From DEPARTMENT OF MICROBIOLOGY, TUMOR AND CELL BIOLOGY
Karolinska Institutet, Stockholm, Sweden

PROINFLAMMATORY FACTOR MEDIATED LYMPHOCYTE ACTIVATION- THE PIVOTAL ROLE OF LEUKOTRIENE B4

Anquan Liu

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In memory of my mother

ABSTRACT

Epstein-Barr virus (EBV) is ubiquitous in the human population. More than 90% of the individuals are virus carriers. The outcome of the first encounter with the virus is highly variable. It can occur unnoticed, but if infection is delayed until adolescence it causes the infectious mononucleosis syndrome in about half of the cases. The decisive mechanism that arrests EBV induced B cell proliferation is attributed to both innate and EBV specific cellular immunity.

EBV specific immunological memory is not transferred from mother to child. Therefore, cord blood mononuclear cells (CBMC) are well suited for analysis of cellular interactions in primary infection. We found that the immunomodulators, PSK and Trx80 inhibited the EBV induced B cell proliferation. Both PSK and Trx80 activated monocytes to produce cytokines in the presence of activated T cells. PSK induced predominantly IL-15 while Trx80 induced IL-12. Both cytokines induced functional activation of the T cells. PI 3-kinase and ROS were involved in the PSK induced activation of monocytes. When the cultures containing activated T cells were restimulated with autologous EBV transformed B lymphocytes, specific cytotoxicity was generated in the cultures. By activation of innate immunological mechanisms it was possible to generate EBV-specific T cell response in CBMC cultures.

In the continuing studies, we found that NK cells were essential for cell mediated inhibition of the proliferation of EBV infected B lymphocytes, through the production of IFN-γ. In the NK cell depleted cultures, the production of IL-15 and IL-12, T cell activation and inhibition of EBV induced B cell proliferation were reduced and cytotoxic T cells could not be generated. These functions were restored by addition of IFN-γ to the NK cell depleted cultures.

Further we demonstrated that leukotriene B_4 (LTB_4) was involved in the effect of the PSK and Trx80. LTB_4 was detected in the medium, and T cell activation was compromised by addition of leukotriene biosynthesis inhibitors. BLT1, the high-affinity receptor of LTB_4 was expressed on T cells in the infected cultures. Moreover, we found that LTB_4 added to infected cultures, which did not receive the PSK and Trx80, induced functional activation of the T cells. LTB_4 activated the monocytes and acted directly on the T cells. In consequence, addition of LTB_4 resulted in the inhibition of the EBV induced B lymphocytes proliferation. Specific cytotoxicity could be generated by restimulation of the T cells. The experiments showed successive stages of T cell activation in acquisition of their immunological effector function. This is orchestrated by complex cellular interactions, and autocrine loops mediated by soluble factors - here IFN-γ, IL-15, IL-12 and LTB_4.

We also studied the function of LTB_4 in B-cell chronic lymphocytic leukemia (B-CLL) cells. B-CLL cells produced LTB_4 and expressed BLT1. Specific leukotriene biosynthesis inhibitors counteracted CD40-dependent activation and CD40-induced expression of CD23, CD54, and CD150. Addition of exogenous LTB_4 reversed the effect of the inhibitors. This study shows that LTB_4 plays an important role in the activation of B-CLL cells. Inhibitors of leukotriene synthesis may be useful for the treatment of B-CLL.

Publications included in the thesis

I. **Liu A**, Klein G, Bandobashi K, Klein E, Nagy N. SH2D1A expression reflects activation of T and NK cells in cord blood lymphocytes infected with EBV and treated with the immunomodulator PSK. *Immunol Lett*. 2002 Mar 1;80(3):181-8.

II. **Liu A**, Arbiser JL, Holmgren A, Klein G, Klein E. PSK and Trx80 inhibit B cell growth in EBV infected cord blood mononuclear cells through T cells activated by the monocyte products IL15 and IL12. *Blood*. 2005 Feb 15;105(4):1606-13.

III. **Liu A**, Holmgren A, Klein G, Klein E. The role of NK cells in the immunological recognition of EBV infected B cells in cord blood mononuclear cell cultures. *J. Leukoc. Biol*. *(under revision)*

IV. **Liu A**, Mahshid Y, Klein G, Claesson HE, Klein E. Leukotriene B_4 activates T cells which inhibit B cell proliferation in EBV infected cord blood derived mononuclear cell cultures. *Blood*. *(under minor revision)*

V. Runarsson G*, **Liu A***, Mahshid Y, Feltenmark S, Pettersson A, Klein E, Bjorkholm M, Claesson HE. Leukotriene B_4 plays a pivotal role in CD40-dependent activation of chronic B lymphocytic leukemia cells. *Blood*. 2005 Feb 1;105(3):1274-9. *contributed equally

CONTENTS

LIST OF ABBREVIATIONS

APCs	Antigen presenting cells
BLT1	LTB_4 receptor
B-CLL	B-cell chronic lymphocytic leukaemia
CBMC	cord blood mononuclear cell
CTL	cytotoxic T lymphocytes
EBNA	EBV nuclear antigen
EBV	Epstein-Barr virus
ERK	extracellular-signal-regulated kinase
FLAP	5-LO activating protein
5-LO	5-lipoxygenase
GM-CSF	granulocyte-macrophage colony-stimulating factor
HLA	human leucocyte antigen
IL	interleukin
IFN	interferon
IM	infectous mononucleosis
JNK	c-Jun N-terminal kinase (JNK)
LCL	lymphoblastoid cell lines
LPS	lipopolysaccharide
LTA_4	leukotriene A_4
LTB_4	leukotriene B_4
MAP	mitogen-activated protein
MTLC	mixed lymphocyte-tumor cell culture
NK cells	natural killer cells
cPLA2	cytosolic phospholipase A2
PBMC	peripheral blood mononuclear cell
PSK	polysaccharide K
PTLD	post transplantation lymphoproliferative disease
ROS	reactive oxygen species
SAP	the SLAM-associated protein
TCR	T-cell receptor
TGF-β	transforming growth factor-beta
Trx 80	truncated thioredoxin with the 80 N-terminal residues
XLP	X-linked lymphoproliferative disease
ZAP-70	zeta-chain associated protein-70

1 INTRODUCTION

1.1 EBV PRIMARY INFECTION

1.1.1 Epstein-Barr virus

Epstein-Barr virus (EBV) was discovered in Burkitt lymphoma in 1964 and since then the cell–virus interaction and the host virus interaction have been widely studied (1).

EBV is a ubiquitous human herpesvirus that is carried by more than 90% of the human population (2). EBV transforms or immortalizes B cells in vitro (3). Despite the transforming properties of the virus, EBV infection is asymptomatic in the vast majority of individuals. A normal, healthy immune response appears to be essential in maintaining the asymptomatic carrier state. Nevertheless, it is associated with a number of malignancies including undifferentiated nasopharyngeal carcinoma, endemic Burkitt's lymphoma, certain Hodgkin's lymphomas, and posttransplant lymphoproliferative disease (PTLD) (1).

1.1.2 Infectious mononucleosis

EBV primary infection can happen in childhood, at which time it is not associated with any specific symptoms. However, if primary infection is delayed until adolescence or adulthood, a half of individuals develop infectious mononucleosis (IM) (4). IM is an acute but self-limiting illness characterized clinically by fever, sore throat, and lymphadenopathy, while hematologically characterized by increased numbers of EBV-infected B cells in peripheral blood and massive oligoclonal expansion of EBV-specific $CD8^+$ T cells (5, 6).

The epidemiology of IM was first established in the 1950s (7), and EBV was identified as its etiological agent in 1968 (8). Classic acute IM resolves in 2 to 6 weeks, but relapses in the first 6 to 12 months after primary infection, and IM, may be linked with a prolonged fatigue syndrome and depression (9).

1.1.3 IM as an immunopathologic disease

In rare cases, subclinical primary EBV infected individuals lack CD8 expansions, even though EBV loads may be high (10). The factors contributing to the development of IM

in adolescence rather than in infancy may be differences in the pre-existing CD8 repertoire (11) and the dose of the infectious virus. It is possible that gene polymorphisms affecting the balance of Th1/Th2 responses and/or cytokine production could affect some individuals to have excessive $CD8^+$ T cell responses (12, 13).
A recent study indicated that HLA class I polymorphisms may affect the susceptibility of patients to develop IM upon primary EBV infection, suggesting that genetic variation in T cell responses can influence the nature of primary EBV infection and the level of viral persistence (14).

