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UNIVERSITY OF CALIFORNIA, SAN DIEGO

The Tumor Microenvironment of B-cell Chronic Lymphocytic Leukemia

A Thesis submitted in partial satisfaction of the requirement
for the degree Master of Science

in

Biology

by

Hsu-Hsiang Chang

Committee in charge:

Thomas J. Kipps, Chair
Stephen M. Hedrick, Co-Chair
Carl F. Ware
Davorka Messmer

2008

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The Thesis of Hsu-Hsiang Chang is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Co-Chair

Chair

University of California, San Diego

2008

DEDICATION

To mom, dad, grandma, and all my family and friends who supported me throughout these years. Above all, to J.

TABLE OF CONTENTS

Signature Page……..... iii

Dedication…… iv

Table of Contents……..... v

List of Abbreviations……... viii

List of Figures……..... x

Acknowledgements…… xii

Abstract…… xiv

Introduction……... 1

A. The Tumor Microenvironment…… 2

B. BAFF and APRIL…… 3

Material and Methods…… 6

A. Cell preparation…….. 7

B. Generation of nurse-like cells and macrophages…… 7

C. Generation of CLL B-cell condition media (CLL-CM)…… 8

D. CLL-CM/CD14+ monocyte co-cultures…… 8

E. Cell separation experiments…… 9

F. Flow cytometry…… 9

G. Measurement of cytokines and chemokines…… 10

H. Blockage of TNF-α……..... 10

I. Measurement of cell viability…… 10

J. Real-time quantitative RT-PCR of BAFF mRNA…… 11

Chapter 1: CLL B-cells produce soluble factor(s) that can induce CD14+ blood monocytes to assume properties of NLCs *in vitro* 13

A. Introduction 14

B. NLCs display higher surface BAFF expression than macrophages 15

C. Soluble BAFF-inducing factors are released in CLL/monocyte co-cultures 17

D. CLL-condition media increase BAFF mRNA levels in monocytes 23

E. Factors secreted from CLL cells induce morphological changes in monocytes 25

F. Monocytes cultured in CLL condition media support CLL cell survival *in vitro* 26

G. Plasma and serum from CLL patients contain factors that cause increased BAFF expression in monocytes 28

H. Discussion 30

Chapter 2: CLL B-cells promote increased BAFF expression in monocytes partially through TNF-α 33

A. Introduction 34

B. CLL B-cell/monocyte co-cultures and CLL-CM contain TNF-α 35

C. Blocking TNF-α in CLL-CM and CLL serum reduced BAFF expression in CD14+ monocytes…… 37

D. Soluble TNF-α and IFN-γ are not sufficient to upregulate surface BAFF in CD14+ monocytes…….... 42

E. Discussion…….. 46

Conclusion…… 49

References……..... 52

LIST OF ABBREVIATIONS

CLL – chronic lymphocytic leukemia

BAFF – B cell activating factor of the TNF family

APRIL – a proliferation-inducing ligand

SDF – stromal cell-derived factor

NLC – nurse-like cell

TAM – tumor-associated macrophage

MΦ – macrophage

CM – condition media

BCMA – B-cell maturation antigen

TACI – transmembrane activator and calcium modulator and cyclophilin ligand interactor

TNF-α – tumor necrosis factor-alpha

IFN-γ – interferon-gamma

IL– interleukin

LT-α – lymphotoxin-alpha

PBMC – peripheral blood mononuclear cell

FACS – fluorescent activated cell sorting

$DiOC_6$ – 3,3'-dihexyloxacarbocyanine

PI – propidium iodine

MFI – mean fluorescence intensity

PBS – phosphate saline buffer

HS – human serum

PSG – penicillin-streptomycin-glutamine

ERK – extracellular signal-regulated kinase

NF-κB – nuclear factor-kappa B

PE – phycoerythrin

CHO – Chinese hamster ovary

FPLC – fast performance liquid chromatography

LIST OF FIGURES

Figure 1-1. Nurse-like cell differentiation and interaction with CLL B-cells…… 16

Figure 1-2. Nurse-like cells express higher surface BAFF levels than macrophages…… 17

Figure 1-3. Soluble factors released as a consequence of CLL B-cell/monocyte cross-talk causes upregulation of BAFF expression in monocytes…… 18

Figure 1-4. Condition media from CLL/monocyte co-cultures increase BAFF expression in monocytes…… 20

Figure 1-5. Condition media from purified CLL B-cell increase BAFF expression in monocytes…… 21

Figure 1-6. Condition media generated from several CLL patients increase BAFF expression in monocytes…….. 22

Figure 1-7. CLL-condition media increase BAFF mRNA levels in CD14+ monocytes…… 24

Figure 1-8. Condition media from purified CLL cells induce morphological changes in monocytes…… 25

Figure 1-9. Monocytes cultured in CLL-CM support CLL survival *in vitro*…… 27

Figure 1-10. Plasma and serum from CLL patients cause increased BAFF expression in monocytes…..…… 29

Figure 2-1. Soluble factors in CLL B-cell/monocyte co-cultures…… 36

Figure 2-2. Levels of TNF-α in CLL condition media correlate with the potency of CLL-CM to induce BAFF upregulation in monocytes……. 37

Figure 2-3. Blockage of TNF-α using decoy receptor, TNF-R-Fc, reduces the BAFF-inducing activity of CLL-CM and CLL serum……. 39

Figure 2-4. Addition of rhTNF-α restores BAFF-inducing activity of CLL-CM…… 41

Figure 2-5. Soluble TNF-α is not sufficient to upregulate surface BAFF in CD14+ monocytes…… 42

Figure 2-6. Soluble TNF-α and IFN-γ are not sufficient to upregulate surface BAFF in CD14+ monocytes…… 44

Figure 2-7. *In vivo* model: CLL microenvironment crosstalk…… 45

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Chapter one, in part, is being prepared for publication to be submitted to *Blood* (D. Messmer, H-H Chang, T. Endo, B. Messmer, Anne-Mette Buhl, Ingo G.H. Schmidt-Wolf, and T.J. Kipps, “TNF-α released from chronic lymphocytic leukemia B cells promotes increased BAFF expression in monocytes which consequently promote leukemic cell survival.”). The thesis author will be the co-author of this paper.

Chapter two, in part, is being prepared for publication to be submitted to *Blood* (D. Messmer, H-H Chang, T. Endo, B. Messmer, Anne-Mette Buhl, Ingo

G.H. Schmidt-Wolf, and T.J. Kipps, “TNF-α released from chronic lymphocytic leukemia B cells promotes increased BAFF expression in monocytes which consequently promote leukemic cell survival.”). The thesis author will be the co-author of this paper.

ABSTRACT OF THE THESIS

The Tumor Microenvironment of B-cell Chronic Lymphocytic Leukemia

by

Hsu-Hsiang Chang

Master of Science in Biology

University of California, San Diego, 2008

Thomas J. Kipps, Chair
Stephen M. Hedrick, Co-Chair

The CLL tumor microenvironment contains cells and cytokines that support CLL survival. CD14+ monocytes when co-cultured with chronic lymphocytic leukemia (CLL) B-cells can differentiate *in vitro* into large, round, adherent cells termed “nurse-like cells” (NLCs) that can support the survival of

CLL cells via the release of B-cell activating factor of tumor necrosis factor family (BAFF). To identify the factor responsible for this property change in CD14+ monocytes, purified CLL cells were cultured for 24hr and collected the cell-free supernatants to generate conditioned media (CLL-CM). CD14+ monocytes of healthy donors cultured in the CLL-CM assumed NLC morphology and acquired high-level BAFF expression similar to CLL/monocyte co-cultures. In contrast, monocytes cultured in non-conditioned media did not acquire such changes. Similar to NLCs, monocytes cultured in CLL-CM were able to support CLL survival *in vitro*.

Furthermore, elevated levels of tumor necrosis factor-alpha (TNF-α) were detected in CLL-CMs. To investigate whether TNF-α is the soluble factor in CLL-CM that causes CD14+ monocytes to assume properties of NLC, a decoy receptor for TNF-α, TNF-R-Fc, was added to the cultures and showed a significant reduction in BAFF expression in monocytes compared to the IgG control. A similar reduction in BAFF expression was observed when CLL-serum was treated with TNF-R-Fc. Addition of rhTNF-α to monocytes was not sufficient to induce BAFF expression in monocytes, suggesting other factor(s) interactions. This finding shows a previously unrecognized symbiosis between CLL cells and their microenvironment and could provide a novel target for therapy of CLL and other indolent B-cell lymphomas.

Introduction

B-cell chronic lymphocytic leukemia (B-CLL), the most common adult leukemia in Western societies, is characterized by the increasing high numbers of mature, monoclonal B cells in the blood, marrow, and secondary lymphoid tissues[1]. B-CLL is a heterogeneous disease in which circulating leukemia cells can avoid cell death or even proliferate through external antigenic receptor stimulatory signals (e.g., B-cell receptors, chemokines and cytokine receptors) and their ligands[2-4]. Although CLL B-cells are thought to have an apoptotic defect *in vivo*, *in vitro* CLL B-cells die rapidly[5,6]. This implies that in *ex vivo* conditions, CLL cells lack the essential survival factors and are not intrinsically resistant to apoptosis[6-9]. Thus, it has been postulated that *in vivo* CLL B-cells receive survival signals from their tumor microenvironment[10,11].

A. The Tumor Microenvironment

Research revolving the tumor microenvironment of various cancers has been increasing and receiving much attention in recent years. Tumor progression has been recognized as the product of an evolving crosstalk between the tumor and cells of its surrounding support tissues[10]. This crosstalk has been implicated in Burkitt's Lymphoma (BL), where the tumor-associated macrophages (TAM) are thought to promote BL cell survival and growth *in situ* via cytokine and protein interactions[16]. Furthermore, researchers have also shown that lymphoma-associated macrophages (LAM) play a critical role as an independent predictor of survival of follicular lymphoma patients[18].

