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Κλινική Ρευματολογίας, Κλινικής Ανοσολογίας και Αλλεργιολογίας
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ΜΕΤΑΠΤΥΧΙΑΚΗ ΔΙΑΤΡΙΒΗ

Μελέτη της έκφρασης και της λειτουργίας αρνητικών ανοσορρυθμιστικών υποδοχέων των Β λεμφοκυττάρων σε υγιείς και ασθενείς με συστηματικό ερυθηματώδη λύκο

ΑΜΑΛΙΑ Γ. ΖΑΜΠΟΥΛΑΚΗ

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Αφιερώνεται στην οικογένειά μου

και σε όσους πίστεψαν, πιστεύουν και θα πιστεύουν σε μένα….

ΠΕΡΙΛΗΨΗ

Ο συστηματικός ερυθηματώδης λύκος (ΣΕΛ) είναι μία συστηματική αυτοάνοση νόσος, με κύριο χαρακτηριστικό την παρουσία υψηλού τίτλου αυτοαντισωμάτων, τα οποία παράγονται από μακρόβια πλασματοκύτταρα. Δεδομένα από *in vivo* και *in vitro* μελέτες έχουν αναδείξει τα ενεργοποιημένα Β λεμφοκύτταρα ως κεντρική συνιστώσα της αιτιοπαθογένεσής του. Η βέλτιστη ενεργοποίηση των Β λεμφοκυττάρων προϋποθέτει συγκλίνοντα σήματα από τον υποδοχέα των Β λεμφοκυττάρων, υποδοχείς τύπου Toll καθώς και συνδιεγερτικά μόρια, ενώ μπορεί να επηρεαστεί από ανασταλτικούς υποδοχείς, όπως ο BTLA (B and T lymphocyte Attenuator), ο PD-1 (Programmed cell death protein 1) και ο PD-L1 (Programmed cell death 1 ligand 1).

Η παρούσα μελέτη αποσκοπεί στη διερεύνηση της έκφρασης και της λειτουργίας των ανασταλτικών υποδοχέων BTLA, PD-1 και PD-L1 σε Β λεμφοκύτταρα από υγιείς δότες και ασθενείς με ΣΕΛ. Η έκφραση των BTLA, PD-1 και PD-L1 εξετάστηκε με κυτταρομετρία ροής σε άωρα Β λεμφοκύτταρα ($CD19^+IgD^+CD27^-$), μεταβατικά Β λεμφοκύτταρα ($CD19^+IgD^+CD27^+$), Β λεμφοκύτταρα μνήμης ($CD19^+IgD^-CD27^+$) και πλασματοκύτταρα ($CD19^+IgD^-CD27^{hi}$) σε βασική κατάσταση και σε απομονωμένα $CD19^+$ Β λεμφοκύτταρα περιφερικού αίματος μετά από ενεργοποίηση. Η έκφραση του συνδέτη του BTLA, HVEM (Herpes Virus Entry Mediator), εξετάστηκε επίσης σε $CD4^+$ Τ λεμφοκύτταρα περιφερικού αίματος. Η ενεργοποίηση, η διαφοροποίηση και ο πολλαπλασιασμός των Β λεμφοκυττάρων, καθώς και η παραγωγή ιντερλευκίνης-6 από αυτά διερευνήθηκαν παρουσία και απουσία του HVEM.

Η ανάλυση των αποτελεσμάτων κατέδειξε πως υπάρχει διαφορετική έκφραση των τριών υποδοχέων στους υποπληθυσμούς των Β λεμφοκυττάρων τόσο υγιών δοτών όσο και ασθενών με ΣΕΛ. Τα επίπεδα του HVEM ήταν σημαντικά αυξημένα στα $CD4^+$ Τ λεμφοκύτταρα ασθενών με ΣΕΛ. Η ενεργοποίηση του υποδοχέα των Β λεμφοκυττάρων οδήγησε σε αύξηση της έκφρασης και των τριών υποδοχέων σε Β λεμφοκύτταρα υγιών, η προσθήκη CpG ολιγοδεοξυνουκλεοτιδίων (συνδέτης υποδοχέα τύπου Toll-9) αύξησε περαιτέρω τα επίπεδα των PD-1 και PD-L1, αλλά όχι και του BTLA, ενώ η προσθήκη ιντερλευκίνης-4, ιντερλευκίνης-10 και ιντερλευκίνης-21 μείωσε τα επίπεδα του BTLA. Τέλος, η προσθήκη του συνδέτη του BTLA, HVEM, σε καλλιέργειες Β λεμφοκυττάρων μετρίασε τα επίπεδα δεικτών

ενεργοποίησης (CD80/CD86), ελάττωσε τη διαφοροποίησή τους σε πλασματοκύτταρα και τον πολλαπλασιασμό τους, αλλά είχε διαφορετική επίδραση στην παραγωγή ιντερλευκίνης-6 από Β λεμφοκύτταρα ασθενών με ΣΕΛ σε σχέση με υγιείς δότες.

Συνοψίζοντας, τα παραπάνω ευρήματα καταδεικνύουν ένα διαφορετικό πρότυπο έκφρασης των τριών υποδοχέων, BTLA, PD-1 και PD-L1, το οποίο δύναται να παίζει σημαντικό ρόλο στη ρύθμιση της ενεργοποίησης και διαφοροποίησης των Β λεμφοκυττάρων. Παρεκκλίσεις στη λειτουργία και έκφραση των ανασταλτικών υποδοχέων των Β λεμφοκυττάρων θα μπορούσαν να οδηγούν σε ενίσχυση της ικανότητας τους για παραγωγή αυτοαντισωμάτων συμβάλλοντας, κατ’επέκταση, στην παθογένεια του ΣΕΛ.

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SCHOOL OF HEALTH SCIENCES
FACULTY OF MEDICINE

GRADUATE PROGRAM IN
THE MOLECULAR BASIS OF HUMAN DISEASE

Laboratory of Autoimmunity and Inflammation
Department of Rheumatology, Clinical Immunology and Allergy
Foundation of Research and Technology
Institute of Molecular Biology and Biotechnology

MASTER THESIS

Study of the expression and function of negative immunomodulatory receptors of B lymphocytes in healthy controls and patients with systemic lupus erythematosus

AMALIA G. ZAMPOULAKI

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ABSTRACT

Systemic Lupus Erythematosus (SLE) is a systemic autoimmune disorder characterized by the presence of high-titer autoantibodies produced by long-lived plasma cells. Evidence from *in vivo* and *in vitro* studies have established activated B cells as an important component of its pathogenesis. Optimal B cell activation and differentiation requires convergent signals from the B cell receptor (BcR), Toll-like receptors (TLRs) as well as costimulatory molecules, whereas B cells can also be influenced by inhibitory immunoreceptors, such as B and T lymphocyte Attenuator (BTLA), Programmed cell death protein 1 (PD-1) and Programmed cell death 1 ligand 1 (PD-L1).

This study aimed at investigating the expression and function of the coinhibitory receptors BTLA, PD-1 and PD-L1 in B cells from healthy donors and active SLE patients. BTLA, PD-1 and PD-L1 expression was examined by flow cytometry in peripheral blood naïve ($CD19^{+}IgD^{+}CD27^{-}$), transitional ($CD19^{+}IgD^{+}CD27^{+}$), memory ($CD19^{+}IgD^{-}CD27^{+}$) and plasma B cells ($CD19^{+}IgD^{-}CD27^{hi}$) at basal state and in purified peripheral blood $CD19^{+}$ B cells following stimulation. BTLA ligand, HVEM (Herpes Virus Entry Mediator), expression was also considered in peripheral blood $CD4^{+}$ T cells. Activation, differentiation, proliferation and IL-6 production of B cells were examined in the presence or absence of the BTLA ligand, HVEM.

Analysis resulted in differential baseline expression of all three receptors studied in B cell subsets both in healthy donors and in SLE patients. HVEM levels were found to be significantly increased in SLE $CD4^{+}$ T cells. BcR activation enhanced the expression all three receptors in normal B-cells; addition of CpG-ODN (TLR9 ligand) further induced PD-1 and PD-L1 –but not BTLA– expression, whereas addition of the cytokines IL-4, IL-10 or IL-21 reduced PD-1 and BTLA levels. *In vitro* cross linking of BTLA with HVEM resulted in decreased expression of activation markers CD80/CD86 and differentiation, in attenuation of B cell proliferation, although it had a differential effect on IL-6 production in B cells from SLE patients compared to healthy controls.

In conclusion, these data demonstrate a different pattern of expression of BTLA, PD-1 and PD-L1 among B cell subsets and upon treatment with stimuli, with important implications for the modulation of B cell activation and differentiation.

Aberrancies in the expression and function of coinhibitory receptors in B cells could contribute to enhanced autoantibody-forming capacity and disease pathogenesis.

INDEX

A. INTRODUCTION

A.1 Innate and Adaptive Immunity

Consisting of both humoral and cellular components, the immune system is a remarkably adaptive biological system having evolved in the vertebrates to provide protection against evading pathogens that would result in a loss of their homeostasis. In order to be effective, it is divided into two major domains that act in a highly interactive and cooperative way, the innate immune system (innate immunity) and the adaptive immune system (adaptive immunity) [1].

The **innate immune system** refers to the non-specific defenses of the vertebrates and consists of anatomic and physiologic barriers (skin, mucous membranes, temperature, pH and chemical mediators) as well as phagocytic cells (blood monocytes, neutrophils and tissue macrophages), which conduct the ingestion of pathogens. This process is of utmost importance and provides the first line of defense immediately after a pathogen enters the host's organism. Normally, most of the pathogens are cleared by this system before they activate the **adaptive immune system**, however, if the pathogen manages to escape from these disease-resistant mechanisms, the specific response of the adaptive immune system is mounted. In this case, lymphocytes (B and T cells) are activated by antigen-presenting cells, which display parts of the antigens on MHC class (Major Histocompatibility Complex) II molecules on their cell surface. After a crosstalk among activated lymphocytes, specific antibodies are produced by differentiated B cells (plasma cells) and specific cytotoxic responses from T lymphocytes begin with the ultimate aim to eliminate the foreign invader. Except for these responses, memory B cells are generated to sustain immunological memory for this pathogen [1].

A.2 B cells as an essential component of the immune system

A.2.1 B cell development

Being a significant cellular component of the adaptive immunity and mediators of antibody (immunoglobulin, Ig) production, B cells are generated prenatally in the yolk sac, the fetal liver and the fetal bone marrow, nevertheless, postnatally their

generation takes place exclusively from pluripotent hematopoietic stem cells in the bone marrow. Maturation occurs as a result of sequential rearrangement of heavy- and light-chain immunoglobulin genes and expression of cell surface molecules and it can be divided into two phases of differentiation, antigen-independent differentiation in the bone marrow and antigen-dependent activation and differentiation in the peripheral lymphoid organs (Figure 1) [2-4].

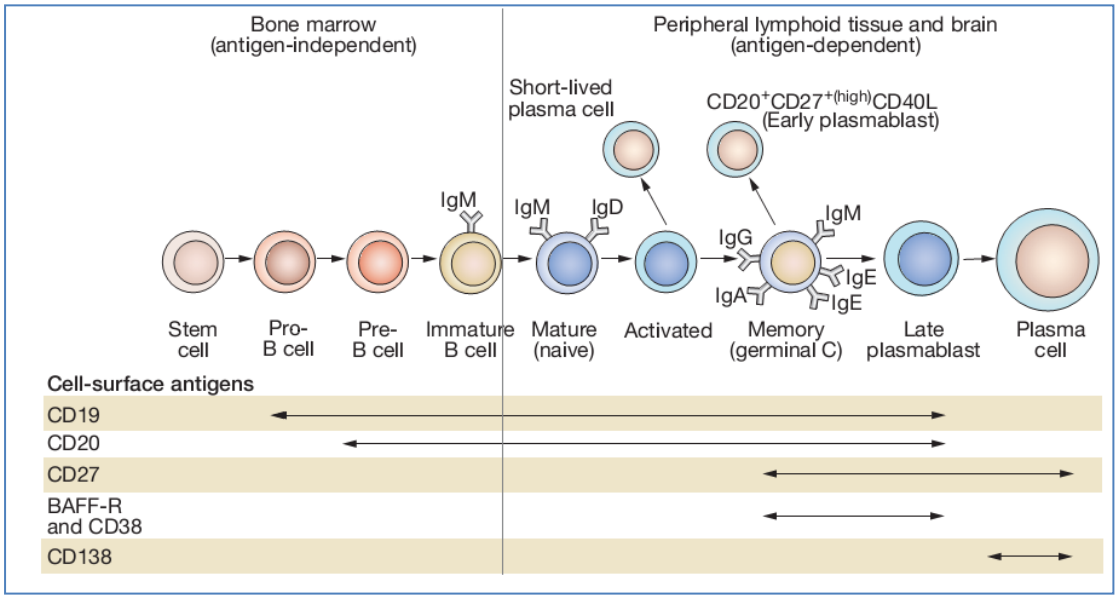


Figure 1 Maturation of B cells. Specific cell surface markers (CD-Clusters of Differentiation) are used to distinguish the different phases of development. Adapted from [3].

In the first phase, the earliest B-lineage cell is a pro-B cell, which matures into a pre-B cell by rearranging its heavy-chain immunoglobulin genes. Next, a pre-B cell develops into an immature B cell, in which IgM is expressed [3-4]. At this stage, immature B cells expressing self-reactive IgM undergo negative selection and are led to apoptosis and clonal deletion [4-5]. The non-auto-reactive immature B cells become naive mature B cells expressing both IgD and IgM. Being potent antigen-presenting cells due to expression of MHC class II molecules, naïve B cells leave the bone marrow, migrate in the periphery and recirculate through secondary lymphoid tissues. They are short-lived, thus, they undergo homeostatic proliferation in order to sustain their numbers. However, once they encounter an antigen, naïve B cells enter the phase of antigen-dependent activation and differentiation [3-5].

From this point on, it has been elucidated that either T-cell independent or T-cell dependent B-cell proliferation exists [5]. In the former case, B cells of the marginal zone of the spleen start a fast T-cell independent response, contrary to the latter case, in which B cells interact with T cells through antigen binding by the B cell receptor

and through binding of co-stimulatory molecules (CD40-CD40L). After B cells are activated, they also express other co-stimulatory molecules, such as B7.1 (CD80) and B7.2 (CD86) which attach to CD28 on the T cell surface. A potent B cell-T cell crosstalk (Figure 2) results in somatic hypermutation of Ig variable regions leading to affinity maturation as well as in Ig class switch recombination to alter the function of the Ig, prior to a second selection process in order to eliminate B cells that fail to bind the inducing antigen with high affinity. As a consequence, the response is highly specific [5]. Except for this, activated B cells undergo massive proliferation in the germinal center and differentiate into memory B cells, late plasmablasts and plasma cells, which can be distinguished according to the expression of specific surface markers. Memory B cells reside in the lymphoid organ where they are rapidly activated upon a second encounter with the inducing antigen, whereas immunoglobulin-producing plasma cells leave the germinal center and migrate to the bone marrow to exert their significant functions [4].

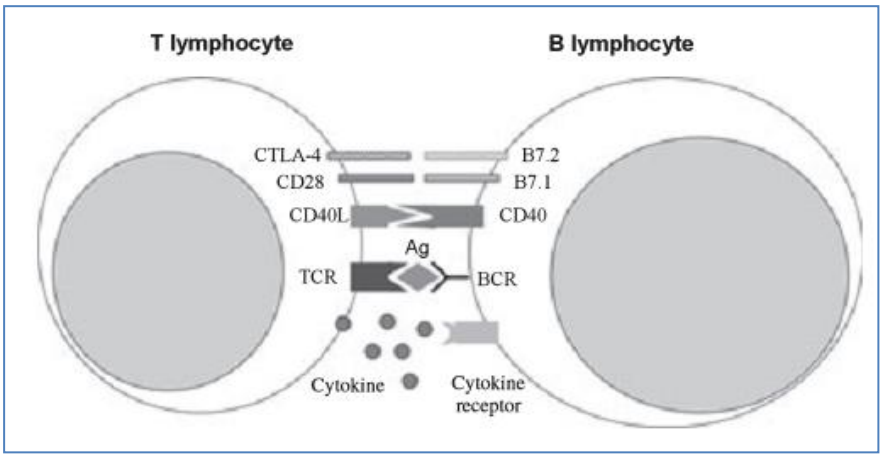


Figure 2 Co-stimulatory molecules involved in B cell-T cell interaction. Adapted from [4].

