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Neutralization of (NK-cell-derived) B-cell activating factor by Belimumab restores sensitivity of chronic lymphoid leukemia cells to direct and Rituximab-induced NK lysis

Running title: BAFF neutralization in CLL

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Abstract

Natural killer (NK) cells are cytotoxic lymphocytes that substantially contribute to the therapeutic benefit of antitumor antibodies like Rituximab, a crucial component in the treatment of B-cell malignancies. In chronic lymphocytic leukemia (CLL), the ability of NK cells to lyse the malignant cells and to mediate antibody-dependent cellular cytotoxicity upon Fc receptor stimulation is compromised, but the underlying mechanisms are largely unclear. We report here that NK-cells activation-dependently produce the tumor necrosis factor family member ‘B-cell activating factor’ (BAFF) in soluble form with no detectable surface expression, also in response to Fc receptor triggering by therapeutic CD20-antibodies. BAFF in turn enhanced the metabolic activity of primary CLL cells and impaired direct and Rituximab-induced lysis of CLL cells without affecting NK reactivity per se. The neutralizing BAFF antibody Belimumab, which is approved for treatment of systemic lupus erythematosus, prevented the effects of BAFF on the metabolism of CLL cells and restored their susceptibility to direct and Rituximab-induced NK-cell killing in allogeneic and autologous experimental systems. Our findings unravel the involvement of BAFF in the resistance of CLL cells to NK-cell antitumor immunity and Rituximab treatment and point to a benefit of combinatory approaches employing BAFF-neutralizing drugs in B-cell malignancies.

Introduction

Natural killer (NK) cells are cytotoxic lymphocytes of the innate immune system that play an important role in the immunosurveillance of hematological malignancies. Their reactivity is governed by the integration of activating and inhibitory signals propagated by a multitude of surface receptors that determine the quality and intensity of NK-cell responses [1]. Owing to their ability to mediate antibody-dependent cellular cytotoxicity (ADCC) upon triggering of the Fcγ Receptor IIIa (CD16), they substantially contribute to the therapeutic success of antitumor antibodies like Rituximab [2]. The latter has become an essential component of most treatment strategies for B-cell malignancies [3,4]. Over the recent years, several studies reported that the ability of NK cells to directly lyse the malignant cells and to mediate ADCC is compromised in chronic lymphocytic leukemia (CLL) [5–15]. The molecular mechanisms underlying these observations, however, remain largely unclear.

In this study, we demonstrate that the resistance of CLL cells to direct and Rituximab-induced NK-cell reactivity is facilitated by the tumor necrosis factor (TNF) family member ‘B-cell activating factor’ (BAFF). BAFF and its close homologue ‘A proliferation inducing ligand’ (APRIL) are mainly produced by immune cells of the myeloid lineage and bind to the two receptors ‘B-cell maturation antigen’ (BCMA) and ‘transmembrane activator and cyclophilin ligand interactor’ (TACI), while BAFF additionally binds to BAFF receptor (BAFFR). Both are critical factors for proliferation, function and survival of peripheral B cells. While at least partially discrepant findings have been reported for APRIL, BAFF plays a prominent pathophysiological role in autoimmune diseases (for reviews see Mackay and Schneider as well as Dillon et al.) [16,17]. Accordingly, a BAFF-neutralizing antibody termed Belimumab (Benlysta) and a BAFF- and APRIL-inhibiting soluble TACI-Ig molecule (Atacicept) have been developed, the first being approved for treatment of systemic lupus erythematosus and the latter being evaluated in advanced phase clinical trials [18,19]. Both BAFF and APRIL also contribute to the pathophysiology of B-cell malignancies by acting as anti-apoptotic and pro-survival factors [20–25]. We demonstrate

that NK cells produce BAFF, in particular upon activation, and this includes triggering of their Fc receptor by immunostimulatory CD20 antibodies used in CLL treatment. We further show that NK-cell-derived BAFF enhances the metabolic activity of CLL cells and notably also diminishes their susceptibility to direct NK-cell lysis and Rituximab-induced ADCC.

Finally, we report that the CLL-protective effects of BAFF can be neutralized by treatment with Belimumab, which sensitizes the leukemia cells to lysis. Our findings not only identify a biological mechanism underling the pronounced resistance of lymphoid malignant cells to direct and Rituximab-induced NK-cell antitumor immunity, they also unravel the potential of BAFF-blocking agents to reinforce NK-cell reactivity for immunotherapy of B-cell malignancies.

Material and Methods

Cells

Peripheral blood mononuclear cells (PBMCs) from CLL patients were obtained at time of diagnosis prior to therapy. All patients declared written informed consent in accordance with the Helsinki protocol, and the study was performed according to the guidelines of the local ethics committee. Polyclonal NK cells (pNKCs) were generated using K562-41BBL-IL15 feeder cells obtained from St Jude´s Children´s Research Hospital as previously described [26]. Resting NK cells and monocytes were purified by MACS isolation (Miltenyi, Bergisch-Gladbach, Germany). Purity of cell preparations was confirmed by fluorescence-activated cell sorting (FACS) and always above 90%. 293-BAFF transfectants and parental controls were described previously [27].

Antibodies and recombinant BAFF

Recombinant human BAFF (rhBAFF) was from Immunotools (Friesoythe, Germany). BAFF mAb 1D6 was from eBioscience (San Diego, CA, USA). The anti-mouse PE-conjugate was from Dako (Glostrup, Denmark). Rituximab and Obinutuzumab were from Roche (Basel, Switzerland), Ofatumumab and Belimumab were from GlaxoSmithKline (Brentford, UK). All other antibodies were from BD Pharmingen (San Diego, CA, USA).

PCR analysis

RT-PCR was performed as described previously [28]. Primers were 5′-CCTCAC GGTGGTGTCTTTCT-3′ and 5′-AAAGCTGAGAAGCCATGGAA-3′ for BAFF and 5′-GAGTCAACGGATTTGGTCGT-3′ and 5′-TTGATTTTGGAGGGATCTCG-3′ for GAPDH.

Flow cytometry

NK cells were incubated with BAFF mAb or isotype control mAb (10 µg/ml each) followed by the secondary phycoerythrin conjugate (1:100). Intracellular staining was performed using the

Cytofix/Cytoperm from BD Biosciences (San Jose, CA, USA) with phycoerythrin-conjugated 1D6 mAb or isotype control according to the manufacturer´s instructions. NK cells and CLL cells were selected by counterstaining for $CD56^+/CD3^-$ and $CD5^+/CD19^+$, respectively. Analysis was performed using a FC500 (Beckmann Coulter, Krefeld, Germany). Specific fluorescence indices were calculated by dividing median fluorescence obtained with specific mAb by median fluorescence obtained with control. Positivity was defined as specific fluorescence index ≥ 1.5.

FACS-based analysis of CLL cell lysis

Rituximab-induced lysis of CLL cells by autologous NK cells was determined by FACS as previously described [29]. In brief, PBMCs of CLL patients were cultivated in the presence or absence of 500 ng/ml BAFF, Belimumab or control antibody (10 µg/ml each) for 48 h. Then, Rituximab (10 µg/ml) was added where indicated for additional 24 h. Afterwards dying and dead $CD19^+/CD5^+$ CLL cells were identified by FACS owing to 7-aminoactinomycin D positivity. Analysis of equal assay volume was ascertained by adding standard calibration beads. Percentage of living cells was calculated from triplicate measurements as follows: 7-aminoactinomycin D negative cells upon treatment/7-aminoactinomycin D negative cells in control x 100.

