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Complex human adenoid tissue-based *ex vivo* culture systems reveal anti-inflammatory drug effects on germinal center T and B cells

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ABSTRACT

Background: Human immunology research is often limited to peripheral blood. However, there are important differences between blood immune cells and their counterparts residing in secondary lymphoid organs, such as in the case of germinal center (GC) T follicular helper (Tfh) cells and GC B cells.
Methods: We developed a versatile *ex vivo* lymphoid organ culture platform that is based on human pharyngeal tonsils (adenoids) and allows for drug testing. We systematically phenotyped Tfh and GC B cell subsets in explant- and suspension cultures using multicolor flow cytometry and cytokine multiplex analysis.
Findings: Phenotypic changes of certain *ex vivo* cultured immune cell subsets could be modulated by cytokine addition. Furthermore, we optimized an activation-induced marker assay to evaluate the response to T cell stimulation. We provide proof-of-concept that Tfh and GC B cells could be modulated in these cultures by different anti-inflammatory drugs in unstimulated states and upon activation with vaccine-derived antigens. For example, GC B cells were lost upon CD40L blockade, and clinically approved JAK inhibitors impacted Tfh and GC B cells, including down-regulation of their key transcription factor BCL6. BCL6 regulation was affected by IL-6 signaling in T cells and IL-4 in B cells, respectively. Furthermore, we demonstrated that JAK signaling and TNF signaling contributed to the stimulation-induced activation of tonsil-derived T cells.
Interpretation: Our optimized methods, assays, and mechanistic findings can contribute to a better understanding of human GC responses. These insights may be relevant for improving autoimmune disease therapy and vaccination efficacy.
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Research in context

Evidence before this study

Potent antibody-mediated immunity to infectious agents and vaccines relies on germinal center (GC) T follicular helper (Tfh) and GC B cells. Since dysregulation of these cells is involved in autoimmune diseases and allergies, GC cells are viable targets for anti-inflammatory therapeutics. These drugs should be tested in relevant settings containing GC cells, and suitable assays are needed since peripheral blood T and B cells differ from their counterparts in secondary lymphoid organs.

Added value of this study

Here, we developed assays for mechanistic studies and drug testing on primary human tonsil-derived material. Through systematic comparison of different *ex vivo* culture systems, we found that GC Tfh and B cells could be cultured *ex vivo* and displayed subset-specific phenotypic changes during suspension and histocultures as well as upon stimulation. In proof-of-concept experiments we validated these cultures for anti-inflammatory drug testing, including GC B cell loss upon blockade of the costimulatory molecule CD40L and BCL6 downregulation in T and B cells upon inhibition of cytokine signaling with JAK inhibitors. Using additional clinically approved anti-inflammatory drugs in our cultures, we provided novel mechanistic insights into the regulation of BCL6 in GC cells, maintenance of which required IL6R signaling in T cells as opposed to IL-4 signaling in B cells. Furthermore, we established a novel assay representing a complex immune response to a vaccine-derived superantigen, pertussis toxin mutant, which triggered robust T cell activation in a B cell-dependent manner. Release of cytokines, which signal through JAKs as well as through TNF receptor, amplified this immune response, providing novel mechanisms and tools for triggering and manipulating human immune responses *ex vivo*.

Implications of all the available evidence

Our data and methods provide the basis for future studies allowing further mechanistic dissection of human GC T and B cell responses *ex vivo* and their modulation by known and novel anti-inflammatory therapeutics. The assays we present could be exploited for drug development by studying human immune responses in a setting that may be more physiologically relevant than widely used assays with human peripheral blood cells.

1. Introduction

Germinal centers (GCs) rely on interactions between T follicular helper (Tfh) cells and GC B cells in secondary lymphoid organs and they are critical for ensuring potent antibody responses [1,2]. GC B cells and Tfh cells both express the transcription factor BCL6 as well as the chemokine receptor CXCR5, which allows both cell types to co-localize within CXCL13-rich B cell follicles. In addition, Tfh cells express high levels of various co-stimulatory and co-inhibitory molecules, including ICOS, CD40L, and PD1, which act on the appropriate receptors expressed by activated B cells [1,3,4]. Furthermore, cytokine signals are involved in GC cell communication, such as Tfh-produced IL-21 and IL-4 that influence B and T cells *via* cytokine receptors and downstream JAK/STAT signaling [1,4,5]. Tfh cell help to B cells is necessary for GC induction and affinity maturation, ultimately leading to long-lived plasma cell and memory B cell formation [1,2]. Activation of Tfh and GC B cells, including immunological memory formation, is the underlying principle exploited in vaccination [6,7]. The GC reaction is tightly regulated, for example by follicular regulatory T (Tfr) cells that share characteristics with Tfh cells yet are immunosuppressive and express the regulatory T cell (Treg) master transcription factor FOXP3 [8,9]. Due to the essential role of Tfh cells in humoral immunity, dysregulated Tfh cell responses contribute to autoimmune diseases, allergies, and cancer [4,6,7,10].

