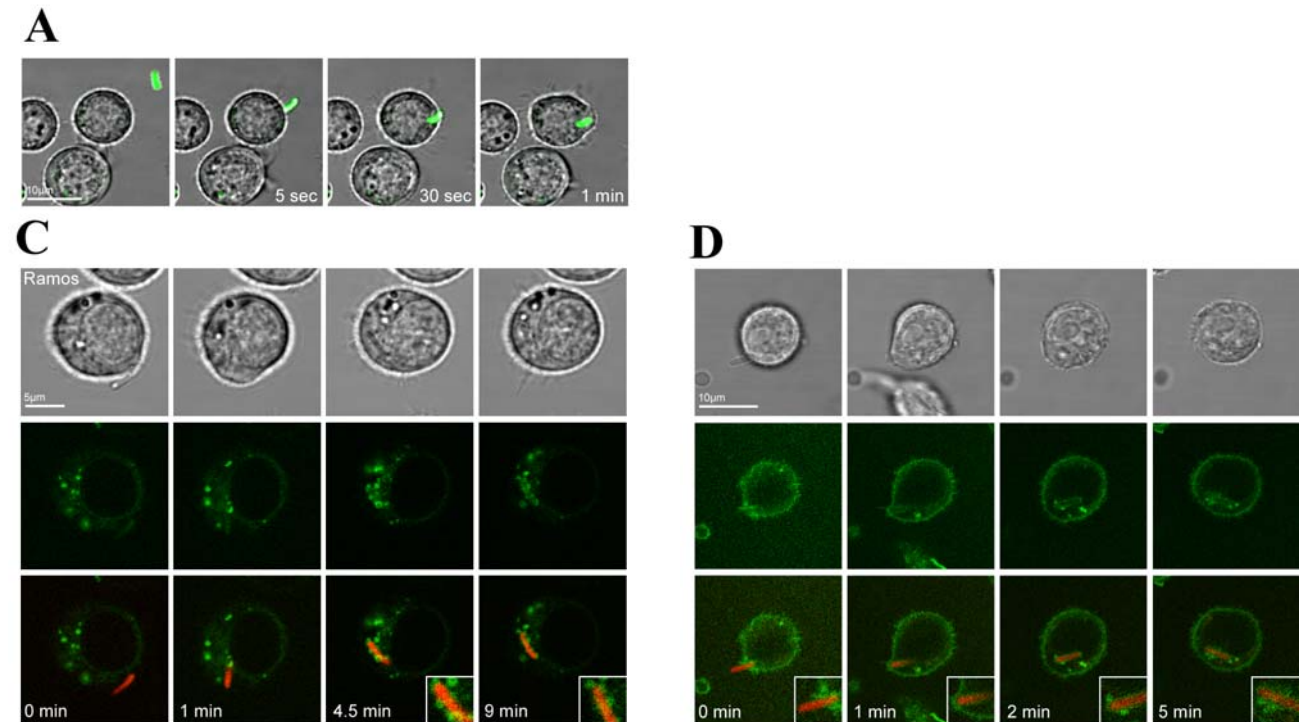


Figure 3B from Chapter 2, page 42

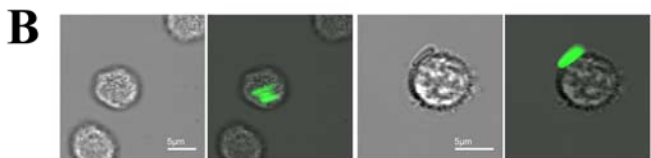


Figure 5A and B from Chapter 2, page 46