Enzyme-linked immunosorbent assay

BAFF levels in supernatants were determined by ELISA according to the manufacturer**'**s instructions using the matched-pair detection set from Adipogen (Liestal, Switzerland). Results are shown as means of triplicate measurements with s.e.m.

Metabolic activity

Primary CLL cells were plated at 2 x 10^6 per well and metabolic activity was measured after 72 h using the Cell Proliferation Reagent WST-1 set from Roche according to the manufacturer's instructions. Results are shown as means of triplicate measurements with s.e.m.

NK-cell activation, degranulation and cytotoxicity

Upregulation of CD69 and CD107a as markers for NK-cell activation and degranulation, respectively, was analyzed by FACS. Direct cytotoxicity and ADCC of NK cells against CLL cells were analyzed by 2 h BATDA Europium assays as described previously [30].

Purification of NK-cell-derived BAFF

Belimumab and Rituximab as control were immobilized on CNBr-activated Sepharose 4B (GE Healthcare, Chalfont St Giles, UK) according to the manufacturer's recommendations. Concentrated NK-cell supernatant was then recirculated on a serial two-column setup overnight at 4 °C followed by elution with sodium citrate pH 2.5. Eluates were neutralized, concentrated and washed with phosphate-buffered saline by ultrafiltration. Eluates of the Belimumab column underwent a second round of purification under identical conditions.

Peptide mass-fingerprint analysis

Eluates from Belimumab-coated columns were pretreated with dithiothreitol and iodacetamide followed by digestion with Trypsin (Sigma Aldrich, St Louis, MO, USA). After ZipTipC18 (Millipore, Schwalbach, Germany) purification and desalting, peptides were analyzed by liquid chromatography-mass spectrometry/mass spectrometry (UltiMate 3000 RSLCnano UHPLC system and LTQ orbitrap XL, Thermo Fisher, Waltham, MA, USA) followed by data processing and peptide annotation against the human proteome as comprised in the Swiss-Prot database (www.uniprot.org), as described previously.31 Search was restricted by enzymatic tryptic specificity. Possible methionine oxidation and carbamidomethyl modifications of cysteines were

taken into account. The false discovery rate was limited to a high confidence to $q < 0.01$ (1% false discovery rate).

Results

BAFF is activation-dependently produced by NK cells as soluble, but not as surface-expressed protein

A previous study reported that activated NK cells express BAFF at the protein level [32], but whether it is expressed on the cell surface or released in soluble form was not studied. After characterizing available reagents for BAFF detection (Supplementary Figure 1A), we performed FACS analyses with resting NK cells within PBMCs of healthy donors as well as pNKCs. We found that NK cells express no or very low levels of surface BAFF (Figure 1a and Supplementary Figure 1B). NK cells were also surface-negative for APRIL, which reportedly is not produced in a membrane-bound form [33]. Notably, mRNA of both BAFF and APRIL was detected by RT-PCR in resting and polyclonal NK cells (Figure 1b and data not shown). Next, we analyzed culture supernatants from isolated resting NK cells and pNKCs for BAFF and APRIL by ELISA. APRIL was never detectable, whereas soluble BAFF was produced by NK cells in all cases with higher levels observed with pNKCs (Figure 1c and data not shown). In line, BAFF protein was detected by intracellular FACS analysis in NK cells among PBMCs of healthy donors, pNKCs and notably also in NK cells of CLL patients, which displayed pronounced BAFF levels (Figure 1d).

To determine whether and how BAFF production by NK cells is regulated, pNKCs were cultured alone or in the presence of the cytokines IL-2 and IL-15 which induce a potent general activation. To mimic CD16 stimulation, we employed culture of the NK cells on immobilized Rituximab, Ofatumumab and Obinutuzumab, the three CD20 antibodies approved for CLL treatment. Although induction of BAFF surface expression was never observed, all stimuli significantly increased the release of soluble BAFF, which was mirrored by enhanced BAFF

mRNA levels in NK cells. Ofatumumab and Obinutuzumab, which mediate more pronounced immunostimulatory effects as compared with Rituximab [34,35], induced higher BAFF release as compared with Rituximab, which in case of Obinutuzumab reached statistical significance. This was mirrored by our results regarding the increase of BAFF protein levels detectable by FACS intracellularly in NK cells (all $P < 0.05$, Student's *t*-test) (Figures 1e, f and data not shown).

BAFF stimulates metabolic activity and protects CLL cells from direct and Rituximab-induced NK lysis

Next, we employed primary CLL cells in WST-1 assays, which revealed a dose-dependent induction of leukemia cell metabolic activity upon exposure to rhBAFF (Figure 2a). Then, we purified BAFF from NK-cell culture supernatants and analyzed this preparation (nkBAFF) by liquid chromatography-mass spectrometry/mass spectrometry. Tryptic peptides in preparations of 293-BAFF transfectants as control covered most of the sequence of soluble BAFF, and some of these fragments were also identified upon analysis of nkBAFF (Tables 1A and B) [36]. It is noteworthy that one of these peptides has a distinct sequence in cows, excluding the possibility that it could originate from fetal calf serum used in these cultures. WST-1 assays with primary CLL cells revealed that the nkBAFF purified using a Belimumab-coated column clearly stimulated the metabolic activity of the leukemia cells (Figure 2b). Next, primary CLL cells cultured in the presence or absence of BAFF were analyzed for their sensitivity to pNKC lysis. BAFF exposure for 24 h significantly (all $P < 0.05$, Student's *t*-test) reduced CLL cell susceptibility to NK-cell attack. Notably, this BAFF-mediated protection of CLL cells was observed for both natural and Rituximab-induced NK lysis and with both rhBAFF and nkBAFF (Figure 2c and d). Finally, to more closely mirror the situation in patients, functional analyses were performed with freshly isolated PBMCs of CLL patients. FACS-based determination of CLL cell viability after incubation in the presence or absence of BAFF and/or Rituximab confirmed that rhBAFF significantly ($P < 0.05$, Student's *t*-test) and sometimes fully protected the malignant

B cells from Rituximab-induced lysis by the patient's own NK cells (Figure 2e). This protective effect was also observed with nkBAFF (Figure 2f).

BAFF reduces CLL cell susceptibility to lysis without affecting NK-cell reactivity

Next, we incubated CLL cells with or without BAFF for 24 h followed by washing of the leukemic cells to exclude an influence of soluble NK-inhibitory factors potentially induced in CLL cells by BAFF exposure. Afterwards, pNKCs were co-cultured with the CLL cells in the absence of BAFF, which further excluded direct effects of BAFF on the effector cells. Neither in the presence nor absence of Rituximab relevant effects of BAFF pretreatment on NK-cell degranulation and activation as revealed by FACS for CD107a and CD69, respectively, were observed. Notably, this held true with allogenic NK cells and also in our autologous experimental setting employing PBMCs of CLL patients directly obtained ex vivo (Figure 3). In line, BAFF exposure of the leukemic cells did not alter the expression of immunoregulatory molecules that reportedly influence NK-cell reactivity against CLL cells (for example, classical and non-classical HLA class I molecules, NKG2D ligands, etc.) or CD20-expression (data not shown). We further observed that BAFF did not affect CLL cell proliferation (at least in our in vitro setting), but significantly (all $P < 0.05$, Student's t-test) protected the leukemic cells from spontaneous apoptosis and sustained their survival. Although no effect on the expression of death receptors (TRAILR, TNFR1 and CD95/Fas) was detected, this protective effect of BAFF for CLL cells was mirrored by a reduction of cleaved PARP as component of their apoptosis machinery (Supplementary Figure 2 and data not shown). These results provide evidence that BAFF protects CLL cells from lysis without affecting NK reactivity per se.

