Analysis of BLyS-dependent signatures and biology in the intestinal mucosa of patients with inflammatory bowel disease

Authors: Victoria Gudiño and Azucena Salas

Date: 29th March 2022

INSTITUT D'INVESTIGACIONS BIOMÈDIQUES AUGUST PI I SUNYER (IDIBAPS)
C/ Rosselló, 149-153
08036 BARCELONA
93.2275400 ext. 2436

INDEX

Attached files:

- Correlation colon CD-controls
- Correlation colon UC-controls
- Correlation ileum CD-controls
- TNFSF13B pathway analysis
- Module analysis all samples
- Network analysis all samples

BACKGROUND

BLyS (*TNFSF13B*) is a cytokine member of the tumor necrosis factor (TNF) ligand superfamily, which is mainly produced by myeloid cells[1]. Upon interaction with either of its three receptors (BAFF-R, TACI or BCMA), it acts as a survival factor for B cells, promoting their proliferation and differentiation into plasma cells[2]. Moreover, it also stimulates the activation of other immune cells such as T cells, monocytes and dendritic cells, thereby participating in inflammatory processes. Indeed, BLyS production is induced during inflammation by cytokines such as interferon gamma (IFNɣ), interferon alpha (IFNα) or transforming-growth factor beta (TGFβ), and its expression is upregulated in immune-mediated diseases such as inflammatory bowel disease (IBD)[3]. IBD is comprised of ulcerative colitis (UC) and Crohn's disease (CD), and we and others have detected an increase in *TNFSF13B* mRNA expression in the intestine of UC and CD patients compared to healthy individuals[3] (*unpublished data*). BLyS-blocking antibodies are commercially available, but their potential therapeutic benefit has not yet been tested in IBD patients. Therefore, the present project aims to investigate the role that BLyS may play in the pathophysiology of IBD. To this end, we plan to identify the processes and signaling pathways modulated by BLyS in intestinal inflammation.

IN SILICO ANALYSIS OF BLYS SIGNATURE

In here, we provide the list of gene transcripts whose expression correlated significantly with that of *TNFSF13B* in human intestinal biopsies from healthy individuals, as well as from UC and CD patients. For this analysis, we used biopsy RNA microarray data from a cohort of patients and healthy controls (see Materials and Methods).

Results

The list of genes positively ($r>0$) or negatively ($r<0$) correlated with *TNFSF13B* per each data set is attached to this report, along with the statistical significance (files: "correlation colon CD-controls", "correlation colon UC-controls", "correlation ileum CD-controls"). The total number of genes that were significantly correlated (adjusted $p < 0.05$) was as follow (Table 1):

	Ileum CD	**Colon CD**	**Colon UC**
Positive corr.	1479	3530	2738
Negative corr.	4865	6463	2706

Table 1: Number of genes positively and negatively correlated with TNFSF13B in each dataset.

The highest positively correlated genes across the three datasets were mainly involved in immune cell activation (*TAGAP*, *LCP2*, *CCR1*, *MNDA, CCL4* or *LAMP3*) or signaling transduction (*RAB31*, *MRAS*, *SNX10* or *PDE4B*). In contrast, most of the inversely correlated genes appeared to be related to catabolic pathways (*BCAT2*, *ACOT11*, *ATP4PD* or *SREBF2*), the cytoskeleton (*CDHR1*, *TLN2*, *CFAP20* or *HEPACAM2*), membrane permeabilization (*AQP8*, *PXMP2* or *GUCA2B*) or acted as DNA-binding transcription factors (*TCEA4*, *HNF4A* or *ZBTB7C*).

Genes that were present in at least two datasets and had an R value > |0.60| were selected for pathway analysis (see attached file "TNFSF13B_pathway analysis")[4]. As expected, immune responses and inflammatory-related processes appeared to be the most significant pathways in the positively correlated genes. However, metabolic and catabolic pathways were among the most representative in the negatively correlated gene list. The same analysis was conducted independently on each of the three datasets and yielded similar results.

To identify direct BLyS target genes, some of the significant biological processes were selected based on previously descriptions of BLyS functionality. The attached excel file ("TNFSF13B_pathway analysis") provides the list of genes involved in these processes. Moreover, signaling pathways (NFκB, MAPK or PI3K) or processes (apoptosis or Th1-Th2 differentiation) thought to be modulated by BLyS activation were assessed for the presence of genes that correlated with *TNFSF13B* expression using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Thus, according to the literature and based on our results, we have selected the following positively correlated genes as potential BLyS targets for further exploration (Table 2):

Gene name	**Function**
MCL1	Anti-apoptotic protein and member of the Bcl2 family. One of the best established anti-apoptotic target genes of BAFF[5,6].
BCL2A1	Anti-apoptotic protein and member of the Bcl2 family. NFκB target gene, essential for lymphocyte survival and activation. Mediates BCR-survival signals[7].
BIRC3	Anti-apoptotic protein. Regulates the NFκB-signaling pathway by binding to TRAF1 and TRAF2. It also regulates immune responses through TLRs and NLRs receptors[8].
TRAF1	TNF-associated factor that mediates TNF-driven NFκB activation by forming a heterodimer with TRAF2[9].
MALT1	Activates the NFκB pathway by binding to TRAF6. It also mediates the induction of Th17 differentiation and is important for T-cell antigen receptor-induced integrin adhesion. Moreover, a study has already shown that BAFF-dependent NFκB activation is impaired upon MALT1 deficiency, as MALT1 interacts and degrades TRAF3, a negative regulator of BAFF signaling[10].

TNFAIP3	A negative feedback regulator of NFκB activation and an inhibitor of cell death[11].
CD40	TNFSF5/CD40L receptor whose interaction serves as a co-stimulator activating B-cell proliferation and differentiation. Activates pathways similar to BAFF such as NFκB, and a study has shown BAFF-driven CD40 upregulation *in vitro*[12].
MAP3K8	Kinase with pro-inflammatory activities via activation of MAPKs and induction of NFκB nuclear translocation. Mediates CD40 signaling, participating in B-cell and T-cell activation[13].
RIPK2	Kinase receptor-interacting protein that activates NFκB signaling and mediates signaling downstream of NOD2[14].
DAPP1	B-lymphocyte adapter protein that promotes BCR signaling, opposite to NAPDH oxidase (NOX). It is regulated downstream of PI3K[15].
PIK3AP1	Also known as B-cell Adapter for Phosphoinositide 3-Kinase (BCAP). Through phosphorylation, it serves as a docking site for PI3K, connecting to BCR and TLR signaling[16].
CSF2RB, CSF1R, CSF3R	Receptors that mediate the production, differentiation and function of macrophages. Upon ligand binding, they activate different signaling pathways. Phosphorylates PIK3R1, activating AKT1 signaling pathways. Also mediates the activation of MAP kinases[17].
PDK1	Kinase that phosphorylates mediators of the PI3K pathway (AKT1, S6K, RSK and PKC), as well as IKKβ, activating the NFκB pathway. It is essential for B-cell survival and activation[18].
PIM2	Kinase induced by NFκB involved in the mTOR pathway through phosphorylation of 4EBP1, affecting protein translation. It has been shown to mediate survival and the cell growth responses of BAFF[5].
TAGAP	Rho GTPase that mediates T-cell activation. It is also required for proinflammatory cytokine production by myeloid cells and for Th17 cell differentiation[19].
CLECL7A	A lectin necessary for activation of NFκB and inflammatory responses to mediate T-helper cell differentiation. Mediates ROS production and enhances cytokine secretion in myeloid cells[20].
CCL4	Also known as Macrophage Inflammatory Protein 1. Chemokine that induces secretion of pro-inflammatory cytokines, in addition to being a chemoattractant for immune cells.
MNDA	Pro-apoptotic protein that promotes the degradation of MCL1. It is thought to be a master regulator of monocytic and granulocytic lineages[21].
ADA	Enzyme involved in humoral and cellular immunity by catalyzing the hydrolysis of adenosine to inosine. Regulates lymphocyte-epithelial cell adhesion by binding to DPP4. Moreover, it activates dendritic cells and enhances $CD4^+$ T-cell differentiation and proliferation[22].
LAMP3	A marker of mature dendritic cells and a responder of IFNγ. Moreover, Vitamin D_3 and IL10 downregulate LAMP3 through the inhibition of NFκB signaling[23].

Table 2: Potential target gene candidates identified in the correlation and pathway analysis.

Module and network expression analyses

Next, we used the same microarray data to perform module and network analysis. Co-expression gene modules ("modules" for short) contain a group of genes with a similar expression pattern. The excel file "Module analysis_all samples" includes the lists of genes in those modules that include one of the four genes - *TNFSF13B*, *TNFRSF13C*, *TNFRSF13B*

and *TNFRSF17* - for all three data sets (colon CD, colon UC and ileum CD). Highlighted genes form part of more than one data set. In addition, we have denoted the membership value for each gene in the module. Highly connected intramodular hub genes tend to have high module membership values. This value ranges between 0 and 1 and reflects how well the expression profile of a given gene matches the average expression profile within the module; in other words, how representative a gene is of the expression profile of the whole module.

Table 3 shows the results of these module analyses, the number of genes within each module containing one of the four genes: *TNFSF13B*, *TNFRSF17*, *TNFRSF13C* or *TNFRSF13B*. Data is shown for all three sample data sets.

		# Genes in the module			
		BLyS - *TNFSF13B*	**BCMA - *TNFRSF17***	**BAFF-R - *TNFRSF13C***	**TACI - *TNFRSF13B***
Microarray dataset	**Ileum CD**	**73**	**6**	**482**	
	Colon CD	**48**			
	Colon UC	**664**		**1209**	

Table 3: Number of genes in each module grouped by datasets.

TNFSF13B (BLyS) is included in a module for all three data sets (Table 3). The genes included in those three modules varied significantly, with less than 100 genes in ileal and colonic CD, and over 600 genes in the UC dataset. In contrast, module analysis only identified those genes that were co-expressed with *TNFRSF13C* (BAFF-R) in UC colon and CD ileum. In contrast, *TNFRSF17* (BCMA) was detected in a small (6-gene) module in the CD ileum, while TACI was found in none.

Next, we submitted the genes within the module of*TNFSF13B* (BLyS) for UC (664 genes) and colonic CD (48 genes) for network analysis as described in the Methods section. The excel file ("Network analysis_all samples") includes the list of genes within the network, their distance to *TNFSF13B*, their correlation with *TNFSF13B* expression, the degree or number of interacting genes within the network (neighbors) and the gene ID of each neighbor. Network representations for colon CD (Figure 1A) and the UC (Figure 1B) are depicted below. For visual simplicity, Figure 1B shows only those genes (107) within the network of the 549 genes having a distance ≤ 2 and a degree ≥ 20.

In both the UC and CD *TNFSF13B* networks we found genes related to inflammation; i.e., macrophage-expressed *CXCL10* and *CXCL11*, *GNB4*, *CSF2RB*, as well as the IL-10 receptor subunit *IL10RA* and the integrin subunit *ITGB2,* the last two both highly expressed by macrophages and subsets of T and B cells. In contrast to those genes that are predominantly found in macrophages, and which lack expression of any of the 3 BLyS receptors, *CD40* is highly expressed by B cells while *FYN* is expressed in a subset of both CD4 and CD8 T cells. Both B and T cells express receptors to BLyS, suggesting that BLyS may directly regulate the expression of these two genes in BLyS-responding cells in the intestinal mucosa. In contrast, the co-expression of BLyS with genes expressed by macrophages may be related to the predominantly macrophage production of BLyS in the context of the inflamed mucosa (Figure 2).

Similarly, the UC-specific network (Figure 1B) includes other cytokines and chemokines (*CXCL1*, *CXCL2*, *CXCL3*, *CXCL6*, *CXCL8*, *CXCL9*, *TNF*, *IL1A*, *IL1B*, *IL6, CCL2, CCL4*), receptors and molecules mediating chemokine and cytokine signaling (*SOCS3*, *SOCS1*, *CXCR2*, *CXCR4*, *IL1R1*, *IL1RAP*, *IL2RB*, *CCR1*, *IL7R*), metallopeptidases (*MMP9)*, or mediators of cytokine response to interferon (*CEBPB*, *IL6, STAT1* , *IRF1*, *IFIT3*, *IFITM3* or *IFI16*), adhesion molecules, etc. Interestingly, besides *CD40*, a co-stimulatory signal essential for T-cell mediated B-cell help, we found other receptors of T- or antigen-presenting cell expressed in the module such as *CD28*, *CD86*, *ICAM1*, *ITGAL* and *ITGB2* (subunits of LFA-1, an ICAM-1 receptor involved in the immunologic synapse), *CD274* (PD1L1) and *ICOS*.