X-linked lymphoproliferative disease (XLP) is characterized by extreme sensitivity to EBV. Primary EBV infection leads to a very severe, often fatal IM, with large polyclonal expansion of EBV-infected B cells and uncontrolled proliferation of cytotoxic T cells. The dysregulated cytotoxic T-cell response and subsequent cytokine release leads to extensive organ damage (15). In more than 60% of cases, EBV infection leads to a fatal aplastic phase with macrophage activation, hemophagocytosis, and the destruction of all lymphoid tissues (16). Most of the survivors of primary infection later develop hypogammaglobulinemia and/or malignant lymphoma.
In 1998, the affected gene *SAP/SH2D1A* on XLP was identified and the gene product was named SAP (the SLAM-associated protein) (17-19). SAP is a short adaptor molecule of 128 amino acids that contains one SH2 domain and is expressed predominantly in activated T and natural killer (NK) cells (20, 21). Identification of the SAP gene has allowed accurate diagnostic testing for the syndrome, which may vary markedly in its clinical presentation.

1.2 IMMUNE RESPONSES TO EBV INFECTION

1.2.1 Humoral immune response

Sera of EBV infected individuals contain antibodies specific to both lytic and latent EBV antigens. However, the cellular immune response is more important for the control of EBV infection. This is supported by the fact that healthy virus carriers harbor multiple EBV strains (22) and immunocompromised individuals can be super-infected with several EBV strains and develop EBV associated PTLD in spite of increased titers of EBV specific antibodies (23).

1.2.2 Cellular immune response

1.2.2.1 NK cells

NK cells are key component of the early innate immune response to many infectious agents. They were initially defined on the basis of their cytotoxic activity (24). NK cells may play an important role in the control of primary EBV infection by eliminating infected B cells. NK cells respond to the lytic infections, because the reduction of HLA class I expression in lytically infected cells increased the sensitivity to NK cell recognition (25, 26). In addition, NK cells could contribute to the development of adaptive immunity through interaction with dendritic cells or T cells (27-29) and via release of immunomodulatory cytokines (30).

Activated NK cells were detected in the blood of infectious mononucleosis patients, and the NK cell number was inversely correlated with the virus load (30). In *in vitro* studies, NK cells were shown to inhibit the EBV induced transformation of B cells. This effect was mediated partially through IFN-γ production (31). Thus IFN-γ can inhibit directly the transformation of B lymphocytes as well. Similarly, tonsil derived NK cells were shown to be IFN-γ producers and in collaboration with IL-12 producing dendritic cells they inhibited in vitro transformation of EBV-infected B lymphocytes (32). However NK cells could not limit the EBV-driven lymphoproliferative disease in the T cell depleted transplant patients (33). It seems that NK cells can inhibit EBV induced B cell transformation in the early stage of infection, but EBV specific T cells are the main effectors in the later stages.

1.2.2.2 Monocytes

By analysis of serum samples from infectious mononucleosis patients, results indicated that monocytes were strongly activated during IM, monocytes and monocyte-derived factors might play an important role in the pathogenesis of IM, and together with T lymphocytes, may be partly responsible for clinical symptoms (34).

1.2.2.3 T cells

The key feature of the immune response in infectious mononucleosis is the marked expansion of activated lymphocytes (35, 36). More than 70% of activated lymphocytes in infectious mononucleosis are oligoclonal expanded $CD8^+$ T cells (37), which express the activation markers such as HLA-DR, CD45RO, CD38 (6, 38, 39). Ex vivo cytotoxic assay (40-42), HLA class I tetramer staining (5), ex vivo ELISpot assay of

IFN-γ release, and single-cell cloning have shown that many of these activated $CD8^+$ T-cells are EBV specific (43-45).

This dramatic $CD8^+$ T-cell response limits the proliferation of EBV-infected B cells, but they also release excessive amounts of cytokines (especially Th1 type cytokines, such as IL-2 and IFN-γ) which are thought to contribute to the symptoms of infectious mononucleosis (46, 47). Recent studies show a correlation between the level of activated T cells and the severity of the symptoms in infectious mononucleosis (48).

Although lytic and latent viral proteins are both expressed during primary EBV infection, early EBV-specific $CD8^+$ T cell responses during acute infection are directed mainly towards lytic proteins (5, 43, 49). After resolution of the acute infection, lytic epitope-specific $CD8^+$ T cells decline in frequency, but most of them remain detectable throughout latency. However, latent epitope-specific $CD8^+$ T cells are not commonly detected in the peripheral blood at presentation with IM (49) but become detectable after several weeks and their frequencies remain stable over long periods of time.

Many EBV-specific $CD8^+$ T cells recognize the epitopes derived from the latent proteins EBNA3A-, -3B-, and -3C (50, 51). Such CD8 responses to EBV latent antigens are of particular interest because of their ability to recognize and kill virus-transformed B cells in vitro and their therapeutic potential against EBV-driven B-lymphoproliferative disease in vivo (52).

Because the T-cells expanded in the IM and also the memory T-cell responses reactivated by in vitro stimulation with LCL are dominated by $CD8^+$ effectors, the HLA class I-restricted CD8 response has attracted the most attention. Data are more limited on the response of other lymphocyte subsets during IM.

The marked lymphocytosis during acute IM involves an expansion of both $CD4^+$ and $CD8^+$ T cells (38). The discovery of an EBV-induced superantigen suggests that the $CD4^+$ T cell expansion may be nonspecific (53). However, recent works show that EBV specific $CD4^+$ T cells responses to lytic and latent viral epitopes are detected early in IM (48, 54, 55). CD4 responses occur also to other pathogens in mice and humans (56). Virus-specific $CD4^+$ T cell numbers in the blood peak within 3 weeks of

presentation in IM patients, up to 2.7%. Thereafter, responses to lytic and latent antigens decline rapidly (46, 54).

The interest in latent antigen specific $CD4^+$ T cell responses is increasing. Firstly, they are likely to be important in the maintenance of virus-specific $CD8^+$ T cell surveillance in the host (57); secondly, because virally transformed B cells express HLA class II molecules and have HLA class II-processing function (1).

In earlier studies, in vitro stimulation of PBMCs with LCLs generated rare $CD4^+$ T cell clones to EBV latent proteins, specific for EBNA1 and EBNA2 derived epitopes respectively (58, 59). Thereafter, $CD4^+$ T cell recall responses to more epitopes have been generated by a variety of protocols. Most work in this area has focused on EBNA1 as a $CD4^+$ T cell target and has produced conflicting reports as to the ability of Ag-specific $CD4^+$ clones to recognize LCL cells presenting peptides from endogenously expressed EBNA1 (60, 61). Recently, a range of epitopes in the EBNA1, EBNA2, EBNA3A, and EBNA3C proteins were found to be recognized in LCL with highly epitope specific (62).

In a very recent study, the specificity of $CD4^+$ T cell lines generated from acutely and persistently infected donors was determined by using a genetically engineered EBV mutant that is unable to enter the lytic cycle and recombinant purified EBV proteins. The T cell lines consistently responded against EBV lytic cycle antigens and autoantigens, but barely against latent antigens. Lytic cycle antigens were predominantly derived from structural proteins of the virus presented on MHC II, via receptor-mediated uptake of released viral particles. These results indicate that structural proteins of EBV are the immunodominant targets of $CD4^+$ T cells in LCL-stimulated T cell populations (63, 64).

1.3 IN VITRO INFECTED CORD BLOOD CELL CULTURES--A MODEL FOR STUDYING PRIMARY EBV INFECTION

EBV specific immunological memory is not transferred from mother to child. Therefore, cord blood mononuclear cells (CBMCs) are well suited for analysis of cellular interactions in primary infection, and the mechanisms on generation of EBV specific cytotoxic T cells.

Infection of cord blood lymphocytes with EBV leads to the outgrowth of EBV infected B cells. However, a number of reports show that the primary responses were induced in fetal cord blood by EBV transformed LCL (65-67).

Several studies of NK cells from freshly isolated seropositive and seronegative adult blood have shown that they can inhibit the outgrowth of EBV-infected cells (25, 68-70). Ex vivo NK cells from fetal cord blood did not inhibit outgrowth of LCL (70), but they could be activated in the EBV infected cord blood mononuclear cell cultures (20, 71) or by culturing them with LCL (65).