Ongoing immune reactions are most commonly studied in mouse models due to the availability of genetically engineered mice and easy access to lymphoid tissues. Nevertheless, differences between murine and human immunity clearly exist [11–14], also regarding Tfh cells [4]. Moreover, drug classes such as biologics specifically targeting human cell surface molecules/cytokines and/or exploiting FcR-mediated effects, may not affect the murine immune system. Human immunology research, however, is often limited to peripheral blood as cell source, and preclinical testing of drugs on blood-derived cells may not sufficiently reflect drug effects on cells that are not frequently found in peripheral blood, such as GC cells. In this regard, circulating $CXCR5^+$ Tfh cells do not entirely mirror their counterparts in lymphoid tissues [1,4,15]. Both human (naso)pharyngeal tonsil (adenoid) as well as palatine tonsil tissue contain a relatively large fraction of *bona fide* Tfh and GC B cells reflecting physiological human GC biology [16], and can be obtained by routine surgery from otherwise healthy donors [17].

Different culture systems for human tonsil-derived cells have been described, either maintaining the original 3-dimensional (3D) structure in small explant tissue blocks cultured at the air-liquid interface (human lymphoid histoculture, "HLH") [18,19], or dense single cell suspension cultures (human lymphoid aggregate culture, "HLAC") [20–22]. The use of tonsil-derived histocultures to study T cell responses has largely been restricted to the context of HIV infection [18,19,21,23–25]. Only few studies implemented both HLH and HLAC in parallel, and both systems supported HIV infection [21,23,24,26]. These studies neither featured deep phenotyping of T helper and B cell subsets nor characterization of anti-inflammatory drug effects in such a system. Considering that the GC reaction is tightly controlled in space and time, we addressed the question of whether 3D-histocultures better support the *ex vivo* phenotype and stimulation ability of GC cells compared to suspension cultures.

We here established assays for phenotyping of human Tfh and B cell subsets using HLAC and HLH cultures in parallel, and we systematically compared these systems to each other and to uncultured tissue. We found that adenoid tissue can be used as a source of primary human *ex vivo* Tfh and GC B cells. Yet, certain subsets specifically changed across all culture conditions compared to uncultured tissue, which is important to consider when applying such methods. We show that established anti-inflammatory drugs specifically affected certain cell subsets such as GC B and Tfh cells in the tonsil cultures, hence providing a versatile platform for the assessment of drug effects on GC cells. Furthermore, we optimized an assay to read-out responses to an antigen contained in childhood vaccine regimens, and demonstrated reproducible cellular responses as measured by activation-induced markers and cytokine secretion, which could be modulated by immune-therapeutic drugs. Together, our methods and data provide a framework for future studies to test drugs or even drug combinations regarding their effects on GC reactions from primary lymphoid tissue in a "human-first" approach.

2. Materials and methods

2.1. Ethics statement and patients

Adenoid (also referred to as (naso)pharyngeal tonsil) tissue was removed during routine adenoidectomies and irreversibly

anonymized (usually, adenoidectomy is performed when children are about 2–6 years old). Indication for surgery was airway obstruction and/or fluid behind the ear drum; acute or recurrent inflammation was an exclusion criterion. The use of this material was approved by the local ethics committee of LMU Munich. The tissue, if not used for research purpose, would otherwise be discarded. We obtained these tissues in an anonymized fashion for research purposes. The local ethics committee had decided that written informed consent does not need to be obtained from the donors or parents under these circumstances.

Data from adenoid material from a total of 28 donors was included in the manuscript. In assays with cryopreserved cells, material from some donors was used in multiple independent sets of experiments. Each figure legend provides details on how many individual donors were analyzed in the given experiment.

2.2. Adenoid cell culture as "HLH" and "HLAC"

Adenoid tissue from surgeries was collected in RPMI medium (without supplements) at 4 °C. Tissue was further processed within 1 to 5 h after surgery as follows. Tissues and cells were cultured as described before as HLH [18,19,25,27] or HLAC [20–22] with modifications. In brief, adenoid-derived tissues and cells were cultured in RPMI medium (with L-glutamine) containing 15% (v/v) FBS (Gibco Fetal Bovine Serum, qualified, heat inactivated) and supplements (0.1 mM MEM-nonessential amino acids, 1 mM MEM sodium-pyruvate, 50 μg/ml gentamicin, 2.5 μg/ml amphotericin B, 0.3 μg/ml ticarcillin, 0.01 μg/ml clavulanate) to obtain "CMT" medium as described [19,27]. Collagen sponges for HLH were used in pre-cut form (1 × 1 × 1 cm; Gelastypt, Sanofi-Aventis Deutschland GmbH), pre-soaked in CMT medium and after the air was pushed out, transferred to 12-well plates with 1 ml CMT medium each. Tissue was washed twice with PBS and dissected in a dish with CMT medium. Bloody, cauterized tissue was discarded and remaining tissue was cut into blocks of ~1 × 1 × 2 mm for HLH. The distribution of tissue blocks was randomized by swirling the dish and subsequently, tissue blocks were immediately transferred onto prepared collagen sponges (4 tissue blocks/sponge/well in a 12-well plate). Remaining tissue and cutting medium were routinely strained while mechanically disrupting with syringe plungers through 40 μm cell strainers immersed in CMT medium. Suspension cells were then washed in CMT medium (500×*g*, 5 min), counted and plated as HLAC with 2×10^6 cells/well in 96U-well plates with 200 μl CMT/well. HLH and HLAC were cultured at 37 °C/5%CO_2 in a water-saturated incubator for three days (60–66 h) unless otherwise stated. For "uncultured" samples, some tissue blocks were also kept in closed tubes filled with CMT medium at 4 °C.