During B cell development, there are cytokines and chemical mediators with a pro-survival effect on B cells. First of all, IL-7, generated by bone marrow stromal cells, acts in favor of the proliferation of immature forms of B cells (pro-B cell). Next, BAFF (B cell Activating Factor) and APRIL (A Proliferation-Inducing Ligand) prevent apoptosis and influence class-switching of immunoglobulins in transitional and mature B cells by binding to three specific receptors on B cell surface, BAFF-receptor (BAFF-R), transmembrane activator and calcium-modulator and cyclophilin ligand interactor (TACI) and the B-cell maturation protein (BCMA) [4, 7-9].

A.2.2 B cell activation

Optimal B cell activation is based on B cell receptor (BcR) stimulation as well as costimulatory receptors and Toll-like receptors activation (Figure 3) [10]. Upon binding the antigen to its surface Ig, B cell receptor (BcR) is phosphorylated mainly by kinase Lyn on tyrosine residues (ITAMs - immunoreceptor tyrosine-based activation motifs). Adaptor molecules [Src homology 2 (SH_2) domain–containing proteins], such as Syk tyrosine kinase, are attached to the phosphorylated tyrosines via their SH_2 domain. This results in transphosphorylation and activation of Syk which leads to phoshorylation and activation of numerous targets, among which are B cell–specific adapter protein (BLNK) and phosphatidyl-inositol 3-kinase (PI3-K) [10-11]. This cascade is dependent on B cell receptor density, antigen affinity and valence, thus it has been proposed that at least 12 B cell receptors are needed for optimal signal transduction. Apart from this, CD19 is a coreceptor on B cell surface, especially on immature B cells. It belongs to Ig superfamily and it functions as a necessary BcR coreceptor so as to recruit important kinases (Lyn, PI3K) ending up in an amplification of BcR signaling [10,12].

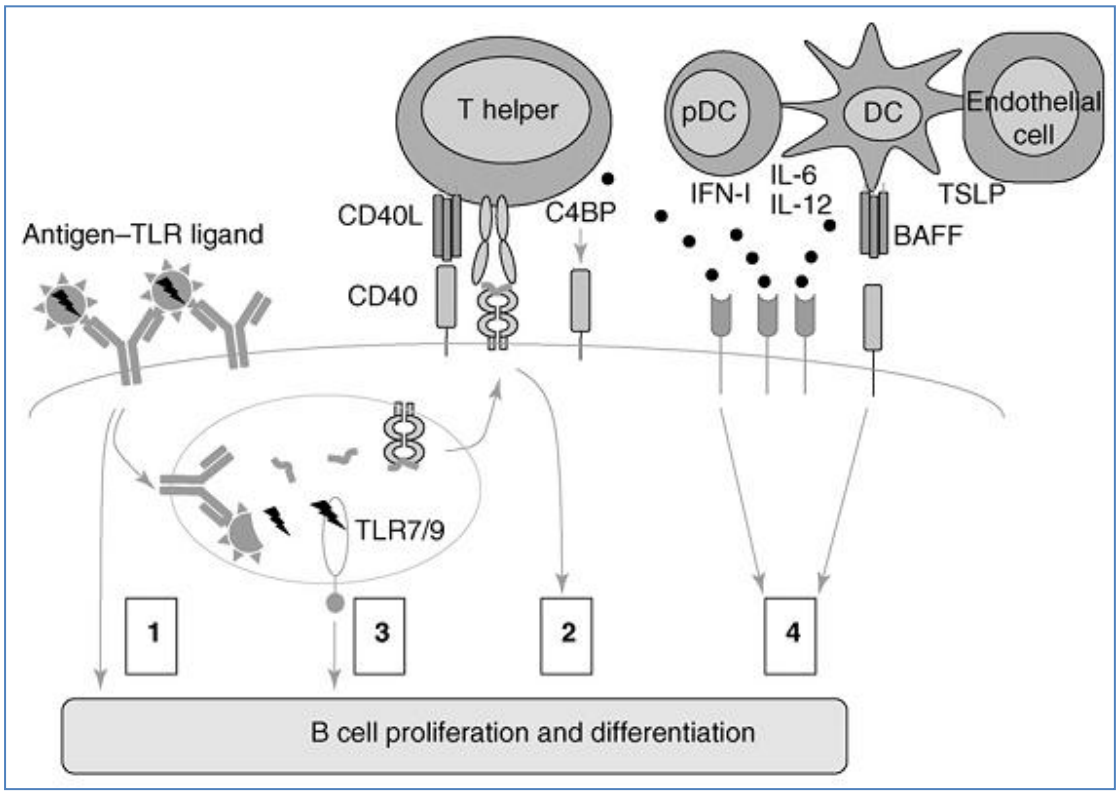


Figure 3 Four types of receptors are needed for optimal B cell activation. (1)B cell receptor, (2) Receptors for collaboration with effector T helper cells, (3) TLRs engagement and (4) Membrane receptors whose ligands are cytokines and survival factors. Adapted from [17].

Furthermore, specific receptors on B cell surface are also crucial for B cell development and function. In vivo trafficking of B cells and B cell survival are

maintained through important receptors, such as chemokine receptor CXCR5, BAFF-R and IL-6R [13]. Moreover, T cell-dependent B cell activation is based on interaction with T helper cells through CD40 and IL-4R (costimulatory receptors) [12, 14]. In addition, Toll-like receptors (TLRs) coupled to B cell receptor and conducting the detection of foreign antigens are widely considered as the third signal for optimal B cell activation. They also enhance proliferation, class switch recombination and differentiation into antibody-producing plasma cells, exerting a vital role both in thymus-dependent and in thymus-independent B cell responses [12, 15-21]. Their presence is so significant that B cells defective in TLR-signaling (MyD88-/-) exhibit a diminished antibody response compared to wild type B cells [22-23].

A.2.3 B cell negative immunomodulatory receptors

Except for the above-mentioned stimulatory receptors, whose coordinated function is the prerequisite for optimal activation, B cells also express a number of coinhibitory receptors which act as the necessary counterbalance in order to maintain B cell homeostasis. Coinhibitory receptors are transmembrane proteins, the majority of which belongs to the immunoglobulin superfamily (Figure 4). Their cytoplasmic domain contains several immunoreceptor tyrosine-based inhibitory motifs (ITIMs), which are phosphorylated by Lyn upon ligation to an immunoreceptor tyrosine-based activatory motif (ITAM) motif. Next, SH_2-containing adaptor molecules (inhibitory phosphatases) are bound with the aim of promoting downstream signaling and modulating cellular inhibition [10, 24-26].

First of all, FcγRIIb (CD32b) is the only Fc receptor expressed in B cells and has low affinity for the Fc part of IgG either in the form of immune complexes or bound to cell membranes [24, 27]. By blocking calcium-dependent signaling, it conducts its inhibitory effect through the B cell receptor. FcγRIIb also promotes apoptosis in the absence of B cell receptor signaling. This effect was shown to be significant in the process of affinity maturation, when low-affinity self-reactive B cells fail to be selected and are led to apoptosis [28]. Numerous studies have demonstrated that FcγRIIb-deficient mice are prone to autoimmunity (collagen-induced arthritis, Goodpasture syndrome, glomerulonephritis), proposing a vital role of this receptor in

the maintenance of peripheral tolerance [29-31].

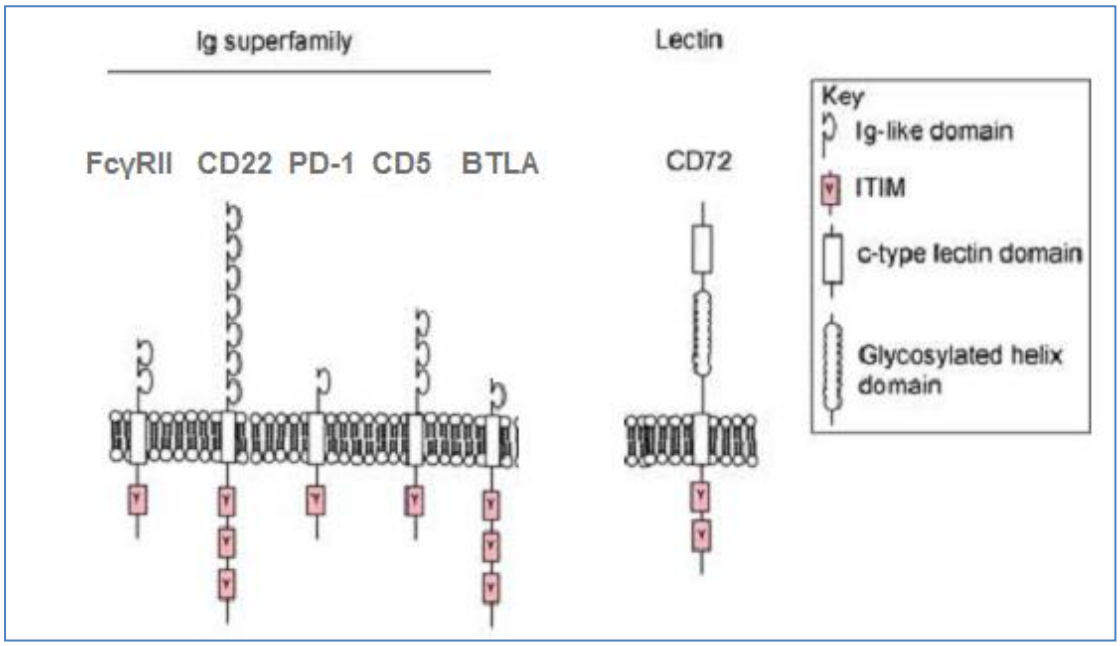


Figure 4 B cell inhibitory receptors. Adapted from [24].

Furthermore, CD22 is a B-cell specific inhibitory receptor, which acts so as to dampen B cell receptor signaling by impeding proximal events through inhibition of MAP kinases ERK2, JNK and p38 and suppression of calcium mobilization [24]. It also plays a role as a homing receptor for recirculating B cells and it is proposed to participate in the prevention of low-affinity of autoreactive B cells [32]. Its ablation in mice results in hyperactive B cells and excessive autoantibody production [33]. Except for this, CD5 is located on the cell surface of IgM-secreting B cells. Upon ligation with CD72, a C-type lectin, it mitigates B cell receptor signaling in order to maintain the activation threshold of B cells [24].

A.2.3.1 PD-1:PD-L1/PD-L2 pathway

Being a CD28 homolog, Programmed cell death protein 1 (PD-1, CD279) is also established as another inhibitory immunoreceptor [10, 24, 34]. It is expressed on B cells, T cells and myeloid cells, nevertheless, it is mainly upregulated upon activation [35]. By clustering with the B cell receptor upon B cell activation, it attenuates downstream signaling by abolishing BcR-mediated proliferation, calcium flux, IL-6 production and tyrosine phosphorylation of CD79b, Syk, PLCγ2 and Erk1/2 [36-37]. In a recent publication, PD-1 was demonstrated to be expressed primarily at naïve and

memory B cells and less at germinal center B cells, suggesting that PD-1 may act putatively to regulate the entry to the germinal center [36]. In addition, PD-1 is significant for B cell selection and survival in the germinal center and influences plasma cell differentiation [38]. Its importance in the maintenance of peripheral tolerance is noted by *in vivo* studies in which PD-1 deficient mice on different backgrounds developed autoimmune disorders (SLE-like glomerulonephritis, deforming arthritis, autoimmune dilated cardiomyopathy) [39-40].

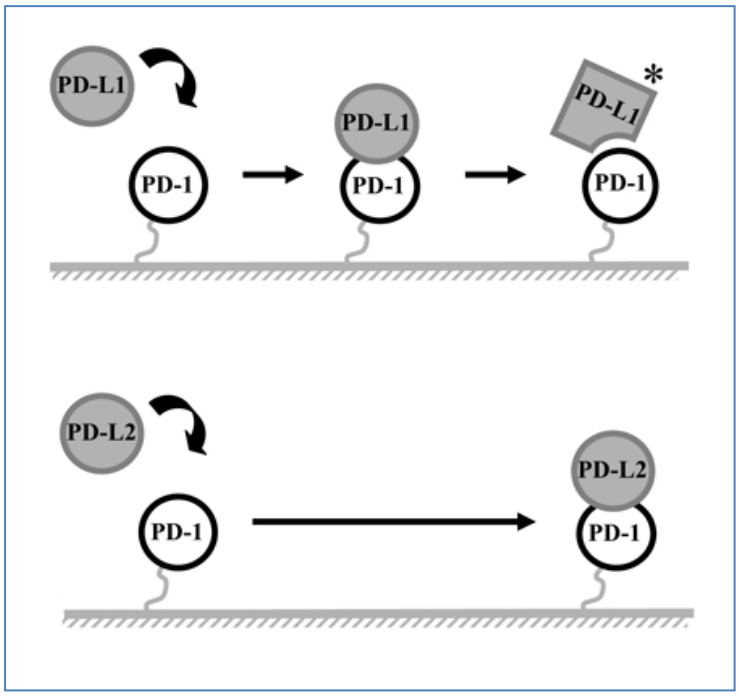


Figure 5 Binding to PD-1 requires conformational change of PD-L1 contrary to the direct interaction of PD-L2 with PD-1. Adapted from [41].

There have been identified two ligands for PD-1, Programmed cell death 1 ligand 1 (PD-L1, CD274) and Programmed cell death 1 ligand 2 (PD-L2, CD273), both of which are B7 homologs and members of the Ig superfamily [24, 34]. They both antagonize for PD-1 ligation, having differential molecular interaction (Figure 5) [41], however PD-L1 is also a ligand for CD80, through which bidirectional signaling is thought to take place [42]. PD-L1 is constitutively expressed on haemopoietic (B cells, T cells, dendritic cells, macrophages) as well as non-haemopoietic cells, whereas PD-L2 expression is inducible and is mainly limited to dendritic cells, macrophages, B1 B cells and memory B cells [43-44]. Upregulation of both ligands is dependent on the cytokine milieu. TNF-α, IL-10, IL-21 result in PD-L1 overexpression, IL-4 and granulocyte/macrophage colony-stimulating factor (GM-CSF) influence PD-L2 and IL-2, IL-7, IL-15 as well as interferons lead to elevated levels of expression of both ligands [45-46]. Specifically, in B cells, it was proven that both B cell receptor stimulation and TLR9 crosslinking with its ligand CpG oligodeoxynucleotides

provoke PD-L1 and PD-L2 upregulation [36]. As for their function, it is now becoming clear that the pathway PD-1:PD-L1/PD-L2 is vital for the establishment of peripheral tolerance, acting so as to regulate responses not only in B and T cells but also in non-haemopoietic tissue cells [44]. As if that was not enough, it has been elucidated that interplay between B cells expressing PD-L1 and PD-L2 and T cells expressing PD-1 mediates memory B cell and plasma cell formation, suggesting a putative effect on B cell biology as well [38].

A.2.3.2 BTLA:HVEM pathway

Another member of the Ig superfamily, B and T Lymphocyte Attenuator (BTLA, CD272), has been recently proposed as a negative regulator of immune cells activation. Its structure (Figure 4) bears great resemblance to the structure of other inhibitory immunoreceptors, having a single Ig-like extracellular domain and three tyrosine residues in its cytoplasmic tail, two of which are found within immunoreceptor tyrosine-based inhibitory motifs (ITIMs) [47-49]. For this reason, it was initially suggested to act as a suppressor, a hypothesis further supported by several studies [50-54]. Expression of BTLA is exclusive on immune cells and it can be constitutively found on the cell surface of B cells, T cells, Natural Killer (NK) cells, dendritic cells and splenic macrophages, however it is upregulated in anergic T cells, T follicular helper cells and resting peripheral B cells [47, 50, 52, 55-56]. A study performed on mice revealed that expression of BTLA reached a maximum at follicular B cells contrary to marginal zone and transitional B cells in the spleen and at mature naïve B cells contrary to immature forms of B cells in the bone marrow [55]. Additionally, the same study demonstrated that BTLA was induced upon BcR stimulation and LPS treatment [55].

While the other inhibitory immunoreceptors interact with members of the Ig superfamily, BTLA represents a unique coreceptor, due the fact that its ligand, Herpes Virus Entry Mediator (HVEM, TNFRSF14, CD270), is a member of the tumor necrosis factor receptor (TNFR) superfamily [57]. HVEM participates in a complex network with stimulatory and inhibitory ligands acting as a bidirectional switch for T cell activation (Figure 6) [58]. Except for BTLA, it also interacts with CD160, glycoprotein D (gD) , LIGHT (**L**ymphotoxin-like, **I**nducible expression, competes with herpes simplex virus **G**lycoprotein D for **H**VEM, a receptor expressed by **T**

lymphocytes) and lymphotoxin α ($LT\alpha_3$) [49, 59]. In terms of structure, HVEM consists of four extracellular cysteine-rich domains (CRDs) to which its ligands are attached leading to differential effect. Specifically, BTLA, CD160 and gD are bound to the distal CRD1 mediating an inhibitory effect, whereas LIGHT and $LT\alpha_3$ anchor to CRD2 and CRD3 resulting in stimulation [52, 60]. Despite the differential effect upon ligand binding, HVEM, altogether, negatively regulates T cell activation [49, 61].