EBV infection in the T cell depleted cord blood led to a larger increase in virus transformation efficiency than either CD16 or CD14 depletion (71). $CD4^+$ T cells may play a critical role in regulating EBV transformation, both by directly killing EBV infected B cells and by secreting IL-2, which induce NK cells to kill infected cells. Vaccination studies in cottontop tamarin model showed that antigen-specific $CD4^+$ T cell cytotoxic response was required for optimum activation of the NK cells (72, 73). Several studies with human lymphocytes have highlighted the cytotoxic activity of $CD4^+$ T cells following stimulation by EBV-transformed LCL in seropositive adults (74, 75) and in fetal cord blood (65-67).

In EBV infected CBMC cultures containing high numbers of activated NK cells and cytotoxic $CD4^+$ T cells the virus transformation efficiency was substantially reduced (71). Using fetal cord blood, $CD4^+$ cytotoxic T cells were generated by restimulation with autologous LCL, and were further cultured with the autologous EBV infected B cells. The $CD4^+$ T cells limited EBV-induced B cell transformation at a high effector/target ratio (10:1), but they potentiated the B cell transformation at the lower effector/target ratio (1:1). Further results showed that Th1 polarized $CD4^+$ T cells were more effectively inhibiting B-cell transformation, but Th2-polarized cell lines did not inhibit LCL growth, and increased the transformation efficiency (76). In line with our findings (77), this study also showed that development of $CD4^+$ cytotoxic T cells needs the presence of monocytes.

The cytokines, interferons, IL-2, IL-12, and IL-15, have been shown to activate NK and T cells, induce them to proliferate, increase the expression of CD95 ligand in the

membrane and enhance their cytotoxic activity in vitro (68, 69, 78). Cytokine secretion by monocytes may be pivotal for NK and T cell activation and cytotoxic activity of EBV-infected cells during a primary immune response.

1.4 IMMUNOMODULATORS

1.4.1 PSK

Polysaccharide K (PSK) is a protein-bound saccharide preparation isolated from *Coriolus versicolor* fungi (79). PSK is used as an immunostimulant for treating cancer patients in Japan for more than 20 years (80). Its antitumor activity has been documented in experimental animal models and beneficial therapeutic effects have been shown in the clinical studies. In experiments performed in our earlier study, lymphocyte response against autologous tumor cells in mixed lymphocyte-tumor cell culture (MLTC), PSK was shown to enhance the generation of cytotoxic response (81). PSK has been shown to elevate the expression of CD40 and CD86 on dendritic cells (82). In this report IL-12 production was measured and detected on the 7^{th} day after the dendritic cells were twice exposed to PSK. Production of IL-15 was not tested.

1.4.2 Trx80

Trx80 is a truncated thioredoxin with the 80 N-terminal residues. It is detectable in plasma and it is secreted from activated cells together with full length thioredoxin (83-85). Truncated thioredoxin was previously shown to be strongly expressed on the cell surface of monocytic cell lines like U937 whereas it was only weakly expressed in other cell lines of B cells, T cells and granulocytes (86).

Trx80 has been shown to stimulate a Th1 response in human PBMC cultures through induction of IL-12 secretion of monocytes (83-85). Compared to thioredoxin, Trx80 has unique functions in activating monocytes. It induces monocyte differentiation with increased expression of CD14, CD40, CD54 and CD86 (83, 84). When it was added to the purified monocyte cell cultures Trx80 induced IL-10 production by monocytes. The mitogen-activated protein (MAP) kinase signaling pathways: p38, ERK and JNK were involved (87).

1.5 CYTOKINES

1.5.1 IL-15

IL-15 is produced mainly by monocytes/macrophages and dendritic cells (88, 89). It was identified as a cytokine having biological properties similar to IL-2 (90, 91).
Although IL-15 shares biologic activities with IL-2, there are several properties of IL-15 that are distinct from those of IL-2. In IL-15 transgenic mice, IL-2 induced activation-induced cell death is inhibited (92). In vitro studies also showed that IL-15 plays an important role in the survival of NK cells by preventing or delaying apoptosis (93). Furthermore, IL-15 promotes the maintenance of $CD8^+$ memory T cells (94, 95).

Viral infections, including EBV, result in enhanced IL-15 gene expression and secretion in the infected peripheral blood mononuclear cells (PBMCs) (96-98). IL-15 inhibited EBV-induced B-cell transformation in adult blood lymphocyte cultures. This growth inhibition was mediated by NK and NKT cells (99).

IL-15 stimulated the generation and proliferation of NK cells in vivo (93). It also activates NK cells and cytotoxic T lymphocytes (100). IL-15 can synergize with IL-12 to produce substantial quantities of IFN-γ, TNF-α and GM-CSF in human PBMC cultures (101).

1.5.2 IL-12

IL-12 is a heterodimer (p70) composed of covalently linked p40 and p35 subunits. Bioactive IL-12 is produced by activated monocytes /macrophages, dendritic cells, and other antigen-presenting cells (102). Its production is positively (IFN-γ and TNF-β) (103) or negatively (IL-4, IL-13, TGF-β, and IL-10) (104, 105) regulated by other immune mediators. Although IL-12 stimulates IFN-γ production, a priming amount of IFN-γ is necessary to get measurable IL-12 levels (103).

IL-12 was discovered as "natural killer–stimulating factor" and as "cytotoxic lymphocyte maturation factor", respectively (106, 107). Initial characterization of its biological activities revealed that IL-12, when added to human peripheral blood lymphocytes, induced IFN-γ production, increased NK cell cytotoxicity as well as T cell proliferation in response to mitogenic lectins and phorbol diesters (102).

Release of this cytokine from antigen-presenting cells directs the differentiation of T cells into effector T helper 1 (Th1) cytokine–producing cells and is a costimulus of activation of Th1 cells, it while suppresses Th2 cell generation (108).

In addition to its noted effects in the priming of Th1 cell responses and IFN-γ production by T and NK cells, more recent studies support its critical role as a third signal for $CD8^+$ T cell differentiation (109, 110), and as an important factor in the reactivation and survival of memory $CD4^+$ T cells (111).

1.5.3 IL-18

IL-18 was initially identified as a potent IFN-γ-inducing factor (112). IL-18 can synergize with IL-12 leading to the activation of *IFN-γ* gene expression (113), and increased production of IFN-γ by $CD4^+$ T cells and NK cells (113).

Furthermore, IL-18 acts as a costimulant for Th1 cells to augment the production of IFN-γ, IL-2, and GM-CSF (114). IL-18R is expressed selectively on Th1 cells (115). $CD4^+$ T cells increased IL-18Rα expression after being stimulated with IL-12, and displayed dose dependent production of IFN-γ and cell proliferation in response to IL-18 (116).

IL-18 increases the cytotoxic actvity of NK and $CD8^+$ T cells (117, 118). It also enhanced the generation of allogeneic cytotoxic T lymphocytes (CTL) in mixed lymphocyte cultures (119).

1.5.4 IFN-γ

The interferons (IFNs) were originally discovered as agents that interfere with viral replication (120). Initially, they were classified by the secreting cell type but are now classified into type I and type II according to receptor specificity and sequence homology. IFN-γ is the sole type II IFN (121).

The IFN system regulates innate and adaptive immunity to viral infection. Viral invasion directly triggers induction of type I IFNs (122). Although both types of IFN are crucial in the immediate cellular response to viral infection, the immunomodulatory

activities of IFN-γ become important later in the response in coordinating the immune response and establishing an antiviral state for longer term control (123, 124).

In vitro studies with EBV-infected adult blood lymphocytes showed that NK cells inhibited the EBV-induced transformation of B cells, and this effect was mediated partially through IFN-γ production (31).

A very recent report showed that IFN-γ and IL-12 suppressed the expression of the lytic cycle initiating BZLF-1 in EBV-infected B lymphocytes. The authors hypothesized that immune activation might contribute to EBV-associated lymphomagenesis by suppressing entry into lytic cycle and in turn promoting latent EBV infection (125).

IFN-γ secretion by NK cells, and possibly professional APCs, is likely to be important in early host defense against infection, whereas T lymphocytes become the major source of IFN-γ in the adaptive immune response (126, 127).