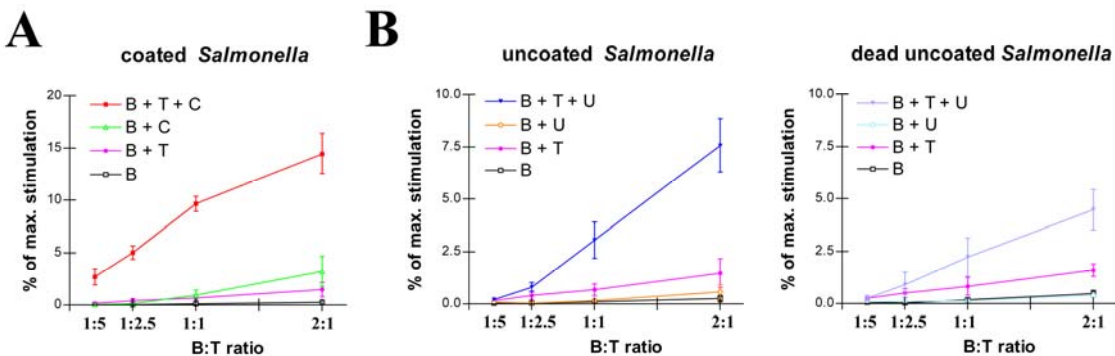


Figure 5C and F from Chapter 2, page 46

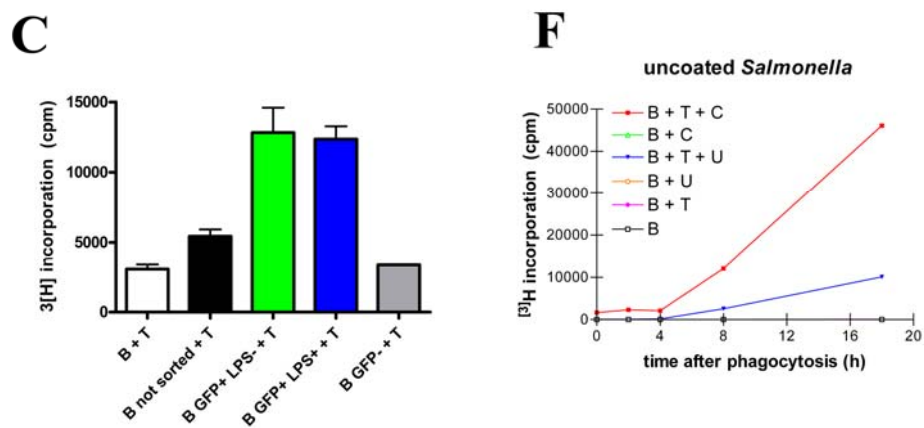


Figure 1A, B and C from Chapter 3, page 62