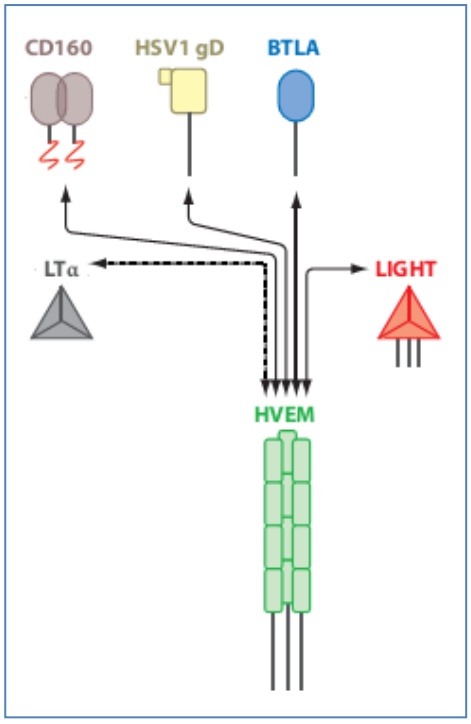


Figure 6 Network of HVEM with its ligands. Adapted from [47].

HVEM has a broad distribution in cells of the immune system (T cells, B cells, NK cells, dendritic cells) [49, 61]. It is upregulated in naïve T cells but its levels decrease as cells become activated only to increase again at the state of quiescence. Contrary to this, regulatory T cells ($CD4^+$ $FoxP3^+$) exhibit an increase in HVEM expression upon activation. Higher levels of HVEM are detected in naïve and memory B cells [47, 62].

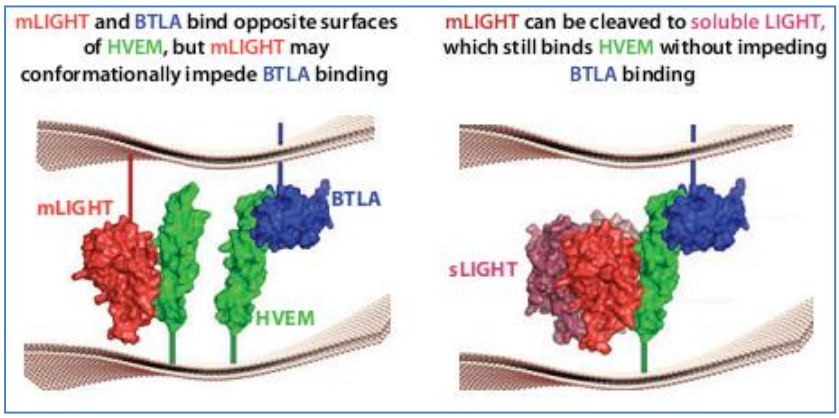


Figure 7 HVEM-BTLA-LIGHT ternary complex. Adapted from [47].

Focusing on BTLA cross linking with HVEM, elucidation of the crystal structure of BTLA-HVEM complex revealed 1:1 stoichiometry [63]. Apart from this, BTLA was bound at the opposite site of LIGHT binding domain creating a ternary complex. In more detail, BTLA binding was accelerated once soluble LIGHT was attached to HVEM, nevertheless membrane LIGHT abrogates BTLA binding noncompetitively. As a consequence, protease cleavage of LIGHT seems to be a regulatory mechanism, in which inhibition by BTLA is abolished by membrane LIGHT and reestablished when LIGHT becomes soluble (Figure 7) [64].

As for downstream signaling through BTLA and HVEM, there is evidence that it can be bidirectional (Figure 8) [47, 61]. It was previously referred that BTLA contains three tyrosine residues, 2 of which are found within ITIMs. Upon BTLA activation, ITIMs recruit SH_2 domain–containing protein tyrosine phosphatase (SHP-1/2) facilitating an inhibitory function for BTLA [52, 65-66]. However the proximal tyrosine was estimated by mass spectrometry to be able to bind to Grb-2 associating with the p85 subunit of phosphatidylinositol 3-kinase (PI3K) resulting in a prosurvival effect [67-68].

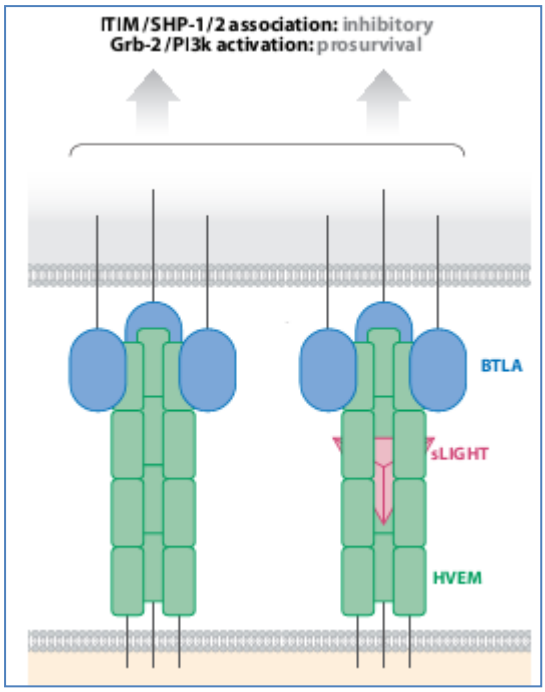


Figure 8 Bidirectional signaling between BTLA and HVEM. Adapted from [47].

In an effort to clarify the role of BTLA in practice, functional studies were performed, which are congruent with a suppressive function of BTLA. BTLA cross linking with HVEM resulted in significant reduction in cytokine production from T cells (IL-2, IL-10, IFNγ) and in attenuation in T cell proliferation [51-53]. In B cells, HVEM stimulation of BTLA decreased the activity of protein tyrosine kinase, Syk and, thus, suppressed the phosphorylation of downstream molecules [B cell linker protein (BLNK), phospholipase Cγ2) and NF-κB nuclear translocation, confirming a role as a negative regulator of B cell activation [54]. This role is further promoted by *in vivo* studies, in which BTLA -/- mice were more susceptible in producing autoantibodies and in developing autoimmune hepatitis-like disease with advancing age [69]. Furthermore, BTLA -/- mice exhibited greater sensitivity in the induction of experimental autoimmune encephalomyelitis (EAE) and enhanced airway inflammation [70-71]. In parallel, HVEM -/- mice were prone to ConA-induced autoimmune hepatitis and induced experimental autoimmune encephalomyelitis (EAE), consistently with HVEM's function as BTLA ligand [72]. Together, these data are convergent with a function for BTLA in favor of the maintenance of the peripheral tolerance and the prevention of autoimmunity.

A.3 Systemic Lupus Erythematosus

Systemic Lupus Erythematosus (SLE) is a systemic autoimmune disorder characterized by a broad variety of clinical manifestations and existence of high-titer of auto-antibodies [73]. The rate of SLE is dependent on country, ethnicity, gender, and changes over time [74]. In a survey performed in central Greece in 2010, its prevalence was estimated at 0.11% [75]. SLE occurs predominantly on women (ratio 9:1), frequently starting at childbearing age. This gender bias has been attributed to estrogens which, upon ligation to estrogen receptor α, mediate immunostimulatory effects and to the fact that the X chromosome contains loci of immunologic interest [76]. Diagnosis is based both on clinical signs and laboratory evidence. In 1982, the American College of Rheumatologists (ACR) established diagnostic criteria being revised in 1997. Presence of 4 out of 11 ACR criteria was estimated to yield a sensitivity of 85% and a specificity of 95% for SLE (Table 1) [77].

CRITERIA	DESCRIPTION
1. Malar rash	Fixed erythema, flat or raised, over the malar eminences, tending to spare the nasolabial folds
2. Discoid rash	Erythematous raised patches with adherent keratotic scaling and follicular plugging; atrophic scarring may occur in older lesions
3. Photosensitivity	Skin rash as a result of unusual reaction to sunlight, by patient history or physician observation
4. Oral ulcers	Oral or nasopharyngeal ulceration, usually painless, observed by physician
5. Non erosive arthritis	Involving 2 or more peripheral joints, characterized by tenderness, swelling, or effusion
6. Pleuritis or pericarditis	1. Pleuritis--convincing history of pleuritic pain or rubbing heard by a physician or evidence of pleural effusion ***OR*** Pericarditis--documented by electrocardigram or rub or evidence of pericardial effusion
7. Renal disorder	1. Persistent proteinuria > 0.5 grams per day or > than 3+ if quantitation not performed ***OR*** 2. Cellular casts--may be red cell, hemoglobin, granular, tubular, or mixed

8. Neurologic disorder	1. Seizures--in the absence of offending drugs or known metabolic derangements; e.g., uremia, ketoacidosis, or electrolyte imbalance ***OR*** 2. Psychosis--in the absence of offending drugs or known metabolic derangements, e.g., uremia, ketoacidosis, or electrolyte imbalance
9. Hematologic disorder	1. Hemolytic anemia--with reticulocytosis ***OR*** 2. Leukopenia--< 4,000/mm^3 on ≥ 2 occasions ***OR*** 3. Lymphopenia--< 1,500/ mm^3 on ≥ 2 occasions ***OR*** 4. Thrombocytopenia--<100,000/ mm^3 in the absence of offending drugs
10. Immunologic disorder	1. Anti-DNA: antibody to native DNA in abnormal titer ***OR*** 2. Anti-Sm: presence of antibody to Sm nuclear antigen ***OR*** 3. Positive finding of antiphospholipid antibodies on: 1. an abnormal serum level of IgG or IgM anticardiolipin antibodies, 2. a positive test result for lupus anticoagulant using a standard method, or 3. a false-positive test result for at least 6 months confirmed by Treponema pallidum immobilization or fluorescent treponemal antibody absorption test
11. Positive antinuclear antibody	An abnormal titer of antinuclear antibody by immunofluorescence or an equivalent assay at any point in time and in the absence of drugs

Table 1 1997 Update of the 1982 American College of Rheumatology revised criteria for classification of systemic lupus erythematosus [77].

SLE activity is assessed by indices created by groups of experienced clinicians with the ultimate aim of objectively measuring disease outcome (disease activity, damage from the disease, health status). A weighted, cumulative index, more

commonly used in clinical trials, is SLEDAI (Systemic Lupus Erythematosus Disease Activity Index). SLE activity is defined as the total sum of weights present at the time of examination (Table 2) [78].

WT	DESCRIPTOR	DEFINITION
8	**Seizure**	Recent onset. Exclude metabolic, infectious or drug cause.
8	**Psychosis**	Altered ability to function in normal activity due to severe disturbance in the perception of reality. Include hallucinations, incoherence, marked loose associations, impoverished thought content, marked illogical thinking, bizarre, disorganized, or catatonic behavior. Exclude uremia and drug causes.
8	**Organic Brain Syndrome**	Altered mental function with impaired orientation, memory or other intelligent function, with rapid onset fluctuating clinical features. Include clouding of consciousness with reduced capacity to focus, and inability to sustain attention to environment, plus at least two of the following: perceptual disturbance, incoherent speech, insomnia or daytime drowsiness, or increased or decreased psychomotor activity. Exclude metabolic, infectious or drug causes.
8	**Visual Disturbance**	Retinal changes of SLE. Include cytoid bodies, retinal hemorrhages, serious exudate or hemorrhages in the choroids, or optic neuritis. Exclude hypertension, infection, or drug causes.
8	**Cranial Nerve Disorder**	New onset of sensory or motor neuropathy involving cranial nerves.
8	**Lupus Headache**	Severe persistent headache: may be migrainous, but must be nonresponsive to narcotic analgesia.
8	**CerebroVascular Accident**	New onset of cerebrovascular accident(s). Exclude arteriosclerosis.
8	**Vasculitis**	Ulceration, gangrene, tender finger nodules, periungual,

WT	DESCRIPTOR	DEFINITION
		infarction, splinter hemorrhages, or biopsy or angiogram proof of vasculitis.
4	**Arthritis**	More than 2 joints with pain and signs of inflammation (i.e. tenderness, swelling, or effusion).
4	**Myositis**	Proximal muscle aching/weakness, associated with elevated creatine phosphokinase/adolase or electromyogram changes or a biopsy showing myositis.
4	**Urinary Casts**	Heme-granular or red blood cell casts.
4	**Hematuria**	>5 red blood cells/high power field. Exclude stone, infection or other cause.
4	**Proteinuria**	>0.5 gm/24 hours. New onset or recent increase of more than 0.5 gm/24 hours.
4	**Pyuria**	>5 white blood cells/high power field. Exclude infection.
2	**New Rash**	New onset or recurrence of inflammatory type rash.
2	**Alopecia**	New onset or recurrence of abnormal, patchy or diffuse loss of hair.
2	**Mucosal Ulcers**	New onset or recurrence of oral or nasal ulcerations.
2	**Pleurisy**	Pleuritic chest pain with pleural rub or effusion, or pleural thickening.
2	**Pericarditis**	Pericardial pain with at least 1 of the following: rub, effusion, or electrocardiogram confirmation.
2	**Low Complement**	Decrease in CH50, C3, or C4 below the lower limit of

WT	DESCRIPTOR	DEFINITION
		normal for testing laboratory.
2	**Increased DNA binding**	>25% binding by Farr assay or above normal range for testing laboratory.
1	**Fever**	>38°C. Exclude infectious cause.
1	**Thrombocytopenia**	<100,000 platelets/mm^3.
1	**Leukopenia**	<3,000 white blood cell/mm^3. Exclude drug causes.

Table 2 SLE disease activity index [78].

With the aim of casting light on SLE pathogenesis and studying its manifestations, models of spontaneous lupus have been developed. The classic model of lupus is the F1 hybrid of New Zealand Black (NZB) and New Zealand White (NZW) strains (NZB/W F1). There also exist its derivatives (NZM2328, NZM2410, MRL/lpr and BXSB/Yaa strains). Studies from these models provide the main source of information concerning cellular and genetic mechanisms resulting in SLE induction [79].

A.3.1 Cellular and molecular pathophysiology of SLE

SLE is considered as a heterogeneous group of diseases, in which genetically predisposed individuals are influenced by environmental stimuli contributing to initiation and perpetuation of the disease. Innate and adaptive immune systems are activated leading to loss of peripheral tolerance as well as the production of autoreactive clones of B and T lymphocytes. Progression and amplification of these procedures result in systemic inflammation, onset of clinical manifestation and target organ damage (Figure 9) [73].

There are three leading immune pathways in the pathogenesis of SLE: defective removal of self-antigens, aberrant innate immune cells activation and expansion of autoreactive B and T lymphocytes (Figure 10) [73, 79-80]. The hallmark of SLE onset is the presence of an overload of nucleic-acid containing cell debris and immune complexes due to defects in apoptotic mechanisms and failure in clearance of apoptotic particles [81-83]. Instead of being phagocytosed normally by macrophages, these remnants stimulate Fc receptors and nucleic-acid specific Toll-like receptors (TLR3, TLR7, TLR8, TLR9) of cells of the innate immune system (plasmacytoid dendritic cells, neutrophils, myeloid dendritic cells), resulting in production of proinflammatory cytokines. Consequently, TLRs ligation in plasmacytoid dendritic cells results in the transcription and release of excessive amounts of interferon α (IFNα) which leads to a further stimulation of innate and adaptive immunity and to endothelial damage [84-86]. In parallel, neutrophils in contact with nucleic-acid containing immunocomplexes become more vulnerable in the formation of neutrophil extracellular traps (NETs) consisting of DNA decorated with small alarmin peptides (cathelicidin LL37). This LL37-DNA complex activates plasmacytoid dendritic cells, further promoting IFNα production [87-88]. Apart from this, TLR stimulation in myeloid dendritic cells redounds to the production of several proinflammatory cytokines (IL-6, IL-12, TNFα, BAFF) bridging innate and adaptive immunity [79].