In turn, IFN-γ induces IL-12 expression in macrophages and monocytes (128). The protective effects of IL-12 in tumor-bearing mice were ablated upon administration of neutralizing IFN-γ specific mAbs (129).

IFN-γ is a major product of Th1 cells and further skews the immune response toward a Th1 phenotype (130). IFN-γ and IL-12 are the prototypic cytokines directing Th1 differentiation during the primary response to antigen, while IL-4 directs differentiation of Th2 populations. IFN-γ induces IL-12 production in phagocytes (128) and inhibits IL-4 secretion by Th2 populations (131), which may further drive Th1 differentiation in vivo.

IFN-γ and IL-12 coordinate the link between pathogen recognition by innate immune cells and the induction of specific immunity, by mediating a positive feedback loop to amplify the Th1 response (130). LPS and other pathogen-associated molecular patterns directly trigger IL-12 production upon recognition by macrophages, DCs, and neutrophils (132), which in turn induces IFN-γ secretion in antigen-stimulated, naive $CD4^+$ T cells and NK cells (133). IL-12-induced IFN-γ participates in a positive feedback by further promoting IL-12 production in macrophages (134, 135).

1.6 LEUKOTRIENE B_4

Leukotriene B_4 (LTB_4) was discovered in 1979 (136), and later was found to be a potent neutrophil chemoattractant (137). The name leukotriene comes from the words leukocyte and triene indicating the compound's three conjugated double bonds (138).

1.6.1 Leukotriene B_4 biosynthesis

LTB_4 is a potent lipid inflammatory mediator derived from membrane phospholipids by the sequential actions of cytosolic phospholipase A_2 ($cPLA_2$), 5-lipoxygenase (5-LO) and leukotriene A_4 (LTA_4) hydrolase. The immediate product of 5-LO is LTA_4 (139), which can be further metabolized into either LTB_4 by LTA_4 hydrolase, or LTC_4 by LTC_4 synthase (140). 5-LO activating protein (FLAP) is critical in leukotriene biosynthesis. It binds arachidonic acid and facilitates the 5-LO reaction.

Biosynthesis of leukotrienes occurs mainly in myeloid cells and B lymphocytes (141). The production of LTB_4 and the biologic effects of this compound on myeloid cells are well characterized. However, much less is known about its effect on B lymphocytes.

In contrast to myeloid cells, normal B cells do not produce LTB_4 after challenge with calcium ionophore A23187 (142, 143). The mechanism of activation of leukotriene biosynthesis in normal B cells is unclear, but there is accumulating evidence that the cellular oxidative status is of importance for the biosynthesis of the leukotrienes (143-145). Furthermore, the p38 mitogen-activated protein kinase appears also to be involved in stress-induced leukotriene synthesis in B cells (146).There is no convincing report demonstrating that T lymphocytes contain 5-LO and can produce leukotrienes. However T lymphocytes do express FLAP, but the function of this protein in T cells is not known (143).

1.6.2 Leukotriene B_4 receptors

The major activities of LTB_4 include the recruitment and activation of leukocytes.
Molecular identification of a receptor for LTB_4 eluded investigators for many years, until the human high-affinity LTB_4 receptor was finally cloned (147), while the mouse ortholog was identified a bit later (148). This high-affinity G-protein coupled receptor

was initially named BLTR, and subsequently renamed BLT1 when a second LTB_4 receptor was identified (149, 150).

By mediating the activities of LTB_4, these receptors participate both in the recruitment and activation of leukocytes. BLT1 is expressed on neutrophils and monocytes (151, 152). It is also expressed on activated T lymphocytes, both $CD8^+$ cells and $CD4^+$ cells (153-155), and on peripheral human non-activated B lymphocytes (156). BLT2 has lower ligand affinity and its tissue distribution is wider than that of the BLT1.

1.6.3 Biological effect of Leukotriene B_4

LTB_4 is an immunomodulator, which has a regulatory role in both natural and adaptive immune responses (157-159). LTB_4 is a potent neutrophil chemoattractant and it also induces the chemotaxis of dendritic cells (160).

LTB_4 enhances activation, proliferation, and antibody production of tonsillar B lymphocytes (161), and stimulates various T cell functions (153-155) .

1.6.3.1 LTB_4 and Monocytes

LTB_4 has been shown to activate monocytes and macrophages by inducing chemotaxis, surface CD11b expression, and phagocytosis. Message for both BLT1 and BLT2 receptors has been detected in $CD14^+$ monocytes (162), and flow cytometric studies of human peripheral blood leukocytes have confirmed BLT1 expression on monocytes, which represent 85–90% of the peripheral monocyte population (163).

LTB4 upregulates on monocytes the avidity of the β1- and β2-integrins for their ligands, and enhances monocyte adhesion (164). It also potentiates the cytokine production of human peripheral blood monocytes (165).

1.6.3.2 LTB_4 and T Lymphocytes

Although LTB_4 is identified as a potent activator of leukocytes of the innate immune system, such as granulocytes and monocytes, LTB_4 has been previously shown to bind to T cells (166) and induce T-cell chemotaxis in vitro (167), as well as T-cell cytokine production (168). Characterization of the BLT1 receptor led to an exploration of its expression on human peripheral blood leukocytes and its identification on T cells (156, 162).

Although BLT1 is not found in naïve T cells, BLT1 expression is significantly upregulated in activated CD4 Th1 and Th2 cells (155), and effector $CD8^+$ cells (153, 154). BLT1 is also expressed in murine T-cell lymphomas (148), suggesting that it may be expressed on peripheral blood T cells in mice as well. Exploration of the functional roles of BLT1 on T cells using mouse models of inflammation has revealed that BLT1 activation plays a critical role in the recruitment of effector CD4 and CD8 cells early in inflammation. In the early phases of an active immunization asthma model, BLT1 $^{-/-}$ mice exhibit defective recruitment of CD4 and CD8 cells in the airways, but have normal levels of airway T cells in an adoptive transfer model, indicating an important role for BLT1-mediated T-cell trafficking in both humoral and adaptive immune responses (155).

A detailed characterization of BLT1 in human resting peripheral blood cells has identified BLT1 expression on a very rare subset of $CD4^+$ and $CD8^+$ T cells (0–1%) (169). This $BLT1^+$ population is enriched for the late activation markers CD38 and HLA-DR, and inflammatory chemokine receptors, and they tend to express higher levels of polarizing cytokines. However, dendritic cell stimulation of CD4 and CD8 cells may lead to a dramatic increase in surface BLT1 expression. Asymptomatic atopic asthmatics demonstrate increased numbers of $BLT1^+$ $CD4^+$ and $CD8^+$ memory T cells in the airways. EBV-specific $CD8^+$ T cells from acutely infected individuals also have increased levels of BLT1 expression compared with those with asymptomatic chronic infection (169).

These studies demonstrate that BLT1 is constitutively expressed on antigen-primed memory/effector T cells. It suggests a primary function of responding rapidly to inflammatory stimuli. However, during infection or T cell activation BLT1 expression is transiently increased on this T cell subpopulation, enhancing their ability to quickly respond to the early phases of infectious and inflammatory processes (170).

1.7 B CELL CHRONIC LYMPHOCYTIC LEUKEMIA

B-cell chronic lymphocytic leukemia (B-CLL) is the most common leukemia in western countries. It is characterized by clonal accumulation of $CD5^+$, $CD23^+$ B lymphocytes (171, 172). B-CLL cells are small cells arrested in the G0/G1 stage of the cell cycle.

B-CLL cells have low expression of a truncated B-cell receptor (CD79a/CD79b) (173). They express MHC-I and -II, CD54 (ICAM-1), CD27, CD40 (174). They do not or express only weakly adhesion or costimulatory molecules, including CD80 and CD86, which are essential for the function of antigen-presenting cell (APC). Chemokine receptor expression on B-CLL cells such as CXCR4, has been implicated in the homing of B-CLL cells to the bone marrow (175).

B-CLL cells secrete immune inhibitory cytokines including IL-10 and TGF-ß (176, 177). T-cells in B-CLL patients are often dysfunctional and proliferate poorly when exposed to mitogens or alloantigens (178).

The immunoglobulin variable heavy chain (IgV_H) mutation status (179, 180), the expression of ZAP-70 (181, 182) and CD38 (183, 184), the p53 functionality (179, 185) were all identified as biologic markers of CLL. The absence of IgV_H mutation, expression of ZAP-70 and CD38, and p53 dysfunction, were seen in patients with the worst clinical outcome.