BAFF neutralization by Belimumab restores CLL cell susceptibility to direct and Rituximab-induced NK lysis

Finally, we determined whether neutralization of BAFF by Belimumab could serve to therapeutically overcome its protective effects on CLL cells. First, primary CLL cells were exposed to rhBAFF in the presence or absence of Belimumab or control mAb for 72 h. Subsequent WST-1 assays revealed that the presence of Belimumab prevented the BAFF-mediated increase of CLL cell metabolic activity (Figure 4a). Second, we studied whether Belimumab also prevented the BAFF-induced resistance of CLL cells to NK-cell attack. Cytotoxicity assays revealed that the addition of Belimumab abrogated the protective effect of BAFF for CLL cells and significantly (all $P < 0.05$, Student's **t**-test) restored both direct and Rituximab-induced lysis by allogeneic NK cells (Figure 4b). Similar results were obtained in our autologous experimental system when NK reactivity was induced by treatment with Rituximab for 24 h: FACS-based lysis assays revealed that Belimumab significantly (all $P < 0.05$, Student's *t*-test) prevented the BAFF mediated resistance of CLL cells to ADCC (Figure 4c). Overall, these results demonstrate that Belimumab treatment may serve to overcome the protective effects of BAFF and to reinforce direct and Rituximab-induced NK lysis in CLL.

Discussion

Owing to their ability to mediate natural and antibody-induced lysis of target cells, NK cells play an important role in antitumor immunity [1,2]. Induction of NK-cell ADCC contributes to the therapeutic effects of antitumor antibodies. It is meanwhile firmly established that this important antibody function largely contributes to the success of Rituximab, which is routinely used for the treatment of B-cell malignancies including CLL [3,4]. However, multiple reports over the last three decades described that in CLL, the ability of NK cells to target the malignant cells is compromised. Both, low susceptibility of the leukemic cells to NK attack and impaired reactivity of patient NK cells have been reported, and notably, this was observed with regard to direct and

antibody-induced cytotoxicity [5–15]. The reportedly higher expression of NK-inhibitory HLA class I as well as lower levels of ligands for activating receptors like CD155 and CD112 in lymphoid as compared with myeloid leukemias contribute to the same [10]. With regard to CLL in particular, not only the down-regulation of activating receptors like NKG2D and NKp30 on patient NK cells, but also release of the NKp30 ligand BAG6 in soluble form as well as pronounced expression of NK-inhibitory molecules like non-classical HLA and various TNF family members by the malignant cells play a role [9,13,29,37–39].

Here, we identified the TNF family member BAFF as a mediator of CLL cell resistance to NK-cell attack: exposure of CLL cells to BAFF profoundly reduced direct and Rituximab-induced NK lysis as revealed by analyses with primary leukemic cells and allogeneic 'healthy' as well as autologous patient NK cells. With regard to this CLL-protective effect, our finding that NK cells themselves produce BAFF is of particular interest. In general, BAFF is mainly expressed and released in soluble form by myeloid cells like monocytes/macrophages, dendritic cells and neutrophils. However, cells of the lymphoid lineage like B and T cells have also been reported to express BAFF, in particular upon activation [16]. Here, we report that NK cells as innate components of the lymphoid lineage produce BAFF as a soluble factor dependent on their activation state, whereas relevant surface expression was never detected. In most cell types, BAFF is expressed on the surface and cleaved by a furin convertase.27 In NK cells, BAFF could be processed intracellularly and released without being expressed in a membrane-bound form, as reported for neutrophils.40 We also detected BAFF protein intracellularly in NK cells of healthy donors and notably also of CLL patients. Both intracellular BAFF levels and release of soluble protein were increased upon general NK-cell activation with the cytokines IL-2 and IL-15, which is in line with a previous study that reported on BAFF expression by NK cells at the protein level by western blot [32].

Notably, enhanced BAFF production was also detected upon CD16 stimulation with Rituximab, and even more pronounced effects were observed with Ofatumumab and Obinutuzumab, the

novel CD20 antibodies approved for CLL therapy. In particular, Obinutuzumab, which is glycol-engineered to enhance its affinity to CD16 [34,35], mediated significantly stronger BAFF induction as compared with Rituximab.

Liquid chromatography-mass spectrometry/mass spectrometry confirmed that BAFF was released by NK cells and was present in the nkBAFF preparations employed in functional assays. Alike rhBAFF, nkBAFF mediated profound resistance of CLL cells to direct and Rituximab-induced NK lysis in allogeneic and autologous experimental assay systems. Notably, the activation and degranulation of allogeneic and autologous NK cells both in the absence and presence of Rituximab were not affected by BAFF pretreatment of CLL cells. It is therefore unlikely that the reduction of NK lysis was due to decreased immunogenicity of the leukemic cells. In line, we did not observe effects of BAFF on the expression of various surface molecules that reportedly influence NK reactivity against CLL cells or CD20, the target for the therapeutic antibodies employed in CLL treatment. Rather, BAFF protects from NK-cell-induced cell death, which is supported by our observation that both rhBAFF and nkBAFF stimulated metabolic activity, decreased apoptosis and sustained survival of CLL cells. The latter is in line with findings of other investigators, which reported that BAFF protects CLL cells from apoptosis and enhances NF-κB activity.22,23 The exact molecular mechanism of how BAFF protects CLL cells from NK lysis, however, was not addressed in our study and remains to be elucidated.

Certainly, NK cells are not the only source of BAFF for CLL cells. Besides components of the microenvironment, the malignant cells themselves can express BAFF [20,22,23]. Nevertheless, nkBAFF may contribute to the reportedly compromised efficacy of NK cells to target CLL cells, as NK-cell activation upon encounter of the malignant cells would result in locally enhanced BAFF levels. Particularly upon antibody treatment, induction of BAFF release by NK cells and the resulting resistance to lysis may be counterproductive for the eradication of the leukemia cells and limit therapeutic efficacy in CLL. On the basis of these considerations, our finding that Belimumab, which is approved for systemic lupus erythematosus treatment, abrogates the CLL-

protective effects of BAFF is of particular interest. Besides increasing the immunostimulatory capacity of an antibody itself, it is rational to enhance its therapeutic efficacy by preventing mechanisms that allow for tumor immune evasion. For CLL, our approach to reinforce antitumor immunity by BAFF neutralization with Belimumab (but likely also other BAFF-blocking reagents) appears very promising. The benefit of BAFF neutralization reported in our study was, like the role of BAFF in NK-cell immunosubversion per se, proven by using primary leukemia cells of CLL patients with allogenic and autologous human NK cells as effectors. The advantage of this experimental model is that it not only precludes artifacts caused by differences of expression and/or function of BAFF or other TNF superfamily members, but also Fc receptor expression/function and the mechanisms and cell types that mediate ADCC in mice and men [30,41–46].