In B-CLL, it has been shown that marrow stromal cells secrete chemokine stromal-cell derived factor-1 (SDF-1, also known as CXCL12) that directs CLL cells to the marrow cellular niches and in turn confers to the CLL cells a survival and growth advantage in the tumor microenvironment[11]. Additionally, a subset of blood cells from B-CLL or normal donors can spontaneously differentiate into large, round, adherent "pancake-like" structures called "nurse-like" cells (NLC) when cultured in the presence of CLL B-cells *in vitro*[13]. Studies have shown that NLC express SDF-1, a proliferation inducing ligand (APRIL), and a B-cell activating factor of the tumor necrosis factor family (BAFF, also known as BlyS, TALL-1, zTNF4, and THANK) that independently and/or in conjunction mediate the survival of CLL B cells *in vitro*[13,14]. When compared to macrophages, NLC consistently express higher levels of CD68[12]. Recent studies have shown that NLC are characterized by an expression profile of surface and cytoplasmic antigens ($CD14^{lo}$, $CD68^{hi}$, $CD83^{-}$, $CD106^{-}$) that is unique from those of monocytes, macrophages, or monocyte-derived dendritic cells[14].

B. BAFF and APRIL

BAFF and APRIL, type II transmembrane proteins of the TNF superfamily, are known for their role in regulating normal B-cell survival, growth and apoptosis; both proteins have been found to be expressed by monocytes, macrophages, dendritic cells, neutrophils, T-cells, follicular dendritic cells, and other non-lymphoid cell types[19]. Although investigators have suggested that BAFF and

APRIL mediate CLL survival via an autocrine pathway, evident of the expression of BAFF and APRIL on the surface of CLL B-cells[20,21], BAFF and APRIL commonly exert its survival and proliferative effects via a paracrine pathway[14] through the three BAFF receptors found on B-CLL cells: BCMA (B-cell maturation antigen), BAFF-R (BAFF receptor), and TACI (transmembrane activator and calcium modulator and cyclophilin ligand interactor)[22]. An additive effect was seen when BAFF and APRIL were administered in different combinations in the recombinant form, in turn showing that BAFF can promote CLL cell survival via the NF-κB pathway distinct from the ERK1/2 and AKT pathway of SDF-1[14,15].

In light of the role of macrophages in the pathogenesis of Burkitt's lymphoma[16], and the increasing amount of CLL patients that are acquiring resistance to fludarabine-based therapies due to support from accessory cells of the CLL microenvironment[17], interfering with the microenvironmental interactions during or before chemotherapy might allow for complete depletion of the CLL cells and/or potentially create an immunosupportive tumor microenvironment to awake/reawake immune cells, suggesting the possibility of immunotherapy[23]. For these reasons, the goal of our study was to investigate in which manner CLL cells manipulate their environment in order to gain survival advantage. Contrary to the previous work[12], we show that direct cell contact of healthy CD14+ blood cells and CLL B cells is not necessary to induce NLC morphology and BAFF expression. Furthermore, we show that the increased BAFF expression in 14+ blood-cell-derived NLC is mediated by TNF-α and other soluble factor(s)

secreted from CLL cells. Thus, blocking the interactions between CLL cells and the microenvironment might provide a novel therapeutic approach for CLL patients.

Material and Methods

A. Cell preparation

Peripheral blood mononuclear cells (PBMC) were isolated from the blood of healthy donors (San Diego Blood Bank) over a Ficoll-Hypaque density gradient. The PBMC interface was collected and washed 4 times using 1X phosphate buffer saline (PBS; GIBCO® Invitrogen, Carlsbad, CA). CD14+ cells were isolated and purified from PBMCs by positive selection using anti-CD14 microbeads (Miltenyi Biotec, Auburn, CA) following the manufacturer's instructions. B-cells were isolated and purified from PBMCs of healthy donors by negative selection using a cocktail of biotin-conjugated antibodies in the B-cell isolation kit II (Miltenyi Biotec) following the manufacturer's instructions. CLL B-cells were isolated and purified from frozen CLL PBMCs by removing CD14 and CD2-positive cells using magnetic beads (Miltenyi Biotec) following the manufacturer's instructions.

B. Generation of nurse-like cells and macrophages

Nurse-like cell: $1.25x10^5$ CD14+ monocytes were co-cultured with 25X concentration of purified CLL cells in RPMI medium containing heat-inactivated 10% pooled human serum (HS; Omega Scientific, Tarzana, CA) and penicillin [10U], streptomycin [10μg], L-glutamine [29.2μg] (Invitrogen, Carlsbad, CA), hepes (1.2g; Invitrogen, Carlsbad, CA) and β-mecaptoethanol (50□M; Sigma-

Aldrich, St. Louis, MO) set at 1mL per well in 24-well Falcon plates (BD Biosciences, San Jose, CA) for 14 days.

Macrophages: PBMC ($2.4x10^7$) were adhered to tissue culture plates (Primaria™, Falcon BD) for 2h. Non-adherent cells were washed off and fresh medium was added to the cells, containing RPMI and 10% human AB serum (Biowhittacker, Lonza Inc, Allendale, NJ). Cells were analyzed after 10 days of culture.

C. Generation of CLL B-cell condition media (CLL-CM)

CLL B-cells were cultured at $1x10^7$cells/mL in RPMI medium containing heat-inactivated 10% pooled HS (Omega Scientific) and PSG, hepes, and β-mecaptoethanol set at 3mL per well in 6-well plates (Falcon). Condition media (cell-free, soluble supernatant only) were collected at the indicated time points and stored in -20°C until further use.

D. CLL-CM/CD14+ monocyte co-cultures

CD14+ monocytes ($2.5x10^5$) were cultured with 1mL CLL-CM in 12-well plates (Falcon) for 7 days at 37°C. Cells were observed for morphological change through microscopy and BAFF expression by flow cytometry on day 7.

E. Cell separation experiments

The role of soluble factors in CLL/monocyte co-cultures was explored by means of Transwell chambers with cell-culture insets of 0.4-μm pore diameter (Corning, Lowell, MA) to separate CLL B-cells and monocytes. Monocytes were cultured ($5x10^5$cells/mL) in the bottom well in RPMI medium containing 10% HS. CLL B-cell suspensions were prepared (25 X of monocyte concentration) in the same medium and cells were added to the upper chamber. CLL B-cells were also co-cultured with monocytes without a porous barrier. After incubation at 37°C for 7 days, monocytes were collected and analyzed for BAFF expression by flow cytometry.

F. Flow cytometry

Cells ($1x10^5$) were labeled by incubating in 100 μl of PBS/5% FCS/0.1% sodium azide staining buffer with 100 μl of (10μg/ml) human IgG (Invitrogen, Carlsbad, CA) for 10 min. Cells were washed in staining buffer and subsequently incubated with 7 μl of IgG_1-PE (isotype control) or anti-human BAFF-PE (R&D Systems, Minneapolis, MN) for 20 minutes on ice. The cells were washed four times using staining buffer, resuspended in 100μL fix solution, and analyzed using the FACSCalibur (Beckon Dickinson). Data was analyzed using the FlowJo 7.2.2 software (Tree Star).

G. Measurement of cytokines and chemokines

The production of cytokines and chemokines in CLL/monocyte co-cultures was measured by ELISA (Pierce Boston Technology Center, SearchLight Proteome Arrays Multiplex Sample Testing Services, Woburn, MA). Soluble TNF-α in CLL-CM (cell-free supernatants) was detected using an anti-human TNF-α ELISA kit (ebioscience, San Diego, CA) according to manufacturer's instruction. The detection range of the kit is 4 - 500pg/mL. Optical density at 450nm were measured with a VersaMax microplate spectrophotometer (Molecular Devices). Data was analyzed using the SoftMax Pro 5 software (Molecular Devices).

H. Blockage of TNF-α

To block the activity of TNF-α, 10ng/mL of TNF-R-Fc (kindly provided to us by Dr. Carl Ware, La Jolla Institute for allergy and immunology) was added to CLL-CM and CLL serum prior to culture with CD14+ monocytes at 2.5 x 10^5cells/mL in 12-well plates (Falcon). CD14+ cells were collected on day 7 and stained for BAFF expression and analyzed by flow cytometry.

I. Measurement of cell viability

Purified CLL B-cells were cultured at 1 x 10^6cells/mL in 24-well plates (Falcon) under various conditions. Determination of CLL cell viability was based on the

analysis of mitochondrial transmembrane potential ($\Delta\psi_m$) using 3,3'-dihexyloxacarbocyanine iodine ($DiOC_6$) and cell membrane permeability to propidium iodine (PI). For viability assays, 100μL of the cell culture was collected at day 2, 5, and 7 and transferred to polypropylene tubes containing 100μL of 60 nM $DiOC_6$ (Molecular Probes® Invitrogen, Carlsbad, CA) and 10μg/mL propidium iodide (PI) in culture media. The cells were then incubated at 37*C for 15 minutes and analyzed within 30 minutes by flow cytometry using a FACSCalibur (Becton Dickinson). Fluorescence was recorded at 525 nm (FL-1) for $DiOC_6$ and at 600 nm (FL-2) for PI. Data was analyzed using the FlowJo 7.2.2 software (Tree Star). The percentage of viable cells was determined by gating on PI negative and $DiOC_6$ bright cells.

J. Real-time quantitative RT-PCR of BAFF mRNA

CD14+ monocytes ($2.5x10^5$) were cultured with 1mL CLL-CM or 10%HS media in 12-well plates (Falcon) at 37^{o}C. Monocytes were collected at day 1, 2, and 5 and combined with 10^5 CHO cells into 350μl RLT buffer (Qiagen, Valencia, CA). Total RNA was immediately isolated using the RNeasy kit from Qiagen (Valencia,CA) according to the manufacturer's instructions. To remove DNA contamination in RNA samples, the isolated RNA was treated with RNase-free DNase (Qiagen) following the manufacturer's instructions. RNA (100 ng per reaction) was used for quantitative QRT-PCR with the 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) using one-step RT-PCR Mix

reagents (AB4309169) as described by the manufacturer. Final primer and probe concentrations were 300 nM probe and 125 nM primer for BAFF, and 200nM and 100nM for β2-microglobulin, and all samples were run in triplicates. Analysis of the sequences of interest was performed by the comparative Ct method of relative quantification using β2-microglobulin as endogenous control. Primers and probes for BAFF were: (forward primer) 5' CGC GGG ACT GAA AAT CTT TG, (reverse primer) 3' CAC GCT TAT TTC TGC TGT TCT GA), (FAM-probe) CCA CCA GCT CCA GGA GAA GGC AAC TC, and for β2-microglobulin: (forward primer) 5'-TGA CTT TGT CAC AGC CCA AGA TA-3', (reverse primer) 5'-AAT CCA AAT GCG GCA TCT TC-3' and (FAM-probe) 5'TGATGCTGCTTACATGTCTCGATCCCA-3'. All probes were TaqMan probes obtained from Applied Biosystems.