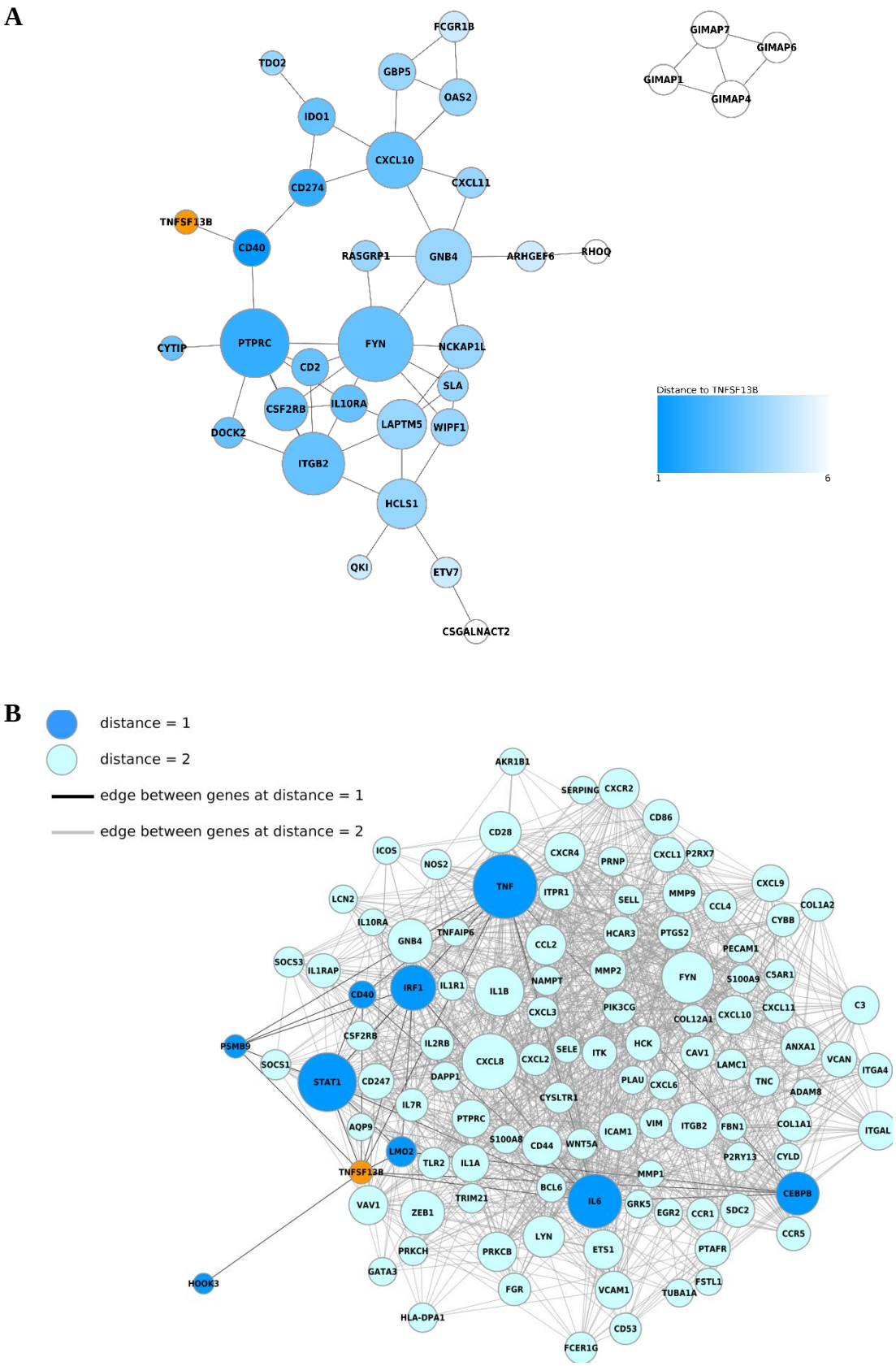


Figure 1: Network of genes within the *TNFSF13B* module of colonic CD and UC biopsies. **A**) Colonic CD *TNFSF13B* module. The blue scale indicates their distance from BLyS, ranging from 1 to 6. **B**) Colonic UC *TNFSF13B* module, which only includes genes with a distance 1 or 2, and a degree (number of neighbors) ≥ 20. The size of the gene circle indicates its degree.

Based on this analysis, we propose a model for BLyS production and its effects on the intestinal mucosa that would explain the variety of genes that correlate with *TNFSF13B* transcription in IBD. These genes include IFN-responding genes, genes expressed by activated macrophages, and those we predict to be direct BLyS targets. As depicted in Figure 2, IFNɣ and IFNα act on macrophages, as well as on fibroblasts and T cells. IFN-activated macrophages are the main source of BLyS in IBD (scRNAseq internal data). This would explain the high expression of the IFN-response and the macrophage genes that correlate with *TNFSF13B* mRNA in mucosal biopsies from IBD patients. Secreted BLyS then acts on target cells (T, B and plasma cells) and drives the expression of another gene set in direct response to BLyS.

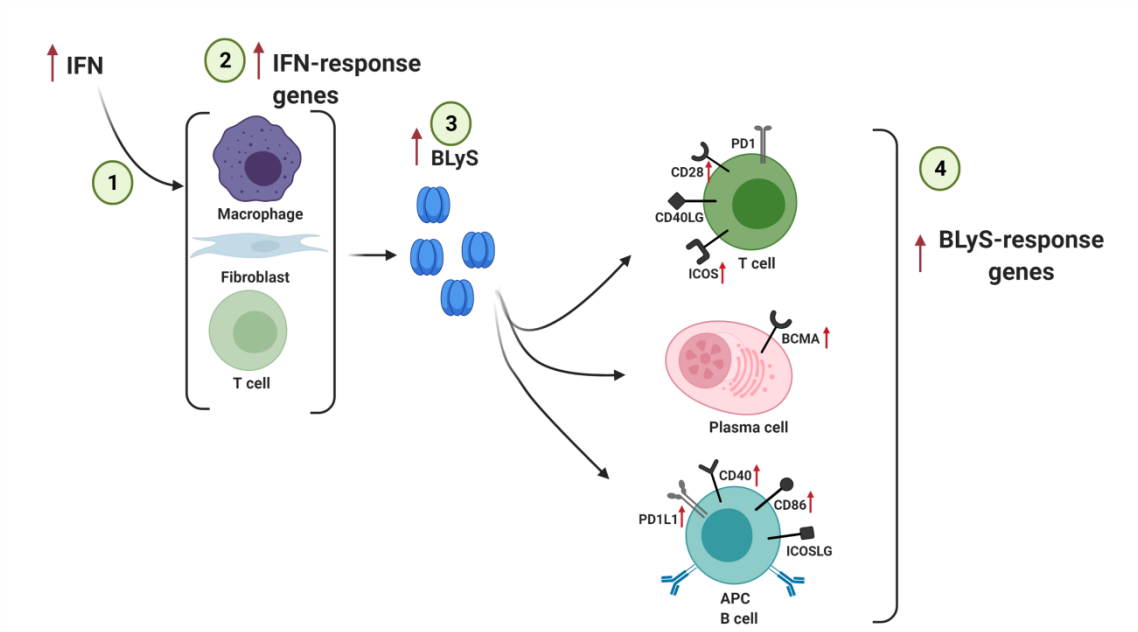


Figure 2: Model of the mechanisms driving BLyS production, as well as the response to BLyS in human intestinal mucosa. (1) IFN is a potent activator of intestinal macrophages. In addition, it can act on other cell types, including those fibroblasts and T cells driving the activation of IFN-responsive genes (2) and the production of BLyS (3). BLyS can then act on T, B and plasma cells that express its receptors on their cell membranes. These cells can in turn upregulate the expression of several key co-stimulatory receptors and of BLyS-responding genes (4). In addition, we believe that BLyS upregulates the expression of its receptor on plasma cells (BCMA).

<u>*In silico* analysis – conclusions</u>

Based on our microarray datasets, the genes encoding for BLyS (*TNFSF13B*) or the BLyS receptors *TNFRSF13C, TNFRSF13B* and *TNFRSF17* are highly upregulated in active UC and CD. We hypothesized that analysis of those genes that significantly correlate with *TNFSF13B* (as previously reported) could reveal potential BLyS targets in intestinal inflammation. We

therefore analyzed microarray data from inflamed and non-inflamed human biopsies. However, this analysis also revealed genes that are co-expressed with BLyS in active IBD, but that may not necessarily be downstream of BLyS signaling.

Expression module analysis using the transcriptional signatures of our sample cohorts identified those genes that were co-expressed with *TNFSF13B* in UC, colonic CD, and ileal CD. In particular, we found a module containing 663 genes that were co-regulated with *TNFSF13B* in UC samples.

Using the lists of genes co-expressed with *TNFSF13B*, we performed a network analysis to reveal inter-gene relationships classified according to their distance from *TNFSF13B,* as well as in relation to the number of "neighbors" that were influenced by that gene within the network. Network analysis of the 664 genes revealed close interactions between *TNFSF13B* and genes key to innate and acquired immune responses. Hence, our next goal was to continue the validation of these gene candidates in BLyS-stimulated biopsies. We argue that validated BLyS targets could then be used as a readout to assess the effects of BLuyS or anti-BLyS antibodies in inflamed biopsies.

FUNCTIONAL INVESTIGATION OF THE BLYS SIGNATURE IN THE INTESTINE

To study the downstream targets modulated by BLyS, we treated intestinal biopsies with human recombinant BLyS (rhBLyS). Activation of the BLyS pathway was monitored by the transcriptional modulation of genes previously identified in the in-silico analysis. Upon identification of potential candidates, the expression of these genes was measured to test the effect of an anti-BLyS antibody. The overall aim was to determine whether inhibition of the BLyS pathway can serve as a therapeutic tool for IBD.

Biological activity of BLyS in PBMC-derived B cells

We first determined the biological activity of rhBLyS using B-cell enriched PBMCs in culture, alone or in combination with an anti-IgM antibody.

We enriched for $CD19^+$ B cells from the buffy coat of a healthy donor using CD19 microbeads (see Methods below). One round of positive selection of PBMCs cells yielded a purity of 75.6% of $CD19^+$ (FACS data not shown). $CD19^+$-enriched PBMCs (100.000 cells/well in quadruplicate) were stimulated with a range of rhBLyS concentrations (50, 100 and 200 ng/ml) alone, or in combination with an anti-IgM antibody (2µg/ml in solution) and cultured for 96 hours. We measured cell viability/proliferation using resazurin, a redox-sensitive dye that changes its absorbance upon being uptaken by viable cells. As shown in

Figure 4, stimulation with rhBLyS alone did not increase proliferation of $CD19^+$-enriched PBMCs compared to the unstimulated control or the anti-IgM conditions. However, when rhBLyS was added in combination with anti-IgM, proliferation significantly increased at all tested rhBLyS concentrations (Figure 3A). Therefore, we concluded that rhBLyS carries biological activity and can be used to stimulated intestinal biopsies.