Taken together, the data presented in this study not only provide the first evidence for the involvement of (NK-cell-derived) BAFF in the impaired susceptibility of CLL cells to direct and Rituximab-induced NK-cell reactivity, they also suggest that combining Belimumab with Rituximab, but likely also with Obinutuzumab, Ofatumumab and other systemic treatment modalities will be beneficial for CLL therapy. Supporting evidence for this notion is derived from a clinical case, where Belimumab sensitized a patient with autoimmune disease for Rituximab treatment [47]. Future combinatorial clinical studies based on the findings presented in this report will be required to validate in vivo our conclusion that Belimumab enhances NK-cell reactivity and ADCC in CLL.

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Conflict of interest

The authors declare no conflict of interest.

References

1 Vivier E, Raulet DH, Moretta A, Caligiuri MA, Zitvogel L, Lanier LL et al. Innate or adaptive immunity? The example of natural killer cells. Science 2011; **331**: 44–49.

2 Ljunggren HG, Malmberg KJ. Prospects for the use of NK cells in immunotherapy of human cancer. Nat Rev Immunol 2007; **7**: 329–339.

3 Adams GP, Weiner LM. Monoclonal antibody therapy of cancer. Nat Biotechnol 2005; **23**: 1147–1157.

4 Keating GM. Rituximab: a review of its use in chronic lymphocytic leukaemia, low-grade or follicular lymphoma and diffuse large B-cell lymphoma. Drugs 2010; **70**: 1445–1476.

5 Jewell AP, Worman CP, Giles FJ, Goldstone AH, Lydyard PM. Resistance of chronic lymphocytic-leukemia cells to interferon-alpha generated lymphokine activated killer-cells. Leukemia Lymphoma 1992; **7**: 473–480.

6 Katrinakis G, Kyriakou D, Papadaki H, Kalokyri I, Markidou F, Eliopoulos GD. Defective natural killer cell activity in B-cell chronic lymphocytic leukaemia is associated with impaired release of natural killer cytotoxic factor(s) but not of tumour necrosis factor-alpha. Acta Haematologica 1996; **96**: 16–23.

7 Kay NE, Zarling JM. Impaired natural killer activity in patients with chronic lymphocytic leukemia is associated with a deficiency of azurophilic cytoplasmic granules in putative NK cells. Blood 1984; **63**: 305–309.

8 Kay NE, Zarling J. Restoration of impaired natural-killer cell-activity of B-chronic lymphocytic-leukemia patients by recombinant interleukin-2. Am J Hematol 1987; **24**: 161–167.

9 Maki G, Hayes GM, Naji A, Tyler T, Carosella ED, Rouas-Freiss N et al. NK resistance of tumor cells from multiple myeloma and chronic lymphocytic leukemia patients: implication of HLA-G. Leukemia 2008; **22**: 998–1006.

10 Pende D, Spaggiari GM, Marcenaro S, Martini S, Rivera P, Capobianco A et al. Analysis of the receptor-ligand interactions in the natural killer-mediated lysis of freshly isolated myeloid

or lymphoblastic leukemias: Evidence for the involvement of the Poliovirus receptor (CD155) and Nectin-2 (CD112). Blood 2005; **105**: 2066–2073.

11 Platsoucas CD, Fernandes G, Gupta SL, Kempin S, Clarkson B, Good RA et al. Defective spontaneous and antibody-dependent cytotoxicity mediated by E-rosette-positive and E-rosette-negative cells in untreated patients with chronic lymphocytic leukemia: augmentation by in vitro treatment with interferon. J Immunol 1980; **125**: 1216–1223.

12 Spitz DL, Zuckerfranklin D, Nabi ZF. Unmasking of cryptic natural-killer (Nk) cell recognition sites on chronic lymphocytic-leukemia lymphocytes. Am J Hematol 1988; **28**: 155–161.

13 Veuillen C, Aurran-Schleinitz T, Castellano R, Rey J, Mallet F, Orlanducci F et al. Primary B-CLL resistance to NK cell cytotoxicity can be overcome in vitro and in vivo by priming NK cells and monoclonal antibody therapy. J Clin Immunol 2012; **32**: 632–646.

14 Ziegler HW, Kay NE, Zarling JM. Deficiency of natural killer cell activity in patients with chronic lymphocytic leukemia. Int J Cancer 1981; **27**: 321–327.

15 Jaglowski SM, Alinari L, Lapalombella R, Muthusamy N, Byrd JC. The clinical application of monoclonal antibodies in chronic lymphocytic leukemia. Blood 2010; **116**: 3705–3714.

16 Mackay F, Schneider P. Cracking the BAFF code. Nat Rev Immunol 2009; **9**: 491–502.

17 Dillon SR, Gross JA, Ansell SM, Novak AJ. An APRIL to remember: novel TNF ligands as therapeutic targets. Nat Rev Drug Discov 2006; **5**: 235–246.

18 Espinosa C, Cervera R. Belimumab, a Blys-specific inhibitor for the treatment of systemic lupus erythematosus. Drugs Today 2010; **46**: 891–899.

19 Kamal A. The efficacy of novel B cell biologics as the future of SLE treatment: A review. Autoimmun Rev 2014; **13**: 1094–1101.

20 Endo T, Nishio M, Enzler T, Cottam HB, Fukuda T, James DF et al. BAFF and APRIL support chronic lymphocytic leukemia B-cell survival through activation of the canonical NF-kappaB pathway. Blood 2007; **109**: 703–710.

21 He B, Chadburn A, Jou E, Schattner EJ, Knowles DM, Cerutti A. Lymphoma B cell evade

apoptosis through the TNF family members BAFF/BLyS and APRIL. J Immunol 2004; **172**: 3268–3279.

22 Kern C, Cornuel JF, Billard C, Tang R, Rouillard D, Stenou V et al. Involvement of BAFF and APRIL in the resistance to apoptosis of B-CLL through an autocrine pathway. Blood 2004; **103**: 679–688.

23 Novak AJ, Bram RJ, Kay NE, Jelinek DF. Aberrant expression of B-lymphocyte stimulator by B chronic lymphocytic leukemia cells: a mechanism for survival. Blood 2002; **100**: 2973–2979.

24 Parameswaran R, Muschen M, Kim YM, Groffen J, Heisterkamp N. A functional receptor for B-cell-activating factor is expressed on human acute lymphoblastic leukemias. Cancer Res 2010; **70**: 4346–4356.

25 Maia S, Pelletier M, Ding JX, Hsu YM, Sallan SE, Rao SP et al. Aberrant expression of functional BAFF-system receptors by malignant B-cell precursors impacts leukemia cell survival. Plos One 2011; **6**: e20787.

26 Imai C, Iwamoto S, Campana D. Genetic modification of primary natural killer cells overcomes inhibitory signals and induces specific killing of leukemic cells. Blood 2005; **106**: 376–383.

27 Schneider P, Mackay F, Steiner V, Hofmann K, Bodmer JL, Holler N et al. BAFF, a novel ligand of the tumor necrosis factor family, stimulates B cell growth. J Exp Med 1999; **189**: 1747–1756.

28 Schmiedel BJ, Arelin V, Gruenebach F, Krusch M, Schmidt SM, Salih HR. Azacytidine impairs NK cell reactivity while decitabine augments NK cell responsiveness toward stimulation. Int J Cancer 2011; **128**: 2911–2922.

29 Buechele C, Baessler T, Schmiedel BJ, Schumacher CE, Grosse-Hovest L, Rittig K et al. 4-1BB ligand modulates direct and Rituximab-induced NK-cell reactivity in chronic lymphocytic leukemia. Eur J Immunol 2012; **42**: 737–748.