1.7.1 CD40 expression on normal B-cells and on B-CLL cells

CD40 belongs to the family of tumor necrosis factor receptors and is expressed throughout B cell development. It is involved in the survival, proliferation and differentiation of B lymphocytes (186, 187). Its physiological ligand CD40L (CD154) is a member of the TNF family (188). CD40/CD40L interaction stimulates B cells, dendritic cells and monocytes to proliferate, differentiate and increase antigen presentation (189-192). Cognate antigen recognition by the TCR induces an upregulation of CD154 on the $CD4^+$ T cell that in turn activated signaling via CD40 and resulted in upregulation of CD80/CD86 expression on the APC. This CD40-CD40L interaction in turn amplifies the activation of the T cells and promotes their differentiation thereby modulating humoral immune responses (193).

In vitro studies with B-CLL cells showed that CD40 ligation induced the expression of CD95, a receptor for apoptotic signals, and provided a strong NF-κB-mediated survival signal (174, 194). Correspondingly, CD40 activation of B-CLL cells reduces fludarabine-induced apoptosis in vitro (194). While generally CD40 activation on B-

cell leukemia and lymphoma cells delivers pro-survival signals, increased apoptosis was shown in the case of multiple myelomas (195).

1.7.2 LTB_4 and CLL

5-lipoxygenase (5-LO), the key enzyme in leukotriene biosynthesis, is abundantly expressed in B-CLL cells (196, 197). B-CLL cells have the capacity to produce leukotriene B_4 (LTB_4) and they expressed its receptor, BLT1 (198). This indicates that LTB_4 might influence the behavior of B-CLL cells in an autocrine and/or paracrine manner.

MK-886 (a specific FLAP inhibitor) and BWA4C (a specific 5-LO inhibitor) both inhibited the DNA synthesis of B-CLL cell stimulated by CD40 ligation. This effect was counteracted by addition of exogenous LTB_4 (198) This is in line with the fact that MK-886 has an antiproliferative effect and induces apoptosis in HL-60 cells (199).

Soluble CD54 and soluble CD23 levels are high in the blood of CLL patients with advanced clinical stage (200, 201). LTB_4 was shown to stimulate CD54 expression on endothelial cells and CD23 on B cells (161), (202) . In the B-CLL cell cultures, MK-886 and BWA4C counteracted the CD40 ligation-induced expression of CD54 and CD23. Exogenous LTB_4 reversed these effects of the inhibitors (198).

LTB_4 played role in the activation of B-CLL cells by CD40L. Leukotriene biosynthesis inhibitors, or BLT1 antagonists, alone or in combination with conventional therapy, might be useful in the treatment of B-CLL.

2 RESULTS AND DISCUSSION

2.1 PSK AND TRX80 INHIBIT B CELL PROLIFERATION AND POTENTIATE T AND NK CELL ACTIVATION IN EBV INFECTED CBMC CULTURES (PAPER I, II)

2.1.1 PSK and Trx80 inhibit B cell proliferation in EBV infected CBMC cultures

Cell compositions in the EBV infected CBMC cultures were detected by flow cytomety. In the PSK and Trx80 containing cultures, the increase of the B cells was less pronounced, while the proportion of T cells increased in comparison to the untreated cultures.

B cell proliferation induced by EBV was assessed by measuring thymidine incorporation in the EBV infected CBMC. The values were lower in the cultures containing PSK and Trx80, indicating that the proliferation of EBV infected B cells was inhibited. PSK and Trx80 had synergistic effect. The inhibition was substantiated by visual examination of the cultures. PSK (25μg/ml) and Trx80 (100nM) did not inhibit the EBV induced proliferation of isolated B cells. The decrease in B cell growth could thus be ascribed to the potentiation of the T, NK cell mediated inhibition.

2.1.2 PSK and Trx80 potentiate T and NK cell activation in EBV infected CBMC cultures

To detect the T and NK cell activation we tested SAP expression since only activated T and NK cells express this protein. We found that introduction of PSK and Trx80 to the culture enhanced SAP expression. The intensity of the SAP band in immunoblot increased only in the EBV infected cultures and only when PSK or Trx80 was added. Presence of both PSK and Trx80 in the infected culture led to higher SAP expression. Since the samples were analyzed on the basis of cell numbers, the higher intensity of the SAP band can be ascribed to the decrease of the proportion of SAP negative B blasts.

2.1.3 The effect of PSK and Trx80 is mediated by monocytes-produced IL-15 and IL-12 respectively (Paper II)

The effect of PSK or Trx80 required the presence of monocytes. In the absence of monocytes, SAP protein was not induced in the PSK and Trx80 containing cultures. Similarly B cell proliferation was not inhibited either. In monocyte reconstitution experiments when PSK and Trx80 treated autologous monocytes were reintroduced into the cultures, SAP expression increased.

Further we showed that the effect of PSK and Trx80 activated monocytes was mediated by IL-15 and IL-12 respecitively. Antibodies against IL-15 prevented the SAP induction and substantially reduced the B cell growth inhibition in the PSK containing cultures. In parallel the Trx80 containing cultures, antibodies against IL-12 inhibited the induction of SAP and reduced the inhibition of B cell proliferation. The specific effect of the antibodies against the cytokines was also shown in the monocyte reconstituted cultures.

We tested the presence of different cytokines in the culture. PSK containing cultures produced IL-15, while Trx80 containing cultures produced IL-12. In the absence of monocytes the supernatants did not contain these cytokines. Similar results were obtained in the monocyte reconstituted cultures.
By intracellular staining we showed that IL-15 and IL-12 were produced by $CD14^{+}$ monocytes.

The monocytes became activated by PSK and Trx80, and produced cytokine IL-15 and IL-12 which acted on T and NK cells. The Trx80 or PSK treated monocytes alone did not produce IL-12 and IL-15.

2.1.4 PI 3-K and ROS were involved in the monocytes activation (Paper II)

PSK-treated monocytes produced ROS. The functional tests substantiated the results. In the cultures which received monocytes treated with the antioxidant and PSK, the level of IL-15 was lower and the SAP band became weaker.

The same experimental strategy was applied on monocytes activated by Trx80. The PI 3-K but not ROS was involve in the response of monocytes to Trx80.

2.1.5 EBV-specific cytotoxic cells can be generated in the PSK and Trx80 containing cultures after restimulation (Paper II)

The PSK and Trx80 containing cultures were restimulated with autologous EBV infected B cells, in the presence of IL-2. The composition of the populations in the 19 day old cultures showed that the population consisted mainly of CD4 positive T cells.

At this time B lymphocytes were removed and the cytotoxic function of the remaining cells was tested against: autologous EBV infected B cells, autologous B cells activated with CD40L and IL-4, allogeneic LCLs and K562 cells. The cells of the PSK and Trx80 containing cultures had appreciable cytotoxicity to the autologous EBV infected B cells. The lysis of autologous target was reduced by HLA class II mAbs CR3/43.

Earlier we have reported similar results in this system when the cultures contained PSK. K562, the classical NK target was also lysed by effector cells originating from the Trx80/PSK cultures, but the effect was weaker than against the specific target. The lysis of K562 cells reflected enrichment of NK cells (Paper I). The composition of the populations indicated also the elevated proportion of CD56 positive NK cells in the Trx80/PSK cultures.

Autologous CD40L-activated B cells were not lysed (203). It is therefore likely that EBV specific T cell cytotoxicity was generated in the cultures.

The results from the PSK and Trx80 treated cultures suggest the following scenario: EBV infected and activated B lymphocytes. They were recognized by T cells that became posed for responding to cytokines produced by monocytes. PSK and Trx80, both activated the monocytes which then produced cytokines in the presence of the primed T cells. PSK induced IL-15 while Trx80 induced IL-12 production. Both cytokines activated the T cells for function (Fig. 1).

Further we studied the contribution of NK cells in the development of adaptive immunity in the EBV infected CBMC populations.