To investigate the transcriptional changes driven by BLyS and/or anti-IgM treatment in $CD19^+$-enriched PBMCs, $CD19^+$ B cells from a different donor were isolated and again stimulated with rhBLyS and/or anti-IgM (2 μg/ml). A single concentration of rhBLyS (100ng/ml) was chosen for this experiment. RNA from these cells (pooled from 12 replicates per each condition) was isolated after 96 hours of incubation and the expression of several genes was measured by qRT-PCR. Expression of BLyS targets, such as *NFKB2*, *PIM2*, *POU2AF* and *CD40,* increased in response to BLyS (Figure 3B). Co-stimulation with anti-IgM, however, had no synergistic effect on the expression of those genes except for CD40. These findings will need confirmation in additional donors. Other genes, such as *RELB* or *BIM,* were not regulated upon rhBLyS stimulation. Interestingly, we observed a pronounced downregulation in *CD3E*, a marker for T cells, together with the concomitant decrease of the T-cell activation markers *FYN*, *CD40LG* or *ICOS*. Anti-IgM induced an increase in *CD79A* and *MS4A1* (B cells), suggesting B-cell proliferation, while *DERL3* (plasma cells) concomitantly decreased. In contrast, rhBLyS alone, or in combination with anti-IgM, did not appear to alter *CD79A, MS4A1 or DERL3* transcription at the times and concentrations studied. Remarkably, when combined with anti-IgM, rhBLyS acted synergistically, further reducing expression of the plasma cell marker *DERL3* and T-cell markers *(CD3E, FYN*, *CD40LG* or *ICOS)*. Altogether, these preliminary (n=1) results suggest that the recombinant BLyS used was active, and when combined via stimulation through the IgM receptor, can induce cell proliferation and T-cell depletion/inactivation. Furthermore, we show that genes such as *NFKB2* and *PIM2* might serve as good targets for measuring the effects of BLyS.

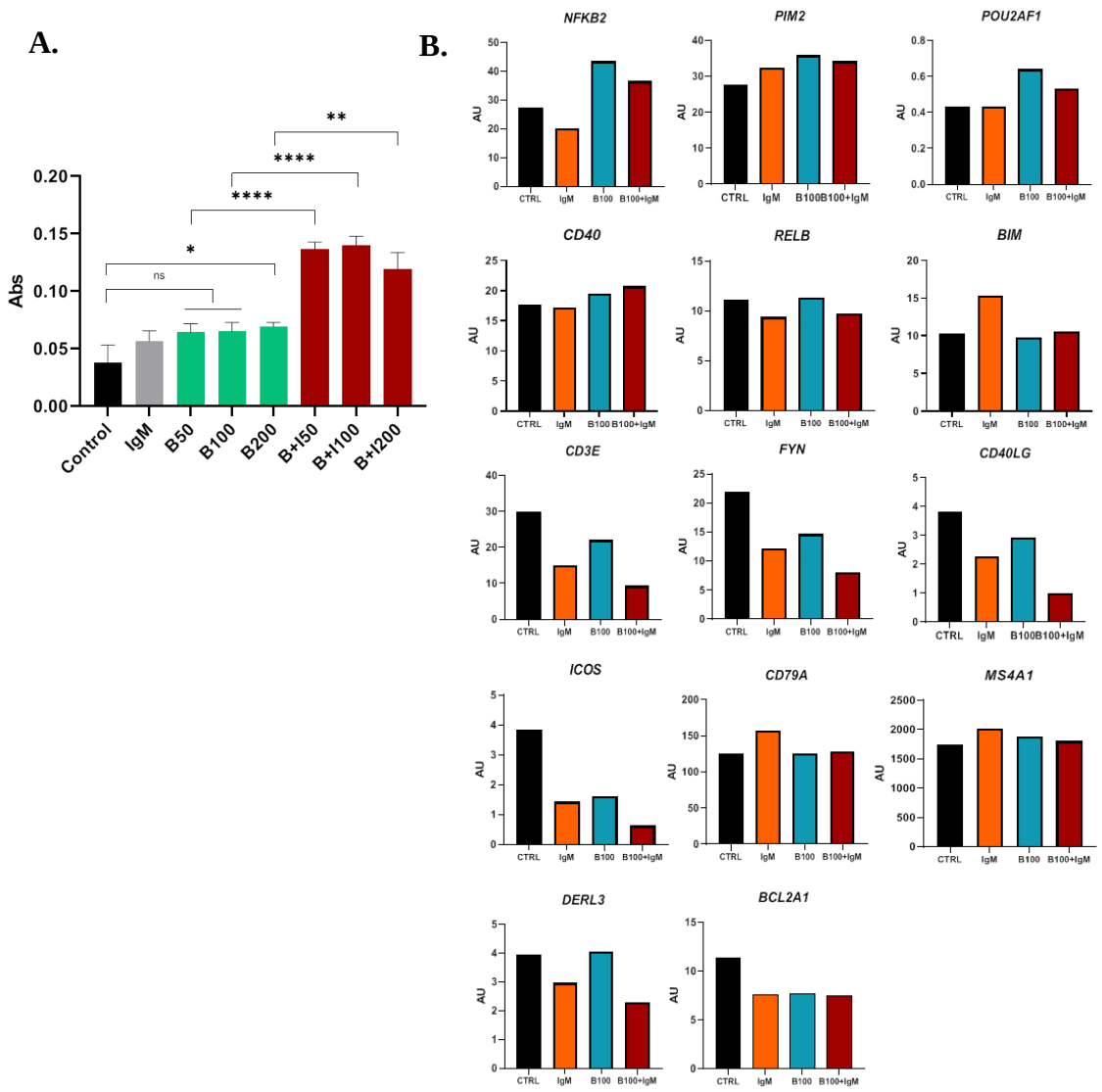


Figure 3: **BLyS is biologically active on peripheral blood cells**. **A**: Proliferation of CD19⁺-enriched PBMCs was measured using resazurin. Cells were treated with media (control), anti-IgM (2 µg/ml), BlyS (50, 100 or 200 ng/ml) or the combination of IgM and BlyS for 96 hours. **B**: qRT-PCR of CD19⁺ cells stimulated with media (control), anti-IgM (2µg/ml), BlyS (100ng/ml) or the combination of both for 96 hours. One-way ANOVA: * = p≤0.05; ** = p≤0.001, **** = p≤0.0001; ns = p≥0.05; n=1.

Expression of BLyS receptors in healthy and inflamed tissue

To determine the ability of intestinal cells to respond to BLyS, we measured the expression of its receptors in inflamed and non-inflamed tissues. BLyS can interact with three different receptors: BCMA (*TNFRSF17*), BAFF-R (*TNFRSF13C*) and TACI (*TNFRSF13B*). Our in-house single-cell RNAseq data on human healthy and IBD colons showed that BCMA is highly expressed by plasma cells, while BAFF-R and TACI are predominantly expressed by B cells, as well as by a few plasma and T cells (Figure 4A). At the RNA level, qRT-PCR analysis

demonstrated that although the expression of all three receptors increased upon inflammation, they were also expressed in non-IBD (healthy) colonic mucosa (Figure 4B).

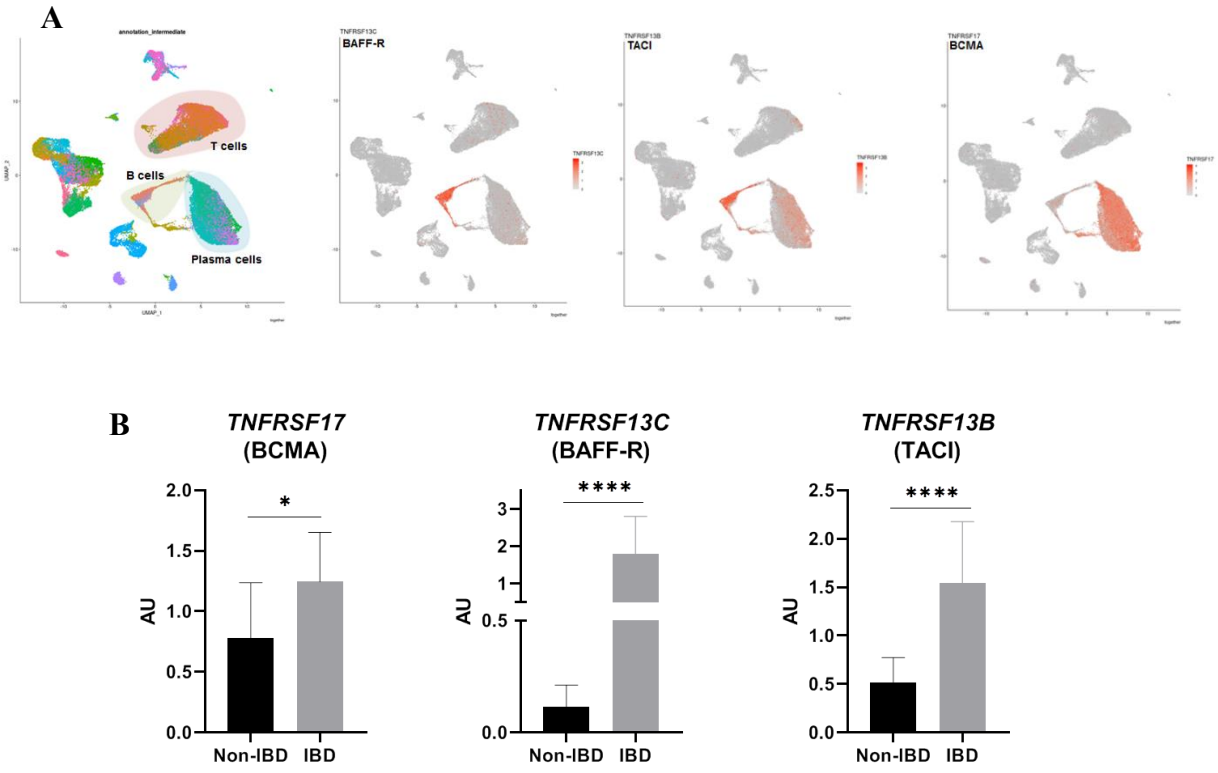


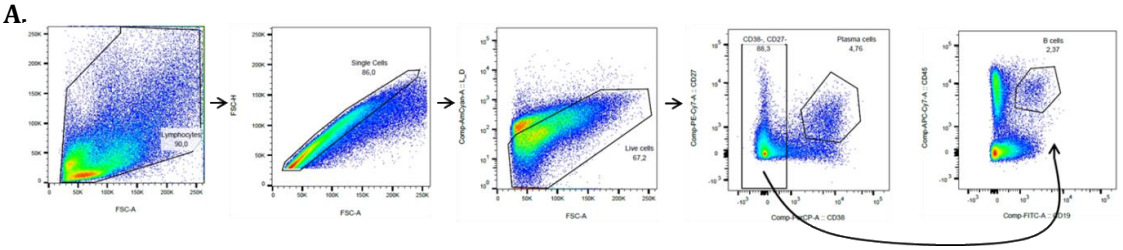
Figure 4: **Expression of BLyS receptors. A:** scRNAseq data on the intestinal mucosa of control, UC and CD colonic cells. UMAPs coloured by (from right to left) cell clusters, expression of *TNFRSF13C*, *TNFRSF13B* and *TNFRSF17*. **B**: qRT-PCR of untreated tissue explants for *TNFRSF17*, *TNFRSF13C* and *TNFRSF13B*. T-test: * = p≤0.05; **** = p≤0.0001; n=12 per condition.

To investigate protein expression, we conducted flow cytometry (FACS) staining on plasma, B- and T-cell subsets from enzymatically digested colonic biopsies. Plasma cells were gated as $CD38^{+}$ and $CD27^{+}$, while B cells were identified as $CD38^{-}CD19^{+}$ cells. T cells were all $CD3^{+}$ cells (Figure 5A, B). We conducted this experiment using three different samples, two inflamed (CD) and one healthy control.

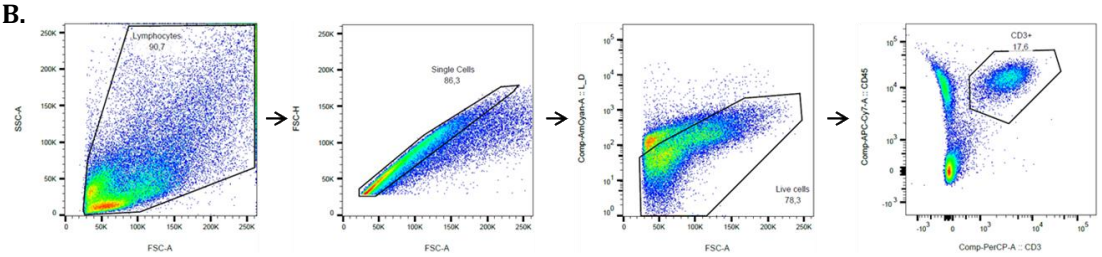
In line with our scRNA-seq results, BAFF-R was highly expressed on B cells, while TACI was also expressed on plasma and T cells. BCMA was characterized as a plasma cell marker, both in homeostasis and disease. However, protein expression was low in general for all three receptors, and we observed significant variability among all three donors.