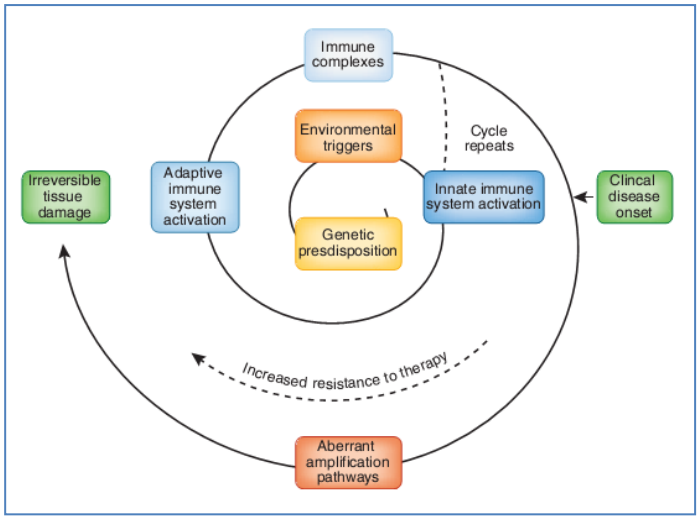


Figure 9 The spiral of disease progression in SLE. Adapted from [73].

In spite of the normal presence of protective tolerogenic mechanisms in lymphocytes (deletion, anergy, receptor editing, suppression by regulatory molecules or cells), defects in peripheral tolerance are the culprits for the aberrant expansion of autoreactive clones of B and T cells [73]. Excessive MHC presentation of peptides, IL-17 production and elevated expression of co-stimulatory and adhesion molecules (CD40 ligand, CD44) result in a lower threshold for T cell activation. Abnormal T cell activation as well as expansion and dysregulation of specific T cell subsets

(follicular T helper cells) support exorbitant B cell proliferation and differentiation into long-lived autoreactive plasma cells leading eventually to the production of high titers of auto-antibodies, the cornerstone of SLE pathogenesis [73, 79-80].

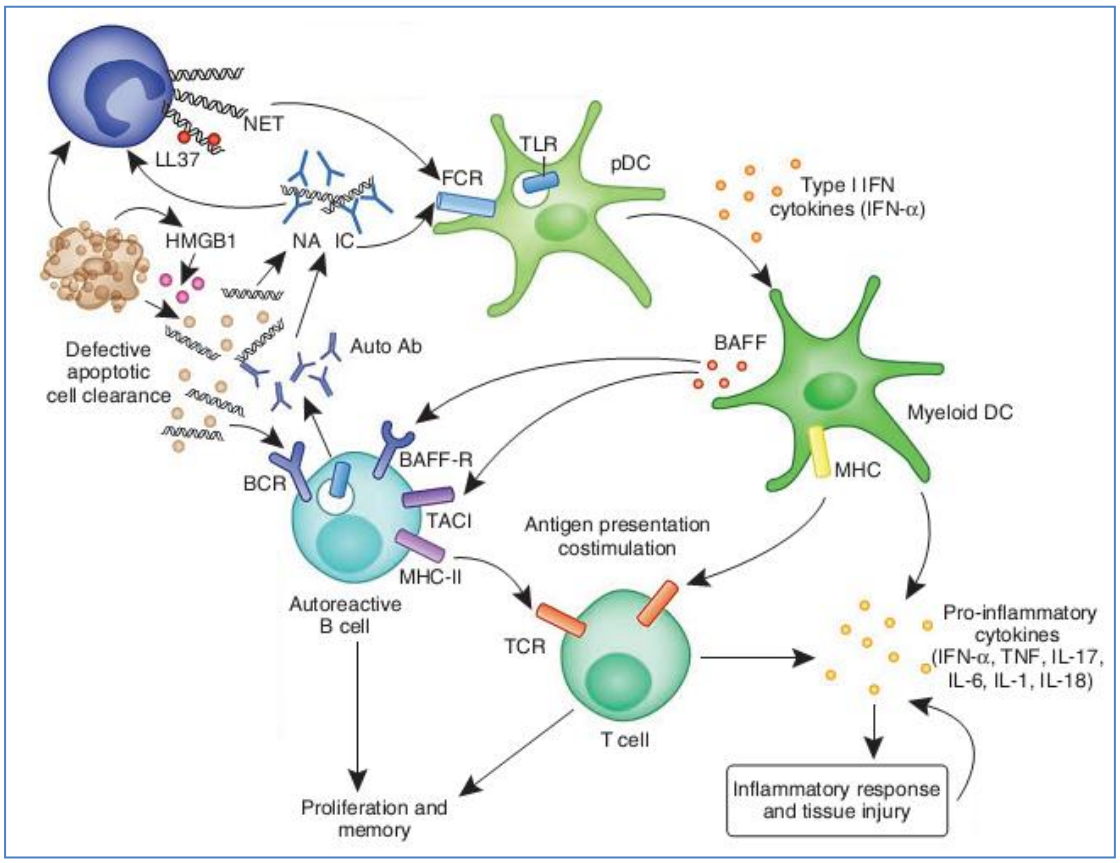


Figure 10 Excess in nucleic-acid containing immunocomplexes leads to aberrant activation of innate and adaptive immune system. Adapted from [73].

A.3.2 The role of B cells in SLE pathogenesis

Attempts to delineate the mechanisms responsible for SLE have placed aberrancies in B cell development, activation and differentiation in the centre of its pathophysiology [89-91]. The current view is that SLE progresses on the ground of polyclonal B cell hyperactivity prompted by abnormal intrinsic properties of B cells, defective immunoregulatory mechanisms and cytokine production as well as effects of an overactive inflammatory environment (overactive or ectopic germinal centers) [91].

First of all, there are genetic studies demonstrating a number of candidate genes associated with SLE and predisposing to a lower activation threshold of B cells [91, 92]. Signaling networks involving PI3K/AKT/mTOR, MEK1/Erk1/2, p38, NF-κB,

Bcl-2 family members and cell-cycle molecules have been found to be overexpressed in B cell from patients with SLE, suggesting that alterations of B cell on cellular level may be the underlying cause for abnormal B cell function [93].

Secondly, frequencies and properties of specific B cell subsets are reported to be disturbed in SLE patients [90-91]. Expansion of memory B cells and plasma cells combined with a decline in naïve B cells is a common finding in active SLE [90]. SLE memory B cells and plasma cells demonstrate significant downregulation of the inhibitory receptor, FcγRIIb, suggesting defects in the regulation of their activation [94-95]. Importantly, SLE activity correlated with increased numbers of circulating plasma cells, antigen-experienced $CD27^{+}IgD^{-}$ memory cells, $CD27^{-}$ IgD^{-} memory cells and $CD27^{-}$ IgD^{-} $CD95^{+}$ memory B cells. $CD27^{-}$ IgD^{-} $CD95^{+}$ population represented a more homogeneous group, in which expression of CD95 (Fas) implied that Fas/FasL interaction may be defective in the germinal center in SLE [96]. Functionally, SLE memory B cells are characterized by abnormal peripheral tolerance (altered selection and receptor editing), excessive somatic hypermutation as well as capacity to act as professional antigen-presenting cells [91, 97-98].

Except for this, regulatory $CD19^{+}CD24^{hi}CD38^{hi}$ B cells, normally producing IL-10 and functioning so as to suppress Th1 and Th2 responses after CD80/CD86 stimulation and CD40/CD40L ligation, exhibited defects in their regulatory capacity and in IL-10 production in SLE patients [99].

Lack of effective central and peripheral checkpoints of tolerance has been proposed as responsible for the existence of autoreactive B cells, however, autoreactive B cells can also be produced *de novo* by somatic hypermutation, as there are studies providing evidence that somatic hypermutation is a prerequisite for the formation of disease-associated anti-nuclear antibodies (ANAs) [91, 100]. Last but not least, activation of T follicular helper cells as well as availability of IL-6, IL-21, CXCL13, TNFβ, and inducible T-cell co-stimulator (ICOS), factors critical for establishing a germinal center, lead to an enhanced germinal center activity and generation of ectopic germinal centers, which provide the essential conditions not only for T-dependent but also for T-independent B cell proliferation and differentiation into long-lived autoantibody-producing plasma cells [90-91]. Thus, multiple abnormalities of B cell development and activation combined with a constitutively hyperactive immune system, loss of tolerance and altered cytokine

profile may create the basis on which autoantibodies against multiple targets are produced resulting in the irreversible peripheral tissue damage featuring SLE.

A.4 Study hypothesis

Research on the cellular and molecular procedures leading to SLE development has led to the conclusion that activated B cells represent a crucial part of its pathophysiology not only owing to their auto-antibody producing capability but also due to their immunostimulatory properties. As a consequence it would be of utmost importance to elucidate the mechanisms and the receptors involved in B cell negative immunomodulation with the ultimate aim of modifying their function so as to suppress B cell activation in favor of attenuating SLE manifestations and pathogenesis.

Thus, main objective of this study was to examine expression and function of the negative immunomodulatory receptors BTLA, PD-1 and PD-L1 in B cells in SLE patients and matched healthy controls. In this context, baseline expression of BTLA, PD-1 and PD-L1 in peripheral blood B cell subsets as well as baseline expression of HVEM in peripheral blood T cell subsets were investigated. Apart from this, regulation of expression of BTLA, PD-1 and PD-L1 in B cells under the effect of cytokines, interferons, and TLR ligands was estimated. Last, functional studies in B cells concerning expression of costimulatory molecules, proliferation, differentiation into plasma cells and IL-6 production were performed under the effect of BTLA ligand, HVEM.

B. PATIENTS, MATERIALS AND METHODS

B.1 Patients

Sixteen (16) active SLE patients diagnosed according to the American College of Rheumatology 1982 criteria and followed up at the Rheumatology Department of the University Hospital of Heraklion, Crete were studied. Active SLE was defined as an SLE Disease Activity Index score (SLEDAI) higher than 8. Patients had not received steroids for at least 24 hours before blood sampling. Healthy age- and sex-matched volunteers from the Department of Transfusion Medicine of the University Hospital of Heraklion, Crete served as controls. All subjects gave written informed consent prior to study.

B.2 Materials and Methods

B.2.1 Peripheral Blood Mononuclear Cells isolation

a. Reagents and Instruments

1. Ficoll-Paque™ PLUS (GE Healthcare Life Sciences, Uppsala, Sweden)
2. Phosphate-buffered Saline (PBS)
3. Trypan Blue (Gibco, Life Technologies, Carlsbad, California, USA)
4. Pasteur pipettes (Sarstedt, Nuembrecht, Germany)
5. Neubauer haemocytometer (Assistent, Germany)
6. Light microscope (Nikon TMS, Tokyo, Japan)

b. Method

Peripheral blood mononuclear cells were isolated from human peripheral blood by Ficoll-Histopaque density gradient centrifugation. Peripheral blood from healthy volunteers or SLE patients, no older than 8 hours and supplemented with anticoagulants (heparin or EDTA), was diluted with 1 volume PBS. The diluted blood was overlayed onto Ficoll-Histopaque (dilution 1:2) and was centrifuged at 1800 rpm, 30 minutes, 22°C with no brake. The mononuclear cell layer (white interphase) was aspirated with a Pasteur pipette and was washed twice with PBS (1500 rpm for 10 min centrifugation). After resuspending the cell pellet in 5 ml

PBS, a volume of cells was diluted with Trypan Blue and placed onto Neubauer haemocytometer so as to be counted by light microscopy.

B.2.2 B cells magnetic separation

a. Reagents and Instruments

1. MACS buffer (PBS, pH 7.2, 0.5% bovine serum albumin (BSA), and 2 mM EDTA by diluting MACS BSA Stock Solution 1:20 with AutoMACS™ Rinsing Solution)
2. CD19 Microbeads (Microbeads conjugated to monoclonal anti-human CD19 antibodies) (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany)
3. Trypan Blue (Gibco, Life Technologies, Carlsbad, California, USA)
4. MACS MS columns and plungers (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany)
5. MiniMACS™ Separator (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany)
6. Neubauer haemocytometer (Assistent, Germany)
7. Light microscope (Nikon TMS, Tokyo, Japan)

b. Method

Isolated peripheral blood mononuclear cells, diluted in MACS buffer, were incubated with appropriate volume of CD19 Microbeads for 15 minutes in the refridgerator (2-8°C). After undergoing one wash with MACS buffer, the cells were resuspended with 500 μl MACS buffer and the suspension was applied onto a MACS MS column, properly placed in the magnetic field of a MiniMACS™ Separator. 3 washes with 500 μl MACS buffer were performed prior to the removal of the column from the separator. The column was then placed into a suitable collection tube and 1 ml MACS buffer was added onto the column. The magnetically labeled CD19^{+} B cells were flushed out by firmly pushing the plunger into the column. B cells were mixed with Trypan Blue and the suspension was placed onto Neubauer haemocytometer in order to be counted by light microscopy. Purity (B cells/total separated live cells) was evaluated by flow cytometry.

B.2.3 B cell cultures

a. Cultures for evaluating the expression of receptors under the effect of different stimuli

i. Reagents and Instruments

1. RPMI medium 1640 (Gibco, Life Technologies, Carlsbad, California, USA)
2. Fetal Bovine Serum (FBS) (Gibco, Life Technologies, Carlsbad, California, USA)
3. Penicillin/Streptomycin Solution 100x (Biosera, Boussens, France)
4. HEPES Buffer Solution (1M) (Gibco, Life Technologies, Carlsbad, California, USA)
5. Sodium Pyruvate (100 mM) (Gibco, Life Technologies, Carlsbad, California, USA)
6. Phosphate-buffered Saline (PBS)
7. AffiniPure $F(ab')_2$ Fragment Goat Anti-Human IgG + IgM (H+L) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA)
8. CpG ODN2006 (InvivoGen, San Diego, California, USA)
9. Recombinant Human IFNγ (PeproTech, Rocky Hill, NJ, USA)
10. Recombinant Human IL-4 (PeproTech, Rocky Hill, NJ, USA)
11. Recombinant Human IL-10 (PeproTech, Rocky Hill, NJ, USA)
12. Recombinant human BAFF (PeproTech, Rocky Hill, NJ, USA)
13. Anti-human CD40 Functional Grade Purified (eBioscience, Inc., San Diego, California, USA)
14. *E.coli* LPS (InvivoGen, San Diego, California, USA)
15. Zymosan (Cell wall from *Saccharomyces cerevisiae*-TLR2 ligand) (InvivoGen, San Diego, California, USA)
16. Poly I:C (polyinosinic:polycytidylic acid) (Sigma, Saint Louis, Missouri, USA)
17. Gardiquimod™ (Imidazoquinoline compound-TLR7 ligand) (InvivoGen, San Diego, California, USA)
18. Costar® Corning® 96-well cell culture clusters, round-bottomed (Corning Inc., NY, USA)

ii. Method

Purified B cells at a concentration of 100.000 cells/well were cultured in duplicate in 96-well round-bottomed plates in RPMI-1640, supplemented with 10% heat-inactivated FBS, 100 IU/mL penicillin, 100μg/mL streptomycin, 10 mM HEPES and 1 mM sodium pyruvate. Cells were stimulated either with 5 μg/ml goat $F(ab')_2$ anti-human IgM combined with CpG ODN2006 (0,1 μM and 5 μM), IFNγ (50 ng/ml), IL-4 (50 ng/ml), IL-10 (50 ng/ml), IL-21 (50 ng/ml), BAFF (500 ng/ml), LPS (100 ng/ml), zymosan (500 ng/ml), polyI:C (500 mg/ml) and gardiquimod (500 ng/ml) or with 5 μg/ml goat $F(ab')_2$ anti-human IgM, CpG ODN2006 (5 μM), IFNγ (50 ng/ml), IL-4 (50 ng/ml), LPS (100 ng/ml) and anti-CD40 (1 μg/ml) as separate stimuli. Incubation lasted for 2 days at 37°C and 5% CO_2.