2.2.1. Definition of technical replicates

For HLH, 4–8 tissue blocks (from 1–2 wells of a 12-well plate) were pooled for a technical replicate, and 2 to 3 technical replicates were analyzed for each donor and condition for HLH. HLH cells egressed to the medium were also analyzed including those cells obtained by gentle squeezing of the collagen sponge after three days of culture and removal of tissue blocks, and technical replicates were defined and pooled from the same wells as for corresponding samples of HLH blocks. For HLAC, a technical replicate was defined as a pool of 2 wells in a 96U-well plate with 2×10^6 cells/well. 1 to 3 technical replicates were analyzed for each donor and condition for HLAC.

2.2.2. Treatment with drugs and cytokines

Where indicated, cultures were treated with the following drugs at the indicated final concentrations: 8 μg/ml anti-CD40L (Ruplizumab, Creative Biolabs) or 8 μg/ml UltraLEAF human IgG1 isotype control (clone QA16A12, BioLegend); 0.5 μg/ml trimerized rhCD40L (BioLegend), 20 ng/ml rhIL-4 (BioLegend) or PBS solvent control; 2 μM JAK inhibitors Tofacitinib or Baricitinib (both: abcr GmbH) or DMSO solvent control; 14.8 μg/ml IL6R blocking antibody Tocilizumab or human IgG1 as isotype control; 0.5 μg/ml (3.3 nM; "low" concentration) or 10 μg/ml (67 nM; "high" concentration) of each TNF blocking reagents Etanercept or Adalimumab, or human IgG1 as isotype control. Further details on plate positioning and drug treatment are provided in Sections 1 and 2 of the Supplemental materials and methods.

2.3. Adenoid cell culture as activation-induced marker (AIM) assay

AIM assays were modified based on Ref. [28]. In brief, cryopreserved adenoid suspension cells prepared as "HLAC" were thawed, and then cultured in 96U-well plates with 1.4×10^6 cells/well in 200 μl CMT medium unless stated otherwise. Cells were either left unstimulated, or were stimulated with Pertussis Toxin mutant (PT; enzymatically inactive point mutant, highly purified and low endotoxin-tested, List Biological Laboratories *via* Biotrend) at 0.1, 1 or 5 μg/ml final concentration. As a control, cells were stimulated with the superantigen Cytostim (Miltenyi Biotec). Cells were cultured for 18–19 h in the AIM assay and subsequently, AIM markers PD-L1, CD25 and OX40 were measured on non-Treg T cells by flow cytometry. For details and variations of the AIM assay, as well as antibody panels, see Supplemental materials and methods Sections 3–6.

2.4. Flow cytometry

Cells were cultured as HLH or HLAC as above, and after the desired time period of culture, cells from each technical replicate were pooled as follows. 4–8 HLH tissue blocks (= 1 technical replicate) were minced between the frosted ends of glass slides in PBS + 2 mM EDTA, and the resulting cell suspension was strained through 50 μm cell strainers. Egressed HLH cells were collected from the culture medium of HLH plates (same wells pooled per replicate where applicable) after removal of HLH tissue blocks and gentle squeezing of the collagen sponge. Uncultured tissue was kept at 4 °C in CMT medium and then processed, as described for HLH tissue blocks, to a single-cell suspension for flow cytometry staining. For HLAC, two wells of a 96U-well-plate (= 1 technical replicate) were pooled before staining unless for AIM assays in which a single well was used per replicate. Cells were centrifuged (500×*g*, 5 min), and the supernatant from HLAC and HLH egressed cells was collected and stored at −20 °C for cytokine measurements. Cells were resuspended in Flow buffer (PBS with 2 mM EDTA and 1% FBS) and each technical replicate was then split into B and T cell panel samples if applicable. Surface, viability, and intracellular staining was performed according to standard procedures, and is detailed together with antibodies and panels in Section 6 of the Supplemental materials and methods. Acquisition was performed on a BD LSRFortessa™ cell analyzer (BD Biosciences) and data were analyzed with FlowJo software version v10.4.2 (FlowJo, LLC).