Despite these limitations, we concluded that BLyS receptors were expressed on intestinal lymphoid cells, both in inflamed and normal tissues.

A.

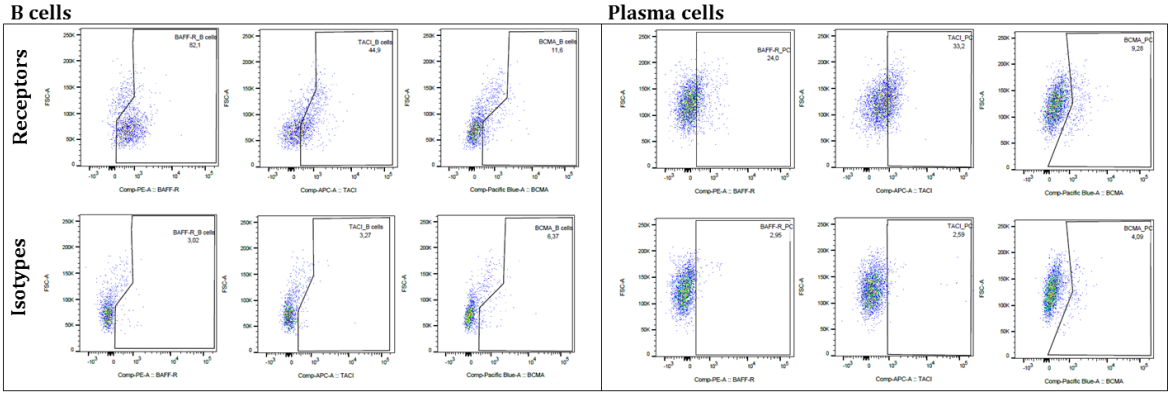


B.

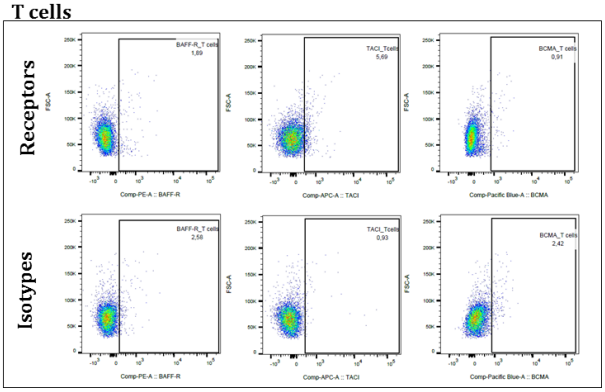


C.

B cells **Plasma cells**

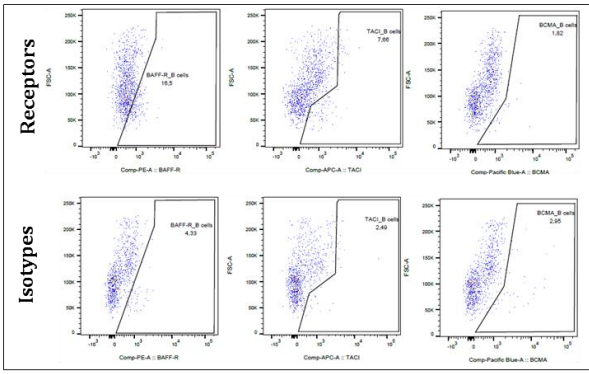


T cells

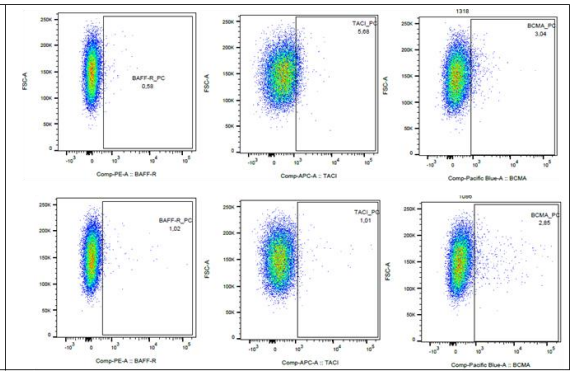


D.

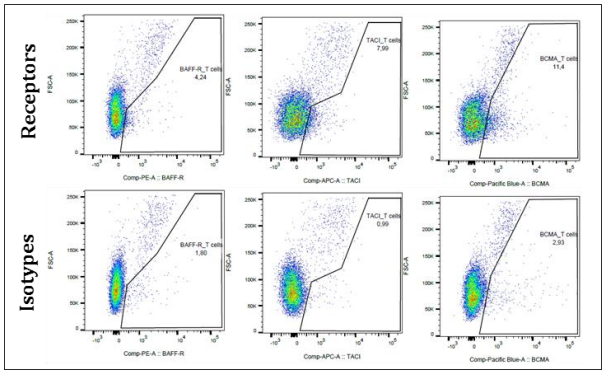
B cells



Plasma cells

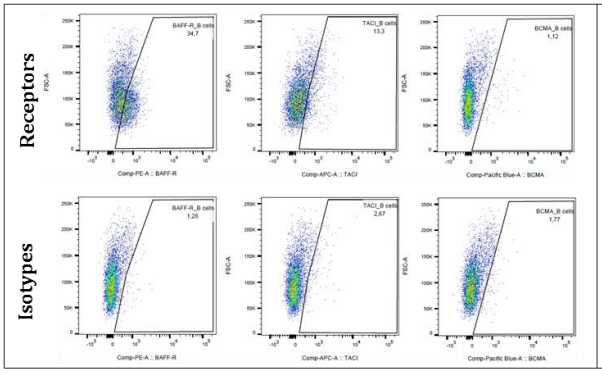


T cells

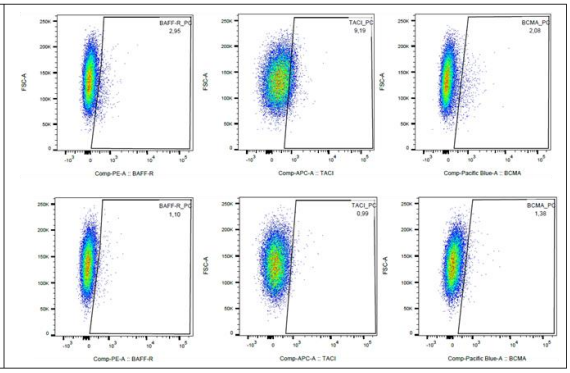


E.

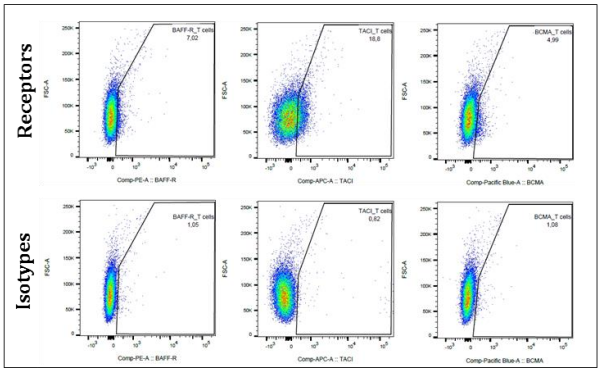
B cells



Plasma cells



T cells



F.

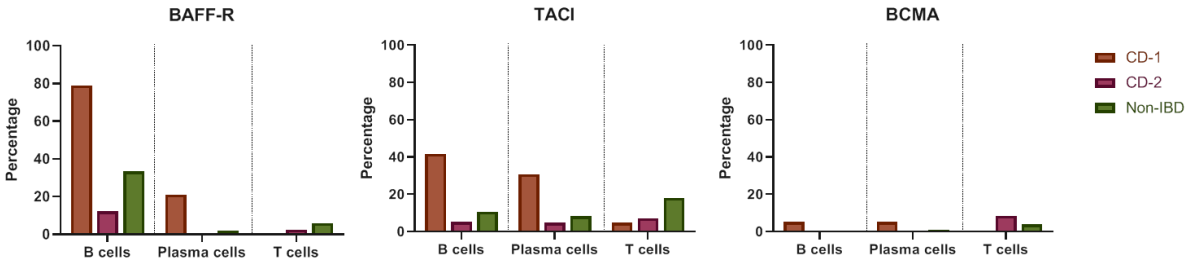


Figure 5: FACS staining for BLyS receptors in intestinal tissue. **A**: Gating strategy for plasma and B cells. **B**: Gating strategy for T cells. C, D and E: Density plots for BAFF-R, TACI, BCMA and their corresponding isotypes for CD-1 (**C**), CD-2 (**D**) and Non-IBD samples (**E**). **F**: Percentage quantification of BAFF-R, TACI and BCMA for each cell population and sample.

Stimulation of the BLyS pathway in tissue explant cultures

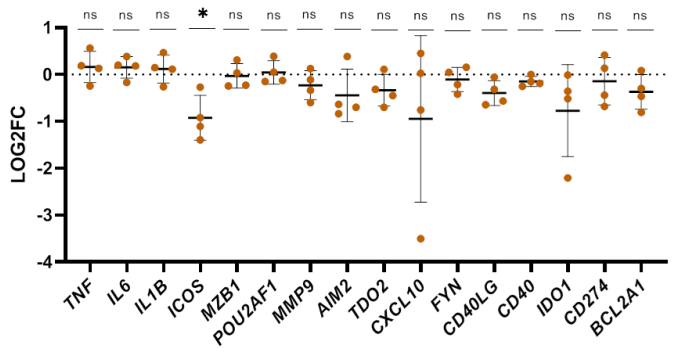
To determine which genes are modulated downstream of BlyS activation, we stimulated intestinal tissue explants with increasing concentrations of rhBLyS. Based on the biological activity of rhBLyS and the ubiquitous expression of BLyS receptors, both in disease and healthy patients, we assumed that tissue explants can respond to rhBLyS stimulation.

Patient-derived intestinal biopsies were stimulated with rhBLyS for varying amounts of time and transcriptional analysis was performed on whole-biopsy RNA. As a positive control, we also stimulated with rhTNFα, whose target genes are well described.

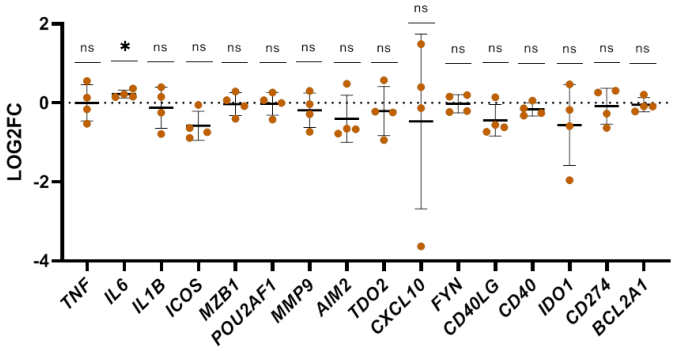
In this project, we conducted a total of 31 human rhBLyS-stimulated explant experiments. Different experimental settings were tested, such as biopsy pre-processing before culture and varying incubation times. Given the heterogeneity we observed in some results, and to avoid potential intrinsic biopsy differences, we decided to chop all biopsies into small pieces instead of culturing one biopsy per condition. Initially, we conducted short-time incubations for 2, 4 and 6 hours. Under these conditions, we observed an upregulation of *TNF*, *IL1β* and *IL6* in biopsies treated with rhTNF, indicating the ability of tissue explants to respond to an external stimulus. Using this system rhBLyS induced a significant decrease in *ICOS* and a slight, but significant, increase in *IL6* under the 100ng/ml condition. Likewise, *AIM2*, *BCL2A1* and *CD40* also showed a slight decrease under certain conditions (Figure 6, illustrating incubation conditions of 2 and 6 hours). However, no significant changes were observed in the other tested genes.

2-hour incubation

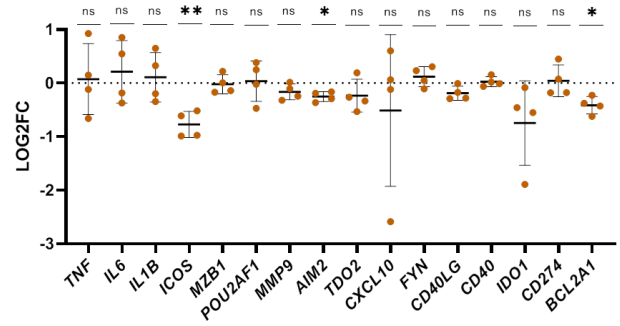
A.