Chapter 1:

CLL B-cells produce soluble factor(s) that can induce CD14+ blood monocytes to assume properties of NLCs *in vitro*

A. Introduction

A subset of CD14+ blood mononuclear cells, when cultured in the presence of CLL B-cells, differentiates into “nurse-like” cells (NLC) that can protect CLL B-cells from apoptosis *in vitro* and possibly *in vivo,* as demonstrated by the elevated CD68 expression and morphological similarities to CD14+ splenocytes of CLL patients[12]. On the contrary, CD14+ mononuclear cells cultured with normal B cells failed to exhibit NLC morphology and the characteristic elevated cytoplasmic CD68 expression, as similar to CD14+ splenocytes of persons without CLL[12]. In light of the role of tumor-associated macrophages (TAM) in promoting Burkitt’s Lymphoma cell survival and growth *in situ*[16], experiments were conducted to investigate the distinct features and functions of NLC and macrophages (MΦ) in the CLL microenvironment. Here we show that CD14+ monocyte-derived NLC display distinctively higher surface BAFF expression than MΦ. Furthermore, in order to elucidate the cellular interactions between CLL cells and monocytes that cause CD14+ monocytes to differentiate into NLC *in vitro*, the goal of this study was to identify whether this differentiation is mediated by cell-cell contact or soluble factor(s) secreted by CLL cells. This study shows that the upregulation of BAFF in NLC is not dependent on direct cell contact with CLL cells. We further demonstrate that soluble factors secreted by CLL cells but not B-cells from healthy donors in the first 24h of culture, the cell-free CLL condition media (CM), can induce CD14+ monocytes to increase BAFF expression. Finally, we show that monocytes cultured in CLL-CM,

similar to NLC, can support CLL cell survival *in vitro*.

B. NLC display higher surface BAFF expression than macrophages

When monocytes isolated from normal donors are cultured in the presence of CLL cells, they differentiate into NLC[12]. In light of the supportive role tumor-associated macrophages play in follicular lymphoma and the role of nurse-like cells (NLC) in supporting CLL cell survival, NLC were compared to MΦ for BAFF expression. Surface BAFF expression profiles became the primary target of investigation as BAFF has been shown to promote CLL cell survival[14]. Here, we recognized a distinctive surface expression profile between NLC and MΦ. When CD14+ monocytes of normal donors were cultured with or without CLL B-cells for 14 days, the monocytes differentiate into NLC and MΦ, respectively (Figure 1-1). Although both morphologically adherent, MΦ exhibit long-spindly structures whereas NLC are characterized by the large, round, pancake-like structures *in vitro*. Cells were harvested on day 14, stained for surface BAFF using PE-conjugated antibodies and BAFF expression was analyzed by flow cytometry. In 11 independent experiments, NLC consistently express significantly higher surface BAFF levels than MΦ (Figure 1-2).

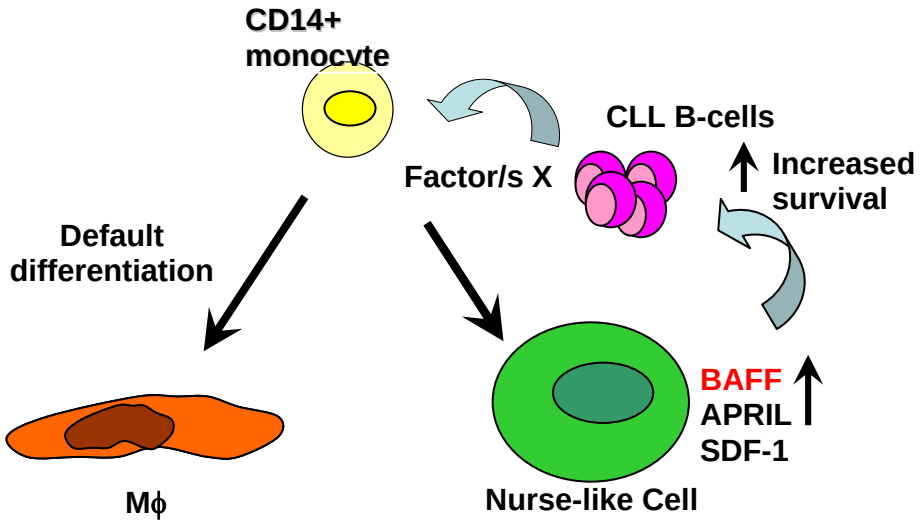


Figure 1-1. Nurse-like cell differentiation and interaction with CLL B-cells. CD14+ monocytes cultured for 14 days in the presence of CLL B-cells differentiate into nurse-like cells (NLC) and in absence of CLL cells differentiate into MΦ. NLC exhibit elevated surface BAFF, APRIL, and SDF-1 expression levels, which in turn cause the increased survival of CLL B-cells *in vitro*.

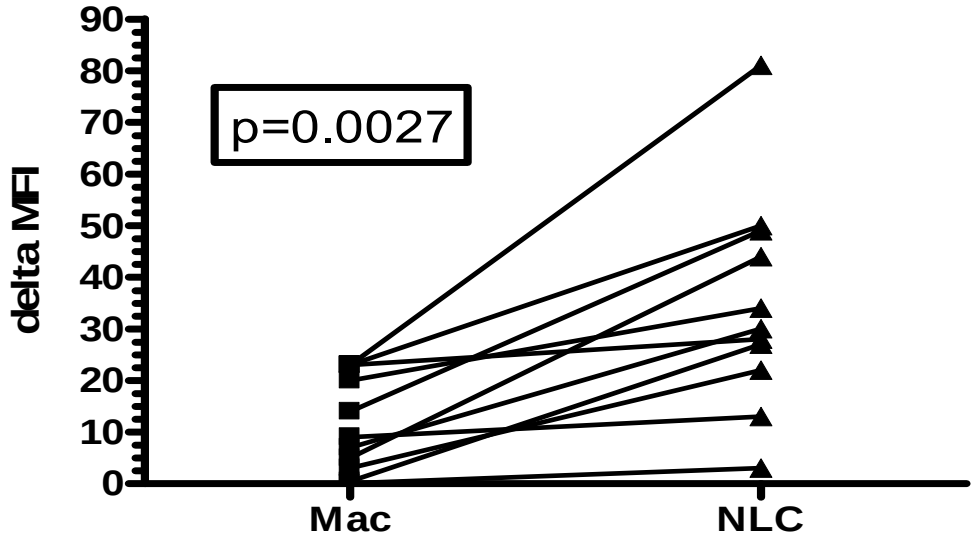


Figure 1-2. Nurse-like cells express higher surface BAFF levels than macrophages. CD14+ monocytes were cultured with/without CLL B-cells to generate NLC and MΦ, respectively. After 14 days of culture, non-adherent cells were washed off with PBS and the adherent NLC and MΦ were collected and analyzed for BAFF expression by flow cytometry. Depicted is the BAFF mean fluorescence intensity of 11 independent experiments using monocytes from different donors. The statistical significance between the two groups was determined using a paired t-test.

C. Soluble BAFF-inducing factors are released in CLL/monocyte co-cultures

To determine whether the increased BAFF expression was due to cell-cell contact with CLL cells or caused by soluble factors secreted from CLL cells, monocytes were cultured in the same well with CLL cells or separated by a trans-

well membrane. After 14 days of culture, the monocytes were harvested, stained for surface BAFF using PE-conjugated antibodies, and BAFF expression was analyzed by flow cytometry. Increased BAFF expression was observed when monocytes were cultured both in the same well and in the trans-well, though slightly lower in the trans-well. The change in BAFF expression levels for same-well and trans-well conditions were substantially higher than the monocyte control (Figure 1-3), suggesting that soluble factor(s) produced as a consequence of CLL/monocyte cross-talk are sufficient to induce upregulation of BAFF in CD14+ monocytes.

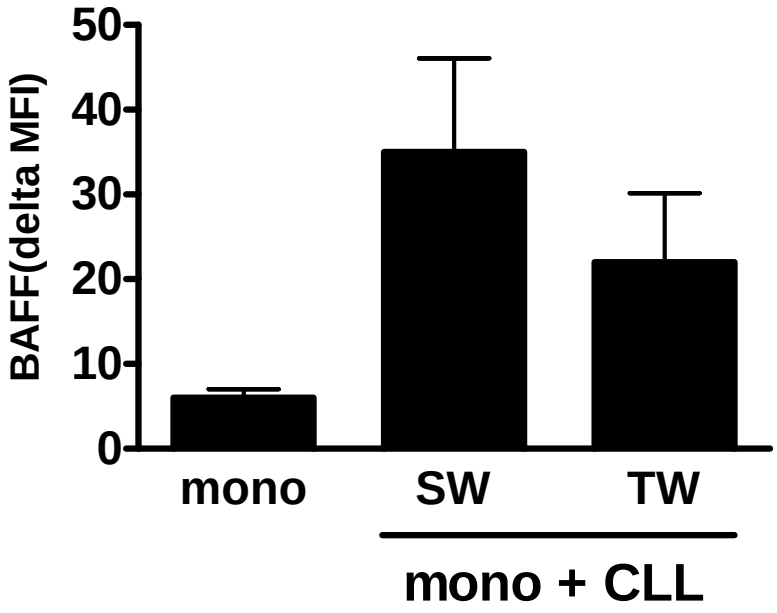


Figure 1-3. Soluble factors released as a consequence of CLL B-cell/monocyte cross-talk causes upregulation of BAFF expression in monocytes. CLL cells were cultured with monocytes for 14 days in same-well (SW) and trans-well (TW). BAFF expression was detected by flow cytometry. Depicted is the mean fluorescence intensity (MFI) +/- SEM of 3 independent experiments using monocytes from different donors.