30 Baessler T, Krusch M, Schmiedel BJ, Kloss M, Baltz KM, Wacker A et al. Glucocorticoid-induced tumor necrosis factor receptor-related protein ligand subverts immunosurveillance of acute myeloid leukemia in humans. Cancer Res 2009; **69**: 1037**–**1045.

31 Berlin C, Kowalewski DJ, Schuster H, Mirza N, Walz S, Handel M et al. Mapping the HLA ligandome landscape of acute myeloid leukemia: a targeted approach toward peptide-based immunotherapy. Leukemia 2015; **29**: 647**–**659.

32 Suzuki K, Setoyama Y, Yoshimoto K, Tsuzaka K, Abe T, Takeuchi T. Effect of interleukin-2 on synthesis of B cell activating factor belonging to the tumor necrosis factor family (BAFF) in human peripheral blood mononuclear cells. Cytokine 2008; **44**: 44**–**48.

33 Lopez-Fraga M, Fernandez R, Albar JP, Hahne M. Biologically active APRIL is secreted following intracellular processing in the Golgi apparatus by furin convertase. EMBO R 2001; **2**: 945**–**951.

34 Bologna L, Gotti E, Da Roit F, Intermesoli T, Rambaldi A, Introna M et al. Ofatumumab is more efficient than rituximab in lysing B chronic lymphocytic leukemia cells in whole blood and in combination with chemotherapy. J Immunol 2013; **190**: 231**–**239.

35 Herter S, Herting F, Mundigl O, Waldhauer I, Weinzierl T, Fauti T et al. Preclinical activity of the type II CD20 antibody GA101 (Obinutuzumab) compared with rituximab and ofatumumab in vitro and in xenograft models. Mol Cancer Ther 2013; **12**: 2031**–**2042.

36 Kuster B, Schirle M, Mallick P, Aebersold R. Scoring proteomes with proteotypic peptide probes. Nat Rev Mol Cell Biol 2005; **6**: 577**–**583.

37 Hilpert J, Grosse-Hovest L, Grunebach F, Buechele C, Nuebling T, Raum T et al. Comprehensive analysis of NKG2D ligand expression and release in leukemia: implications for NKG2D-mediated NK cell responses. J Immunol 2012; **189**: 1360**–**1371.

38 Reiners KS, Topolar D, Henke A, Simhadri VR, Kessler J, Sauer M et al. Soluble ligands for NK cell receptors promote evasion of chronic lymphocytic leukemia cells from NK cell anti-tumor activity. Blood 2013; **121**: 3658**–**3665.

39 Buechele C, Baessler T, Wirths S, Schmohl JU, Schmiedel BJ, Salih HR. Glucocorticoid-induced TNFR-related protein (GITR) ligand modulates cytokine release and NK cell reactivity in chronic lymphocytic leukemia (CLL). Leukemia 2011; **26**: 991**–**1000.

40 Scapini P, Nardelli B, Nadali G, Calzetti F, Pizzolo G, Montecucco C et al. G-CSF-stimulated neutrophils are a prominent source of functional BLyS. J Exp Med 2003; **197**: 297**–**302.

41 Levings MK, Sangregorio R, Sartirana C, Moschin AL, Battaglia M, Orban PC et al. Human CD25+CD4+ T suppressor cell clones produce transforming growth factor beta, but not interleukin 10, and are distinct from type 1 T regulatory cells. J Exp Med 2002; **196**: 1335**–**1346.

42 Tuyaerts S, Van Meirvenne S, Bonehill A, Heirman C, Corthals J, Waldmann H et al. Expression of human GITRL on myeloid dendritic cells enhances their immunostimulatory function but does not abrogate the suppressive effect of CD4+CD25+ regulatory T cells. J Leukoc Biol 2007; **82**: 93**–**105.

43 Liu B, Li Z, Mahesh SP, Pantanelli S, Hwang FS, Siu WO et al. GITR negatively regulates activation of primary human NK cells by blocking proliferative signals and increasing NK cell apoptosis. J Biol Chem 2008; **283**: 8202**–**8210.

44 Baessler T, Charton JE, Schmiedel BJ, Grunebach F, Krusch M, Wacker A et al. CD137 ligand mediates opposite effects in human and mouse NK cells and impairs NK-cell reactivity against human acute myeloid leukemia cells. Blood 2010; **115**: 3058**–**3069.

45 Bruhns P. Properties of mouse and human IgG receptors and their contribution to disease models. Blood 2012; **119**: 5640**–**5649.

46 Nimmerjahn F, Ravetch JV. Fc gamma receptors as regulators of immune responses. Nat Rev Immunol 2008; **8**: 34**–**47.

47 De Vita S, Quartuccio L, Salvin S, Picco L, Scott CA, Rupolo M et al. Sequential therapy with belimumab followed by rituximab in Sjogren's syndrome associated with B-cell lymphoproliferation and overexpression of BAFF: evidence for longterm efficacy. Clin Exp

Rheumatol 2014; **32**: 490**–**494.

Figure legends

Figure 1. BAFF expression and regulation in NK cells.

(A) $CD56^{+}CD3^{-}$ NK cells within PBMCs of healthy donors (resting NK cells) and pNKCs were analyzed for BAFF surface expression by FACS. Combined results obtained with a total of 10 donors each depicted as individual specific fluorescence index levels with medians of results (−) are shown.

(B, C) MACS-isolated NK cells and pNKCs from three representative donors were investigated for (b) BAFF mRNA expression and (c) release in soluble form by RT-PCR and ELISA, respectively. BAFF-transfected 293 cells as well as IFN-γ-treated monocytes and parental 293 cells as well as Raji cells served as positive and negative controls, respectively.

(D) Intracellular BAFF protein expression in CD56+CD3– NK cells within PBMCs of healthy donors, pNKCs and NK cells of CLL patients was determined by FACS. Combined results obtained with a total of at least 10 donors depicted as individual specific fluorescence index levels with medians of results (−) are shown.

(E, F) pNKCs were left untreated (untr.) or incubated for 72 h with IL-2 (200 U/ml), IL-15 (10 ng/ml) or on Rituximab (R), Ofatumumab (Ofa) or Obinutuzumab (Obi) after immobilization to plastic.

(E) BAFF levels in supernatants were analyzed by ELISA. To account for the substantial variation observed with NK cells of different donors, results obtained in two exemplary experiments (left) and combined data from analyses with six independent donors depicted as relative increase compared with untreated NK cells (set to 1 in each individual data set) with s.e.m. (right) are shown.

(F) Intracellular protein levels were determined by FACS and are shown as specific fluorescence

index levels obtained with NK cells of three independent donors with s.e.m. of results. Statistically significant differences ($P < 0.05$, Student's *t*-test) are indicated by *.

Figure 2. Effects of BAFF on CLL cell metabolic activity and susceptibility to direct and Rituximab-induced NK cell lysis.

(A, B) Metabolic activity of primary CLL cells was measured by WST-1 assays after 72 h of incubation with (A) the indicated concentrations of rhBAFF or (B) nkBAFF obtained as described in the methods section. Flow through and eluates from a Rituximab affinity column served as controls.

(C, D) Primary CLL cells were incubated with BAFF for 24 h followed by washing and analysis in Europium cytotoxicity assays with pNKCs in the presence or absence of Rituximab (10 µg/ml).