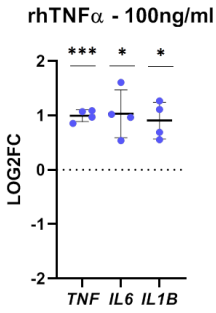
B.



C.

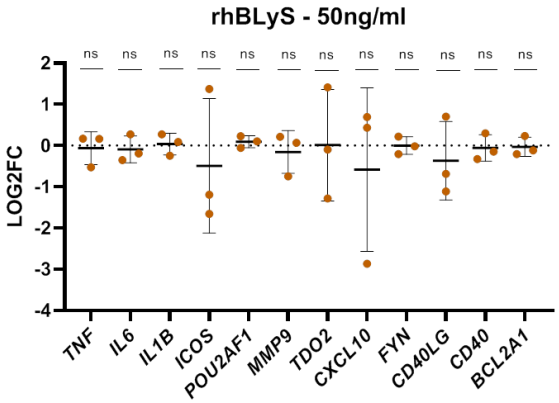


D.

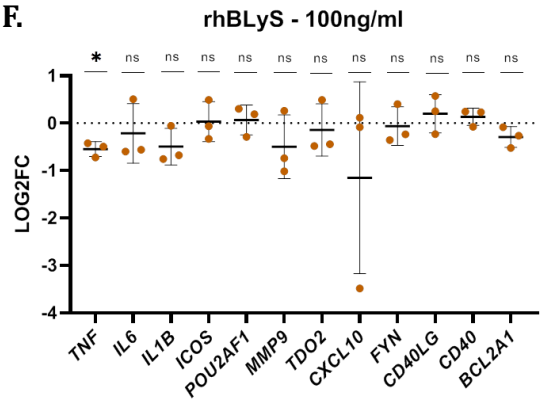


6-hour incubation

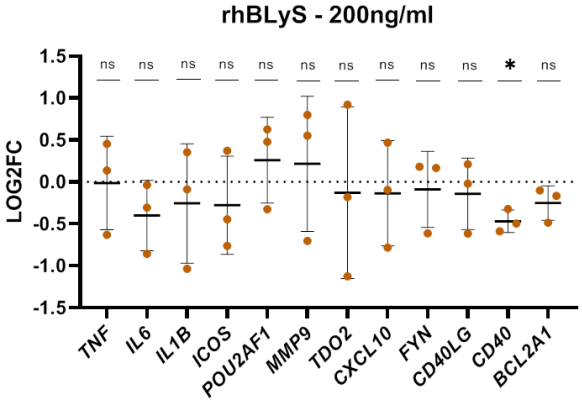
E.



F.



G.



H.

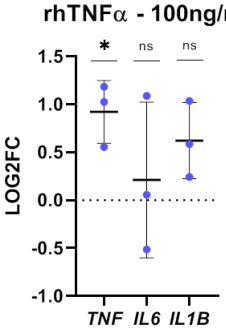


Figure 6: Gene regulation after cytokine stimulation in healthy human intestinal explant cultures. qRT-PCR from biopsies stimulated with 50ng/ml (**A**), 100ng/ml (**B**) and 200ng/ml (**C**) of rhBLyS and with 100ng/ml of rhTNFα (**D**) for two hours. qRT-PCR from biopsies stimulated with 50ng/ml (**E**), 100ng/ml (**F**) and 200ng/ml (**G**) of rhBAFF and with 100ng/ml of rhTNFα (**H**) for six hours.
n=4 (A, B, C and D) and n=3 (E, F, G and H). Data is shown as the logarithm in base 2 of the fold change compared to the control condition (just media). One sample t test; *p≤0.05, **≤0.01, ns ≥ 0.05.

Potential reasons that might explain the limited effects observed in rhBLyS-stimulated biopsies could be the selected incubation time and genes, or the lack of a co-stimulatory stimulus to enhance BLyS signaling. Therefore, we conducted a new set of explant experiments (n=5 healthy donors) in which we increased the incubation time to 24 hours. In addition, rhBLyS was used in combination with anti-IgM, based on our results using isolated CD19⁺-enriched PBMCs (Figure 3). Moreover, we included some other genes in the transcriptional analysis based on a literature review (*MCL1* and *OTUD7)*[5,24]. Although none of the changes compared to the unstimulated conditions proved significant, we now observed a trend towards an increase in *NFKB2*, *BCL2A1* and *CD40* when tissues were stimulated with rhBLyS and anti-IgG (Figure 7).

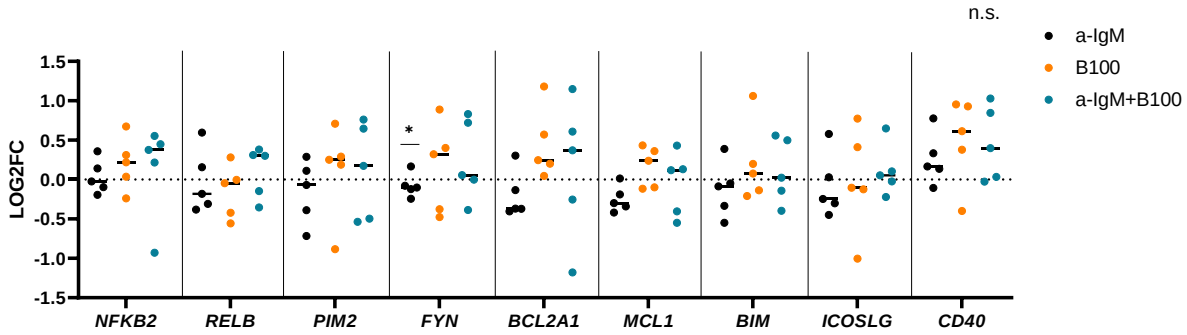


Figure 7: Overnight incubation of healthy human intestinal explants. Tissue was cultured with either anti-IgM (2µg/ml), rhBlyS (100ng/ml) or in combination with both for 24 hours. Data is shown as the logarithm in base 2 of the fold change compared to the control condition (just media). n=5. One sample t test; *p≤0.05, ns ≥ 0.05.

<u>Lamina propria mononuclear cells (LPMCs) from intestinal biopsies as an alternative method for investigating the BLyS pathway</u>

Given the difficulties we encountered in identifying genes that were modulated by rhBLyS in tissue explant cultures, we thought of using isolated LPMCs as an alternative method to investigate BLyS signaling. Although less physiologic, this approach provides additional readouts to measure pathway modulation such as cell proliferation and/or phenotyping and analysis of cell populations by FACS. In addition, it might also provide better access to

antibodies in experiments in order to test their effects in culture. We conducted two different experiments in which LPMCs derived from healthy donor biopsies were isolated and cultured with rhBLyS alone, or in combination with other molecules. In the first experiment, we designed a gating strategy to distinguish two states of $CD27^{+}CD38^{+}$ plasma cells (long-lived and short-lived plasma cells) based on the expression of CD19 (long-lived plasma cells lose CD19 expression). We also measured the percentage of total plasma cells, B cells and T cells. LPMCs were cultured for 20 hours with rhBLyS, anti-IgM, FAB GAM (anti-IgG+IgA+IgM) and the last two in combination with rhBLyS. We observed a slight increase in the percentage of plasma cells (due to an expansion on long-lived plasma cells) and B cells upon rhBLyS treatment, although no synergic effect was observed when rhBLyS was combined with anti-IgM or FAB GAM (Figure 8).

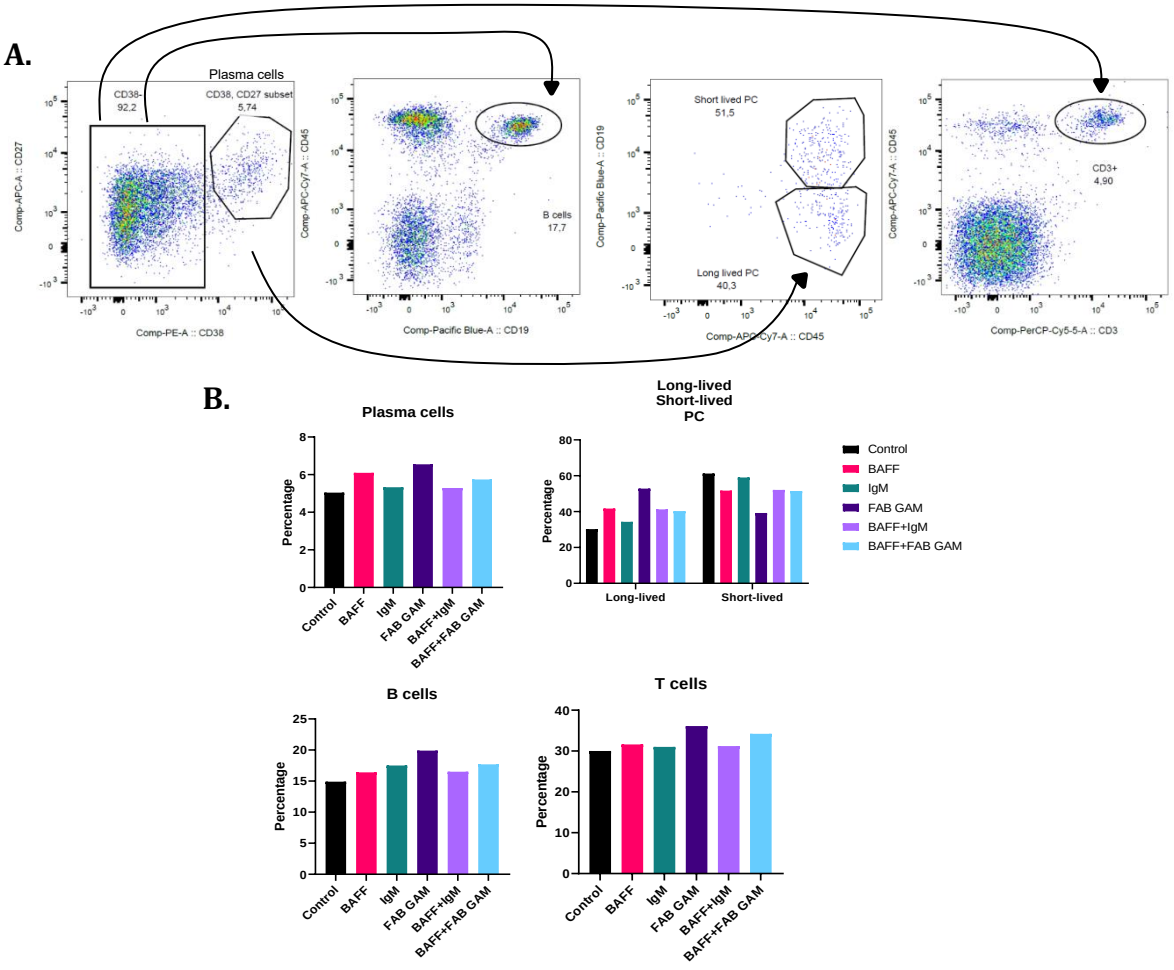


Figure 8: FACS analysis of stimulated LPMCs. A: Gating strategy for plasma cells ($CD38^{+}$, $CD27^{+}$), long-lived ($CD19^{-}$) and short-lived ($CD19^{+}$) plasma cells, B cells ($CD19^{+}$ from $CD38^{-}$ subset) and T cells ($CD3^{+}$), from total live cells. **B**: Bar plots representing percentages of plasma cells, long-lived and short-lived plasma cells, B cells and T cells of LPMCs non-treated (control) or treated with rhBLyS (200ng/ml), anti-IgM (2µg/ml), FAB GAM (10µg/ml) or the combination of anti-IgM and FAB GAM with rhBLyS.