To further confirm that soluble factor(s) are responsible for BAFF upregulation, condition media were collected from CLL/monocyte co-cultures and tested for their ability to upregulate BAFF. In addition, to identify the time course with the most potent BAFF-inducing activity, condition media were collected from the co-cultures at days 2, 4, 6, and 8. At these time points cells were collected, spun down, and the cell-free supernatants (=condition media) were added to freshly isolated monocytes for 7 days, at which point they were collected and analyzed for BAFF expression. We found that CM from CLL/monocyte co-cultures that were collected at day 6 induced the strongest increase of BAFF expression in monocytes (Figure 1-4).

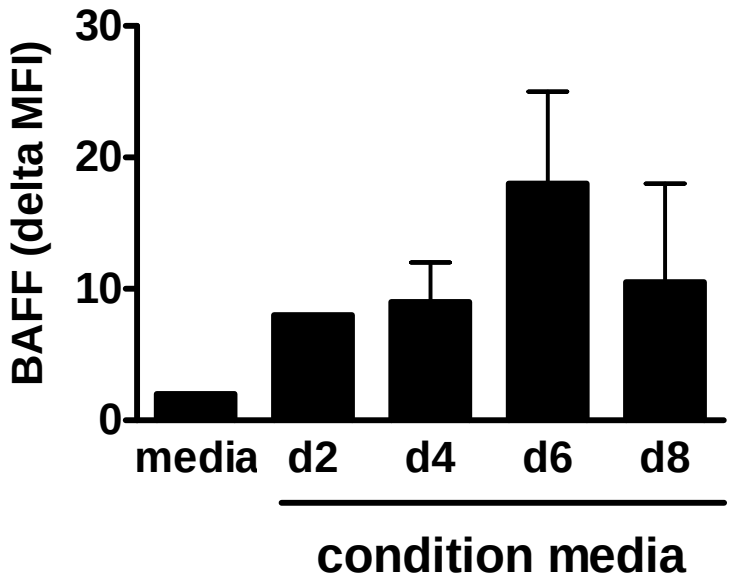


Figure 1-4. Condition media from CLL/monocyte co-cultures increase BAFF expression in monocytes. Monocytes were cultured with CLL/monocyte condition media that was collected at day 2, 4, 6, and 8 of culture. 7 days after exposure to the condition media BAFF expression was measured by flow cytometry. The data shown is the mean +/- SEM of 2 independent experiments using monocytes from 2 different donors.

Though this result was promising it is difficult to identify which cell in the co-culture has secreted the soluble factor. Furthermore, there can be considerable cross-talk between the cells that complicates the identification as well. Therefore, we tested whether condition media from purified CLL B-cell can increase BAFF expression of monocytes. CLL B cells were purified by negative selection to avoid stimulation and cultured for 2, 4, 6, and 8 days at which point the cells were harvested, spun down and the cell-free supernatants (=condition

media) were collected and added to freshly isolated monocytes for 7 days. CLL condition media collected at all time points induced increases in BAFF expression of monocytes. However, in contrast to CM from co-cultures, the earlier time points showed the most dramatic increase (Figure 1-5).

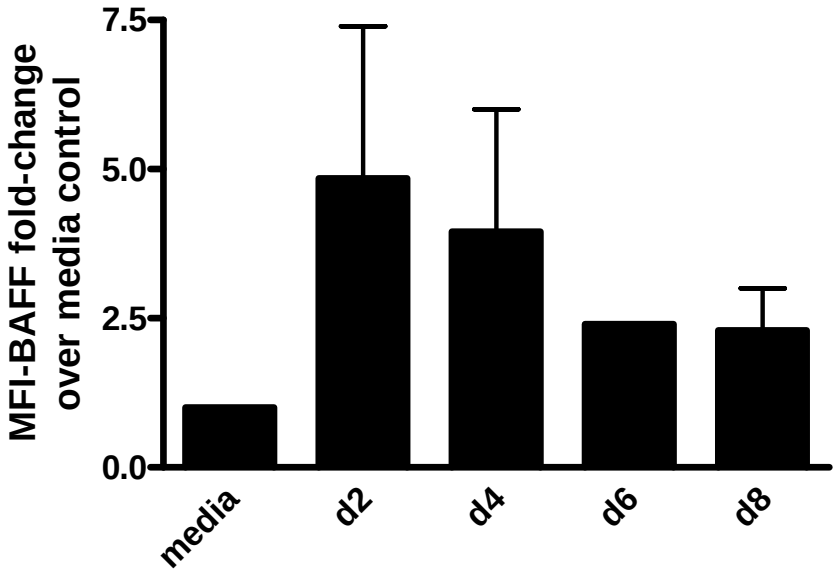


Figure 1-5. Condition media from purified CLL B-cell increase BAFF expression in monocytes. Condition media generated from purified CLL cell collected at day 2, 4, 6, and 8 was added to monocytes. After 7 days of culture the monocytes were analyzed for BAFF expression by flow cytometry. BAFF "fold-change" was recorded compared to media control, which was set as 1. The data shown is the mean +/- SEM of 2 independent experiments using monocytes from 2 different donors.

To further validate our observation that soluble factor(s) secreted from CLL cells increase BAFF expression in monocytes, condition media were generated from several different CLL patients, collected at 24h after culture and added to monocytes for 7 days (Figure 1-6). All CLL condition media (CLL-CM) tested so far induced increased BAFF expression in monocytes. This effect was consistently observed with 24h CLL-CM. Most importantly, the effect was exclusively observed in CLL-CM; 24h CM generated from peripheral blood B-cells isolated from healthy individuals were not able to induce upregulation of BAFF in monocytes (Figure 1-6).

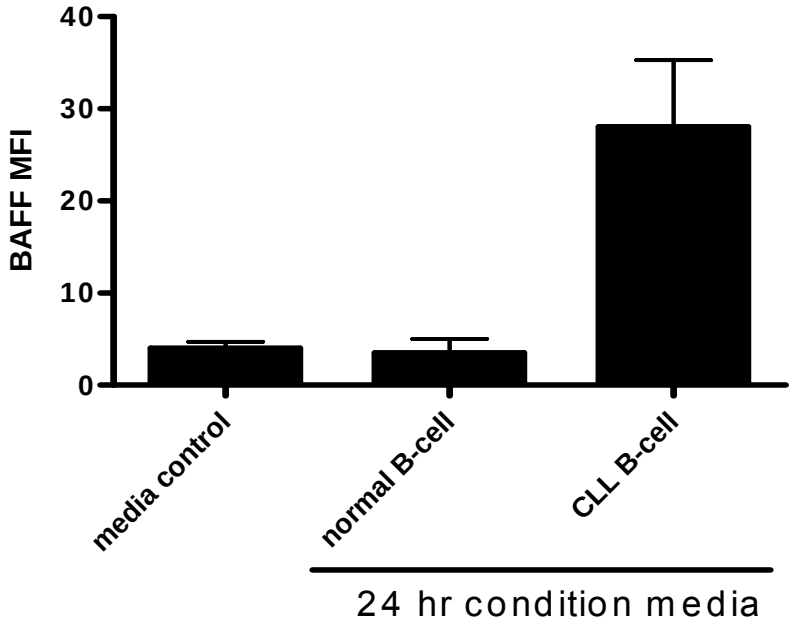


Figure 1-6. Condition media generated from several CLL patients increase BAFF expression in monocytes. Monocytes were cultured in CM collected from normal and CLL donors at 24h for 7 days. Media control is 10%HS RPMI. The data shown is the mean +/- SEM of 2 independent experiments using monocytes from 2 different donors, normal CM from 2 different donors, and CLL-CM from 12 different CLL patients.

D. CLL-condition media increase BAFF mRNA levels in monocytes

To determine the mode by which the CLL-CM upregulates BAFF expression in CD14+ monocytes, monocytes were cultured in CLL-CM or control media. Cells were collected on days 1, 2, and 5 and combined with filler (Chinese hamster ovary; CHO) cells. Total RNA was isolated and used for real-time quantitative RT-PCR analysis. The strongest increase (17-fold) in BAFF mRNA levels was detected in monocytes cultured in CLL-CM (conditioned monocytes) over monocytes cultured in control media (control monocytes) on day 1. At day 2 and 5 of culture, monocytes in CLL-CM showed a 2-fold increase over control monocytes (Figure 1-7).

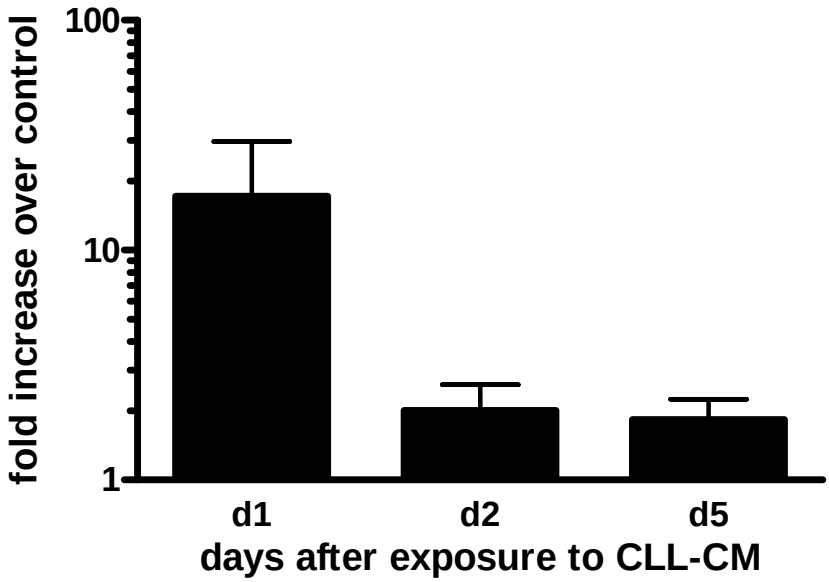
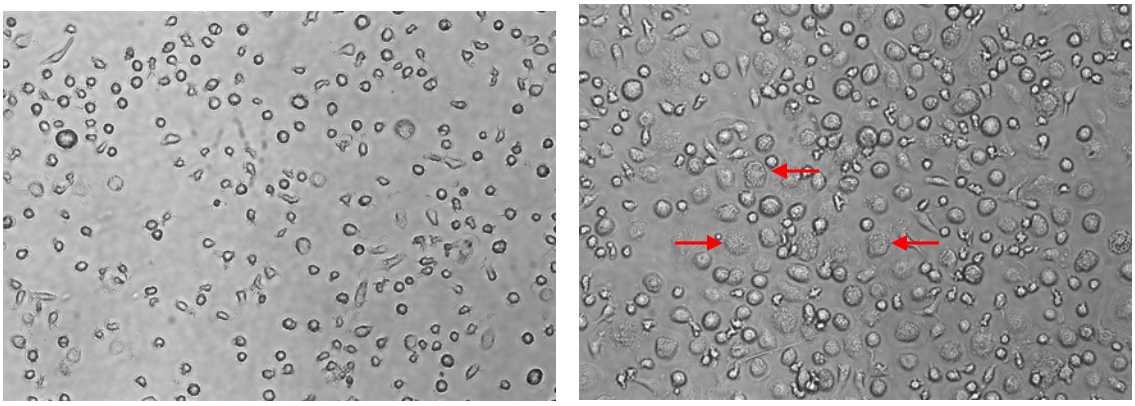


Figure 1-7. CLL-condition media increase BAFF mRNA levels in CD14+ monocytes. CD14+ monocytes were cultured for 1, 2, 5 days in CLL-CM or control media. Monocytes were collected and combined with 10^5 CHO cells at the indicated time points. Total RNA was isolated and used for real-time quantitative RT-PCR analysis. The values shown are the fold increase at each time point in relation to the CLL-CM or control media-treated monocytes at the same time point. Data represent the mean +/- SEM of 2 independent experiments.