b. Cultures for evaluating the expression of receptors under the effect of sera

i. Reagents and Instruments

1. RPMI medium 1640 (Gibco, Life Technologies, Carlsbad, California, USA)
2. Fetal Bovine Serum (FBS) (Gibco, Life Technologies, Carlsbad, California, USA)
3. Penicillin/Streptomycin Solution 100x (Biosera, Boussens, France)
4. HEPES Buffer Solution (1M) (Gibco, Life Technologies, Carlsbad, California, USA)
5. Sodium Pyruvate (100 mM) (Gibco, Life Technologies, Carlsbad, California, USA)
6. Phosphate-buffered Saline (PBS)
7. AffiniPure $F(ab')_2$ Fragment Goat Anti-Human IgG + IgM (H+L) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA)
8. Blood collection tubes, supplemented with clot activator and gel for serum separation
9. Costar® Corning® 96-well cell culture clusters, round-bottomed (Corning Inc., NY, USA)

ii. Method

Peripheral blood from healthy volunteers and SLE patients was placed in blood collection tubes, supplemented with clot activator and gel for serum separation, and left to coagulate for 25-30 minutes. Next, coagulated blood was centrifuged at 2.500 rpm for 15 minutes at room temperature and the yellow upper phase containing the serum was aspirated, aliquoted and stored at -80°C. Purified B cells at a concentration of 100.000 cells/well were cultured in duplicate in 96-well round-bottomed plates in RPMI-1640, supplemented with 100 IU/mL penicillin, 100μg/mL streptomycin, 10 mM HEPES and 1 mM sodium pyruvate. Cells were also supplemented with 20% heat-inactivated FBS, 5 μg/ml goat F(ab')$_2$ anti-human IgM, 20% serum from healthy volunteers and 20% serum from SLE patients at various combinations. Incubation lasted for 2 days at 37°C and 5% CO_2.

c. Cultures for evaluating the effect of BTLA ligand, HVEM, on B cell functions

i. Reagents and Instruments

1. RPMI medium 1640 (Gibco, Life Technologies, Carlsbad, California, USA)
2. Fetal Bovine Serum (FBS) (Gibco, Life Technologies, Carlsbad, California, USA)
3. Penicillin/Streptomycin Solution 100x (Biosera, Boussens, France)
4. HEPES Buffer Solution (1M) (Gibco, Life Technologies, Carlsbad, California, USA)
5. Sodium Pyruvate (100 mM) (Gibco, Life Technologies, Carlsbad, California, USA)
6. Phosphate-buffered Saline (PBS)
7. AffiniPure F(ab')$_2$ Fragment Goat Anti-Human IgG + IgM (H+L) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA)
8. Anti-human CD40 Functional Grade Purified (eBioscience, Inc., San Diego, California, USA)
9. Recombinant Human HVEM/Fc Chimera (R&D Systems, Inc., Minneapolis, MN, USA)
10. Mouse IgG2a K Isotype Control Functional Grade Purified (eBioscience, Inc., San Diego, California, USA)

11. Costar® Corning® 96-well cell culture clusters, round-bottomed (Corning Inc., NY, USA)

ii. Method

Purified B cells at a concentration of 100.000 cells/well were cultured in duplicate in 96-well round-bottomed plates in RPMI-1640, supplemented with 10% heat-inactivated FBS, 100 IU/mL penicillin, 100μg/mL streptomycin, 10 mM HEPES and 1 mM sodium pyruvate. Plates were initially coated with recombinant human HVEM Fc chimera at a low (2 μg/ml) and at a high (10 μg/ml) concentration or with equal amount of mouse IgG2a K Isotype at 37°C for 2 hours. After the plates have undergone 2 washes with PBS, purified B cells were placed in the wells, stimulated with 5 μg/ml goat F(ab')$_2$ anti-human IgM and incubated for 2 days at 37°C and 5% CO_2. Additionally, purified B cells, stained with CFSE, were placed in the wells, stimulated with 5 μg/ml goat F(ab')$_2$ anti-human IgM combined with 1 μg/ml anti-CD40 and incubated for 3 days at 37°C and 5% CO_2.

B.2.4 CFSE proliferation assay for B cells

a. Reagents and Instruments

1. Phosphate-buffered Saline (PBS)
2. Fetal Bovine Serum (FBS) (Gibco, Life Technologies, Carlsbad, California, USA)
3. Bovine Serum Albumin Fraction V (7,5%) (Gibco, Life Technologies, Carlsbad, California, USA)
4. CellTrace™ CFSE Cell Proliferation Kit (Invitrogen, Carlsbad, California, USA)

b. Method

Purified B cells were resuspended in 1 ml pre-warmed PBS/BSA 0.1% /maximum 5 x 10^6 B cells in a 15-ml falcon tube. In this tube, 1 μl of CFSE(5 mM)/10^6 B cells in a drop of 100 μl PBS was added. The tube was covered immediately with foil and the cell suspension was mixed vigorously. After incubating the cells at 37°C for 10 minutes, 5 ml ice-cold PBS/FBS 5% were added and the cells were centrifuged at 1400 rpm for 10 minutes at room

temperature. Cells were resuspended in complete medium in suitable volume and added to the culture.

B.2.5 Flow cytometry

a. Reagents and Instruments

1. Phosphate-buffered Saline (PBS)
2. Fetal Bovine Serum (FBS) (Gibco, Life Technologies, Carlsbad, California, USA)
3. Monoclonal anti-human antibodies
   a. Fluorescein isothiocyanate (FITC)-conjugated CD20, Peridinin Chlorophyll Protein complex (PerCP)-conjugated CD19, phycoerythrin (PE)-conjugated BTLA, fluorescein isothiocyanate (FITC)-conjugated CD25, phycoerythrin (PE)-conjugated HVEM (Biolegend, San Diego, California, USA)
   b. PE-Cy7-conjugated CD27, phycoerythrin (PE)-conjugated PD-L1, phycoerythrin (PE)-conjugated CD80, phycoerythrin (PE)-conjugated CD86, fluorescein isothiocyanate (FITC)-conjugated PD-1, phycoerythrin (PE)-conjugated BAFF-R, fluorescein isothiocyanate (FITC)-conjugated IgG2a Isotype Control (eBioscience, Inc., San Diego, California, USA)
   c. Fluorescein isothiocyanate (FITC)-conjugated IgD, phycoerythrin (PE)-conjugated PD-1 (BD Pharmingen, San Diego, California, USA)
   d. PE-Cy5-conjugated CD4, PE-Cy5-conjugated CD38, PE-Cy5-conjugated IgG1, phycoerythrin (PE)-conjugated IgG1 (Beckmann Coulter, Brea, California, USA)
4. Flow cytometer (Cytomics FC 500 model) (Beckmann Coulter, Brea, California, USA)

b. Method

Cells (either freshly isolated PBMCs or B cells from culture well plates) were washed once with PBS by undergoing centrifugation at 1400 rpm for 10 minutes at room temperature. Next, they were stained with appropriate volume of monoclonal anti-human antibodies according to the manufacturer's protocol in different combinations depending on the experimental settings. After incubating the cells for 20 minutes at room temperature in the dark, the cells were washed

with 1 ml PBS/FBS 5% and centrifuged at 1400 rpm for 10 minutes at room temperature. Then, the cells were subjected to flow cytometry on a Cytomics FC 500 flow cytometer. Analysis was performed with FCS Express V3 and GraphPad Prism 5 software.

B.2.6 RNA isolation from B cells

a. Reagents and Instruments

1. PureLink® RNA Mini Kit [containing Spin Cartridges (with collection tubes), Lysis Buffer, Wash Buffer I, Wash Buffer II, RNase–free Water)] (Ambion, Life Technologies, Carlsbad, California, USA)
2. 70% ethanol
3. Nanodrop 2000 Spectrophotometer (Thermo Scientific, Waltham, Massachusetts, USA)

b. Method

Purified B cells from healthy controls or SLE patients were centrifuged at 1400 rpm for 10 minutes at room temperature and the pellet was resuspended and homogenized in appropriate amount of Lysis Buffer with 1% 2-mercaptoethanol as a reducing agent according to the manufacturer's protocol. After being transferred in spin cartridges and added equal volume of 70% ethanol in order to provide suitable binding conditions, the samples were centrifuged at 12000g for 15 seconds at room temperature. Next, the spin cartridges were washed three times (once with Wash Buffer I and twice with Wash Buffer II containing ethanol) and the membranes in the spin cartridges with the RNA attached were centrifuged at 12000g for 2 minutes at room temperature so that the membrane was dry. 30 μl of RNase–free Water were added to the centre of the membrane and left to incubate for 4 minutes at room temperature followed by centrifugation at 12000g for 2 minutes at room temperature so as for the RNA to be eluted. RNA concentration was quantified by using Nanodrop 2000 Spectrophotometer according to manufacturer's protocol and the samples were stored at -80°C.

B.2.7 Reverse Transcriptase reaction –Real-time quantitative Polymerase Chain Reaction

a. Reagents and Instruments

1. PrimeScript™ 1st strand cDNA Synthesis Kit (containing PrimeScriptTM RTase (200 U/µl), 5x PrimeScript™ Buffer, RNase Inhibitor (40 U/ µl), dNTP Mixture (10 mM), Oligo dT Primer (50 µM), RNase-free water) (Takara, Otsu, Shiga, Japan)
2. KAPA SYBR® FAST Universal 2x qPCR Master Mix (Kapa Biosystems Inc., Woburn, MA, USA)
3. Betaine
4. PCR machine (Veriti 96 well thermal cycler) (Applied Biosystems, Life Technologies, Carlsbad, California, USA)
5. RT-PCR machine (CFX Connect™, Real-Time System) (BioRad, Hercules, California, USA)

b. Method

cDNA was prepared from isolated RNA using PrimeScript™ 1st strand cDNA Synthesis Kit according to manufacturer's protocol. 1 µg of RNA was used as a template for every reaction and was mixed initially with appropriate volume of Oligo dT primers and mixture of dNTPs. After incubation for 5 minutes at 65° C at a Veriti 96 well thermal cycler, the samples were cooled immediately on ice. Next, appropriate volumes of 5x PrimeScript buffer, RNase inhibitor, PrimeScript Reverse Transcriptase and RNase-free water were added and the samples were incubated at 50° C for 45 minutes and at 95° C for 5 minutes at a Veriti 96 well thermal cycler, followed by cooling on ice. cDNA was stored at -20° C.

PCR amplification of the resulting cDNA samples was performed using appropriate volumes of KAPA SYBR® FAST Universal 2x qPCR Master Mix, betaine and specific for each gene primers at a CFX Connect™, Real-Time System. The target sequences for human BTLA were 5'-TTGGGTCTTCTTCTTAATCC-3'(forward) and 5'-ACTTGATCTTACGGGACA-3' (reverse), for human PD-1 were 5'-GACAACGCCACCTTCACCT-3'(forward) and 5'-TCGTTGGTCTGCCTGTTCG-3' (reverse) and for human PD-L1 were 5'-GCCGAAGTCATCTGGACAAG-3'(forward) and

5'-TACACTGGTCGTGTGACTCT-3' (reverse). The following PCR conditions were used: 95°C for 3 minutes, 39 cycles at 95°C for 15 seconds and specific for each gene annealing temperature and time (*btla* 64°C/1 minute, *pd-1* 64°C/1 minute, *pd-l1* 58°C/1 minute). Data were analyzed with the Livak method ($\Delta\Delta C_T$), using β-actin as a reference gene.

B.2.8 Enzyme Linked ImmunoSorbent Assay (ELISA)

a. Reagents and Instruments

1. Human IL-6 ELISA Ready-SET-Go! ® [containing 250x Capture Antibody, 250x Detection antibody, 250x Detection enzyme (Avidin-HRP), ELISA/ELISPOT Coating Buffer Powder, 5x concentrated Assay Diluent, Human IL-6 Standard] (eBioscience, Inc., San Diego, California, USA)
2. 1x Tetramethylbenzidine (TMB) Substrate Solution (eBioscience, Inc., San Diego, California, USA)
3. Stop Solution (2N H_2SO_4)
4. Wash Buffer (1x PBS, 0.05% Tween-20)
5. 96-well plate (Nunc C8 White LockWell MaxiSorp) (Thermo Scientific, Waltham, Massachusetts, USA)
6. Model 680 Microplate Reader (BioRad, Hercules, California, USA)

b. Method

A 96-well plate (Nunc C8 White LockWell MaxiSorp) was coated with 100 μL/well of 1x Capture Antibody in Coating Buffer (prepared by diluting ELISA/ELISPOT Coating Buffer Powder in appropriate volume of distilled water) and left to incubate overnight at 4° C. The plate was washed three times with Wash Buffer and it was blocked with 200 μL/well of 1X Assay Diluent followed by incubation at room temperature for 1 hour. Next, the plate was washed once with Wash Buffer and the samples as well as the standards (diluted according to manufacturer's protocol) were added (100 μL/well) and left to incubate overnight at 4° C on a rocker. After four washes with Wash Buffer, incubation of 100 μL/well of 1x Detection antibody for 1 hour at room temperature, four washes with Wash Buffer and subsequent incubation of 100 μL/well of 1x Detection enzyme (Avidin-HRP) for 1 hour at room temperature, 100 μL/well of 1x Tetramethylbenzidine (TMB) Substrate Solution to each well

were added and the plate was left to incubate at room temperature for 15 minutes. The reaction was stopped by adding 50 μL of Stop Solution to each well and the plate was read at 450 nm and 570 nm at a Model 680 microplate reader. Values at 570 nm were subtracted from those of 450 nm and data were analyzed by Graph Pad Prism 5 software.

B.2.9 Statistical analysis

Statistical analysis was performed using Graph Pad Prism 5 software and p value < 0.05 was considered as indicative of statistical significance.

C. RESULTS

C.1 Baseline expression of negative immunomodulatory receptors and activation markers in peripheral blood B cells from healthy controls and SLE patients .

C.1.1. Comparable peripheral blood B cell subpopulations in healthy controls and patients with SLE.

Freshly isolated PBMCs from healthy controls and patients with SLE were subjected to flow cytometry and B cells were categorized according to membrane expression of CD19, IgD and CD27. CD19 is a B cell-specific marker, IgD is a marker of naïve B cell subpopulations and CD27 is a marker of more mature B cell subpopulations [3]. Accordingly, B cells were classified into **$CD19^{+}CD27^{-}IgD^{+}$ naïve B cells**, **$CD19^{+}CD27^{+}IgD^{+}$ transitional B cells**, **$CD19^{+}CD27^{+}IgD^{-}$ memory B cells** and **$CD19^{+}CD27^{++}IgD^{-}$ plasma cells** (Figure C.1.1).

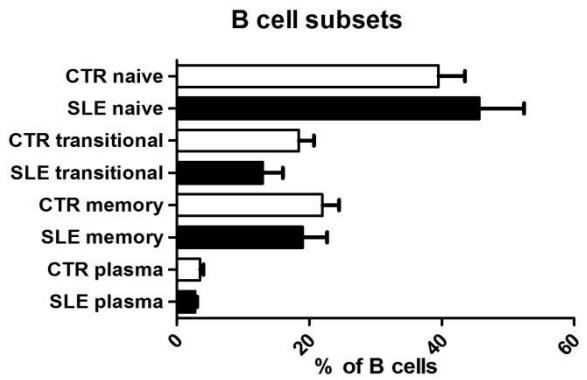


Figure C.1.1
B cell subpopulations as determined by flow cytometry in PBMCs from healthy controls (n=9) (CTR) and SLE patients (n=9)(SLE).

As seen in figure C.1.1, naïve B cells represent the majority of B cells in both healthy controls and SLE patients (39.5-45%), whereas plasma cells account for only 2.8-3.6% of B cells. No significant difference was noted between healthy controls and SLE patients.

C.1.2. Baseline expression of negative immunomodulatory receptors BTLA, PD-1 and PD-L1 in B cell subsets in PBMCs from healthy controls and patients with SLE.

Expression of BTLA, PD-1 and PD-L1 by means of mean fluorescence intensity (MFI) was evaluated by flow cytometry in the above-mentioned B cell subsets in controls and SLE patients (Figure C.1.2). Expression of BTLA was relatively high. It was shown to be significantly higher in naïve and transitional B cells (163.2±9.8 and 165±13, mean±SEM, respectively) compared to memory B cells (115.8±8) from SLE patients and the same trend was observed in healthy controls, without any significant difference between the subsets of each group (Figure C.1.2).