2.5. Measurement of secreted cytokines and IgA

Cytokines in HLH, HLAC and AIM assay supernatants were measured by Human Magnetic Luminex Assays using a Premixed Multiplex assay (for detection of IFN-γ, IL-2, IL-4, IL-6, IL-17A, IL-21, IL-22, TNF-α) from R&D Systems according to the manufacturer's recommendations. Samples were diluted 1:2. Acquisition was performed on a MAGPIX system and data were analyzed with Milliplex Analyst or xPONENT software (Luminex, Merck Millipore). For sample sets in which IL-6 concentrations exceeded the standard, IL-6 was measured by IL-6 ELISA (Thermofisher Scientific) in accordingly diluted samples as per manufacturer's instructions. Secreted IgA was measured by ELISA (IgA human uncoated ELISA kit, Thermofisher Scientific) in appropriately diluted samples as per manufacturer's instructions.

2.6. Data analysis

Statistical analyses and data visualization were performed in R programing language or GraphPad Prism (version 7). Means of technical replicates per donor and culture condition and treatment were calculated and used for statistical analysis. Normal distribution of data was confirmed by Shapiro-Wilk normality test and significance was calculated by two-tailed paired *t*-test (pairing within a donor). Differences were considered significant if $p<0.05$.

3. Results

3.1. Systematic comparison of HLH and HLAC reveals comparable T and B lymphocyte fractions

Only few studies have compared HLH (tonsil tissue blocks cultured on collagen sponges at air:liquid interface) and HLAC (tonsil suspension cell) cultures [21,23,24,26], and none has provided detailed phenotyping of T and B cell subsets that form the complex structural organization of GCs [2]. Indeed, the size of adenoid HLH tissue blocks ($1 \times 1 \times 2$ mm) permitted the presence of entire B cell follicles and GCs (Supplemental Fig. 1a). We systematically compared the phenotype of T and B cells derived from adenoid HLH and HLAC cultures. In HLH cultures, we studied cells in tissue blocks along with those egressed into the medium after three days of culture (experimental setup, see Supplemental Fig. 1b). We also compared the cultured cells to *ex vivo* "uncultured" tissue to determine potential changes occurring during culture. All flow cytometric analyses were performed after strict gating on viable cells. This is especially important for HLH in which the viability was decreased particularly in cells residing in tissue blocks (Fig. 1a). Despite differing viability, the fractions (within live cells) of B cells, T cells and $CD4^+$ T helper cells were similar across culture conditions (Fig. 1b–d), indicating that HLH cultures can be used for cellular sub-phenotyping when live cell gating is used. While T cell:B cell ratios increased in all culture conditions compared to uncultured tissue (Fig. 1b-c), the fraction of T helper among T cells remained largely unchanged (Fig. 1d).

3.2. Detailed phenotyping of T helper and B cell subsets uncovers specific culture-related changes

Next, we focused on a more detailed analysis of $CD4^+$ T cell and B cell subsets within the cultures. We established comprehensive flow cytometry phenotyping panels with a focus on Tfh and GC B cells (Supplemental Figs. 2 and 3). General gating on follicular T cells based on high CXCR5 and PD1 expression identified distinct cell populations (Fig. 2a). However, gating solely *via* CXCR5 and PD1 cannot sufficiently denote Tfh cells, as Tfr cells may also be contained in this gate and can only be excluded by intracellular staining for FOXP3 [9]. To exclude Tregs and Tfr cells, but include activated T cells that can up-regulate low levels of FOXP3 in humans [29], we examined a gating strategy commonly used for human blood [30] to distinguish naïve Tregs, activated Tregs, and conventional T cells based on FOXP3 and CD45RO (Supplemental Fig. 2a). Since $CXCR5^{hi}PD1^{hi}$ cells lacked high FOXP3 expression but displayed a slight shift in FOXP3 levels (Supplemental Fig. 2b and c), we included a part of the $CD45RO^+FOXP3^{low}$ population (so-called "fraction III" [30]) in our pre-gating on non-Treg memory T cells (here called "non-Treg Tmem"; Supplemental Fig. 2a and c). We further ensured (Supplemental Fig. 2d) that after pre-gating on non-Treg Tmem, within the GC Tfh gate, virtually no $Helios^+FOXP3^+$ cells were present that would represent Tfr cells, which are believed to be of thymic origin hence expressing Helios [4,9].

Along with GC Tfh cells, we also gated on mantle Tfh (mTfh) and non-Tfh cells as described [28,31] based on CXCR5 and PD1 expression, in comparison to gating based on CXCR5 levels only (Supplemental Fig. 2a). Notably, we observed that in any culture condition, compared to uncultured cells, fractions of the memory T helper cell population expressing intermediate levels of CXCR5 were decreased while those of the $CXCR5^{hi}$ population were simultaneously increased (Fig. 2a and b). However, these $CXCR5^{hi}$ cells did not all represent *bona fide* GC Tfh cells as they down-regulated PD1 and ICOS and switched from an activated to quiescent state [4,10,15] (Fig. 2a–c). Interestingly, $CXCR5^{hi}$ cells emerging during culture displayed a Tfh17-like phenotype based on expression of CCR6 and CXCR3 [4,10,15,32] (Supplemental Fig. 4). Culture-related changes also occurred in the B cell compartment, most notably a strong reduction in GC B cell frequencies was observed when gating these cells as $BCL6^+CXCR5^+$ or $CD95^+CD38^+$ (Fig. 2d; Supplemental Fig. 3).