This experiment was repeated with another healthy donor to measure LPMC proliferation and transcriptomic changes (Figure 9). As a co-stimulus, we used anti-IgM and IL-6[25]. Moreover, we also tested pre-coating of rhBLyS within the plate instead of adding it directly to the culture medium. Proliferation was measured with resazurin, and no significant changes were observed under any of the conditions (data not shown). RNA from LPMCs was isolated after 24 hours in culture and the expression of previously selected genes was analyzed (Figure 9). Interestingly, we observed a pronounced upregulation in the B-cell genes *MS4A1* (CD20) and *CD40* under the rhBLyS-coated condition, but not with soluble BLyS or IL-6. In contrast, genes such as *NFKB2*, *RELB, BCL2, PIM2, OTUF7* and *ICOSLG* were upregulated by coated and uncoated BLyS, though apparently to a higher degree under uncoated conditions. Similarly to BLyS, IL-6 increased the expression of *NFKB2, RELB* and *ICOSLG,* but none of the other related genes. We also observed a slight decrease in *CD3E* and *ICOS*, a T cell co-stimulator, which suggested a decrease in T cells with BLyS or IL-6 stimulation. However, overall, we did not observe a synergistic effect of BLyS when combined with either anti-IgM or IL6.

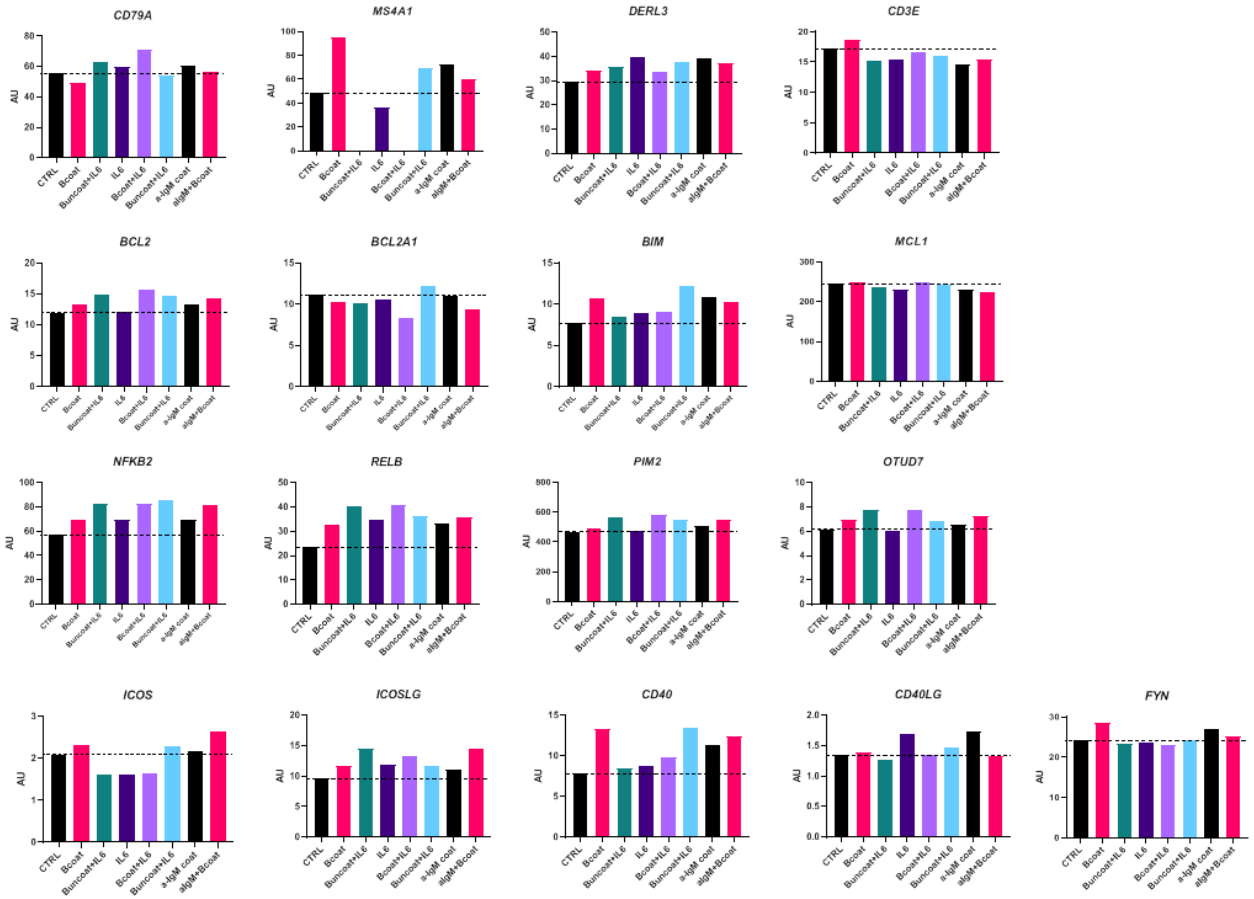


Figure 9: Cytokine stimulation of LPMCs. Gene expression analysis of LPMCs treated for 20 h with rhBLyS (coated, 10µg/ml, and uncoated, 200ng/ml), IL6 (1ng/ml), anti-IgM (2µg/ml) or in combination with IL6 or anti-IgM.

Based on this experiment, we concluded that coating rhBLyS in the plate prior to culturing LPMCs might be the best approach to stimulate BLyS signaling. Transcriptionally, we observed an increase in B cells (*MS4A1* and *CD40*) and activation of the NFκB pathway (*NFKB2* and *RELB*). This could serve as a readout to test the effects of anti-BLyS.

Inhibiting BLyS signaling in intestinal cells

Transcriptomic analysis

First, we wanted to determine the feasibility of utilizing an antibody in the explant setting. As proof-of-concept we used an anti-TNFα (infliximab).

A surgical section from the ascending colon of a CD patient with active disease was used. We performed both explant and LPMC cultures from the same donor. The mucosa was cut into 2-mm pieces and incubated with infliximab or control IgG (10µg/ml) in 4 replicates per condition. After 24 hours, explant RNA was isolated. In parallel, isolated LPMCs were cultured under the same conditions for 20 hours and the RNA was collected.

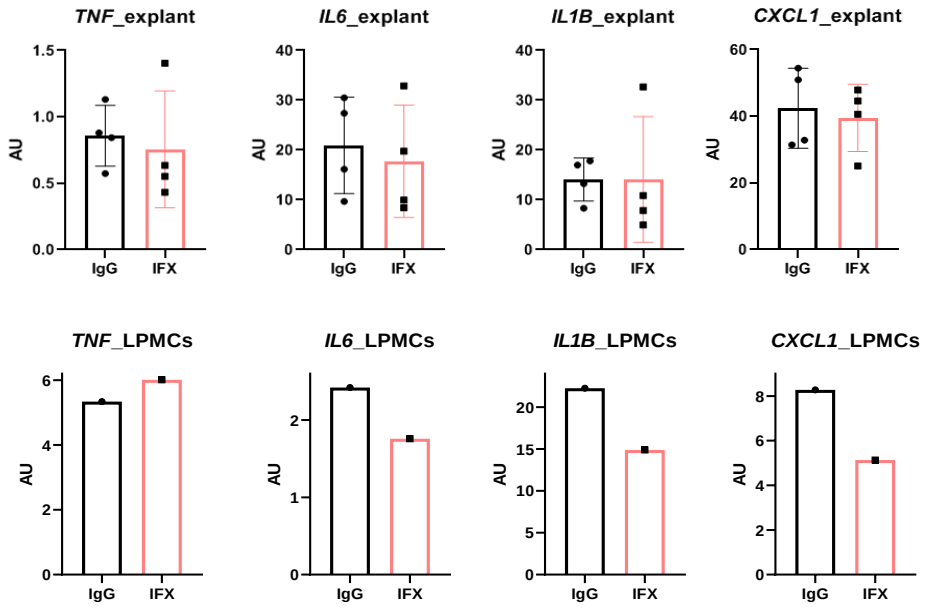


Figure 10: Infliximab treatment in human inflamed intestinal mucosa. qRT-PCR of explants (top panel) and LPMCs (bottom panel) from a Crohn's disease patient treated with infliximab (IFX) (10µg/ml) or IgG control for 24 hours.

qPCR-analysis from tissue explants revealed high variability in gene expression between replicates, regardless of treatment (Figure 10A). This high inter-biopsy heterogeneity may render any comparisons between groups unfeasible.

In addition to favoring antibody access during culture, using LPMCs may ensure homogeneous sample distribution among different wells. Indeed, infliximab induced the downregulation of *IL6*, *IL1B* and *CXCL1* in LPMC cultures, while no changes were observed in tissue explants (Figure 10). Based on these results, we propose using LPMCs, rather than explants, to test the effects of inhibitory antibodies.

Biopsies from three IBD (one CD and two UC) patients were digested and LPMCs were cultured for 24 hours with anti-BLyS or an anti-IgG control. A panel of potential BLyS target genes and markers of cell populations (*MS4A1*, *CD79A, DERL* and *CD3E*) were used to assess the effects of anti-BLyS by qRT-PCR (Figure 11). Despite the low number of replicates (n=3) detected, there was a subtle, but significant, downregulation in the expression of both the ICOS receptor and the ligand (*ICOS* and *ICOSLG*, respectively). However, no significant differences were observed in any of the other genes analyzed.

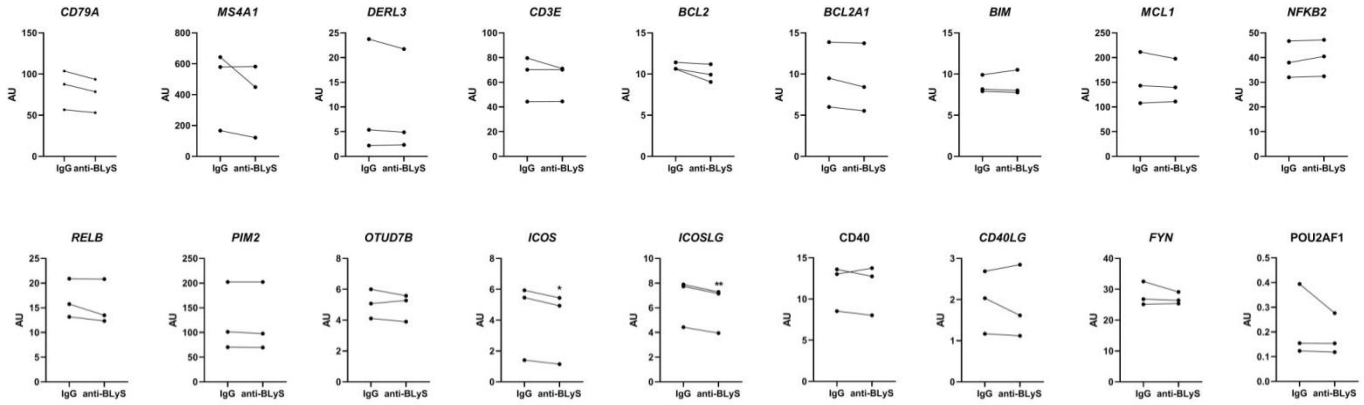


Figure 11: Inhibition of BLyS in LPMCs. qRT-PCR of LPMCs from inflamed tissue, cultured with either IgG or anti-BAFF (6µg/ml) for 24 hours. T-test: * = p≤0.05; ** = p≤0.01; n=3.

Immunoglobulin production

BLyS is a B-cell survival factor that exerts its functions by binding to three different receptors (BAFF-R, TACI and BCMA). Several studies have shown that during the generation of long-lived plasma cells, there is an increase in the expression of BCMA, suggesting that this process partly depends on this receptor[26,27]. Interestingly, a recent paper has shown that

small intestine biopsies cultured as tissue explants for a period of up to 32 days can continue to secrete immunoglobulins, demonstrating the existence of long-lived plasma cells in the intestine[28]. In addition, they showed that stimulation of the biopsies with APRIL, another TNF family ligand that shares receptors with BLyS, can enhance IgA secretion, while inhibition of BCMA downregulates its production. This finding is also supported by increasing evidence pointing towards a requirement for APRIL to generate these long-lived plasma cells, as well as to a similar contribution by BLyS[25,29,30]. Taking these findings together, we propose measuring IgA/IgG secretion as a potential readout to test the effects of an anti-BLyS antibody in the *ex vivo* culture system.

A surgical section from the ascending colon of a UC patient was cultured as a tissue explant with anti-BLyS for 9 days and immunoglobulin production was measured on days 1, 3 5, 7 and 9. No changes were observed in the release of IgA and IgG by explants treated with anti-BLyS compared to IgG controls (Figure 12). We also isolated LPMCs from this tissue resection and measured both proliferation and immunoglobulin production. However, no changes were detected in any of the measurements.