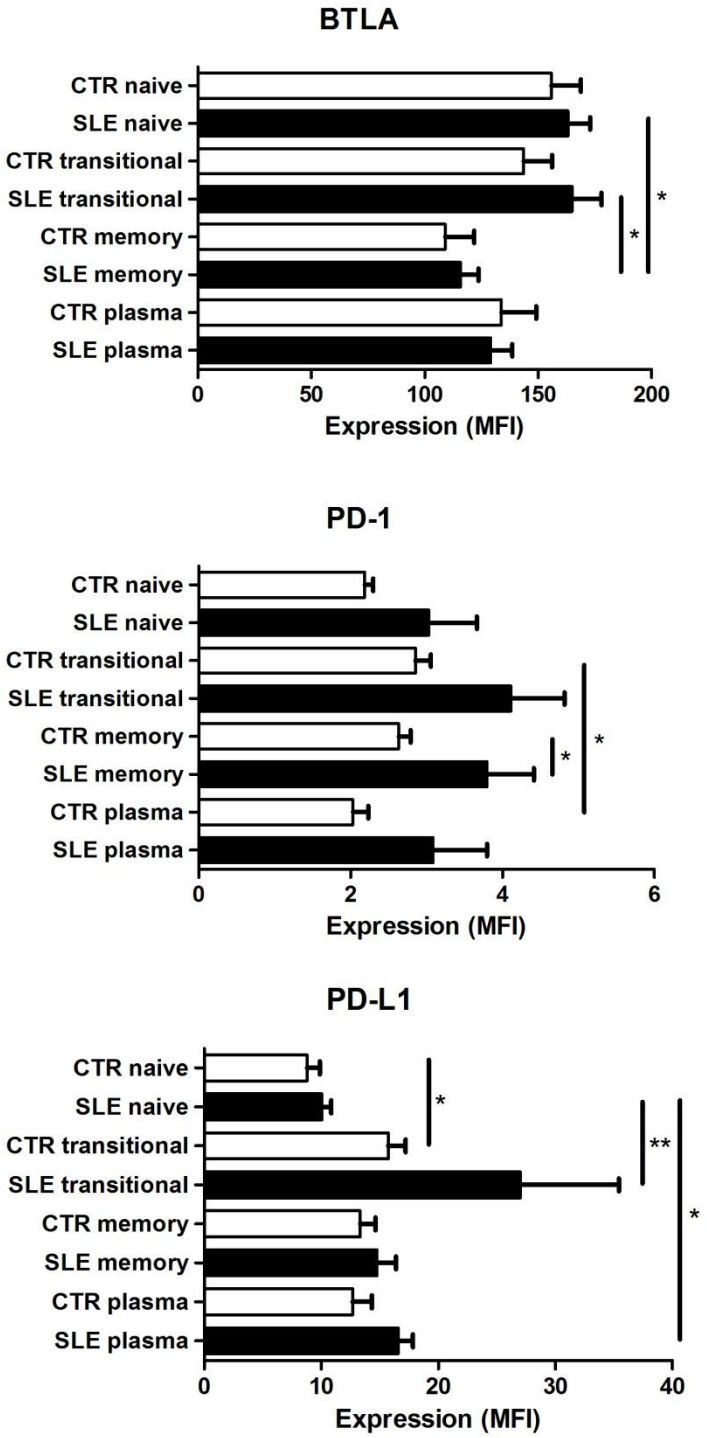


Figure C.1.2
Baseline expression of BTLA, PD-1 and PD-L1 in B cell subsets ($CD19^{+}CD27^{-}IgD^{+}$ naïve B cells, $CD19^{+}CD27^{+}IgD^{+}$ transitional B cells, $CD19^{+}CD27^{+}IgD^{-}$ memory B cells and $CD19^{+}CD27^{++}IgD^{-}$ plasma cells) in PBMCs from healthy controls (n=9)(CTR) and patients with SLE (n=9) (SLE)as determined by flow cytometry (*p<0.05, **p<0.01, Mann-Whitney test).

Baseline PD-1 expression was generally low in all subsets in both groups and even lower in naïve B cells and plasma cells in each group, nevertheless PD-1 was expressed at a higher level in B cells from SLE patients, specifically memory B cells from SLE patients exhibited a significant higher expression (3.8±0.6) compared to controls (2.6±0.2) (Figure C.1.2). PD-L1, having a moderate baseline expression, was primarily expressed on transitional B cells. Significant differences were recognized between control transitional B cells (15.7±1.5) compared to control naïve B cells (8.8±1) as well as in SLE transitional B cells (27±8.4) compared to SLE naïve B cells (10±0.8) (Figure C.1.2).

To validate the results of the flow cytometric analysis, mRNA from freshly isolated B cells (purity 85-96%) from healthy controls and SLE patients was obtained and quantitative RT-PCR was performed. BTLA expression was 1.9–fold increased (range 0.6-6.1) and PD-L1 was 1.9-fold upregulated (range 0.8-4.3) in SLE compared to control B cells, in consistency with the results of the flow cytometry. PD-1 expression was comparable between the two groups [0.9 fold expression (range 0.4-2) in SLE compared to control B cells] (Figure C.1.3).

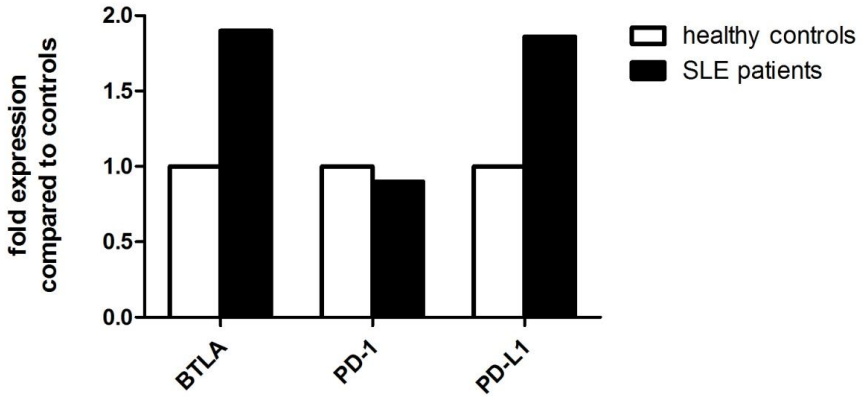


Figure C.1.3 Expression of BTLA, PD-1 and PD-L1 in B cells freshly isolated from healthy controls (n=5) and SLE patients (n=5) as determined by RT-PCR. β-actin was used as a reference gene and average expression in SLE patients was calibrated with average expression of healthy controls.

C.1.3. Baseline expression of costimulatory molecules CD80, CD86 and CD40 in B cell subsets in PBMCs from healthy controls and patients with SLE.

CD80 (B7-1) and CD86 (B7-2) on B cell surface serve as ligands for CD28 on T cell surface, thus maintaining B-T cell interaction and mediating the antigen-presenting function of B cells [5, 91]. This interaction is further improved by binding

of CD40 on B cell surface to CD40L on T cell surface, allowing for optimal B cell activation [14]. Levels of all three costimulatory molecules were assessed by flow cytometry in the above-mentioned B cell subsets in freshly isolated PBMCs from healthy controls and SLE patients (Figures C.1.3.1-2).

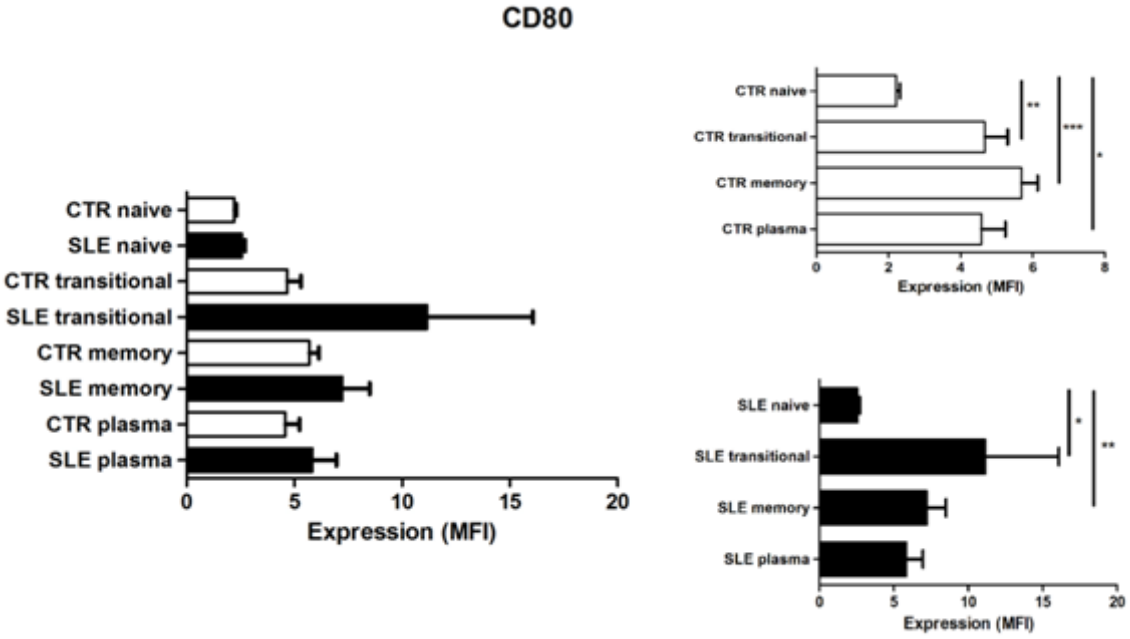
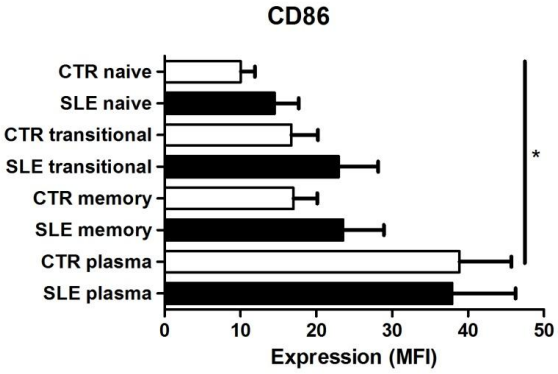


Figure C.1.3.1
Baseline expression of CD80 and CD86 in B cell subsets ($CD19^{+}CD27^{-}IgD^{+}$ naïve B cells, $CD19^{+}CD27^{+}IgD^{+}$ transitional B cells, $CD19^{+}CD27^{+}IgD^{-}$ memory B cells and $CD19^{+}CD27^{++}IgD^{-}$ plasma cells) in PBMCs from healthy controls (n=9) (CTR) and patients with SLE (n=9)(SLE) as determined by flow cytometry (*$p<0.05$, **$p<0.01$,***$p<0.001$, Mann-Whitney test).



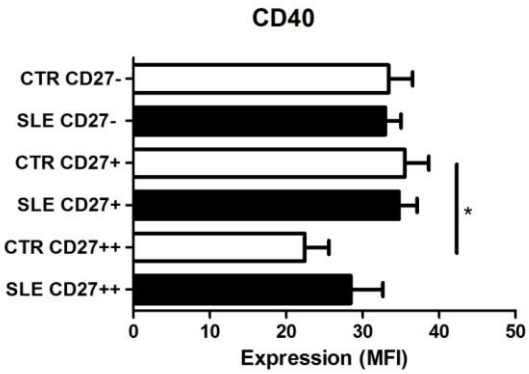


Figure C.1.3.2
Baseline expression of CD40 in B cell subsets ($CD19^{+}CD27^{-}$, $CD19^{+}CD27^{+}$ and $CD19^{+}CD27^{++}$)in PBMCs from healthy controls (n=9) (CTR) and patients with SLE (n=9) (SLE) as determined by flow cytometry (*$p<0.05$, Mann-Whitney test).

As shown in figure C.1.3.1, CD80 was found to have a higher expression on more mature forms of B cells, specifically in memory B cells in controls and in transitional B cells in SLE patients. CD86, following the same trend, was expressed primarily in plasma cells in both groups and a significant increase in CD86 expression in plasma cells (38.9±6.8) was noticed compared to naïve B cells (10±1.9) in healthy controls (Figure C.1.3.1). As for CD40, it exhibited a moderate expression, which was lower in $CD19^{+}CD27^{++}$ B cells in both groups. However, in this subset, its expression tended to be higher in SLE patients compared to healthy controls (Figure C.1.3.2).

C.1.4. Increased baseline expression of HVEM in peripheral blood T helper cells from patients with SLE.

Freshly isolated PBMCs from healthy controls and patients with SLE were subjected to flow cytometry and expression of HVEM was evaluated in subpopulations of T cells according to the expression of CD4 and CD25. HVEM was significantly overexpressed in all subsets of $CD4^{+}$ T cells in SLE patients.

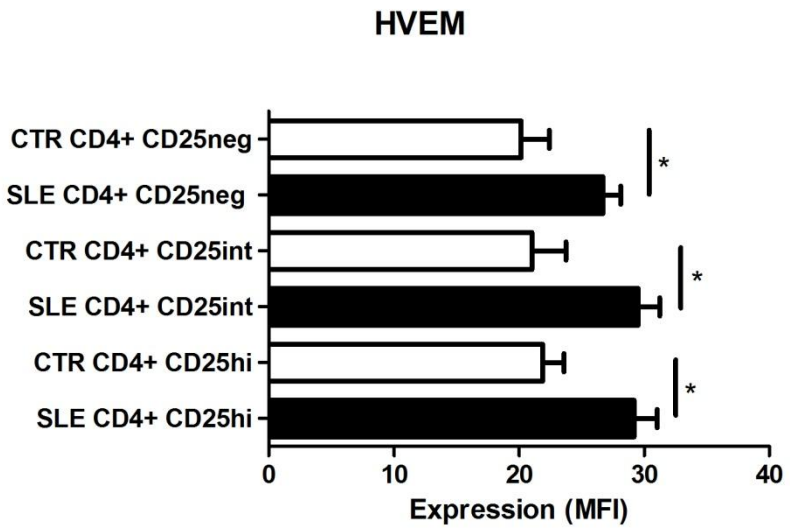


Figure C.1.4 Baseline expression of HVEM in T cell subsets ($CD4^{+}CD25^{neg}$, $CD4^{+}CD25^{int}$ and $CD4^{+}CD25^{hi}$) in PBMCs from healthy controls (n=7) (CTR) and patients with SLE (n=12) (SLE) as determined by flow cytometry (*p<0.05, Mann-Whitney test).

C.2 Regulation of expression of negative immunomodulatory receptors and activation markers in purified B cells from healthy controls.

In an attempt to determine the stimuli resulting in alteration of expression of negative immunomodulatory receptors (BTLA, PD-1, PD-L1) and costimulatory

molecules (CD86) on B cell surface, purified healthy B cells were treated with a variety of stimuli, including BcR activation with anti-IgM, interferons (IFNγ), Th2 cytokines (IL-4, IL-10), TLR ligands [zymosan (TLR2 ligand), polyI:C (polyinosinic:polycytidylic acid-TLR3 ligand), LPS (TLR4 ligand), gardiquimod (imidazoquinoline compound-TLR7 ligand), CpG ODN (TLR9 ligand)], B cell survival factors (BAFF), B cell costimulatory molecules (anti-CD40) and cytokines with pathophysiological relevance to SLE (IL-21) [101]. Treatment lasted for 48 hours followed by flow cytometry of the cultured B cells. Separate cultures were set in which stimuli were added with or without BcR stimulation. Apart from this, cultures of purified B cells were set in which the influence of serum from patients with SLE was evaluated.

C.2.1 BTLA was overexpressed upon BcR stimulation, but Th1/Th2 cytokines and IL-21 reduced this effect.

BTLA was significantly upregulated upon BcR stimulation (134.3±13.8 vs 84.8±10.9 in untreated B cells), nevertheless, treatment with IFNγ, IL-4, IL-10 and IL-21 attenuated this effect (Figure C.2.1.1).

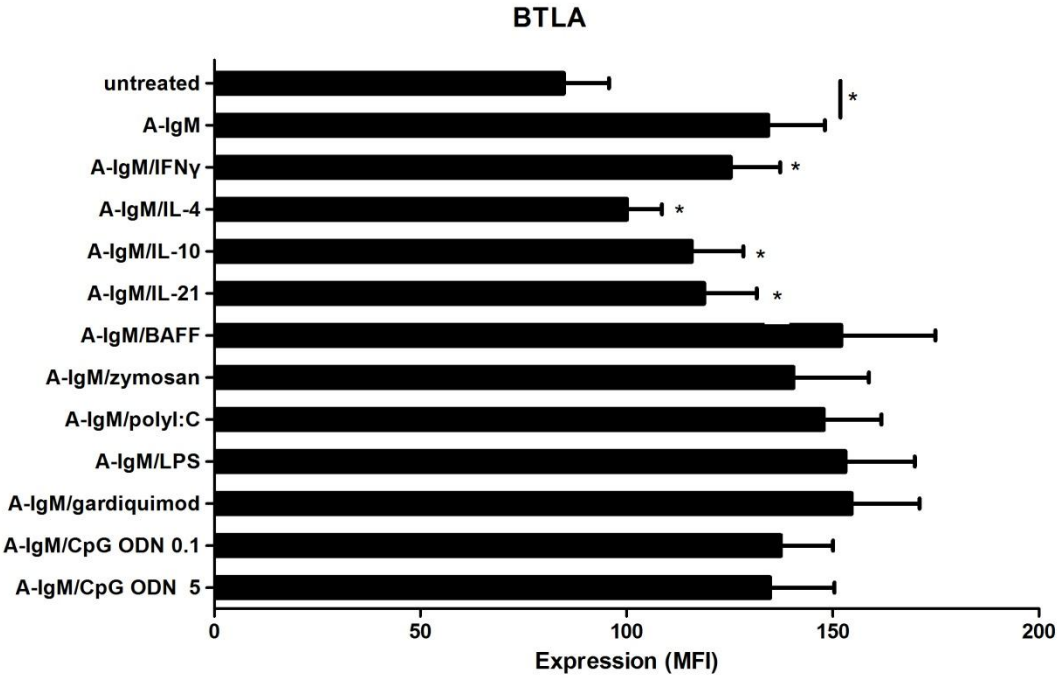


Figure C.2.1.1 Expression of BTLA in purified B cells from healthy donors (n=7) after 48-hour-treatment with various cytokines and TLR-ligands upon BcR activation as determined by flow cytometry (*p<0.05, comparison with A-IgM, unless otherwise indicated, Wilcoxon signed rank test).