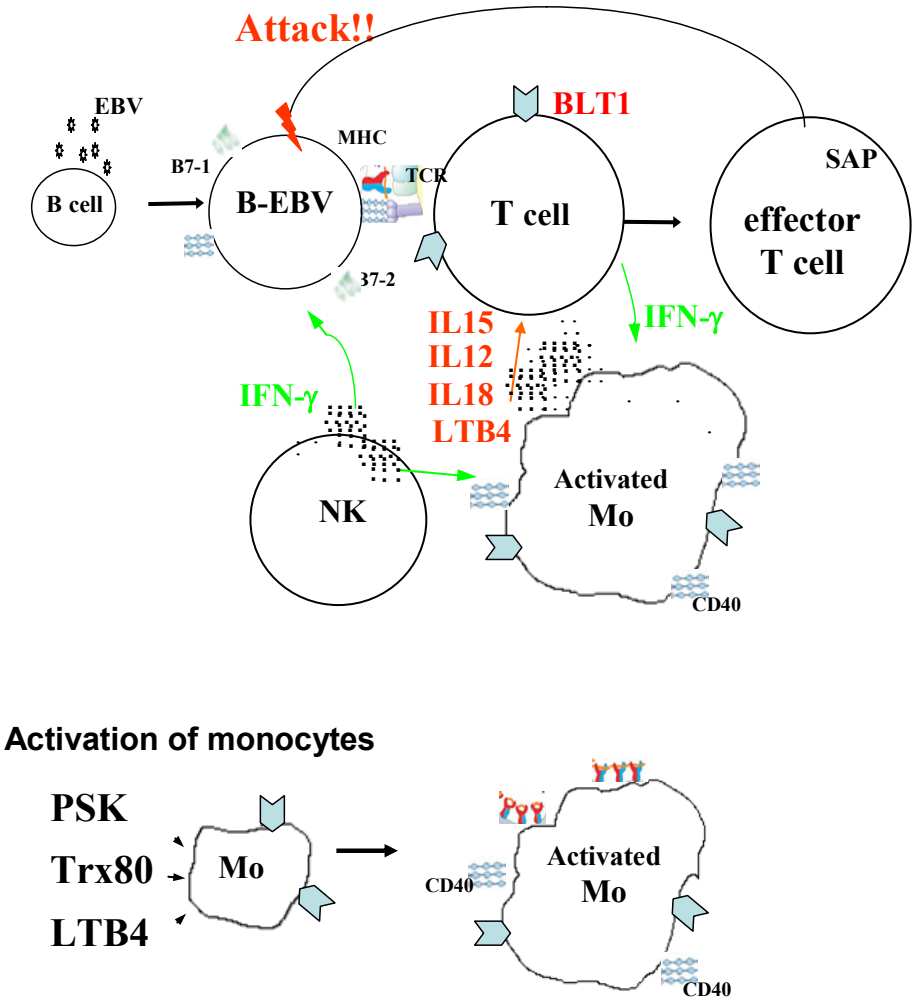

Fig. 1. Interactive autocrine/ paracrine loops in the EBV infected CBMC cultures.

-B cell are activated by EBV
-T cells are activated by the activated B cells
-Activated T cells express BLT1
-Monocytes are activated by PSK, Trx80 or LTB_4
-Activated monocytes get in contact with activated T cells
-Activated monocytes produce lymphokines and LTB_4
-Lymphokines and/ or LTB_4 impose functional activation of T cells
-NK cells are also required for IFN-γ production

2.2 NK CELLS ARE ESSENTIAL FOR THE IMMUNOLOGICAL RECOGNITION OF EBV-INFECTED CORD BLOOD B CELLS (PAPER III)

2.2.1 T cell activation, lymphokine production and B cell growth inhibition were reduced in the EBV infected cord blood cell cultures in the absence of NK cells

NK cell depleted populations, in parallel with the total mononuclear cells, were infected with EBV and the immunomodulators, PSK and Trx80 were added.

The expression of CD69 and SAP protein was used to indicate the activation of T cells. A low proportion of $CD3^+$ T and $CD56^+$ NK cells expressed CD69 in the ex vivo samples. Their frequency was higher in the virus infected, and it was markedly elevated in the immunomodulator containing cultures. The frequency of CD69 positive T cells was considerably reduced in the absence of NK cells.

SAP expression of T cells separated by negative selection was tested by immunoblot. In line with the increase of the activation marker CD69, the SAP band was stronger in the T cells derived from the immunomodulator containing cultures. In the absence of NK cells, T cell activation was less pronounced, as indicated by the relatively weaker SAP band of the T cells.

We confirmed our previous findings that the supernatant of PSK and Trx80 treated CBMC cultures contained IL-15 and IL-12, respectively. Their level was reduced by 71±5% and 77 ± 4% in the NK cell depleted cultures.

The EBV induced B cell proliferation was reduced in the immunomodulator treated cultures. In the cultures initiated at 2×10^5 cells/ml, the mean values of B cell growth inhibition in the PSK and Trx80 containing cultures were 56%, 49% respectively. In the absence of NK cells the growth inhibition was weaker, it was 15%, 17% respectively.

2.2.2 NK and T cells produced IFN-γ, which played a role in lymphokine production, T cell activation and B cell growth inhibition

IFN-γ production was detected on the single cell level. In the 3-day-old EBV infected cultures, IFN-γ was expressed in a high proportion of $CD56^+$ NK cells, but not in the

$CD3^+$ T cells. In the PSK and Trx80 treated cultures, a high proportion of both cell categories were IFN-γ positive. But in the NK cell depleted cultures IFN-γ producing T cells were not detected. In accordance, the supernatant of infected cultures contained IFN-γ and it was higher in the immunomodulator treated cultures. In the supernatant of the cultures initiated with NK cell depleted populations, IFN-γ was not detectable. These results showed that NK cells were the essential producers of IFN-γ in the virus infected cultures.

IFN-γ was required for IL-12 and IL-15 production of monocytes. Cultures that received IFN-γ neutralizing antibody contained lower amounts of IL-15 and IL-12. Moreover, addition of antibody against IFN-γ to the immunomodulator containing cultures reduced the intensity of the SAP band to the control level in the lysates of T cells. Consequently the inhibition of B cell proliferation was abolished by addition of the antibody against IFN-γ. These results are in line with the reduction of the T cell activating lymphokine production in these cultures.

2.2.3 Added IFN-γ compensated the absence of NK cells

The levels of IL-15 and IL-12 were reduced in the NK cell depleted cultures, but exogenous IFN-γ restored the production of IL-15 and IL-12 in PSK and Trx80 treated cultures.

SAP expression of T cells was reduced in the NK cell depleted cultures, but addition of IFN-γ or IL-15 and IL-12 compensated it to same extent. The results show that IFN-γ induced T cell activating lymphokine production of the monocytes, and the activation of T cells could be directly induced by added IL-15 and IL-12.

In accordance with these findings, in the absence of NK cells, the inhibition of EBV induced B cell growth was considerably lower in the immunomodulator containing cultures, but this was restored by added IFN-γ. The inhibition of B cell proliferation showed a dose response for the added IFN-γ.

In line with the demonstration that monocytes produce these lymphokines, IL-15 and IL-12 were not induced by IFN-γ treatment in the monocyte depleted cultures. Inhibition of B cell proliferation was not restored in the monocyte depleted cultures.

2.2.4 NK cells were required for the generation of cytotoxic T cells in the EBV infected cultures

T lymphocytes were separated by negative selection from the 10 day old cultures, and expanded in culture with irradiated allogeneic CBMC, IL-2 and IL-15 (added 3 times). On the 27th day the cytotoxic function of the cells was tested against: autologous EBV infected B cells, autologous B cells activated with CD40L and IL-4, K562 cells and allogeneic LCL. Autologous EBV infected B cells, but not CD40L-activated B cells were lysed, indicating that T cells recognizing the EBV infected B cells were generated in the cultures (203).

T lymphocytes separated from cultures initiated without NK cells, did not acquire cytotoxic function when exposed the same strategy.
In the cultures initiated with the total population to which antibodies against IFN-γ were added there was no cytotoxic function detected either.
The absence of NK cells in the initial population could be compensated by addition of IFN-γ but only if monocytes were present.

These results showed that cytotoxic T cells were generated in the primary EBV infected cultures, but only if the initial population contains NK cells. IFN-γ production was the main contribution of NK cells as shown by restoring these function by addition of IFN-γ.

Results in this paper show the following steps: EBV infects and activates B cells. NK and T cells respond to the encounter of activated B cells and acquire the capacity to respond to the activating signals. NK cells produce IFN-γ. Addition of the PSK and Trx80 activate the monocytes, which in the presence of the primed T cells and IFN-γ are induced to produce cytokines IL-15 and IL-12. This creates a positive feedback, the primed T cells respond to the cytokines with acquisition of functional activity, and inhibit B cell proliferation. Further incubation of the cultures induces selection of EBV specific T cells and leads to the generation of cytotoxic T cells (Fig. 1).