(C) Representative results (left) and combined data obtained with rhBAFF (500 ng/ml) in six independent experiments with cells of different patients at an E:T ratio of 20:1 (right).

(D) Representative results of an experiment with nkBAFF out of three with similar results.

(E, F) PBMCs of CLL patients with moderate leukemia cell counts (40–80%) were left untreated or incubated with BAFF in the presence or absence of Rituximab (10 µg/ml). After 72 h, lysis of $CD19^+CD5^+$ CLL cells was determined by FACS. Results are depicted as means of triplicate measurements with s.e.m.

(E) Exemplary results (left) and combined data of seven experiments with cells of different patients (right) and rhBAFF.

(F) Exemplary results of one representative experiment with nkBAFF out of three with similar results. Statistically significant differences (Po0.05, Student's t-test) are indicated by *.

Figure 3. NK-cell activation and effector function are not altered upon pretreatment of CLL cells with BAFF

(A) Primary CLL cells of six different patients were incubated with or without rhBAFF (500 ng/ml)

for 24 h followed by washing and culture with allogeneic pNKCs in the presence or absence of Rituximab (10 **µ**g/ml). Expression of CD107a and CD69 on CD56+CD3− NK cells were monitored by FACS after 4 and 24 h, respectively.

(B) PBMCs of five different CLL patients with moderate leukemia cell counts (40**–**80%) were left untreated or incubated with rhBAFF in the presence or absence of Rituximab, followed by analysis of CD107a and CD69 expression on autologous CD56+CD3− NK cells by FACS after 4 and 24 h, respectively. Results of untreated cells were set to 1 in each individual data set, and data are shown as relative surface expression with medians and s.e.m. of results. Statistical significance was calculated by Student's *t*-test; ns, not significantly different.

Figure 4. Effects of BAFF neutralization with Belimumab on metabolic activity as well as direct and Rituximab-induced NK-cell lysis of CLL cells.

(A, B) Primary CLL cells from patients with >80% lymphocyte count were incubated with or without rhBAFF (500 ng/ml), Belimumab or an isotype control mAb (10 **µ**g/ml each) as indicated.

(A) After 72 h, metabolic activity was measured by WST-1 assays. Representative results obtained with cells from one patient from a total of at least six with similar results are shown.

(B) After 24 h, Europium cytotoxicity assays were performed with pNKCs (E:T ratio 20:1) in the absence (left panels) or presence (right panels) of Rituximab (10 µg/ml). Combined data of six independent experiments with cells of different patients are shown. Results obtained with untreated CLL cells were set to 1 in each individual data set.

(C) CLL cells of patients with moderate leukemia cell counts (40**–**80%) were cultured for 48 h with or without rhBAFF (500 ng/ml), Belimumab (10 µg/ml) or isotype control antibody. Then Rituximab (10 µg/ml) was added where indicated for additional 24 h followed by analysis of CD19+CD5+ CLL cell lysis by FACS. Results are depicted as means of triplicate measurements with s.e.m. Combined data of six experiments with cells of different patients are shown.

Statistically significant differences ($P < 0.05$, Student's *t*-test) are indicated by *.

Table 1A. Tryptic peptides identified by MS/MS in positive control (FDR of >1%).

Peptide Sequence		Position [a]	Q-value
#1	TYAMGHLIQR	205–214	0
#2	LEEGDELQLAIPR	253–265	0
#3	AVQGPEETVTQDcLQLIADSETPTIQK	134–160	0
#4	cIQNMPETLPNNScYSAGIAK	232–252	0
#5	TYAmGHLIQR	205–214	0.001
#6	VHVFGDELSLVTLFR	217–231	0
#7	GSYTFVPWLLSFK	161–173	0.002
#8	cIQNmPETLPNNScYSAGIAK	232–252	0

Abbreviations: c, carbamidomethyl-cysteine; FDR, false discovery rate; m, methionine sulfoxide; MS/MS, spectrometry/mass spectrometry.

[a] Amino acid residue position within the BAFF sequence according to Uniprot.org (accession Q9Y275 or TN13B_HUMAN).

Table 1B. Tryptic peptides identified by MS/MS in three replicates of purified and concentrated cell culture supernatants of pNKCs (FDR of >1%).

Replicate	Peptide	Q-value
#1	LEEGDELQLAIPR	0
	AVQGPEETVTQDcLQLIADSETPTIQK	0
#2	LEEGDELQLAIPR	0
#3	LEEGDELQLAIPR	0

Abbreviations: c, carbamidomethyl-cysteine; FDR, false discovery rate; MS/MS, spectrometry/mass spectrometry; pNKCs, polyclonal NK cells.

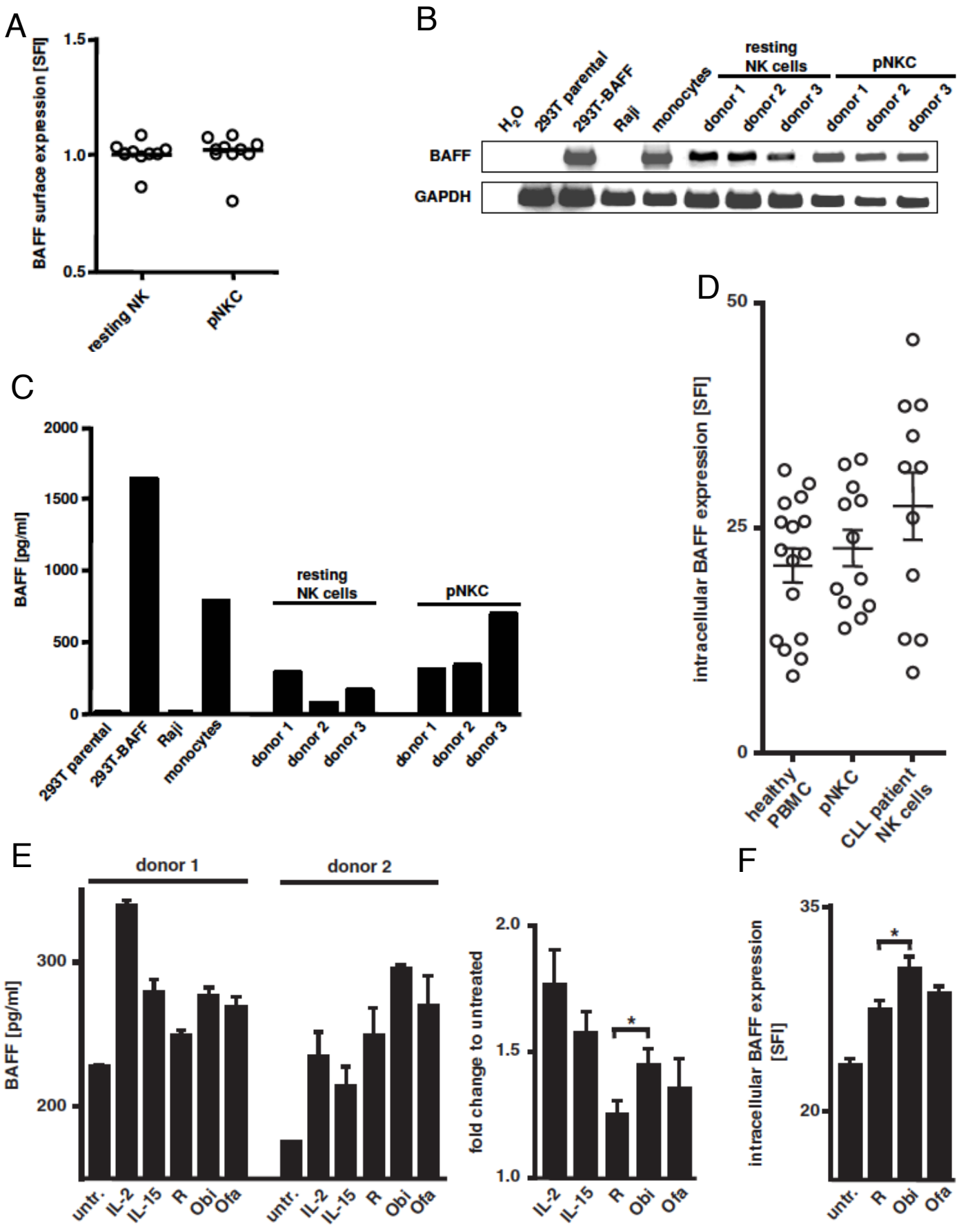
A
BAFF surface expression [SFI]
1.5
1.0
0.5
resting NK
pNKC
B
H2O
293T parental
293T-BAFF
Raji
monocytes
resting NK cells
pNKC
donor 1
donor 2
donor 3
donor 1
donor 2
donor 3
BAFF
GAPDH
C
BAFF [pg/ml]
2000
1500
1000
500
0
resting NK cells
pNKC
293T parental
293T-BAFF
Raji
monocytes
donor 1
donor 2
donor 3
donor 1
donor 2
donor 3
D
intracellular BAFF expression [SFI]
50
25
0
healthy PBMC
pNKC
CLL patient NK cells
E
donor 1
donor 2
BAFF [pg/ml]
300
200
untr.
IL-2
IL-15
R
Obi
Ofa
untr.
IL-2
IL-15
R
Obi
Ofa
fold change to untreated
2.0
1.5
1.0
*
IL-2
IL-15
R
Obi
Ofa
F
intracellular BAFF expression [SFI]
35
20
*
untr.
R
Obi
Ofa