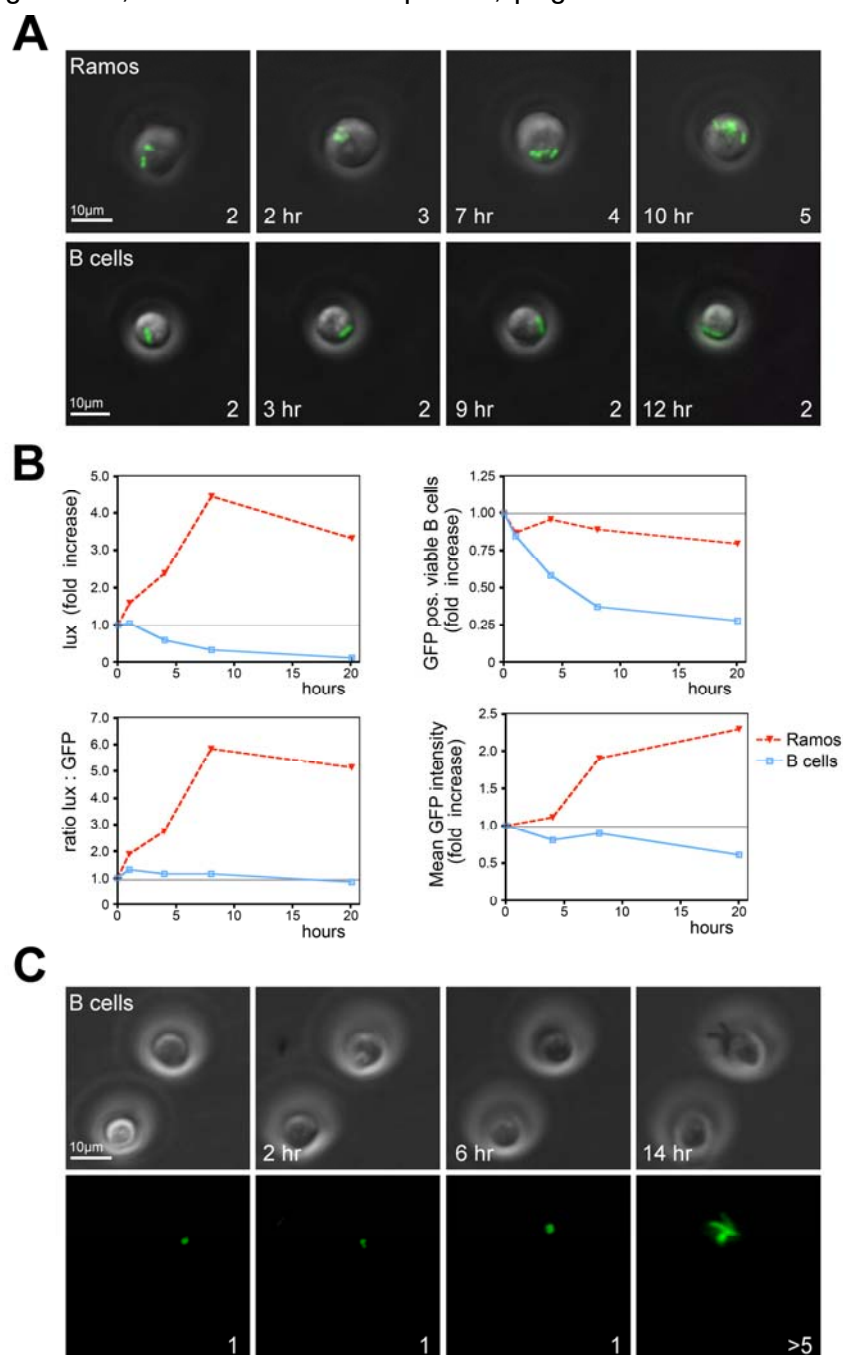


Figure 2A and B from Chapter 3, page 64

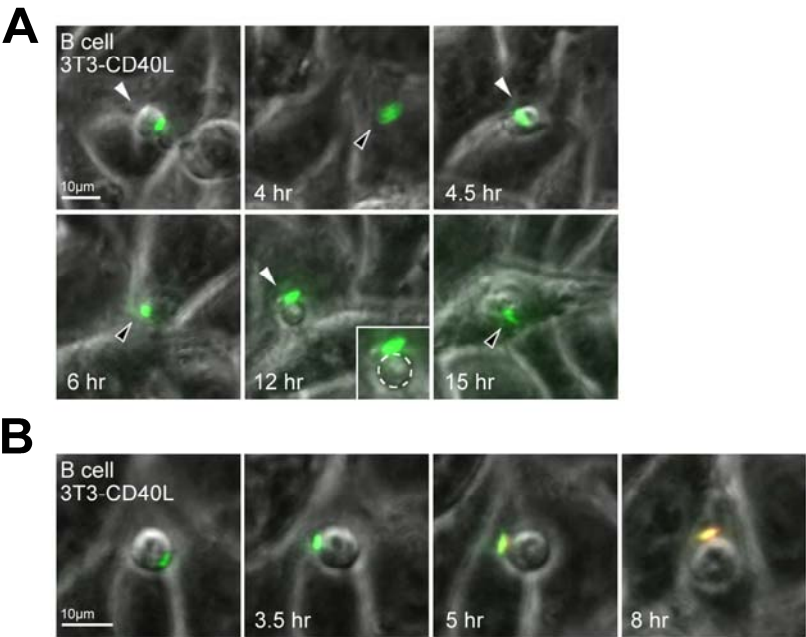


Figure 3A, B and C from Chapter 3, page 66