2.3 LEUKOTRIENE B_4 PLAYS A PIVOTAL ROLE IN LYMPHOCYTE ACTIVATION (PAPER IV)

2.3.1 Involvement of LTB_4 in T and NK cell activation and B cell growth inhibition in EBV infected cord blood cell cultures

In accordance with our earlier results, the samples of the EBV infected cultures containing immunomodulator (PSK or Trx80) showed an increase of the SAP protein. The SAP level increase induced by the immunomodulator was prevented by the leukotriene biosynthesis inhibitors MK886 or BWA4C. Addition of LTB_4 to such cultures restored the intensity of the SAP bands. Inhibition of the endogenous LTB_4 production could thus be counteracted by introduction of the leukotriene from the outside, confirming the contribution of LTB_4 to the activation of T and NK cells.

Addition of MK886 or BWA4C reduced the production of IL-15 and IL-12 in the PSK and Trx80 containing cultures. Cytokine production was lower in presence of both inhibitors. Similarly to the reestablishment of SAP expression in the cells, the cytokine levels were restored to the control values when LTB_4 was added to the cultures.

EBV induced B cell proliferation was inhibited in the cultures containing the immunomodulators. MK886 or BWA4C reduced the growth inhibitory effects of PSK and Trx80. In the presence of both inhibitors, thymidine incorporation was similar to that of the control.

In summary, these results confirm our earlier findings that activation of T and NK cells in the EBV infected CBMC cultures can be detected by SAP expression, by the production of cytokines and by the inhibition of B cell proliferation. The new aspect that emerges from the present experiments is the essential role of LTB_4 in the activation of the effector cells.

2.3.2 LTB_4 and IFN-γ production in the EBV infected cultures

LTB_4 was detected in the EBV infected cultures that received the immunomodulators. LTB_4 production was considerably reduced when any of cytokines IFN-γ, IL-15 or IL-12 were neutralised by specific antibodies.

Its production also required the presence of monocytes. This is consistent with the identification of monocytes as the main LTB_4 producing cells under our culture conditions. Granulocytes are strong LTB_4 producers, but they were not present in our experiments. Activation of monocytes by the immunomodulators was essential for LTB_4 production, as shown earlier for the production of IL-15 and IL-12.

IFN-γ was produced in the infected cell population and its level increased in the immunomodulator containing cultures.

These results show the activation circuit between monocytes, NK and T cells. The latter cells assist in the activation of monocytes for LTB_4 production with provision of IFN-γ, but they require IL-15 or IL-12 produced by the monocytes.

2.3.3 Expression of BLT1, the LTB_4 receptor on the cells in the EBV infected cultures

In accordance with earlier reports, *ex vivo* T cells did not express BLT1, the LTB_4 receptor (168, 198). The frequency increased in the virus infected, and it increased further in the immunomodulator containing cultures. Interestingly, the activated NK cells did not express BLT1.

BLT1 is known to be expressed on 50% of *ex vivo* B lymphocytes (198). In *in vitro* cultures the CD19 subset did not express the receptor.

The majority of *ex vivo* T cells expressed chemokine receptor CXCR4 and it was downregulated in the infected cultures. Rare *ex vivo* T cells expressed CXCR3 and CCR5, but they increased to 51 and 54 % respectively in the infected cultures.

2.3.4 LTB_4 mediated potentiation of T cell activation in the EBV infected cultures

The production of LTB_4 in the immunomodulator containing cultures and the restoration of lymphocyte activation by addition of exogenous LTB_4 following inhibition of its endogenous synthesis prompted us to test the effect of LTB_4 added to the cultures, in the absence of PSK and Trx80.

Immunomodulators were omitted and LTB_4 was added to the EBV infected cultures. SAP expression was increased in a dose dependent manner. Specificity controls

included addition of LTC_4, LTD_4 and 5 (S),12 (S)-DiHETE that were all inactive. LTB_4 did not increase SAP expression in the uninfected cultures. Thus LTB_4 has a similar effect as the immunomodulators, PSK and Trx80.

The number of B cells increased, and the T cells decreased proportionally in the EBV infected cultures. In the presence of LTB_4, however, the B cell enrichment was impaired. The proportion of NK cells remained unchanged. Proliferation of the B lymphocytes, were in line with the composition of the cell populations in that the values were lower in the cultures containing LTB_4.

The growth inhibition in the cultures could be attributed to the activated effector cells, because LTB_4 did not affect the proliferation of separated B lymphocytes infected by the virus. Taken together, these data suggest that LTB_4 activated the T cells in the cultures and they inhibited EBV induced B cell proliferation.

Leukotriene B_4 raised the production of IFN-γ in the cultures. The supernatants of the monocyte-depleted cultures contained less IFN-γ. Low amounts of IL-15 and IL-12 were produced when the concentration of added LTB_4 was high. However LTB_4 induced IL-18 even when applied in lower amounts, and IL-18 production required also the presence of monocytes.

2.3.5 LTB_4 stimulates the production of LTB_4 and lymphokines by monocytes that activate T cells in EBV infected cultures

A fraction of T cells expressed BLT1 in the infected cultures. Therefore addition of LTB_4 had a direct effect on these cells, and induced elevation of SAP expression even in the absence of monocytes. However, the SAP inducing capacity of LTB_4 was tenfold higher in the presence of monocytes.

Reconstitution of monocyte-depleted cultures with LTB_4 treated monocytes could induce SAP expression, which was further increased when LTB_4 was added.
In monocyte reconstitution experiments, LTB_4 treated monocytes to the cultures of monocyte-depleted, EBV-infected cell populations. SAP was expressed. Inhibition of LTB_4 biosynthesis by adding MK886 and BWA4C to these reconstituted cultures reduced SAP expression considerably.

LTB_4 activated monocytes may produce IL-18, IL-15 and IL-12. Anti-IL-18 reduced SAP induction considerably while a mixture of anti-IL15 and anti-IL12 reduced SAP expression to a moderate extent. This was in line with the presence of these cytokines in the supernatants and showed that among these three cytokines LTB_4 predominantly induced production of IL-18.

Thus we found that LTB_4 could activate monocytes and they produced LTB_4. They also produced IL-18, IL-15 and IL-12 and they all contributed to the activation of T cells primed to respond to these mediators in EBV infected cultures.

We have shown earlier that SAP can be expressed both by activated T and NK cells. Next, we posed the question whether the increase of SAP protein detected in the immunoblots could be contributed by both cell subsets. For this we used two strategies. In the first, LTB_4 was added to monocyte-depleted cultures and it induced SAP expression. Lysates were also prepared from these cultures after removal of the T cells. These samples did not show stronger SAP band, indicating that T cells contributed to the increase of SAP expression. After removal of the T cells the population should still contain NK cells, but apparently their SAP expression was not changed by LTB_4. This was in line with the absence of the LTB_4 receptor on the NK cells. In the second strategy, the infected cultures were set up with monocyte and T cell depleted populations. In these cultures, containing B and NK cells, LTB_4 had no effect on SAP expression.

In conclusion, LTB_4 added to the EBV infected CBMC culture could activate both the monocytes and the T cells. Thus, in these cultures T cells can be activated both by the added LTB_4 and by the cytokines produced by the LTB_4 activated monocytes.

2.3.6 Generation of EBV-specific cytotoxic cells

We have shown earlier that the immunomodulator treated cultures could be restimulated by autologous EBV infected B cells to yield specific cytotoxicity. We tested whether this was also possible when LTB_4 was used as immunomodulator.

Therefore, the cultures initiated with added LTB_4 were stimulated twice with autologous EBV infected B cells. The LTB_4 treated culture contained mainly CD4 T cells. After removal of B lymphocytes the residual cells were assayed for cytotoxic

function. They showed appreciable cytotoxicity against autologous EBV infected B cell targets. In accordance with the preponderance of CD4 T cells in the culture, the lytic capacity was reduced by HLA class II mAbs CR3/43. The effect was specific, because activated autologous B cells, K562 or allogeneic LCLs (CBM1 and LCL2996) were not killed.