Figure 1

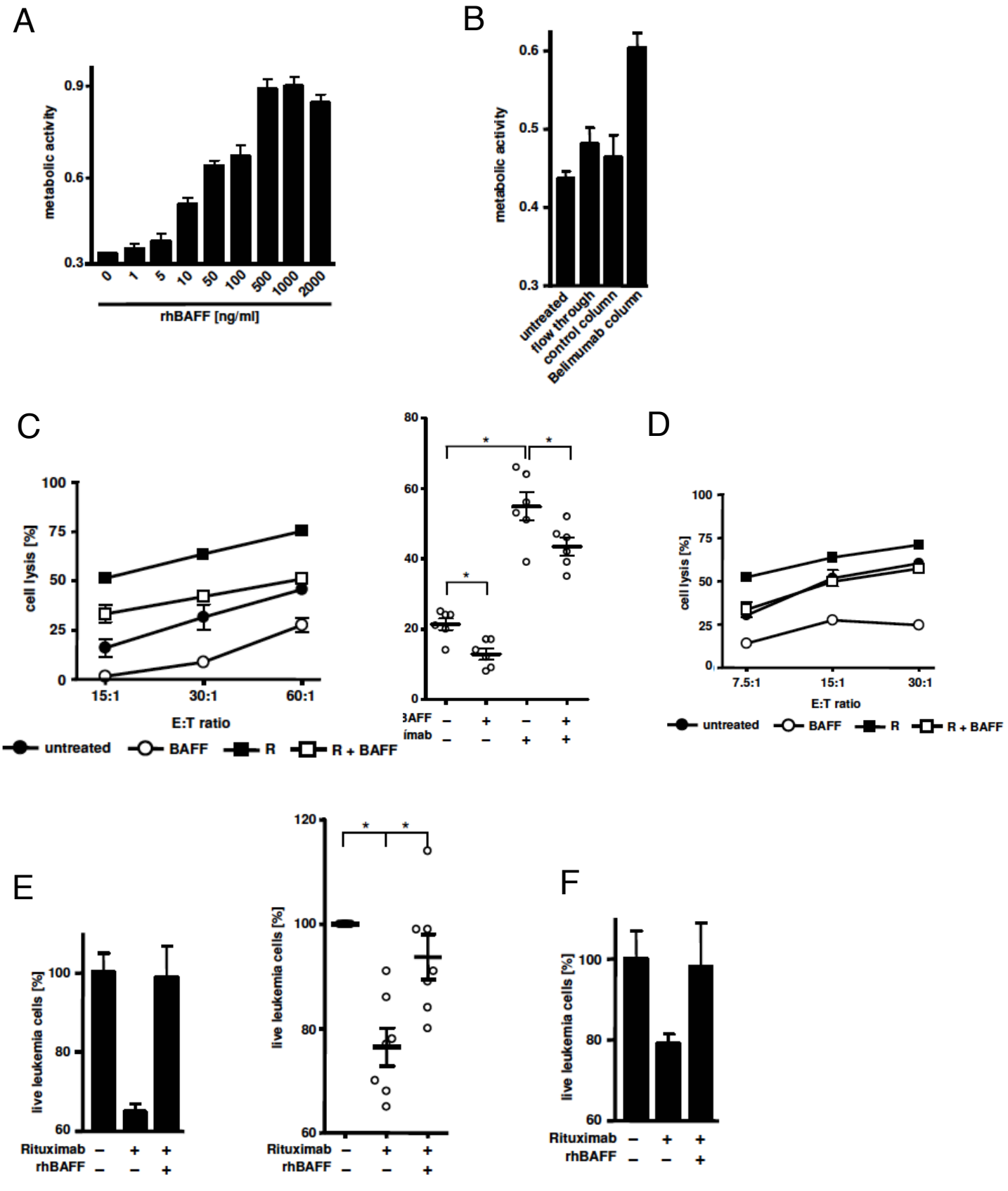
A
metabolic activity
0.9
0.6
0.3
0
1
5
10
50
100
500
1000
2000
rhBAFF [ng/ml]
B
metabolic activity
0.6
0.5
0.4
0.3
untreated
flow through control column
Belimumab column
C
cell lysis [%]
100
75
50
25
0
15:1
30:1
60:1
E:T ratio
untreated
BAFF
R
R + BAFF
80
60
40
20
0
*
*
*
BAFF – + – +
ímab – – + +
D
cell lysis [%]
100
75
50
25
0
7.5:1
15:1
30:1
E:T ratio
untreated
BAFF
R
R + BAFF
E
live leukemia cells [%]
100
80
60
Rituximab – + +
rhBAFF – – +
120
100
80
60
*
*
live leukemia cells [%]
Rituximab – + +
rhBAFF – – +
F
live leukemia cells [%]
100
80
60
Rituximab – + +
rhBAFF – – +