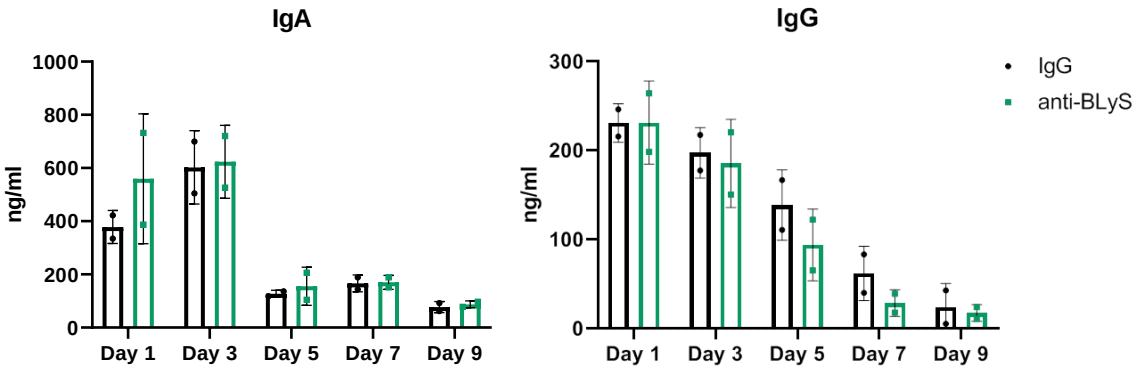


Figure 12: Immunoglobulin secretion in explants treated with anti-BLyS. Tissue from a UC patient cultured as explants for nine days. Supernatants were collected on day 1 and then every two days.

CONCLUSIONS

BLyS is a B-cell survival cytokine whose expression increases in immune-mediated diseases, such as IBD. However, little is known about the downstream signaling pathways that BlyS is involved in during IBD and whether inhibition of its function could serve as a therapeutic strategy in that setting. Therefore, in this project, we have aimed to characterize the function of BLyS in IBD through computational and functional analysis.

As signature of BLyS in IBD is poorly understood, we first conducted co-expression analysis of BLyS (*TNFSF13B*) and the three BLyS receptors (TACI – *TNFRSF13B*, BAFF-R – *TNFRSF13C*, BCMA – *TNFRSF17*) in a microarray dataset of ileal and colonic healthy and inflamed intestinal mucosa. Co-expression analysis was performed twice. First, we looked for individual genes that positively or negatively correlated with the expression of BLyS or BLyS receptors. Second, we conducted module analysis with group genes that presented a similar expression pattern. Importantly, as *TNFSF13B* expression increased with inflammation, many of these genes co-expressed with TNFSF13B reflected the inflammatory state rather than being direct targets of BLyS. This could be the case with cytokines released by macrophages, as myeloid cells do not express BLyS receptors. However, genes expressed by B and T cells were considered as potential target candidates, since lymphocytes and plasma cells express BLyS receptors and respond to it. Based on these analyses and the literature, we selected up to twenty potential BLyS target genes to use in our functional analysis. These genes were mainly involved in lymphocyte activation and survival processes, as well as in the NFκB pathway.

To investigate the impact of modulating the expression of BLyS in human intestinal mucosa, we used an *ex vivo* tissue explant setting. Colonic biopsies were cultured in the presence of different stimuli for different time periods and RNA was isolated for transcriptome analysis. We first stimulated biopsies with rhBLyS to identify those genes downstream of BLyS activation, which could then be used as readouts to monitor the effects of blocking BLyS expression with an anti-BLyS antibody. As an experimental control, we stimulated with rhTNFα, which drove a significant upregulation of its known target genes, even at early time points (2h). In contrast, stimulation of explants with rhBLyS led to subtle differences in some of the genes we measured. After testing different experimental setups, overnight incubation appeared to be necessary to detect changes in gene expression. In this scenario, we detected a trend towards an increase in *NFKB2*, *BCL2A1* and *CD40* expression, although none of these changes were significant (n=3). Potential reasons to explain the limited and delayed effect with rhBLyS included over-activation of the pathway by endogenous BLyS or the lack of BLyS receptors in the tissue. However, BLyS was virtually undetectable in explant supernatants measured by ELISA. In addition, the expression of TACI, BCMA and BAFF-R was detected in healthy and inflamed biopsies by both RNA and protein.

In addition, we explored alternative approaches to investigate BLyS functionality in mucosal responses. On one side, we measured long-term immunoglobulin secretion by tissue explants when cultured with either rhBLyS or anti-BLyS, as has been previously published; i.e., APRIL stimulation promotes, while BCMA inhibition diminishes, immunoglobulin secretion on intestinal explants[28]. On the other, we monitored changes in pro-inflammatory

cytokine release by these explants, such as IL1B. Furthermore, we digested biopsies to isolate LPMCs and cultured them with either rhBLyS or anti-BLyS. The culturing of LPMCs was intended to 1) ensure homogeneity across sample replicates and 2) to facilitate antibody access to targeted cells compared to tissue explants. However, these experiments will require further optimization, including testing lower doses of antibodies (both anti-BLyS and isotype controls) and increasing the number of replicates.

Therefore, we conclude that BLyS can regulate the expression of identified target genes in human colonic mucosa and that the culturing of tissue explants can serve as a viable model for these studies. In contrast, the culturing of explants is not useful for testing the effects of antibodies *ex vivo*, and LPMCs instead may represent a viable option that in any case requires further exploration.

Bearing in mind the enhanced expression of BLyS in inflammation, and the effects of BLyS reported herein, deciphering its implication during inflammatory diseases such as IBD is a topic of interest, and we hope these experiments could help guide the design of future projects investigating the function of BLyS.

MATERIALS AND METHODS

Correlation analysis

Microarray data was processed as previously described (Deliverable 1_2 report). The final sample composition of the study was as follows:

	CD	**UC**	**Control**
Colon	45	18	22
Ileum	26	-	6

Module expression and network analysis

We used weighted gene co-expression network analysis (WGCNA) software[31] to calculate the correlations between expression levels of all possible pairs of genes. Genes with multiple probes were assigned the average expression value of all their probes. We used the WGCNA "signed"-type network, meaning that inverse correlations were not considered. We also used a large value for the WGCNA parameter minKMEtoStay (0.9 for colon CD, and 0.85 for colon UC and ileum CD) to obtain modules of tightly co-expressed genes. We plotted the interactome corresponding to the genes in the module containing TNFSF13B using

combined interactomic data from three sources: the Reactome database (predicted interactions excluded)[32], the STRING database (interactions with a score < 700 were excluded)[33], and the PathwayCommons database[34]. The combined interactome connects two genes if there is a functional relationship between them (e.g., regulatory, metabolic, physical interaction, etc.). Interactomes were plotted using Cytoscape software[35].

Explant biopsy culture

Human colonic tissue obtained from endoscopy or from a surgically resected intestine was washed twice with complete medium (RPMI, Fetal Bovine Serum, Glutamine, PSA -Penicillin, Streptomycin and Amphotericin B-, Hepes and Gentamicin) for 10 min on a shaker at RT. When a surgical piece was used for a tissue explant, it was first cut into 2-mm fragments and twice washed with complete medium. After washing, biopsies were chopped using a blade and merged together, and divided evenly between wells. Each well contained 500 µl of complete medium with 2µl/ml of Normorcin (cat# Nol-42-06, Invivogen). Cytokines or antibodies used were as follow: rhBLyS (cat# 7537-BF. R&D systems), anti-IgM (cat# 109-006-129, Jackson Immuno Research), affinity purified F(ab')2 goat anti-human IgA+IgG+IgM (H+L) (cat# 109-006-064, Jackson Immuno Research), IL-6 (cat# 570802, BioLegend), Infliximab (Remicade), Infliximab – IgG control (IgG1 Kappa, human, cat#I5154, Sigma-Aldrich), anti-BLyS (provided by GSK) and anti-BLyS – IgG control (IgG1K, mouse, cat# 553447, BD Pharmingen). Biopsies were incubated for 2, 4, 6 and/or 24 hours. For immunoglobulin and cytokine production, longer incubation time points were sometimes used (up to 9 days). In such cases, two-thirds of the supernatant was replaced with fresh media every 2 days. RNA was isolated only in explants cultured for up to 24 hours.

Isolation of LPMCs

Intestinal tissue was cut into 1-mm sections and treated with 50µl of DTT 1M in 10 ml of HBSS for 15 min at RT on a rocker. The tissue was then washed for 10 min in complete medium at RT on a rocker. After washing, biopsies were finely cut with a blade and divided into Eppendorf tubes containing 489 µl complete medium, 10 µl of Liberase and 1µl of DNAse. The tissue was then incubated for 1 hour at 37ºC, and the shaker was set to 250 rpm. After digestion, cells were pipetted up and down with a p1000 and passed through a 50-µm filter. Filters were washed with PBS buffer supplemented with 1mM EDTA, 25mM Hepes and 2% FBS and cells were passed again through a 50-µm filter. Cells were then centrifuged at 400*g* for 5 min at 4º C and the pellet resuspended in 2 ml of PBS to count the cells. Cells were plated on a 96-well plate at a confluency of 100,000 cells per well, in completed media alone or supplemented with the cytokines or antibodies as indicated in each experiment. To

measure cell proliferation, resazurin was added after 17 hours of culture, and absorbance measurements were made at 21, 23 and 24 hours of culture. For RNA isolation, we followed the steps explained in the "CD19+ cell culture" protocol.

Peripheral blood mononuclear cell isolation from buffy coat

Ten ml of a buffy coat was diluted in 90 ml of PBS at RT and carefully divided into three 50 ml falcon tubes containing 16 ml of Ficoll (Polymorphprep, cat# 04-03-9393101, Palex). Cells were carefully delivered to the bottom of each tube, allowing Ficoll to form a separate layer above the diluted cell suspension. Tubes were centrifuged for 20 min at 690*g* at 20ºC without acceleration/deceleration. The white interface containing PBMCs was carefully aspirated, transferred to a 50 ml falcon tube and topped with RT PBS up to 50 ml. Cells were centrifuged for 10 min at 700*g* at RT. Supernatant was discarded, the pellet was washed with 10 ml of PBS at 4º C and was centrifuged for 5 min at 400*g* at 4º C. This washing step was repeated three times. The cell pellet was then resuspended in 50 ml of PBS and the cells were counted.

CD19+ cell isolation from PBMCs

After PBMC isolation, $2x10^8$ cells were filtered through a 40-µm cell strainer, centrifuged at 300*g* at 4º C for 10 min and resuspended in 1.6ml of buffer (provided by the isolation kit) and 400 µl of CD19 magnetic beads (CD19 microbeads, human, cat#130-050-301, Miltenyi Biotec). Cells were incubated with beads for 15 min at 4ºC. After incubation, cells were washed with 30 ml of buffer and centrifuged at 300*g* for 10 min at 4º C. Meanwhile, we prepared an LS column (cat#130-042-401, Miltenyi Biotec) by washing with 3 ml of buffer. The cell pellet was resuspended with 1 ml of buffer and cells were passed through the column. The column was washed three times with 3 ml of buffer. To collect $CD19^+$-labelled cells, we added 5 ml in the column and cells were eluted with a plunger.

To measure the percentage of $CD19^+$ cells after magnetic bead enrichment, 100,000 unlabeled ($CD19^-$ fraction) and 100,000 labeled ($CD19^+$ fraction) cells were stained with a-$CD19^+$ antibody (cat#302234, BioLegend) and Zombie Aqua Fixable Viability kit (cat# 423101, BioLegend, 1/1000). The percentage of viable $CD19^+$ cells was measured with a BD FACSCanto II flow cytometer (Becton Dickinson) and analyzed with FlowJO software (Becton Dickinson).

$CD19^+$ cell culture

The $CD19^+$-enriched cell suspension was plated at 100,000 cells per well on a 96-well plate, using five replicates per condition. Cells were cultured in completed media alone or were supplemented with anti-IgM, rhBLyS or anti-IgM+BLyS for 96 hours. After 72 hours of

culture, 10 µl of resazurin (cat#AR002, R&D) was added to the media and absorbance was measured 24 hours later.

After 96 hours of culture, wells were scrapped and centrifuged at 400*g* for 10 min. Supernatants were discarded, and RNA was isolated using GeneJET RNA Cleanup and a Concentration Micro kit (cat#K0842, Thermo scientific) following the manufacturer's instructions.