By analyzing tissue immediately after surgery or after storage for three days at 4 °C (defined as "uncultured" tissue), we confirmed that the observed changes indeed occurred during HLH and HLAC cultures, but not in uncultured tissue that had been stored at 4 °C until analysis (Supplemental Fig. 5a–c). The outcome was the same whether "uncultured" tissue was stored at 4 °C in tissue chunks or as cell suspension prepared according to the HLAC protocol (data not shown). Accordingly, we confirmed by kinetic analysis that the observed changes occurred progressively over time during HLH and HLAC culture (Supplemental Fig. 5d) and hence are not due to differences in cell recovery during preparation of HLAC single cells *versus* uncultured cells.

3.3. Manipulation of CD40L and IL-4 signals alters GC B cell maintenance in human lymphoid tissue cultures

As a first proof-of-concept of our *ex vivo* platform for drug testing, we next assessed whether GC cells could be manipulated by anti-inflammatory drugs that target relevant and well-known cytokine and signaling pathways. CD40L is an important molecule in the interaction of Tfh and GC B cells, indispensable for GC reactions and GC B cell survival [33–35]. The therapeutic antibody Ruplizumab blocks CD40:CD40L interactions and was developed for treatment of rheumatic diseases such as systemic lupus erythematosus (SLE), but despite promising outcomes regarding SLE disease activity, clinical studies were halted due to thromboembolic events when used systemically [36]. Recently, alternative CD40L blocking proteins that are Fc receptor silent to prevent such side effects [37–39] have been designed for future clinical development, and the results further confirm the promise of CD40L blocking to treat human diseases. When treating HLAC cultures with Ruplizumab for three days, GC B cell frequencies were specifically reduced, irrespective of the gating strategy used to define GC B cells (Fig. 3a and b). Importantly, and supporting specific effects on GC B cells, frequencies of gated parent B cell populations were not affected (Supplemental Fig. 6a). Interestingly, despite GC B cell decrease, GC Tfh cell frequencies (and most other T cell subsets) remained unchanged (Supplemental Fig. 6b and data not shown). When further inspecting the full T cell panel for changes induced by CD40L blocking, we detected a significant decrease in $OX40^+$ fractions within CD4 T cells (Supplemental Fig. 6b), suggesting the possibility of back-signaling from CD40L into T cells or indirect effects caused by GC B cell decrease.

Since blocking of CD40L reduced GC B cell frequencies (Fig. 3b), which also decreased during culture in general (Fig. 2d), we asked whether providing exogenous CD40L could rescue GC B cell frequencies. Indeed, addition of CD40L (together with IL-4) rescued BCL6-expressing GC B cells in HLAC cultures, reaching levels similar to uncultured tissue (Fig. 3c). Interestingly, this effect specifically concerned BCL6 expression in GC B cells, as neither frequencies of GC B cells defined by BCL6-independent gating strategies nor BCL6 in Tfh cells were enhanced by the addition of CD40L + IL-4 (Supplemental Fig. 6c and d). While combination of CD40L and IL-4 rescued BCL6 expression in GC B cells, CD40L or IL-4 alone had lesser effects