Once stimuli were added without additional BcR stimulation, an increase in BTLA expression was noticed upon IFNγ, IL-4, LPS and anti-CD40 treatment compared to a-IgM treatment. As for the effect of SLE sera, BTLA was shown to be elevated in B cells treated with serum from patients with low SLEDAI compared to B cells treated with serum from patients with high SLEDAI (Figure C.2.1.2).

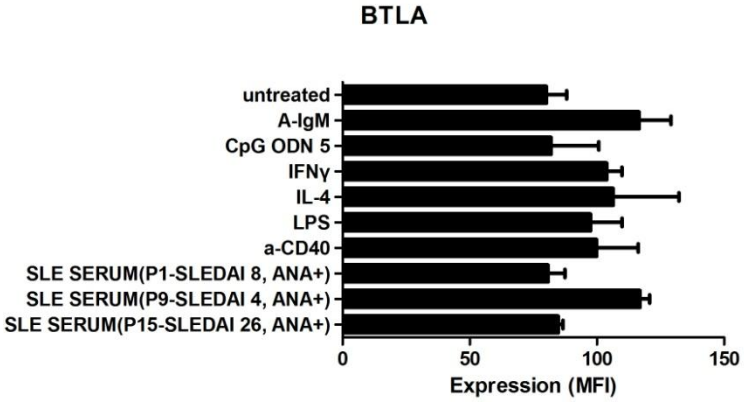


Figure C.2.1.2 Expression of BTLA in purified B cells from healthy donors (n=3) after 48-hour-treatment with various cytokines, TLR-ligands and SLE sera as determined by flow cytometry.

C.2.2 Significant upregulation of PD-1 and PD-L1 due to combined a-IgM/CpG ODN treatment.

Compared to the expression upon a-IgM treatment, both PD-1 and PD-L1 displayed significantly increased expression in B cells upon BcR stimulation and TLR9 cross linking with CpG ODN (16.34±1.7 vs 13.27±1.6 and 57.47±4.2 vs 25.6±2.6, respectively). The rest of the stimuli with or without combination with a-IgM did not alter the expression of either of them, whereas serum from patients with high SLEDAI resulted in a mild decrease of the levels of both receptors in B cells compared to serum from patients with low SLEDAI (Figures C.2.2.1-3).

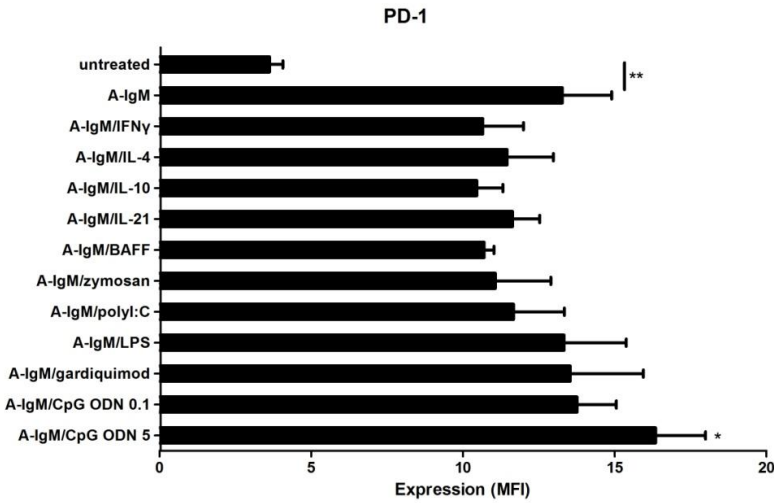


Figure C.2.2.1 Expression of PD-1 in purified B cells from healthy donors (n=7) after 48-hour-treatment with various cytokines and TLR-ligands upon BcR activation as determined by flow cytometry (*p<0.05, comparison with A-IgM, unless otherwise indicated, Wilcoxon signed rank test).

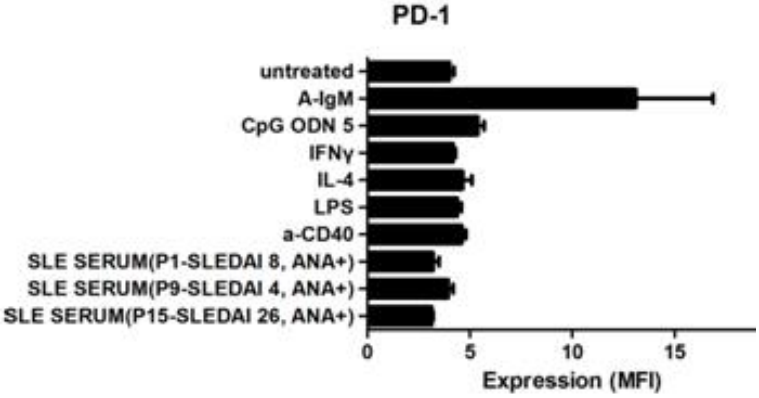


Figure C.2.2.2 Expression of PD-1 in purified B cells from healthy donors (n=3) after 48-hour-treatment with various cytokines, TLR-ligands and SLE sera as determined by flow cytometry.

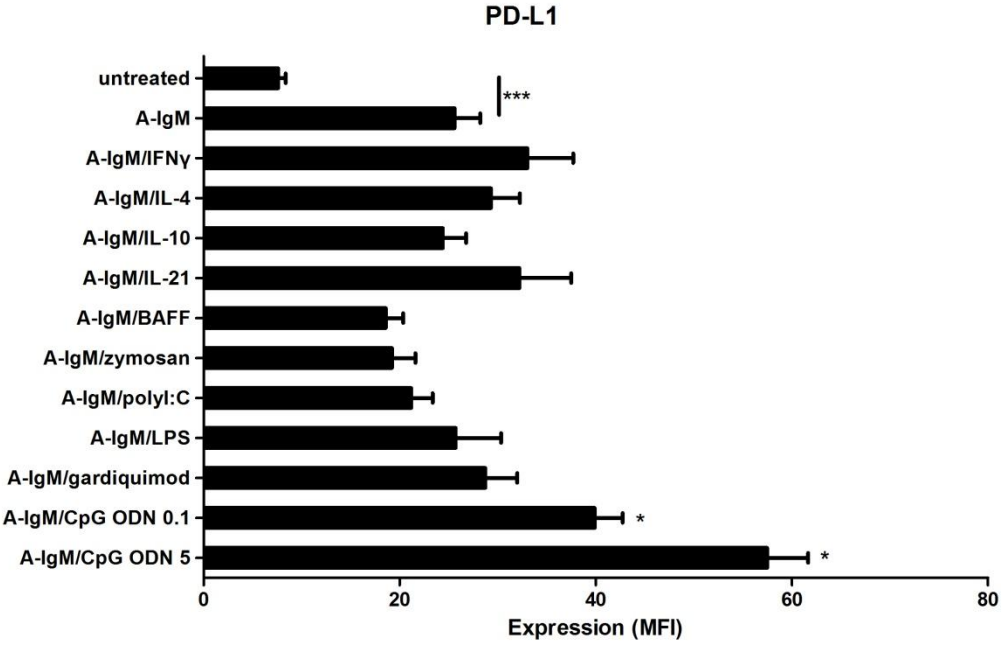


Figure C.2.2.1 Expression of PD-L1 in purified B cells from healthy donors (n=7) after 48-hour-treatment with various cytokines and TLR-ligands upon BcR activation as determined by flow cytometry (*p<0.05, comparison with A-IgM, unless otherwise indicated, Wilcoxon signed rank test).

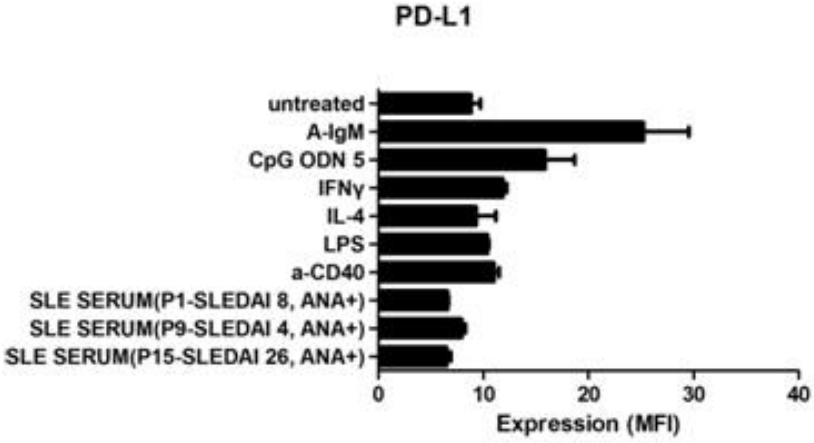


Figure C.2.2.3 Expression of PD-L1 in purified B cells from healthy donors (n=3) after 48-hour-treatment with various cytokines, TLR-ligands and SLE sera as determined by flow cytometry.

C.2.3 CD86 was elevated in the presence of a-IgM, IL-4 and CpG ODN.

CD86 expression was also evaluated and found to be significantly increased in BcR-stimulated B cells (63.7±11 vs 18±3.4) compared to untreated B cells. Furthermore, CD86 was even more increased in the presence of IL-4 (97.2±12.6) and TLR9 ligand (75.4±9) (Figure C.2.3).

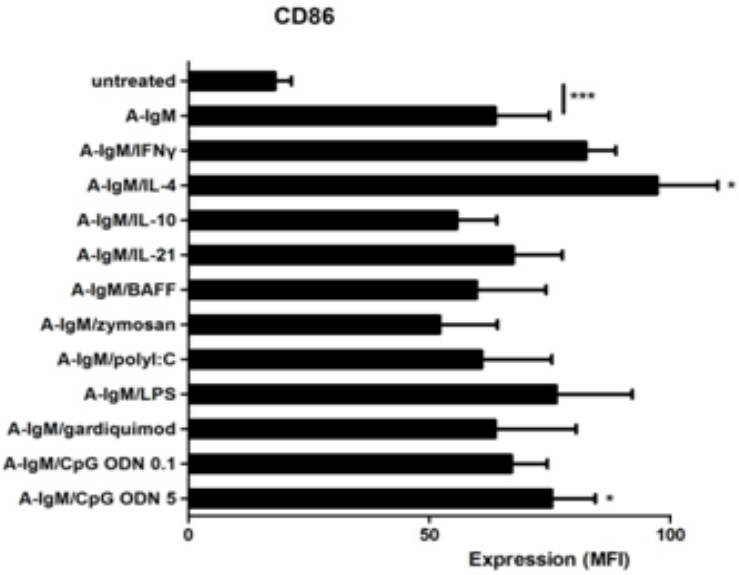


Figure C.2.3 Expression of CD86 in purified B cells from healthy donors (n=7) after 48-hour-treatment with various cytokines and TLR-ligands upon BcR activation as determined by flow cytometry (*p<0.05, comparison with A-IgM, unless otherwise indicated, Wilcoxon signed rank test).

C.3 Effect of BTLA on B cell activation, differentiation, proliferation and cytokine production

With the aim of clarifying the effect of BTLA ligation on B cell biology (activation, proliferation, differentiation and cytokine production), purified B cells from healthy controls and patients with SLE were cultured in the presence or absence of plate-bound BTLA ligand, HVEM, upon BcR stimulation. After treatment for 2-7 days, cultured B cells were subjected to flow cytometry.

C.3.1 In vitro BTLA cross linking with its ligand, HVEM, redounded to a fall in activation markers levels in B cells and in diminished plasma cell numbers.

To validate the role of BTLA on B cell activation, expression of CD80 and CD86 was evaluated. Both molecules displayed a mild decrease upon treatment with HVEM in B cells (Figure C.3.1.1).

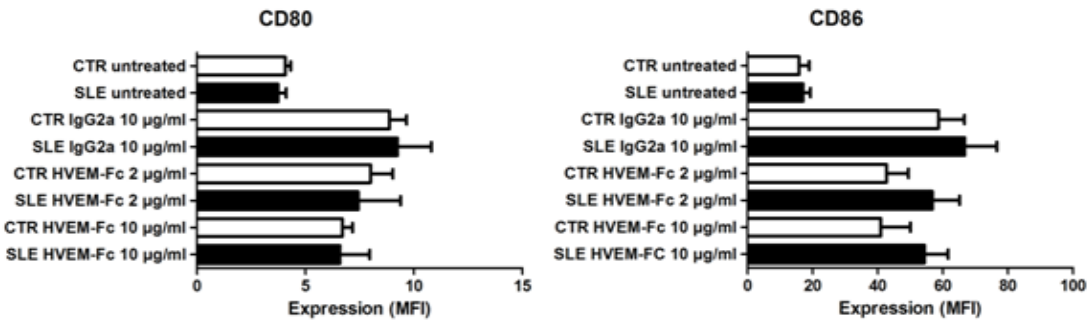


Figure C.3.1.1 Expression of CD80 and CD86 was examined by flow cytometry in purified B cells from healthy donors (n=5) (CTR) and SLE patients (n=7) (SLE) upon BcR activation for 48 hours in the presence or absence of BTLA ligand, HVEM. IgG2a was used as an isotype

Furthermore, differentiation into plasma cells, characterized by simultaneous expression of CD27 and CD38, was examined by flow cytometry in B cells cultured for 2 days or 7 days. In 2-day cultures, a modest reduction in the number of plasma B cells was observed at any concentration of HVEM both in controls and in SLE patients (Figure C.3.1.2). However, B cell treatment with low concentration of HVEM for 7 days resulted in enhanced numbers of plasma cells contrary to the decreased numbers observed with HVEM at a high concentration in both groups (Figure C.3.1.2).

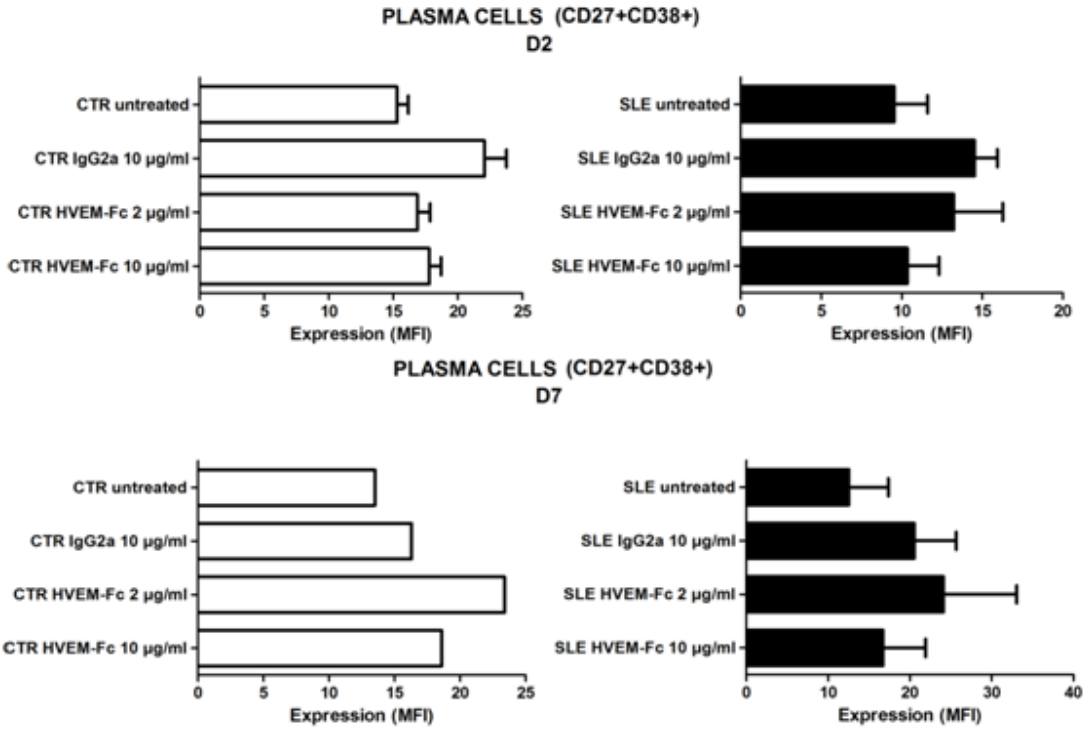


Figure C.3.1.2 Differentiation into plasma cells ($CD27^{+}CD38^{+}$) was examined by flow cytometry in purified B cells from healthy donors (n=5) (CTR) and SLE patients (n=5)(SLE) upon BcR activation for 2 days in the presence or absence of BTLA ligand, HVEM. Additional similar long-term (7 days) experiments were performed with purified B cells from 1 healthy donor and 4 SLE patients. IgG2a was used as an isotype control.