RNA extraction and qPCR analysis

After culturing, explants were resuspended in RLT lysis buffer (Qiagen RNeasy Mini Kit) and homogenized in a Bullet blender 24 (Next Advance) using stainless steel beads (Next Advance) for 7 min at maximum speed. Total RNA was then isolated using the Qiagen RNeasy Mini Kit assay following the manufacturer's instructions. RNA was transcribed to cDNA using the reverse-transcriptase high-capacity cDNA Archive RT kit (Applied Biosystems). qRT-PCR was conducted using TaqMan Assays (Applied Biosystems) with the following predesigned gene primers:

Gene	**Reference**
TNF	Hs01113624_g1
AIM2	Hs00915710_m1
BCL2A1	Hs00187845_m1
IL1B	Hs01555410_m1
MZB1	Hs00414907_m1
POU2AF1	Hs01573369_m1
TDO2	Hs00194611_m1
FYN	Hs00176628_m1
IL6	Hs00985639_m1
CD40LG	Hs00163934_m1
MMP9	Hs00234579_m1
CXCL10	Hs01124251_m1
IDO1	Hs00984148_m1
CD274	Hs00174517_m1
ICOS	Hs00359999_m1
ICOSLG	Hs00323621_m1
RELB	Hs00232399_m1
NFKB2	Hs00174517_m1
BIM	Hs00197982_m1
PIM2	Hs00179139_m1
DERL3	Hs00405322_m1
CD79A	Hs00998119_m1
CD3E	Hs01062241_m1

MS4A1	hs00544819_m1
CD40	Hs01002915_g1
BCL2	Hs00608023_m1
OTUD7B	Hs00902945_g1
MCL1	Hs03043899_m1
CXCL1	Hs00605382_m1
TNFRSF13B	Hs00900358_m1
TNFRSF17	Hs00171292_m1
TNFRSF13C	Hs00606874_g1
ACTB	Hs99999903_m1

ACTB was used as an endogenous control for all genes.

ELISA

Tissue explants and LPMC supernatants were collected at the indicated times and frozen at -20ºC until use. Protein concentration in supernatants was determined using the following commercial kits: CXCL1 (Human CXCL1/GRO alpha DuoSet ELISa, cat# DY275, R&D), IL1B (Human IL-1 beta/IL-1F2 DuoSet ELISA, cat# DY201, R&D), IgA (IgA Human Uncoated ELISA Kit, cat#88-50600-88, Invitrogen) and IgG (IgG Total Human Uncoated ELISA kit, cat# 88-50550-22, Invitrogen).

FACS staining

After 20 hours in culture, LPMCs were scrapped off and centrifuged at 400*g* for 5 min at 4º C. Cell viability was detected using a Zombie Aqua Fixable Viability kit (cat# 423101, BioLegend, 1/1000). Cells were also incubated with Fc block (cat# 422302, BioLegend, 1/50). Antibodies were prepared in a volume of 50 µl FACS buffer (Sorter buffer without FBS) using the following concentrations:

Antibody	**Clone**	**Cat#**	**Company**	**Concentration**
Epcam - FITC	9C4	324204	BioLegend	5/100
CD38 – PE	HIT2	303506	BioLegend	10/100
CD3 – PerCP	OKT3	317336	BioLegend	1.25/100
CD27 – APC	M-T271	558664	BD Pharmingen	5/100
CD45 – APC-cy7	2D1	348795	BD Biosciences	5/100
CD19 – BV421	HIB19	302234	BioLegend	2.5/100
CD8 – FITC	RPA-T8	301006	Biolegend	5/100
BAFF-R – PE	11C1	316905	Biolegend	10/100

mIgG1 – PE	MOPC-21	400114	Biolegend	2.5/100
CD4 – PE-Cy7	OKT4	317414	Biolegend	5/100
TACI – APC	1A1	311911	Biolegend	10/100
ratIgG2a – APC	R35-95	554690	BD Biosciences	10/100
BCMA – BV421	19F2	357519	Biolegend	5/100
mIgG2a – BV421	MOPC-173	400260	Biolegend	15/100

Cells were incubated with antibodies for 20 min at 4º C in the dark, washed with 3 ml of FACS buffer and centrifuged at 300*g* for 5 min at 4º C. Supernatant was discarded, and cells were fixed with BD stabilizing fixative (cat# 339860, BD Bioscience). Samples were analyzed using a BD FACSCanto II flow cytometer (Becton Dickinson) and analyzed with FlowJO software (Becton Dickinson).

Statistical analysis

GraphPad Prism version v8.3 software was used for all statistical analyses. Comparison between two groups was assessed using the Student's T-test, 2-tailed parametric and unpaired analysis. One-way ANOVA was used for comparisons of more than two groups. For explant culture analysis, fold changes relative to the control condition were transformed to logarithm in base 2, and values were assessed by a parametric One-sample T test. P values < 0.05 were considered statistically significant. Only significant values are labelled in the graphs. Levels of significance are labelled as follows: * = $p < 0.05$; ** = $p < 0.001$; *** = $p < 0.001$; **** $p \leq 0.0001$.

REFERENCES

1. Mackay, F. & Schneider, P. Cracking the BAFF code. *Nat. Rev. Immunol.* **9**, 491–502 (2009).
2. Smulski, C. R. & Eibel, H. BAFF and BAFF-Receptor in B Cell Selection and Survival. *Front. Immunol.* **9**, 2285 (2018).
3. Uzzan, M., Colombel, J.-F., Cerutti, A., Treton, X. & Mehandru, S. B Cell-Activating Factor (BAFF)-Targeted B Cell Therapies in Inflammatory Bowel Diseases. *Dig. Dis. Sci.* **61**, 3407–3424 (2016).
4. Xia, J., Gill, E. E. & Hancock, R. E. W. NetworkAnalyst for statistical, visual and network-based meta-analysis of gene expression data. *Nat. Protoc.* **10**, 823–844 (2015).

5. Woodland, R. T. *et al.* Multiple signaling pathways promote B lymphocyte stimulator–dependent B-cell growth and survival. *Blood* **111**, 750–760 (2008).
6. Wensveen, F. M., Slinger, E., van Attekum, M. H., Brink, R. & Eldering, E. Antigen-affinity controls pre-germinal center B cell selection by promoting Mcl-1 induction through BAFF receptor signaling. *Sci. Rep.* **6**, 35673 (2016).
7. Sochalska, M. *et al.* Conditional knockdown of BCL2A1 reveals rate-limiting roles in BCR-dependent B-cell survival. *Cell Death Differ.* **23**, 628–639 (2016).
8. Bertrand, M. J. M. *et al.* cIAP1/2 Are Direct E3 Ligases Conjugating Diverse Types of Ubiquitin Chains to Receptor Interacting Proteins Kinases 1 to 4 (RIP1–4). *PLoS One* **6**, e22356 (2011).
9. Xie, P. TRAF molecules in cell signaling and in human diseases. *J. Mol. Signal.* **8**, 7 (2013).
10. Tusche, M. W. *et al.* Differential requirement of MALT1 for BAFF-induced outcomes in B cell subsets. *J. Exp. Med.* **206**, 2671–2683 (2009).
11. Vereecke, L., Beyaert, R. & van Loo, G. The ubiquitin-editing enzyme A20 (TNFAIP3) is a central regulator of immunopathology. *Trends Immunol.* **30**, 383–391 (2009).
12. Zhang, F. *et al.* BAFF upregulates CD28/B7 and CD40/CD154 expression and promotes mouse T and B cell interaction in vitro via BAFF receptor. *Acta Pharmacol. Sin.* **37**, 1101 (2016).
13. H, C. & JC, R. TRAF-dependent association of protein kinase Tpl2/COT1 (MAP3K8) with CD40. *Biochem. Biophys. Res. Commun.* **328**, 198–205 (2005).
14. Chirieleison, S. M., Kertesy, S. B. & Abbott, D. W. Synthetic Biology Reveals the Uniqueness of the RIP Kinase Domain. *J. Immunol.* **196**, 4291–4297 (2016).
15. Richards, S., Watanabe, C., Santos, L., Craxton, A. & Clark, E. A. Regulation of B-cell entry into the cell cycle. *Immunol. Rev.* **224**, 183 (2008).
16. Okada, T., Maeda, A., Iwamatsu, A., Gotoh, K. & Kurosaki, T. BCAP: the tyrosine kinase substrate that connects B cell receptor to phosphoinositide 3-kinase activation. *Immunity* **13**, 817–27 (2000).
17. Hamilton, J. A. Colony-stimulating factors in inflammation and autoimmunity. *Nat. Rev. Immunol.* **8**, 533–544 (2008).
18. Baracho, G. V. *et al.* PDK1 regulates B cell differentiation and homeostasis. *Proc. Natl. Acad. Sci.* **111**, 9573–9578 (2014).
19. Chen, J. *et al.* TAGAP instructs Th17 differentiation by bridging Dectin activation to EPHB2 signaling in innate antifungal response. *Nat. Commun.* **11**, 1913 (2020).
20. Gringhuis, S. I. *et al.* Dectin-1 directs T helper cell differentiation by controlling noncanonical NF-κB activation through Raf-1 and Syk. *Nat. Immunol.* **10**, 203–213

(2009).

21. Milot, E., Fotouhi-Ardakani, N. & Filep, J. G. Myeloid nuclear differentiation antigen, neutrophil apoptosis and sepsis. *Front. Immunol.* **3**, 397 (2012).
22. Martinez-Navio, J. M. *et al.* Adenosine deaminase potentiates the generation of effector, memory, and regulatory CD4 T cells. *J. Leukoc. Biol.* **89**, 127–136 (2011).
23. Malaguarnera, L., Marsullo, A., Zorena, K., Musumeci, G. & Di Rosa, M. Vitamin D3 regulates LAMP3 expression in monocyte derived dendritic cells. *Cell. Immunol.* **311**, 13–21 (2017).
24. Hu, H. *et al.* OTUD7B controls non-canonical NF-κB activation through deubiquitination of TRAF3. *Nature* **494**, 371–374 (2013).
25. Jourdan, M. *et al.* IL-6 supports the generation of human long-lived plasma cells in combination with either APRIL or stromal cell-soluble factors. *Leukemia* **28**, 1647–1656 (2014).
26. O'Connor, B. P. *et al.* BCMA Is Essential for the Survival of Long-lived Bone Marrow Plasma Cells. *J. Exp. Med.* **199**, 91–97 (2004).
27. Laurent, S. A. *et al.* γ-secretase directly sheds the survival receptor BCMA from plasma cells. *Nat. Commun.* **6**, (2015).
28. Mesin, L., Di Niro, R., Thompson, K. M., Lundin, K. E. A. & Sollid, L. M. Long-Lived Plasma Cells from Human Small Intestine Biopsies Secrete Immunoglobulins for Many Weeks In Vitro. *J. Immunol.* **187**, 2867–2874 (2011).
29. Thai, L. H. *et al.* BAFF and CD4 1 T cells are major survival factors for long-lived splenic plasma cells in a B-cell–depletion context. *Blood* **131**, 1545–1555 (2018).
30. Avery, D. T. *et al.* BAFF selectively enhances the survival of plasmablasts generated from human memory B cells. *J. Clin. Invest.* **112**, 286–297 (2003).
31. Peter Langfelder, S. H. WGCNA: an R package for weighted correlation network analysis. *BMC Bioinforma.* **9**, 559 (2008).
32. Fabregat, A. *et al.* The Reactome Pathway Knowledgebase. *Nucleic Acids Res.* **46**, D649–D655 (2018).
33. Szklarczyk, D. *et al.* STRING v11: protein–protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. *Nucleic Acids Res.* **47**, D607–D613 (2019).
34. Rodchenkov, I. *et al.* Pathway Commons 2019 Update: Integration, analysis and exploration of pathway data. *Nucleic Acids Res.* **48**, D489–D497 (2020).
35. Paul Shannon, Andrew Markiel, Owen Ozier, Nitin S Baliga, Jonathan T Wang, Daniel Ramage, Nada Amin, Benno Schwikowski, Trey IdekerPaul SGenome Res hannon 1, Andrew Markiel, Owen Ozier, Nitin S Baliga, Jonathan T Wang, Daniel Ramage, Nada

Amin, Benno Schw, T. I. Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res.* **11**, 2498–504 (2003).