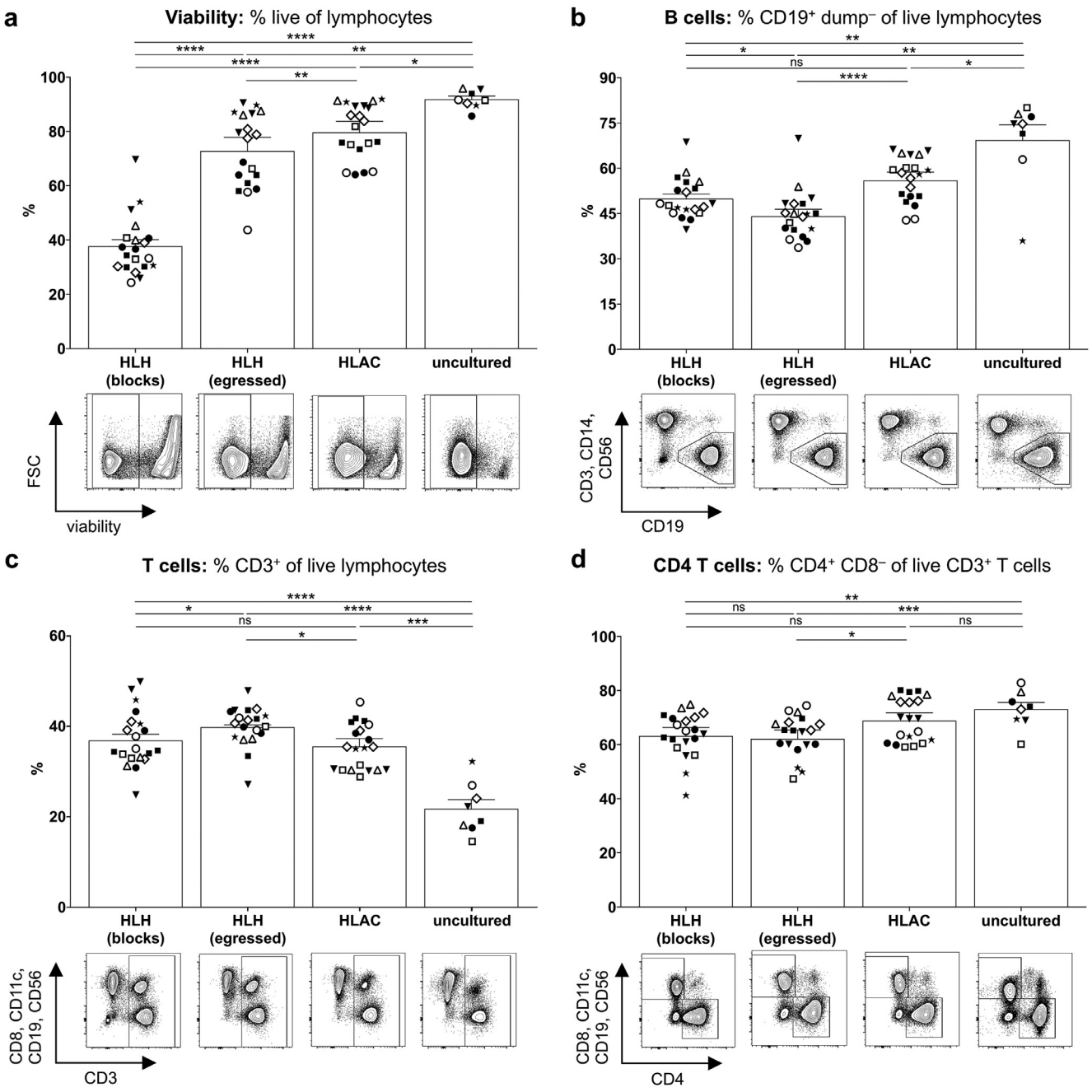

Fig. 1. Overall T and B cell frequencies are comparable in HLH and HLAC *ex vivo* cultures despite different viability. (a–d) Adenoid tissues were cultured as HLH or HLAC (see text) for 3 days. From HLH cultures, cells that had remained in the tissue blocks after culture ("blocks"), as well as those cells that had egressed from the tissue blocks into the medium ("egressed") were studied in parallel. As a comparison, tissue analyzed after surgery without further culture (storage at 4 °C) is shown as "uncultured". Cells were analyzed by flow cytometry, always pre-gating on live cells by viability dye as in (a). For full gating strategy, see Supplemental Figs. 2 and 3. Data is quantified in the bar charts. Intra- and inter-donor variation is displayed as follows: Same symbols (shape and color) represent the same donor, with multiple identical symbols representing technical replicates. A technical replicate is defined as follows: for HLH as a pool of 4–8 tissue blocks derived from 1–2 wells in 12-well culture plates; for HLAC as a pool of two wells from a 96U-well plate. Each technical replicate was then split into two panels. The same symbol shape (not color) represents experiment (batch). For statistical analysis, the mean of all technical replicates was calculated per donor and culture condition, and then a two-tailed paired *t*-test was performed with $n = 8$ donors (pairing within a donor; significance indicated by following symbols: *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$; ****: $p<0.0001$; ns: not significant). The bars and error bars indicate mean values and corresponding SEM (of all donors, using mean of technical replicates per donor as input). Representative contour plots are depicted for each indicated culture condition below the bar charts.

(Fig. 3c; Supplemental Fig. 6e). Despite high fractions of GC Tfh cells, IL-4 and IL-21 levels in the cultures were barely above detection limit (Supplemental Fig. 7a), concurring with the well-described low-level cytokine production by GC Tfh cells whose function is mainly to influence B cells in close proximity [16,28]. This may explain the effect of IL-4 supplementation, although short-range cytokine signals upon direct cell contact may still occur at low overall concentrations. IL-4, IL-21, and other cytokines measured by multiplex assay were not significantly affected by CD40L blocking (Supplemental Fig. 7a and data not shown). Unlike in peripheral blood [40] and as described by others for tonsils [28,31], we confirmed that adenoid T cells displayed baseline expression of CD40L (Supplemental Fig. 7b). Nevertheless, addition of exogenous CD40L conferred improved maintenance of GC B cells (Fig. 3c). As GC B cells are tightly linked to differentiation of