This paper showed that LTB_4 can act as immunomodulator in EBV infected cord blood lymphocyte cultures. It activates the monocytes and also the T cells that by the encounter of EBV infected B lymphocytes enter in a responsive state. The activated T cells then control the EBV induced B lymphocyte proliferation (Fig. 1, 2).

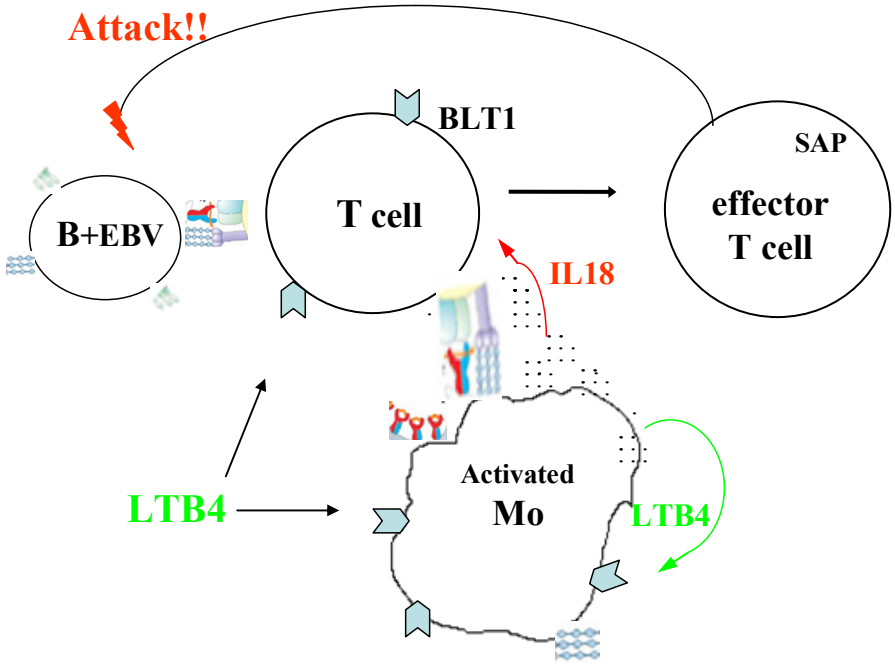

Fig. 2. Added leukotriene B_4 activates T cells and monocytes

-T cells are activated by the activated B cells
-Activated T cells express BLT1
-Monocytes are activated by added LTB_4
-Activated monocytes produce IL18 and LTB_4
-LTB_4 and /or IL18 impose functional activation of T cells

2.4 LTB_4 PLAYS A PIVOTAL ROLE IN CD40-DEPENDENT ACTIVATION OF CHRONIC B LYMPHOCYTIC LEUKEMIA CELLS (PAPER V)

2.4.1 Biosynthesis of leukotrienes in B-CLL cells

B-CLL cells did not produce detectable amounts of leukotrienes after challenge with either A23187 or arachidonic acid alone. But activation of the cells with both A23187 and arachidonic acid led to the formation of LTB_4. Preincubation of intact cells with the thiol-reactive agent diamide, prior to the addition of calcium ionophore and arachidonic acid, led to a markedly increased production of LTB_4 compared with untreated intact cells. Similar amounts of LTB_4 were produced in sonicated cells, incubated with arachidonic acid. There was no obvious correlation between the capacity to produce leukotrienes and the clinical stage of the disease.

Taken together, the results demonstrated that all investigated B-CLL clones had the capacity to produce LTB_4 and that all B-CLL clones contained substantial amounts of 5-LO, which could be activated under certain conditions.

2.4.2 BLT1 expression on B-CLL cells

Peripheral blood leukocytes from healthy donors were analyzed with FACS for the expression of BLT1. Virtually all granulocytes and monocyte expressed BLT1. There was no expression of BLT1 on peripheral nonactivated $CD4^+$ or $CD8^+$ T lymphocytes. These are in agreement with the previous findings. In contrast, 30%-50% of the peripheral B lymphocytes stained positively for BLT1.

BLT1 expression varied from about 15% to 85% in the investigated B-CLL clones (average expression, 42%).

2.4.3 Effects of leukotriene synthesis inhibitors on DNA synthesis in B-CLL cells

In order to determine whether leukotrienes are of importance for proliferation of B-CLL, the cells were cultivated in the presence of leukotriene biosynthesis inhibitors. B-CLL cells were cultivated together with CD40L-L cells or control L cells in the absence or presence of MK-886 or BWA4C. MK-886 and BWA4C markedly inhibited DNA synthesis induced by CD40L stimulation. LTB_4 alone did not amplify CD40-induced

thymidine incorporation. However, exogenous LTB_4 reversed the inhibitory effect of MK-886 and BWA4C on thymidine incorporation.

Taken together, these data demonstrate that specific inhibitors of leukotriene synthesis cause a pronounced inhibition of DNA synthesis that could be reversed by addition of exogenous LTB_4.

2.4.4 Effects of leukotriene biosynthesis inhibitors on CD23, CD54, and CD150 expression in B-CLL cells

MK-886 and BWA4C markedly counteracted the CD40 ligation induced expression of CD23, CD54, and CD150. LTB_4 alone did not cause any significant effect on the expression of the investigated antigens. However, addition of LTB_4 counteracted the inhibitory effect of the inhibitors on antigen expression. These results show that LTB_4 is involved in the expression of these antigens, which are associated with activation of B-CLL cells.

In summary, this study demonstrates that LTB_4 plays an important role in the activation of B-CLL cells. The present report indicates that leukotriene biosynthesis inhibitors, LTA_4 hydrolase inhibitors, or BLT1 antagonists, alone or in combination with conventional therapy, might also be useful in the treatment of B-CLL.

3 CONCLUSIONS

EBV specific immunological memory is not transferred from mother to child. We studied the cellular interactions in *in vitro* EBV infected cord blood mononuclear cell (CBMC) cultures. The EBV induced B lymphocyte proliferation was inhibited when the resident monocytes were activated with immunomodulators, PSK and Trx80. Feedback between the activated T cells and activated monocytes has led to IL-15 or IL-12 production, and the T cells acquired functional capacity. The proliferation of EBV infected B cells was inhibited. EBV specific cytotoxicity could be generated in these cultures by restimulation with EBV transformed autologous B cells.

Further we found that NK cells were essential for cell mediated inhibition of the proliferation of EBV infected B lymphocytes, through the production of IFN-γ.
Cytotoxic T cells could be generated in the primary EBV infected cultures initiated with the cell population containing NK cells. Results indicated that by activation of innate immunological mechanisms it was possible to generate EBV-specific T cell response in CBMC cultures.

The model system is reminiscent in several aspects to the clinical picture in the primary EBV infection, the symptoms of infectious mononucleosis, such as T and NK lymphocytosis and elevation of IFN-γ level in the serum. The intensity of innate immunity mechanisms is highly variable in the patients but it leads regularly to the development of EBV specific immunological memory.

We further demonstrate that leukotriene B_4 (LTB_4) is involved in the effect of the immunomodulators. Moreover, we found that LTB_4 added to infected cultures, induced functional activation of the T cells. LTB_4 activated the monocytes and acted directly on the T cells. In consequence, addition of LTB_4 inhibited the EBV induced proliferation of B lymphocytes. Specific cytotoxicity could be generated by restimulation of the T cells. The experiments showed successive stages of T cell activation in acquisition of their immunological effector function. This is orchestrated by complex cellular interactions, and autocrine loops mediated by soluble factors - IFN-γ, IL-15, IL-12, IL-18 and LTB_4.

Primary EBV infection may be silent or may lead to mononucleosis. The severity of the symptoms is highly variable. Severe cases could be mollified by reducing the extensive immune activation. These considerations motivate detailed analysis of the role of LTB_4 in the development of cellular immune responses and the possibility for its modification by LTB_4. EBV infected cultures of mononuclear cells as experimental model seems to provide a good system for such studies.

We also studied the function of LTB_4 in B-cell chronic lymphocytic leukemia (B-CLL) cells. B-CLL cells produced LTB_4 and expressed BLT1. By using specific inhibitors of leukotriene biosynthesis we showed that LTB_4 played a pivotal role in the activation of B-CLL cells. LTB_4 might influence the function of B-CLL cells in an autocrine and/or paracrine manner. Inhibitors of leukotriene synthesis may be useful for the treatment of B-CLL.

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