Figure 2

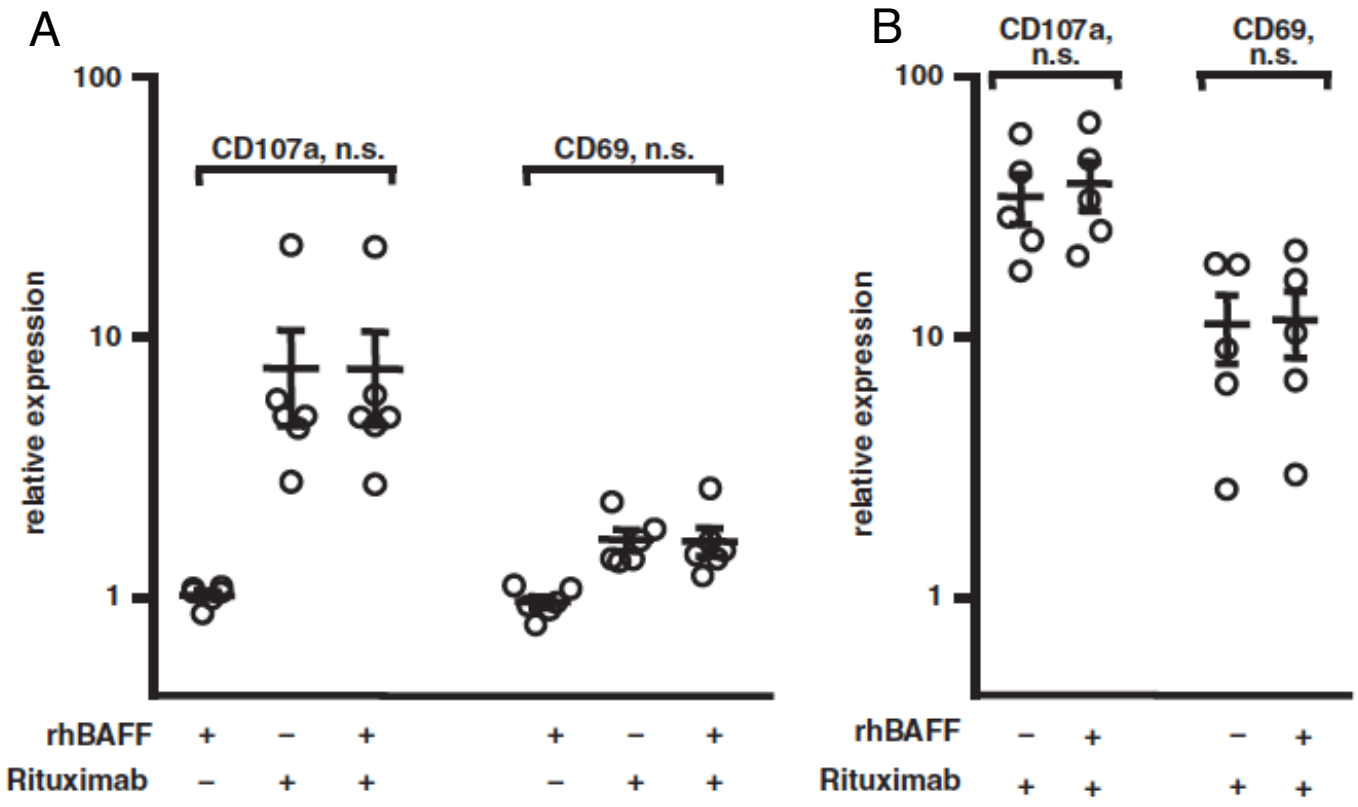
A
100
10
1
relative expression
CD107a, n.s.
CD69, n.s.
rhBAFF + − + + − +
Rituximab − + + − + +
B
CD107a, n.s.
CD69, n.s.
100
10
1
relative expression
rhBAFF − + − +
Rituximab + + + +

Figure 3

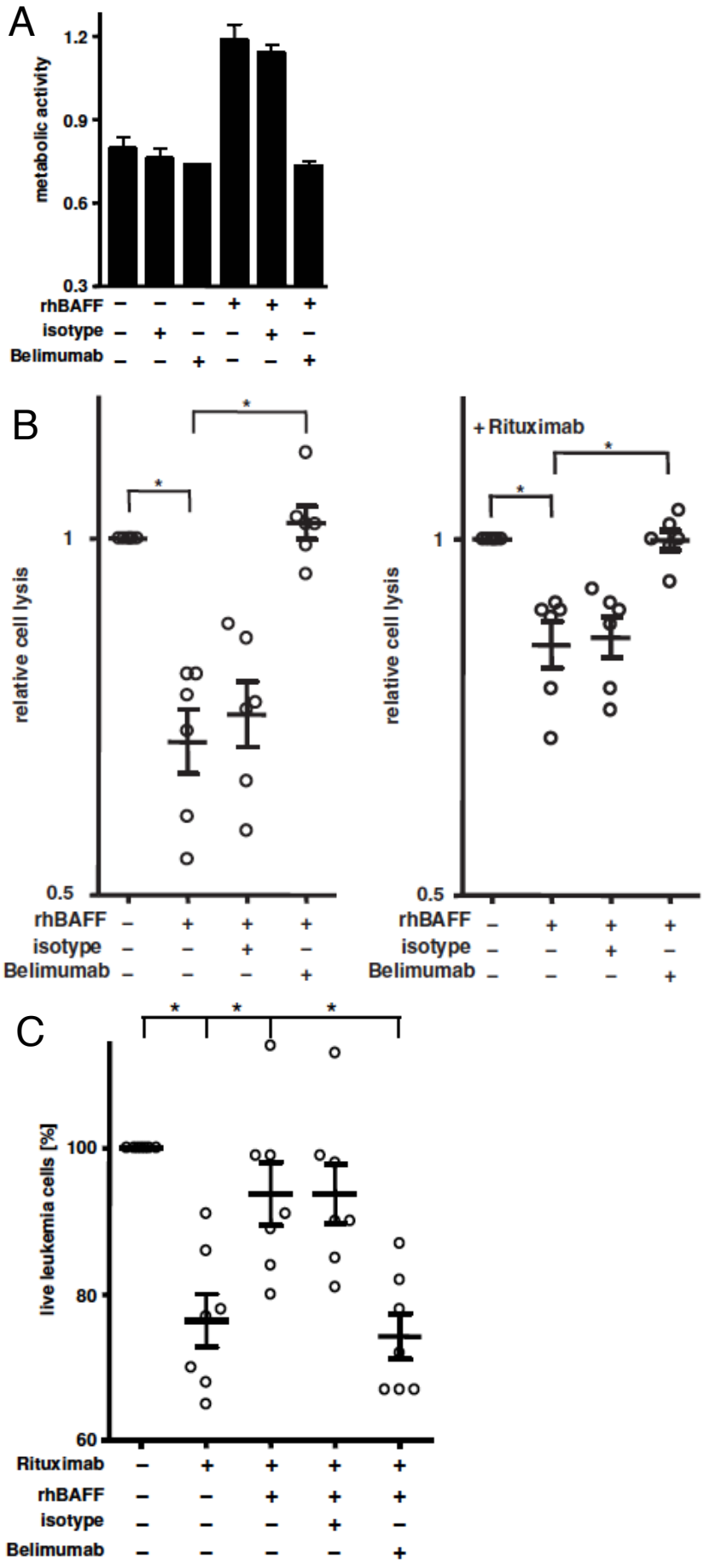
A
metabolic activity
1.2
0.9
0.6
0.3
rhBAFF – – – + + +
isotype – + – – + –
Belimumab – – + – – +
B
relative cell lysis
1
0.5
rhBAFF – + + +
isotype – – + –
Belimumab – – – +
+ Rituximab
relative cell lysis
1
0.5
rhBAFF – + + +
isotype – – + –
Belimumab – – – +
C
live leukemia cells [%]
100
80
60
Rituximab – + + + +
rhBAFF – – + + +
isotype – – – + –
Belimumab – – – – +

Figure 4