E. Factors secreted from CLL cells induce morphological changes in monocytes

Monocytes cultured for 9 days in CM from purified CLL cells were analyzed under brightfield microscopy. Significant morphological changes were observed. Compared to monocytes cultured in control media the monocytes cultured with CLL-CM displayed larger, pancake-like adherent structures (see arrows) which resemble previously described NLC (Figure 1-8). Though there is variation between experiments, we observed that in most cases not all monocytes differentiate into NLC, recognized by the large adherent pancake-like structures, but many remain non-adherent and small.



Media **CLL condition media**

Figure 1-8. Condition media from purified CLL cells induce morphological changes in monocytes. Monocytes were cultured in CLL-CM or control media for 9 days. Cells were observed under the inverted microscope in brightfield. Red arrows indicate differentiated cells resembling NLC. Image was taken at 40X using the Nikon Eclipse TE300 inverted epi-fluorescent microscope.

F. Monocytes cultured in CLL condition media support CLL cell survival *in vitro*

In addition to the BAFF upregulation and morphology resembling NLC, we tested whether monocytes cultured in CLL-CM were able to support CLL survival *in vitro* as has been previously described for NLC[13,14]. "Conditioned monocytes" were generated by culturing CD14+ monocytes of normal donors with CLL-CM or media for 7 days at which point the monocytes were used for CLL cell survival assays, Purified CLL B-cells were co-cultured with "conditioned monocytes" or control monocytes (cultured in media only) for 7 days. Monocytes cultured in CLL-CM consistently provided increased survival benefits to CLL cells as compared to control monocytes (Figure 1-9).

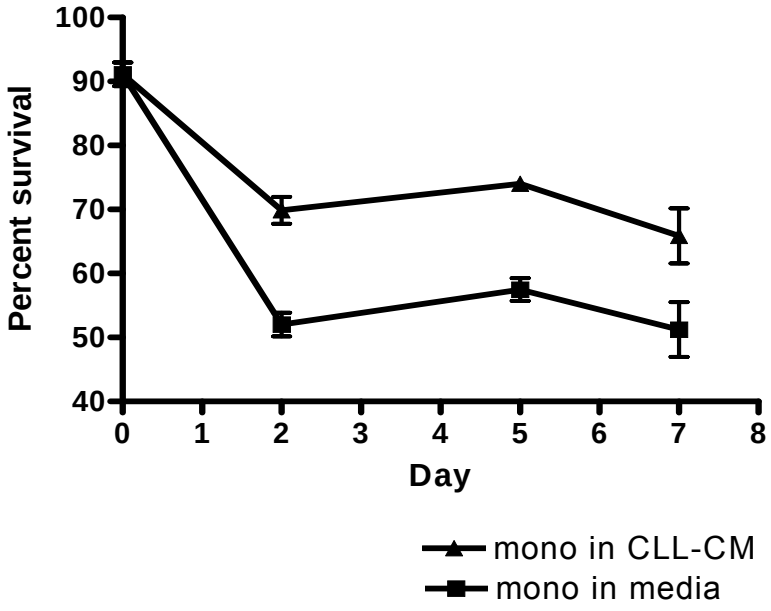
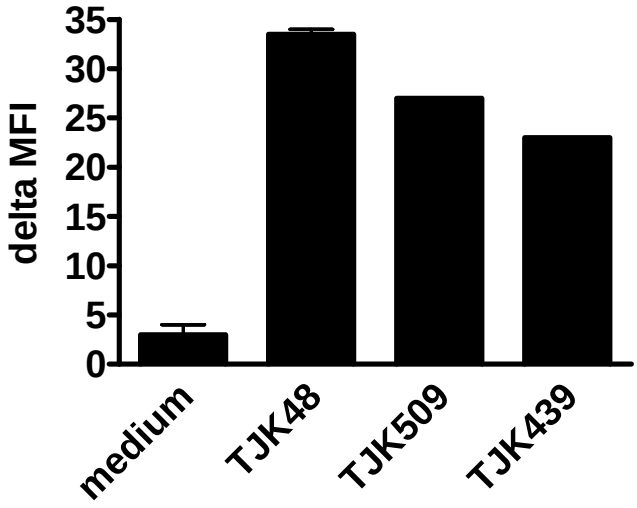


Figure 1-9. Monocytes cultured in CLL-CM support CLL survival *in vitro*. Monocytes were cultured in CLL-CM or control media for 7 days prior to the addition of purified CLL B-cells. CLL cells were harvested and stained with $DiOC_6$/PI on days 0, 2, 5, 7 and analyzed by flow cytometry. The data shown is based on gated small lymphocytes and $DiOC_6$ bright PI negative cells (=viable cells). The mean +/- SEM of 6 independent experiments using CLL cells from 2 different patients is shown.

G. Plasma and serum from CLL patients contain factors that cause increased BAFF expression in monocytes

Since the previous experiments were performed with *in vitro* cultured CLL cells, we investigated whether the BAFF-inducing factor(s) are also present in CLL plasma isolated from patient blood. Monocytes were cultured in 25% CLL plasma for 12 days and analyzed for BAFF expression. A 4- to 7-fold increase in BAFF expression was observed in monocytes cultured in CLL plasma when compared to control media (Figure 1-10A). When monocytes were cultured in 50% or 10% serum from CLL patients increased BAFF expression was consistently observed as compared to monocytes cultured in human serum from normal donors (Figure 1-10B).

A)



B)

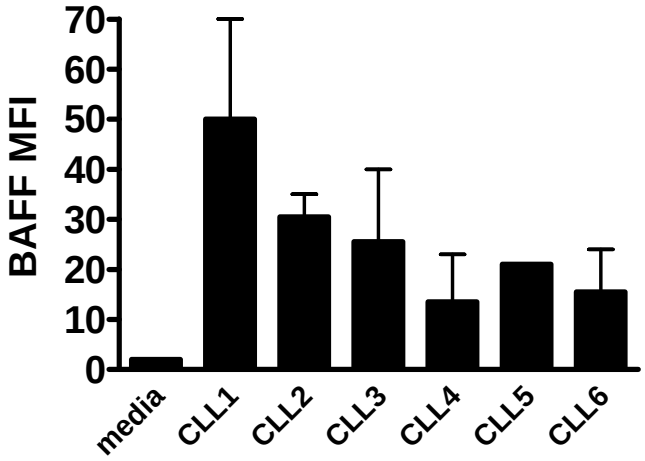


Figure 1-10. Plasma and serum from CLL patients cause increased BAFF expression in monocytes. (A) Monocytes were cultured in CLL plasma for 12 days and cells were collected and analyzed for BAFF expression by flow cytometry. Depicted is the BAFF expression level (Mean + SEM) of three CLL plasma donors compared to plasma from a healthy donor using monocytes from two different individuals. **(B)** Monocytes were cultured in 50% pooled human serum from normal donors (media) or 50% serum from six different CLL patients (CLL 1-6). Cells were collected and analyzed for surface BAFF expression by flow cytometry at day 7. Data shown are BAFF expression levels (Mean +/- SEM) of two independent experiments using monocytes from different donors.

I. Discussion

It has previously been shown that CLL cells when cultured with monocytes, cause the monocytes to differentiate into NLC, which in turn support the survival of CLL cells[12-14]. Work has been done on how the NLC support CLL cell survival, but little is known about how CLL cells cause monocytes to differentiate into NLC. We have shown that NLC exhibit higher BAFF surface expression levels than MΦ (Figure 1-2), thus further distinguishing them apart. To further characterize the factor(s) that induce this upregulation of BAFF in monocyte-derived NLC, CLL cells were co-cultured with monocytes in same-well and trans-well conditions. The results allowed us to conclude that CLL cells secrete soluble factor(s) that were able to cross the membrane and induce BAFF expression in monocytes (Figure 1-3). The results also indicate that cell-cell contact between CLL B-cells and monocytes was not necessary to induce upregulation of BAFF in monocytes. Furthermore, soluble factors secreted from CLL B-cells but not normal B-cells in the first 24h of culture, can cause upregulation of BAFF in monocytes and induce monocytes to undergo morphological changes into NLC (Figure 1-6, 1-8); the NLC can be distinguished from the long & spindly-like MΦ under the microscope (data not shown).

To investigate the mode of regulation in which CLL-CM caused monocytes to upregulate BAFF expression, real-time/quantitative RT-PCR experiment was conducted to detect BAFF mRNA levels in monocytes cultured in CLL-CM. A 17-fold increase in BAFF mRNA levels in monocytes cultured in CLL-CM over

monocytes cultured in control media was detected on day 1. Interestingly, the strongest increase in BAFF expression was observed at day 1 (Figure 1-7). At day 2 and 5 monocytes cultured in CLL-CM showed a 2-fold increase in BAFF mRNA levels over media control. This is interesting, because surface BAFF protein levels are not detected at early time points, but instead increase with time, whereas the differences in mRNA levels are most dramatic early on. It is possible that BAFF protein is expressed at high levels early on, but retained within the cells and the trafficking to the cell surface occurs at a slower pace. This possibility could be tested by performing intracellular staining for BAFF at different time points and if our hypothesis is correct, one would expect high intracellular BAFF levels at days 1-2 matching the high levels of mRNA.