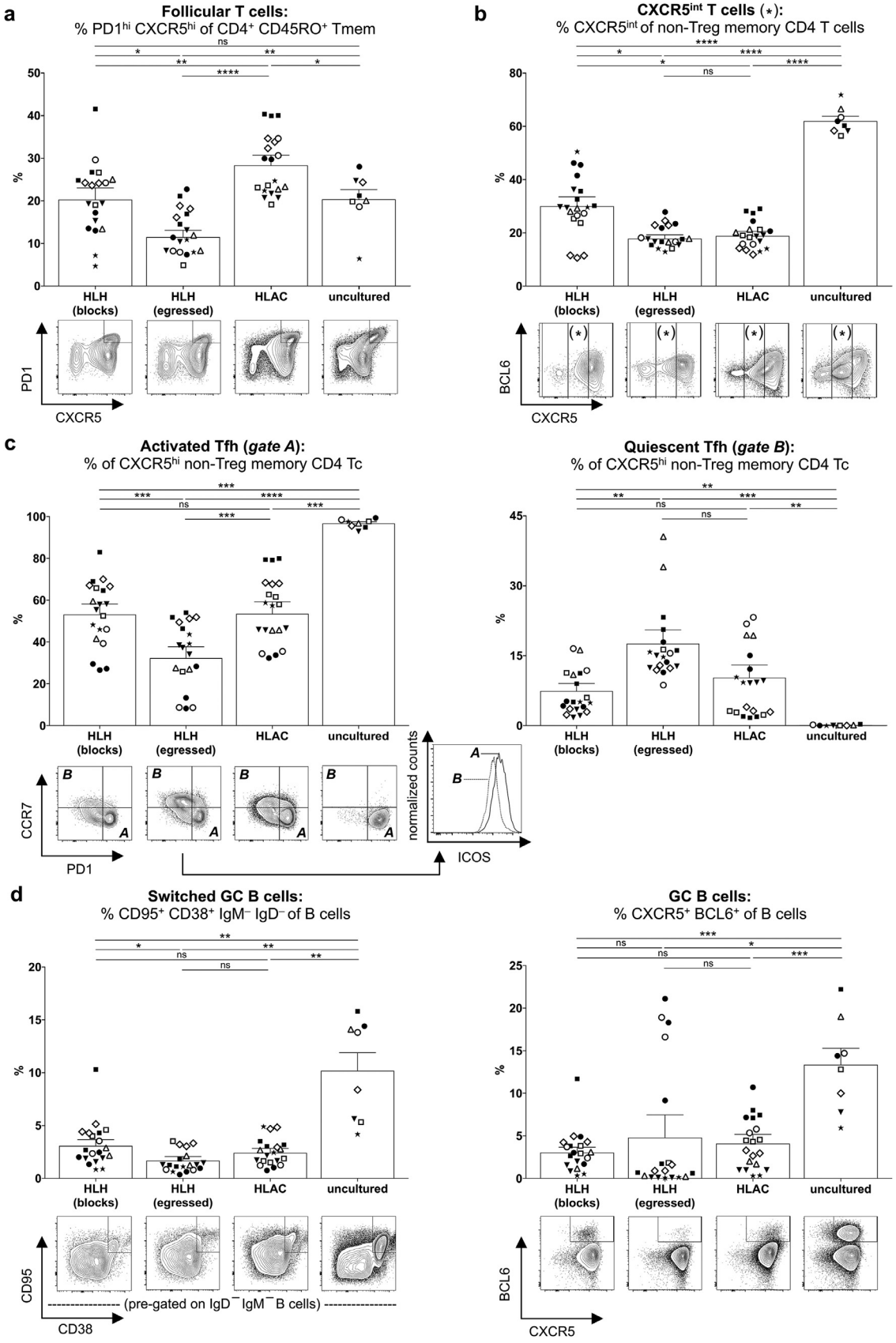

Fig. 2. Tfh and GC B cell populations in HLH and HLAC cultures display specific changes compared to uncultured tissue. (a–d) Adenoid tissues were cultured and analyzed by flow cytometry as in Fig. 1. Asterisks "(*)" in contour plots in (b) denote the CXCR5^{int} T cell population. For (c), the contour plots are representative for both the left and right panels representing data derived from gates *A* and *B*, respectively (indicated by labels "*A*" and "*B*"). The histograms show ICOS expression, overlaid for gates *A* and *B*, exemplarily shown for cells egressed from HLH blocks. For (d), contour plots in the left panel are shown for CD95 *versus* CD38 after pre-gating on non-naive switched (IgD$^-$ IgM$^-$) B cells, and the fraction of these cells of all (CD19$^+$) B cells was calculated and summarized in the bar charts. Data is represented and was statistically analyzed as in Fig. 1.