C.3.2 HVEM treatment led to a dose-dependent reduction in B cell proliferation.

CFSE proliferation assay was used to assess proliferation in purified B cells from healthy controls and SLE patients stimulated with a-IgM and anti-CD40 in the presence or absence of BTLA ligand, HVEM. CFSE dilutions determined by flow cytometry were considered as cell divisions. Despite the fact that SLE B cells exhibited a higher tendency to proliferate compared to controls, BTLA cross linking with HVEM led to dose-dependent abolishment of proliferation of B cells both in healthy controls and in SLE patients,. Specifically, in SLE patients, the percentage of B cells under division was significantly diminished when the cell were treated with high concentration of HVEM compared to isotype control (14.9%±4.8 vs 30.2%±4) (Figure C.3.2).

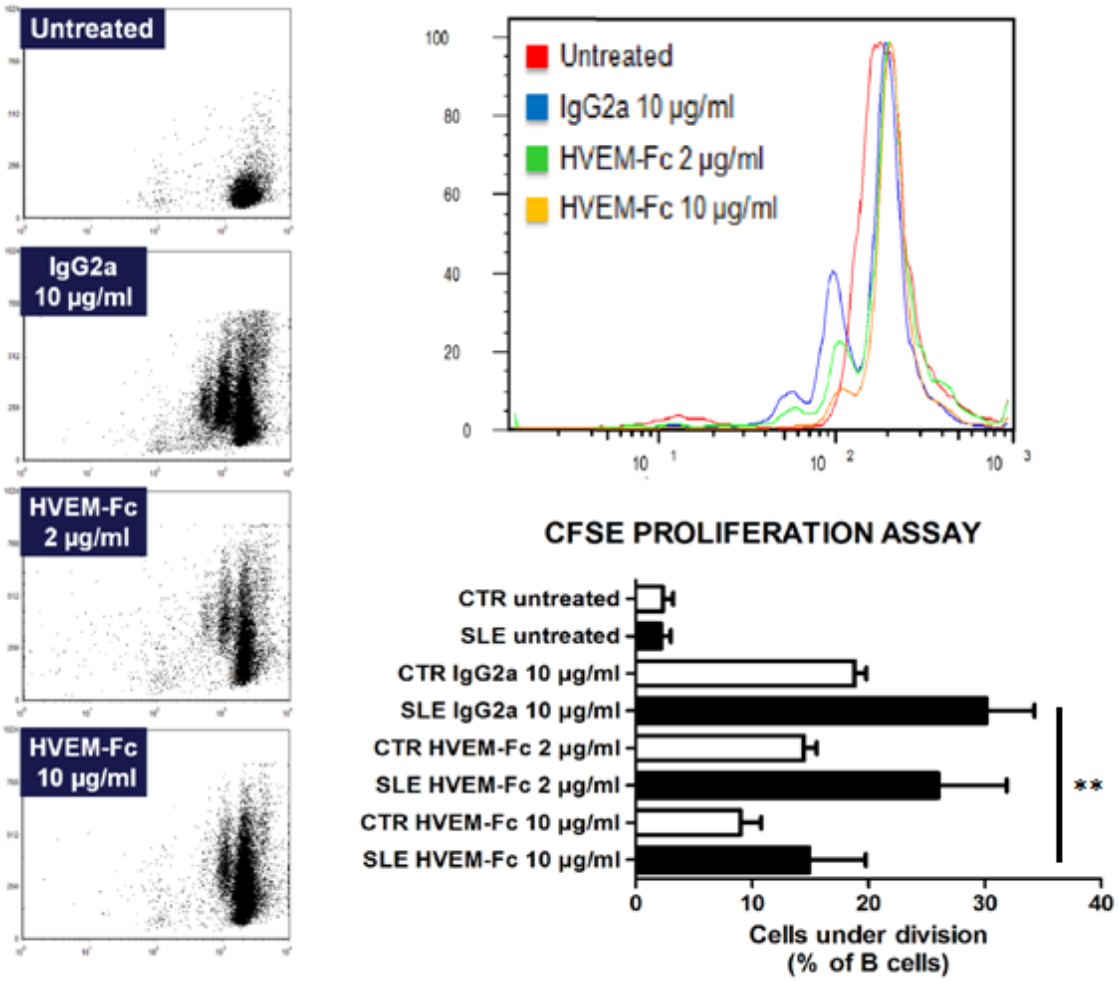


Figure C.3.2 Evaluation of proliferation of purified B cells from healthy donors (n=5) (CTR) and SLE patients (n=8)(SLE) by flow cytometry. B cells were labeled with CFSE and stimulated with anti-IgM and anti-CD40 upon BTLA cross linking with its ligand, HVEM-Fc for 3 days. IgG2a was used as isotype control. Representative dot plots and histogram are shown (**p<0.01, Wilcoxon signed rank test).

C.3.3 HVEM treatment influences differentially IL-6 production from control and SLE B cells.

IL-6 is a cytokine with a profound impact on B cell differentiation into antibody-producing plasma cells [102], as a result it seemed important to analyze its production by purified B cells upon BcR stimulation in the presence or absence of BTLA cross linking in short-term (day 2) and long-term cultures (day 7) (Figure C.3.3.1-2). Although treatment with HVEM for 2 days led to a modest reduction of IL-6 production from cultured control B cells, IL-6 production from cultured SLE B cells was enhanced upon HVEM treatment (Figure C.3.3.1). The same phenomenon, even more overt, was noticed in SLE B cells treated with HVEM for 7 days (Figure C.3.3.2).

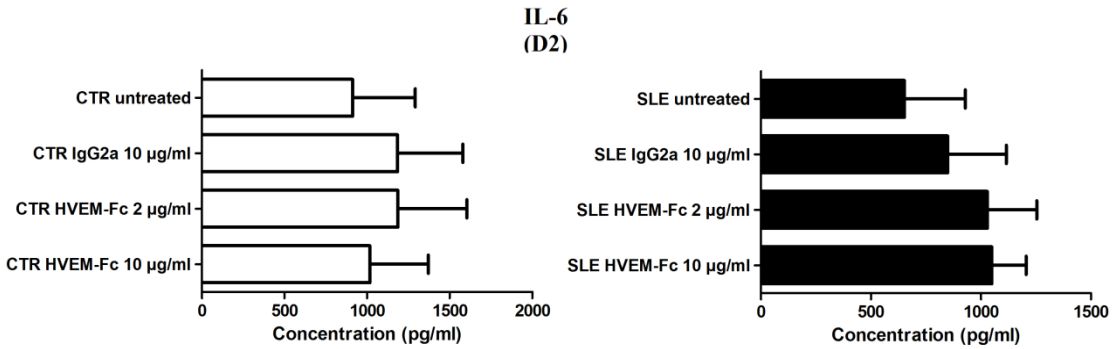


Figure C.3.3.1 IL-6 production from purified B cells from healthy donors (n=4)(CTR) and SLE patients (n=6) (SLE) measured by ELISA after 48-hour culture.

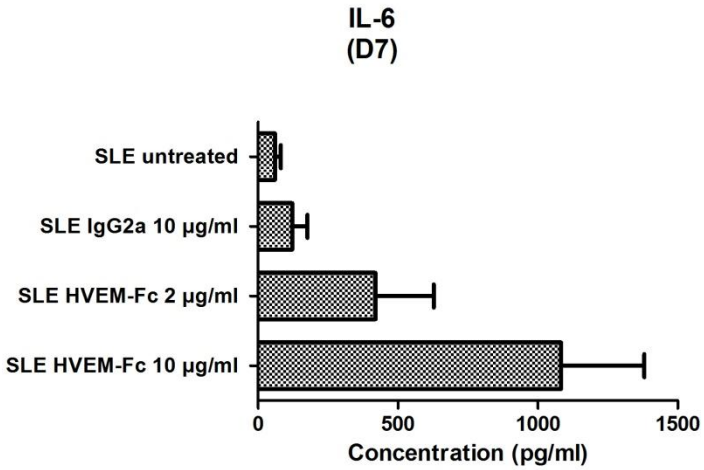


Figure C.3.3.2 IL-6 production from purified B cells from SLE patients (n=2) measured by ELISA after 7-day culture.

To summarize, the conducted study came to the following conclusions:

- The three major immunomodulatory receptors BTLA, PD-1 and PD-L1 are differentially expressed among B cell subsets both in healthy controls and SLE patients.
- Comparable expression of BTLA, PD-1 and PD-L1 on B cells from healthy controls and SLE patients was shown.
- The BTLA ligand, HVEM, is significantly overexpressed at $CD4^+$ T cells from SLE patients compared to healthy controls.
- BTLA, PD-1, PD-L1 and CD86 are induced upon BcR activation and exhibit further significant increase upon treatment with specific cytokines and the TLR9 ligand, CpG ODN. Th2 cytokines (IL-4, IL-10) attenuate the BcR-induced overexpression of BTLA.
- In B cells, BTLA cross-linking through HVEM resulted in reduced expression of activation markers, diminished differentiation into plasma cells and dose-dependent reduction in cellular proliferation, but failed to inhibit IL-6 production in SLE patients compared to healthy controls.

D.DISCUSSION

Numerous studies have emphasized the **central role of B cells in SLE pathogenesis**, not only due to the production of high-titer autoantibodies but also owing to their ability to be potent antigen-presenting cells and to differentiate into long-lived plasma cells [89-91]. Loss of B cell tolerance represents a hallmark of SLE [73]. For this reason, a study on the **negative immunomodulatory receptors of B cells**, such as BTLA, PD-1 and PD-L1, would provide an insight on the specific cytokine milieu and other factors that determine their expression as well as on their function upon cross linking with their ligands. All three receptors were examined in terms of baseline expression and regulation of expression in B cells, however, there was a focus on BTLA function, as this is a recently discovered immunoreceptor with unique properties whose function in B cells remained under investigation.

First of all, B cells were categorized into subpopulations (naïve B cells, transitional B cells, memory B cells, plasma cells) and percentages were measured in healthy controls and SLE patients. Although evidence from various studies showed that there was expansion of memory B cells and plasma cells in SLE patients [90], this was not confirmed in this study, possibly due to the small number of samples and the heterogeneity of patients' disease activity, as determined by SLEDAI. Next, **baseline expression of BTLA, PD-1, PD-L1, CD80** and **CD86** was examined in the above-mentioned subpopulations. BTLA expression was overall high, but it was even higher in more naïve forms of B cells (naïve and transitional B cells), consistent to what has already been known for BTLA [47, 50]. On the other hand, PD-1 exhibited very low expression in all B cell subsets both in healthy controls and in SLE patients, suggesting that the two receptors mediate their inhibitory function in separate states of activation. BTLA could modulate B cell activation at a basal state but PD-1 could be the central inhibitory immunoreceptor at activated B cells. PD-L1 was moderately expressed in B cell subsets, except for transitional B cells, where it was primarily found. **Transitional B cells comprise a distinct checkpoint at which auto-reactivity is censored** [103], as a result this finding may demonstrate that PD-L1 represents a regulatory mechanism by which potent and alloreactive B cell are chosen to survive through this selection process. CD80 and CD86, markers of ability of B cells for antigen presentation were found to be increased in more mature forms of B

cells, as expected, whereas CD40 was almost equally expressed in all subsets. In addition, the significant **upregulation of HVEM, BTLA ligand, in SLE helper T cells** needs to be investigated. To the best of our knowledge, this is the first time that this effect was reported and it may suggest a possibility that, in the context of SLE, HVEM functions as a stimulatory molecule by attaching to other ligands in its complex ligand network, such as LIGHT.

Except for baseline expression of BTLA, PD-1 and PD-L1, another important study objective was their expression under the effect of a variety of stimuli. B cell receptor stimulation elevated the levels of expression of all three receptors, although its effect on PD-1 and PD-L1 was even greater. Apart from a-IgM, the rest of the stimuli influenced differentially the expression of all three receptors under study. **BTLA was downregulated by Th2 cytokines** (IL-4, IL-10) and **IL-21**. Effect of Th2 cytokines on BTLA expression needs to be further studied, as BTLA has been shown to be involved in the termination of Th2 responses [70]. Both PD-1 and PD-L1 were upregulated in the presence of CpG ODN, a result consistent with findings from a recent publication [36].

Functional assays in this project focused on expression of antigen-presentation markers by B cells (CD80/CD86), differentiation into plasma cells, proliferation and IL-6 production, properties of utmost significance for proper B cell function and homeostasis. A higher and a lower concentration of HVEM were used with the aim of discriminating dose-dependent effects. In this analysis, CD80/CD86 exhibited a modest, but consistent, reduction of their levels upon treatment with both concentrations of HVEM in healthy controls and in SLE patients, suggesting that antigen presentation is not one of the main targets of BTLA suppressive capacity. When differentiation into plasma cells under the effect of HVEM was checked, the results were interesting. 48-hour treatment with both concentrations of HVEM resulted in a slight decrease in the numbers of plasma cells in healthy controls and SLE patients, however, 7-day treatment with low concentration of HVEM led to increased plasma cell numbers in both groups. This finding may be suggestive of a **differential influence of HVEM in B cells at an acute and at a chronic basis**, as it is in the case of autoimmune disorders, and indicative of a stimulatory function. As for B cell proliferation, it was attenuated upon treatment with HVEM in a dose-dependent manner in both groups, an effect consistent to what has already been elucidated not only for B cells but also for T cells [51-53]. Last, IL-6 production,

although diminished in healthy B cells, was elevated upon HVEM treatment in SLE B cells. This effect was even more profound when B cells were treated with HVEM in long-term cultures (7 days), suggesting once again a stimulatory function of HVEM at a chronic basis. Thus, the perspective of inhibiting HVEM signaling in SLE patients through administration of a neutralizing antibody against HVEM may be of great importance for the attenuation of its immunostimulatory properties and the subsequent amelioration of SLE manifestations.

E. CONCLUSIONS – FUTURE PERSPECTIVES

This project aimed at casting light on the expression of BTLA, PD-1 and PD-L1 in B cells in the presence or absence of specific stimuli, so as to delineate the factors responsible for an upregulation or downregulation of these three immunoreceptors. Alterations in their expression result in suppressed or enhanced B cell activation respectively, an effect with direct correlation to SLE pathogenesis. Flow cytometry revealed a consistent and significant elevation of HVEM baseline expression in SLE T helper cells which requires further study, as there is possibility that HVEM binds to other than BTLA ligands of its complex ligand network (LIGHT, CD160, $LT\alpha_3$, gD) [52, 60]. Among them, LIGHT seems to be the most relevant candidate due to the fact that LIGHT and BTLA are the only HVEM ligands expressed in B cells, as a consequence it would be useful to examine its expression in B cells at a basal state and after stimulation both in healthy controls and in SLE patients. Effect of HVEM cross linking with BTLA was studied particularly, nevertheless the exported results were controversial and do not demonstrate a definite suppressive function for HVEM. This could be attributed to the complexity and divergence of B cell biology, in which properties, such as antigen-presenting capacity, differentiation into plasma cells, proliferation and production of crucial cytokines (IL-6) are not necessarily influenced in parallel and need to be separately examined. Another important consideration is the use of specific stimulating anti-BTLA antibody, instead of HVEM, with the aim of confirming the specific effect of BTLA activation on B cell properties.

In conclusion, different patterns of expression of negative immunomodulatory receptors in B cells were demonstrated suggesting separate roles in attenuation of B cell activation. Functional assays concerning BTLA-HVEM were not confirmative of a suppressive function in B cells in the context of SLE, suggesting that aberrant expression may lead to defects in inhibition of B cell activation and contribution to SLE pathogenesis.

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