Even though all the experiments have been conducted *in vitro*, the use of a short time-point of primary leukemia cells provides a close mimicry to *in vivo* conditions. In addition, not only did primary CLL cells release the BAFF-inducing factor during a relatively short culture period of 24hr, but CLL plasma and serum also contain factor/s that can increase BAFF expression in monocytes (Figure 1-10). This is intriguing in that it further suggests that soluble factor(s) are being secreted by CLL cells *in vivo*.

As monocytes cultured in CLL-CM were able to acquire properties of NLC *in vitro*, functional studies were conducted on these “conditioned monocytes” to see whether they support CLL cell survival *in vitro* to a similar extent as conventional NLC (generated from CLL/monocyte co-cultures). A notable survival advantage was seen in CLL B-cells co-cultured with monocytes conditioned in

CLL-CM as opposed to the monocytes cultured in plain media (Figure 1-9).

This chapter, in part, is being prepared for publication to be submitted to *Blood* (D. Messmer, H-H Chang, T. Endo, B. Messmer, Anne-Mette Buhl, Ingo G.H. Schmidt-Wolf, and T.J. Kipps, “TNF-α released from chronic lymphocytic leukemia B cells promotes increased BAFF expression in monocytes which consequently promote leukemic cell survival.”). The thesis author will be the co-author of this paper.

Chapter 2:

CLL B-cells promote increased BAFF expression in monocytes partially through TNF-α

A. Introduction

Tumor necrosis factor-alpha (TNF-α) is a cytokine that possesses an array of biological activities on different cell types such as inducing inflammation, apoptosis, cell differentiation and proliferation[24]. CLL B-cells have been shown to produce TNF-α, which can in turn promote cancerous cell growth via an autocrine and paracrine manner[25]. Furthermore, elevated TNF-α levels were detected in CLL serum and plasma and have been implicated as a predictor of the survival rate of CLL patients[25]. Despite these findings, little is known on the mechanism in which TNF-α acts and the role it plays in the CLL tumor microenvironment. We have detected TNF-α in supernatants from CLL/monocyte co-cultures and in CLL-CM at levels similar to those reported in CLL serum. Through blocking TNF-α using decoy receptor, TNF-R-Fc, we identified TNF-α as one of the soluble factors that contribute to monocyte differentiation into NLC. In light of the role of TNF-α and IFN-γ on fibroblast-like synoviocytes of rheumatoid arthritis patients[26], we investigated the effects of administering these cytokines alone and in combination to CD14+ monocytes and conclude that soluble TNF-α and IFN-γ were not sufficient to induce upregulation of BAFF in monocytes.

B. CLL B-cell/monocyte co-cultures and CLL-CM contain TNF-α

In an attempt to identify the soluble factor(s) produced by CLL cells, supernatants of CLL/monocyte co-cultures were collected and tested for the presence of various pro-inflammatory and anti-inflammatory cytokines (IL-10, TNF-α, IL-8, IFN-α, TRAIL, IL-6, and IFN-γ) at various time points. These cytokines were chosen either because they have been shown to be present in elevated levels in CLL patient or because of their ability to induce BAFF expression in other systems. All cytokines were present at all the time points with the exception of IFN-γ, which was not detected in the CLL/monocyte supernatants at any time point (Figure 2-1).

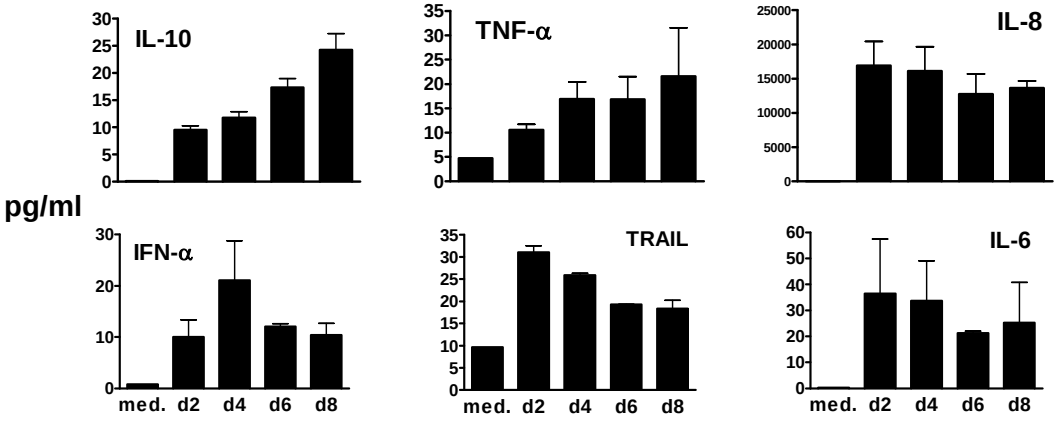


Figure 2-1. Soluble factors in CLL B-cell/monocyte co-cultures. CLL B-cells were co-cultured with monocytes; supernatants were collected at day 2, 4, 6, and 8, and analyzed for the presence of IL-10, TNF-α, IL-8, IFN-α, TRAIL, IL-6, and IFN-γ by ELISA. All cytokines were present at various time points except INF-γ, which was not detected. The data represent the mean +/- SEM of 2 independent experiments using supernatants from 2 different CLL/monocyte co-cultures.

Despite detecting various soluble cytokines in CLL/monocyte co-culture supernatants, it was difficult to ascertain which cell type is producing what factor as a constant cross-talk exist between CLL cells and monocytes. Our focus was on TNF-α because of the published data on TNF in CLL. To answer whether TNF-α is produced by the CLL cells, we tested CLL-CM for the presence of TNF-α by ELISA and found that TNF-α is present in 24h CLL-CM (Figure 2-2A). Preliminary results indicate that TNF-α levels tend to correlate with the potency of CLL-CM to upregulate surface BAFF expression in CD14+ monocytes (Figure 2-2B).

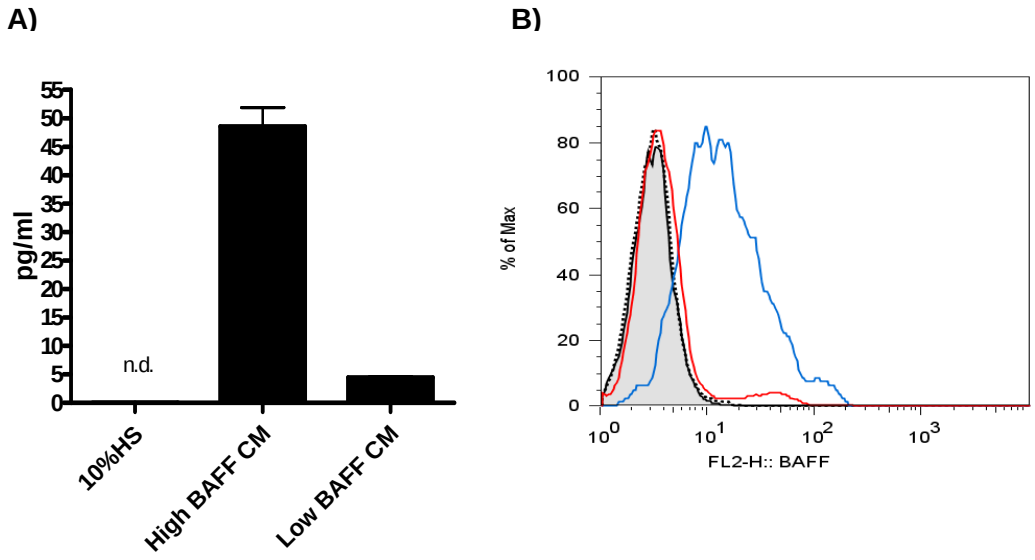


Figure 2-2. Levels of TNF-α in CLL condition media correlate with the potency of CLL-CM to induce BAFF upregulation in monocytes. (A) Detection of TNF-α levels in CLL-CM by ELISA. The detection range was 4pg/ml – 500pg/ml. The data shown is the mean +/- SEM of 2 CLL-CM of high BAFF-inducing activity and 1 CLL-CM of low BAFF-inducing activity. TNF-α was not detected in control media (10% HS). **(B)** CD14+ monocytes were cultured in CLL-CM with high TNF levels (blue line), CLL-CM with low TNF levels (red line) or media control (solid line/shaded region) for 7 days and analyzed for surface BAFF by flow cytometry.

C. Blocking TNF-α in CLL-CM and CLL serum reduced BAFF expression in CD14+ monocytes

In order to test whether the presence of TNF-α in the CLL-CM is responsible for the increased BAFF expression in CD14+ monocytes, experiments were conducted to block TNF-α in CLL-CM and CLL serum. CLL-CM and CLL serum were pretreated with the decoy receptor TNF-R-Fc or human

IgG control 1h prior to the addition of CD14+ monocytes into culture. After 7 days in culture, the monocytes were collected and analyzed for BAFF expression by flow cytometry. CD14+ monocyte cultured in CLL-CM and CLL serum both showed a significant reduction in surface BAFF expression when treated with TNF-R-Fc. In CLL-CM, TNF-R-Fc reduced monocyte BAFF expression to about half when compared to cultures treated with human IgG (control) (Figure 2-3A). Seven out of 9 CLL sera tested demonstrated a similar reduction in monocyte BAFF expression when treated with TNF-R-Fc (Figure 2-3B).

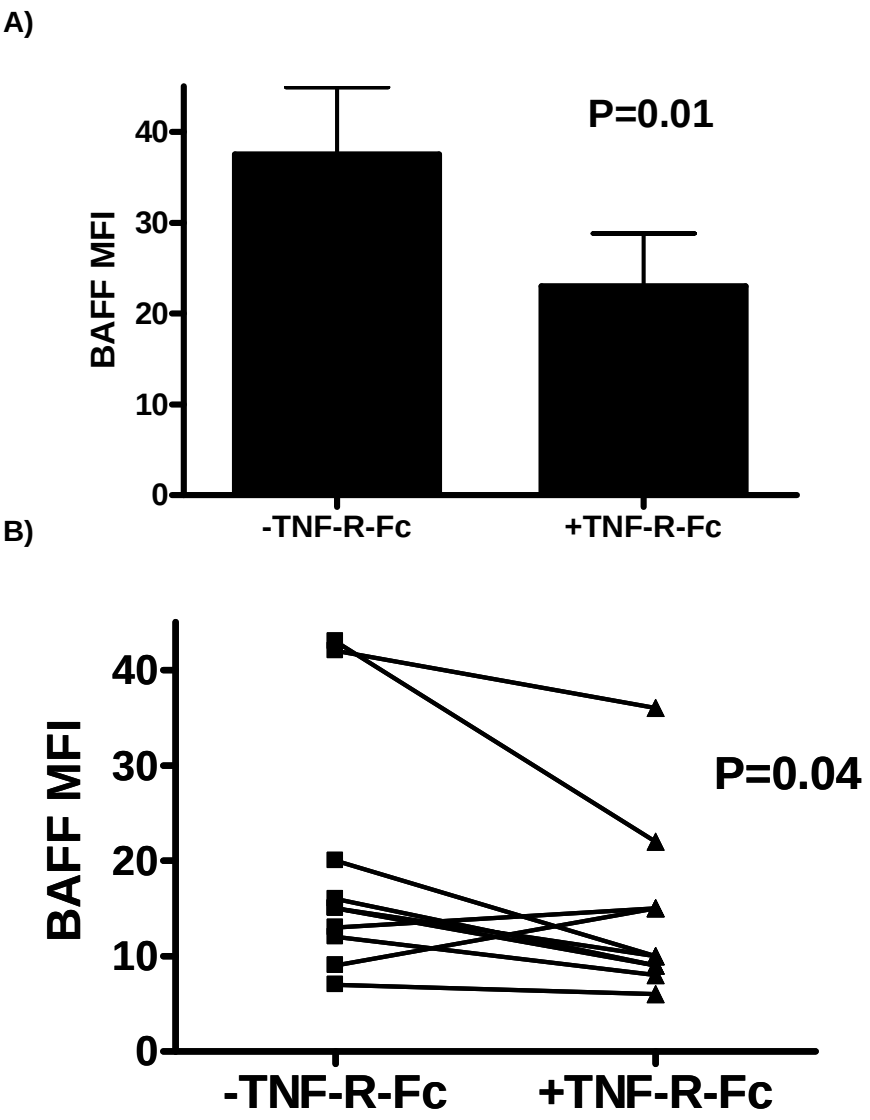


Figure 2-3. Blockage of TNF-α using decoy receptor, TNF-R-Fc, reduces the BAFF-inducing activity of CLL-CM and CLL serum. 80nM TNF-R-Fc was used to block TNF-α in **(A)** 4 different CLL-CM and **(B)** 9 different CLL serums. CLL-CM and CLL serum were pretreated with TNF-R-Fc for 1h prior to the addition CD14+ monocytes into culture. 80nM human IgG was used a control (-TNF-R-Fc). Monocytes were collected and stained with anti-BAFF-PE and isotype control after 7 days of culture. Samples were analyzed by flow cytometry. The mean fluorescence intensity (MFI) is depicted and the data shown above is the mean +/- SEM. Statistical significance was determined using the paired t-test.

To confirm that the blocking experiment using TNF-R-Fc is the result of a specific TNF-α blocking effect and not due to interaction of the decoy recdeptor with other factors, we reconstituted the TNF-R-Fc treated CLL-CM with recombinant human TNF-α (rhTNF-α). TNF-R-Fc and rhTNF-α were added simultaneously to CLL-CM 1h prior to the addition of CD14+ monocytes into culture. After 7 days in culture, monocytes were collected and analyzed for BAFF expression by flow cytometry. The addition of rhTNF-α to TNF-R-Fc treated CLL-CM demonstrated a restoration of BAFF-inducing ability of the CLL-CM (Figure 2-4).

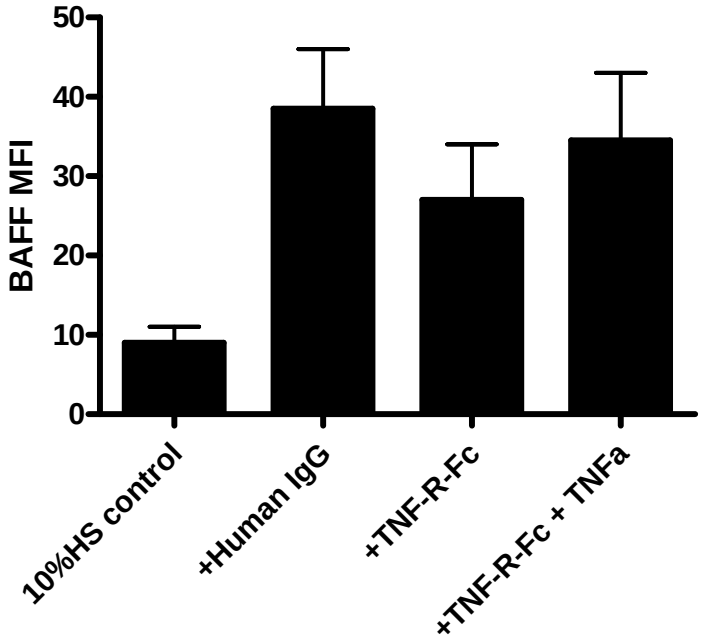


Figure 2-4. Addition of rhTNF-α restores BAFF-inducing activity of CLL-CM. Monocytes were cultured in control media (10% HS controls), CLL-CM treated with 80nM of human IgG, 80nM TNF-R-Fc alone or in combination with 30pg/ml rhTNF-α for 1h prior to the addition of CD14+ monocytes into culture. Monocytes were collected and analyzed for BAFF expression on day 7 by flow cytometry. The mean fluorescence intensity (MFI) is depicted and the data shown represents the mean +/- SEM of 2 independent experiments.

D. Soluble TNF-α and IFN-γ are not sufficient to upregulate surface BAFF in CD14+ monocytes

Since the addition of rhTNF-α to TNF-R-Fc treated CLL-CM restored the BAFF-inducing activity of the CLL-CM, we investigate whether adding rhTNF-α to 10% HS media will induce BAFF expression in CD14+ monocytes. Various concentrations (0.1-10ng/ml) of rhTNF-α were added to monocytes at day 0. CLL-CM was added to the monocytes as positive control. No increase in BAFF expression was observed in monocytes after 7 days (Figure 2-5).

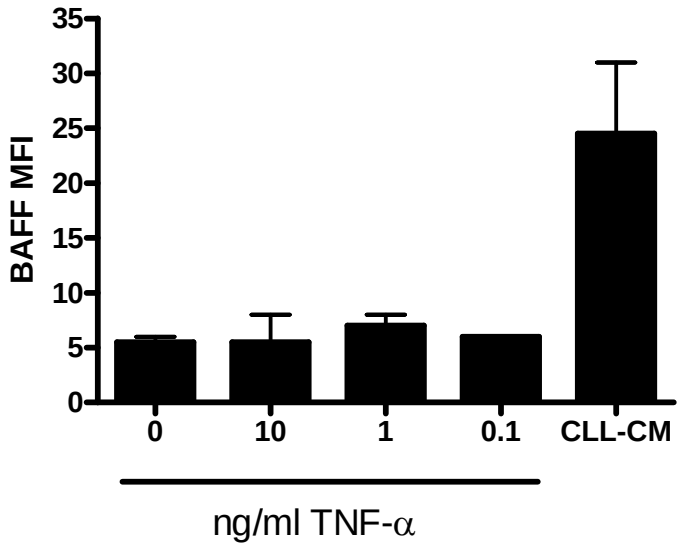


Figure 2-5. Soluble TNF-α is not sufficient to upregulate surface BAFF in CD14+ monocytes. rhTNF-α was added to monocytes at titrating doses as indicated. Cells were collected after 7 days of culture and analyzed for BAFF expression by flow cytometry. The mean fluorescence intensity (MFI) is depicted and the data represent the mean +/- SEM of 3 independent experiments.

Previous studies in rheumatoid arthritis have shown that soluble TNF-α and INF-γ can induce significant increases in BAFF mRNA and protein levels in fibroblast-like synoviocytes[26]. In light of the role of TNF-α and IFN-γ on fibroblast-like synoviocytes, we investigate whether administering soluble TNF-α and IFN-γ alone or in combination at various concentrations can induce BAFF expression in CD14+ monocytes in 7 day cultures. Recombinant cytokines were added to monocytes on day 0. Compared to media control and CLL-CM (positive control for BAFF induction), we did not observe upregulation of BAFF expression in monocytes after 7 days at the conditions tested (Figure 2-6).

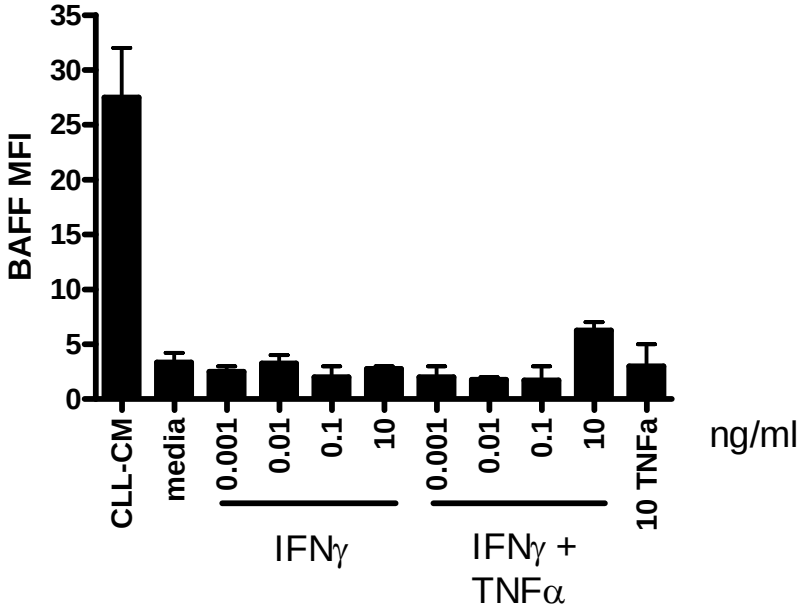


Figure 2-6. Soluble TNF-α and IFN-γ are not sufficient to upregulate surface BAFF in CD14+ monocytes. rhIFN-γ was added to monocytes at (0.001-10 ng/ml) either alone or in combination with 10ng/ml rhTNF-α as indicated. Cells were collected after 7 days of culture and analyzed for BAFF expression by flow cytometry. The mean fluorescence intensity (MFI) is depicted. The data represent the mean +/- SEM of 3 independent experiments using monocytes from different donors.

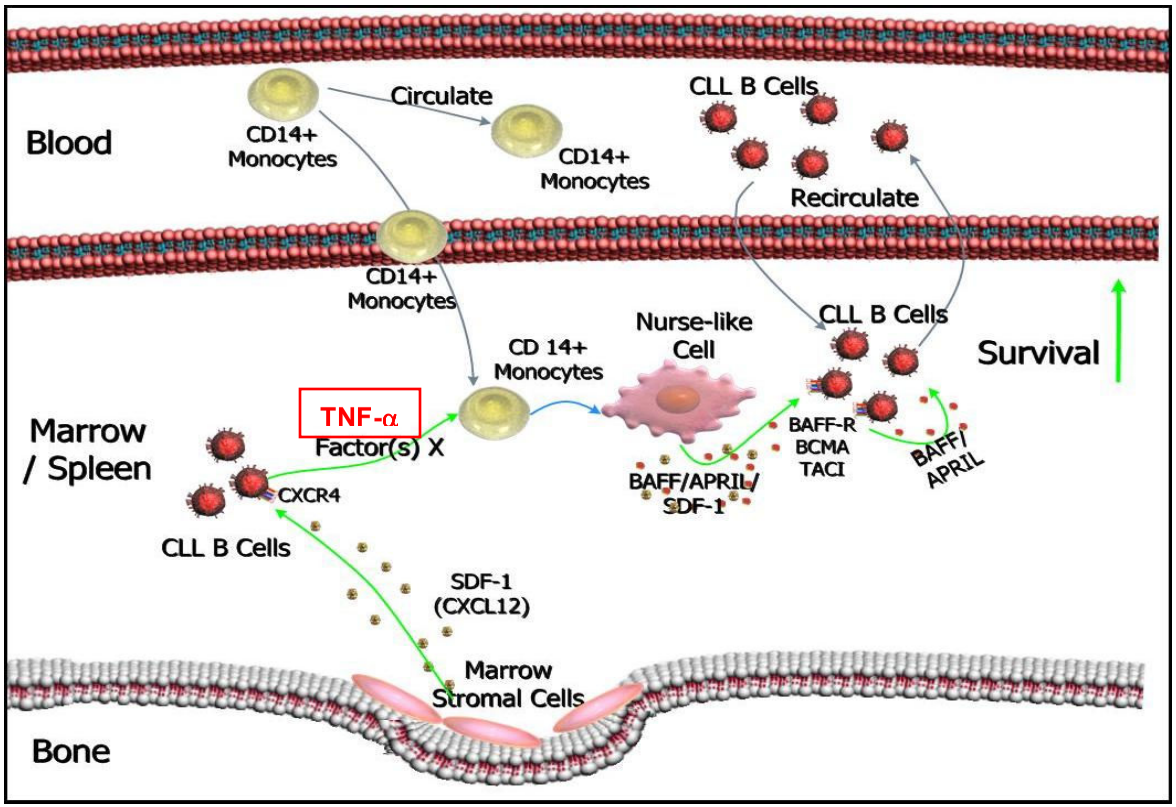


Figure 2-7. *In vivo* model: CLL microenvironment crosstalk. CLL B-cells are commonly found in lymphoid organs such as the bone marrow and spleen. CLL B-cells express CXCR4, BAFF-R, BCMA, and TACI receptors on its surface, which can interact with SDF-1 (CXCL12), BAFF and APRIL to gain survival advantage. CD14+ blood monocytes can migrate into the lymphoid organs. Upon encounter with CLL B-cells, soluble factor(s) X and TNF-α released by the CLL cell cause CD14+ monocytes to differentiate into NLCs, which in turn support CLL cell survival. Green, blue, gray arrows indicate cellular interaction, cell differentiation, and migration, respectively.

E. Discussion

Molecular markers such as zeta-chain associated protein-70 (ZAP-70) expression, CD38 expression, cytogenetic abnormalities, and immunoglobulin V (IgV) gene mutational status allow for the prognosis of CLL patients[33-35]. Several studies have reported the presence of TNF-α in serum of CLL patients[31] and some found a correlation between disease progressing[25] ZAP-70 expression and increased levels of TNF-α[32]. Interestingly we have detected TNF-α in supernatants of CLL/monocytes co-cultures and CLL-CM at levels similar to those described in serum of CLL patients[24].

IFN-γ and TNF-α work synergistically to induce BAFF expression in fibroblast-like synoviocytes and macrophages[26]. Our data show that a TNF-α decoy receptor, TNF-R-Fc, when added to condition media as well as CLL serum reduced the BAFF expression by approximately half. The addition of rhTNF-α into TNF-R-Fc treated CLL-CM partially restored BAFF expression in monocytes. This suggests that TNF-α plays a role in increasing BAFF expression in those monocytes and that TNF-α is likely one of the soluble factors released into CLL-CM by CLL B-cells. However, when rhTNF-α was added to monocytes alone or in combination with rhIFN-γ, no increase in BAFF expression was observed. Thus, TNF-α is not sufficient to induce BAFF in monocytes under our experimental conditions suggesting that the presence of other factors/cofactors is required. The remaining factors working together with TNF-α remain to be identified.

To identify and characterize the soluble factor(s) responsible for the monocyte differentiation into NLC, 24h CLL-CM was generated in serum-free media for fractionation by fast performance liquid chromatography (FPLC). Unfortunately, most of the CLL-CM generated in serum-free media did not induce BAFF expression in CD14+ monocytes when analyzed by flow cytometry. To little avail, experiments utilizing size fractionation spin columns, DNase, or proteinase K on CLL-CM provided little insight on the properties of the soluble factor(s) as the lost of serum factors to the columns or proteinase K deemed CD14+ monocytes unviable for BAFF analysis.

Instead of the direct FPLC approach to identify the soluble factor(s), we optioned for an indirect blocking method with several targets in mind (IL-6, IL-8, IL-10, IFN-α, TNF-α…etc). As the decoy receptor for TNF-α[36], TNF-R-Fc, became available to us through Dr. Carl Ware, TNF-α blocking experiments were conducted on CLL-CM and CLL serum and showed a significant BAFF-reducing effect on CD14+ monocytes cultured in the respective media (Figure 2-3). As TNF-R-Fc is also known to block lymphotoxin-alpha (LT-α), a soluble homotrimer of distinctive immunological roles from TNF-α[37], one cannot preclude the possibility that LT-α might be mediating the upregulation of BAFF in CLL cells. To test this, one can see whether the addition of recombinant LT-α can induce BAFF expression in monocytes. Although the addition of rhTNF-α alone or in combination with rhIFN-γ to monocytes was not sufficient to induce BAFF expression, preliminary results indicate that TNF-α's ability to induce BAFF

expression in monocytes is enhanced in the presence of macrophage colon-stimulating factor (M-CSF; data not shown). Further experiments need to be conducted to conclude whether LT-α and/or M-CSF are soluble factors produced by CLL B-cells.

Ultimately, the ability to produce potent CLL-CM in serum free media will allow us to perform FPLC on the CLL-CM. Once the big batch of CLL-CM is fractionated into small 1mL-fractions, CD14+ monocytes can be cultured in each fraction for 7 days and analyzed for morphology and BAFF expression. We will also characterize each fraction by gel electrophoresis and sequencing (mass spec/Edman degradation). Once the factor(s) are identified, we will quantify its presence in unfractionated condition media by ELISA. We will also determine the percentage of CLL cells that express the factor(s) by intracellular FACS/cytospin. If available, we will also purchase recombinant protein and blocking antibody against the factor(s) to confirm that the identified factor(s) is indeed responsible for inducing BAFF expression in monocytes.

This chapter, in part, is being prepared for publication to be submitted to *Blood* (D. Messmer, H-H Chang, T. Endo, B. Messmer, Anne-Mette Buhl, Ingo G.H. Schmidt-Wolf, and T.J. Kipps, “TNF-α released from chronic lymphocytic leukemia B cells promotes increased BAFF expression in monocytes which consequently promote leukemic cell survival.”). The thesis author will be the co-author of this paper.

Conclusion

In recognition of the growing attention to the tumor microenvironment, many recent studies on B-CLL have been focused on characterizing the surrounding support cells in the CLL microenvironment. To this end, accessory cells such as marrow stromal cells[5,7,11], fibroblasts[27], follicular dendritic cells[28,29], and regulatory T-cells[30] have all been implicated to contribute to CLL cell survival *in vitro*. Blood-derived nurse-like cells have also been shown to support CLL survival via antigen-ligand interactions between SDF-1, BAFF, APRIL and their receptors on the CLL cell[11-14]. Although tumor-associated macrophages have been shown to play a supportive role in follicular lymphoma, the significance of macrophages in the CLL microenvironment is diminished when compared to the higher BAFF-expressing NLC. Although many previous studies have demonstrated the increased CLL survival capacity provided by NLC, few have addressed the question as to how the CLL cell manipulates the microenvironment to its survival advantage. It was thought that cell contact between CLL cells and CD14+ cells were required to generate NLC *in vitro*[12]. Yet, we show that cell contact is not necessary and that soluble factor(s) produced by CLL cells is sufficient to induce CD14+ cells to differentiate into NLC *in vitro* (Figure 1-3).

Since cells of the microenvironment can protect CLL cells from chemotherapy-induced apoptosis *in vitro*, it is conceivable that in certain *in vivo* niches like the spleen or bone marrow such interaction and protection may occur (Figure 2-7). In conjunction with current CLL therapeutic drug delivery regimens, unraveling the interaction between CLL cells and cells of their microenvironment

may represent a novel target for therapy and should allow for a combination of treatment options in the future. This will likely enhance the therapeutic drug effects by interfering with the protective effect while killing CLL cells, potentially allowing the complete depletion of cancerous cells. TNF-α blocking agents such as infliximab, a monoclonal antibody used for rheumatoid arthritis treatments, could present new targets for combinational therapy in CLL.

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