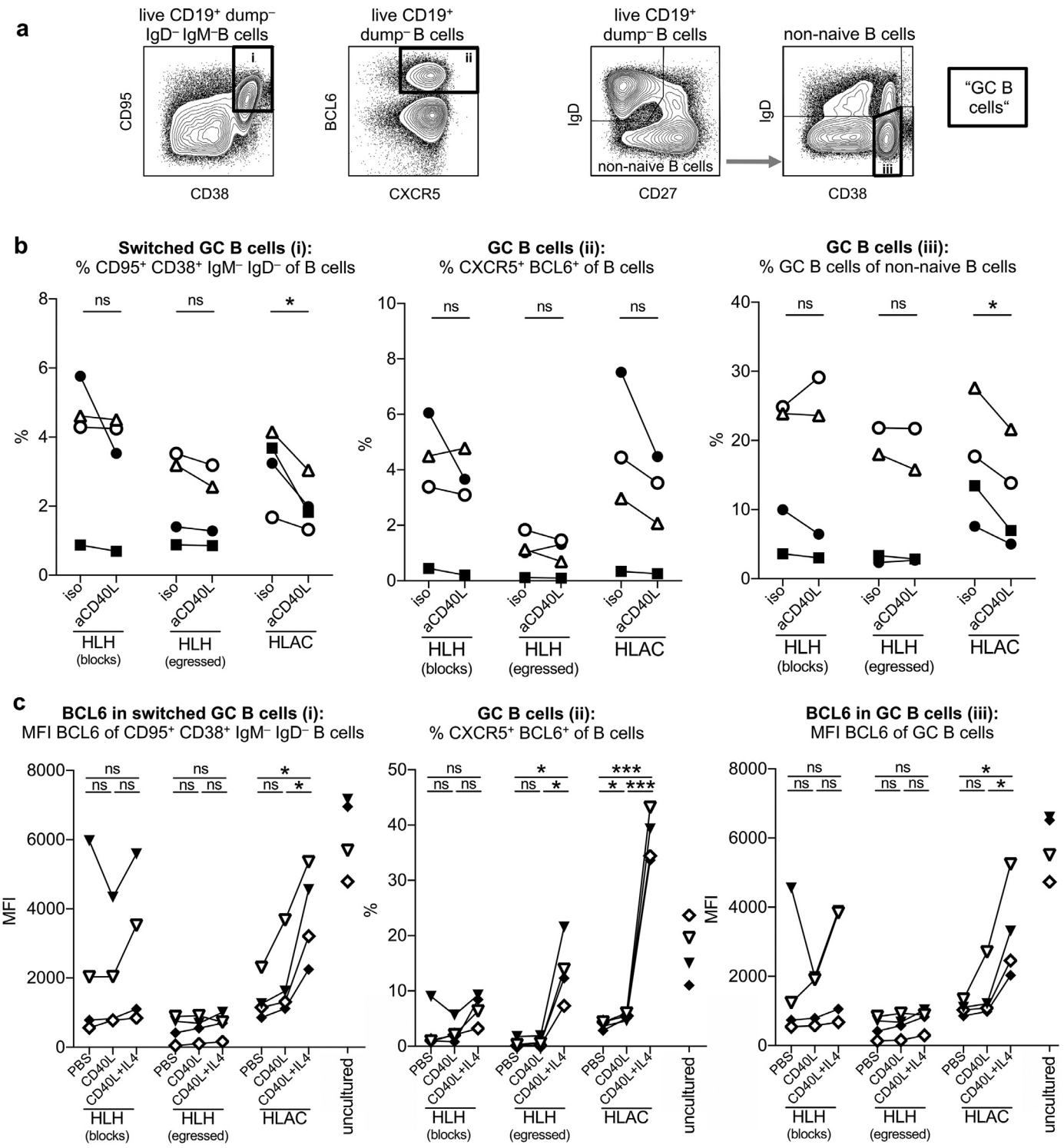

Fig. 3. Blocking of CD40L reduces GC B cells in HLAC cultures, while CD40L + IL-4 improve GC B cell maintenance. (a) Gating strategy to define GC B cells by different alternative marker combinations (referred to as "i", "ii", and "iii" and indicated by bold gate outlines). The "dump" channel included CD3, CD14 and CD56. For full gating strategy, see Supplemental Figs. 2 and 3. (b) Adenoid tissues were cultured as HLH and HLAC for 3 days in the presence of isotype control antibody ("iso") or CD40L blocking antibody ("aCD40L") and then analyzed by flow cytometry. 2–3 technical replicates were plated per donor and condition (as described in Fig. 1, with 4–8 tissue blocks pooled for HLH and 2 × 96 U wells pooled for HLAC), and the mean values of technical replicates were calculated and are indicated by the symbols. Each symbol type represents one donor ($n = 4$ donors in 3 independent experiments). (c) Adenoid tissues were cultured for 3 days as HLAC or HLH in the presence of CD40L or CD40L + IL-4, or PBS as control. Uncultured tissue from the same donors is shown on the right of each graph. Symbols represent means of technical replicates per donor (2 technical replicates each with 4 or 6 tissue blocks pooled for HLH; 1 or 2 replicates each for HLAC). Each symbol type represents one donor ($n = 4$ donors in 2 independent experiments). Significance was calculated by two-tailed paired t-test and is indicated by labels as described in Fig. 1.

memory B cells and long-lived antibody-producing plasma cells, we also measured the secretion of IgA in the supernatants. Although class-switched antibodies were secreted at high amounts, there was no significant difference in IgA levels upon CD40L blocking (Supplemental Fig. 7c) at least at the relatively early time point of culture we studied (3 days). Plasma cells expressed high levels of intracellular

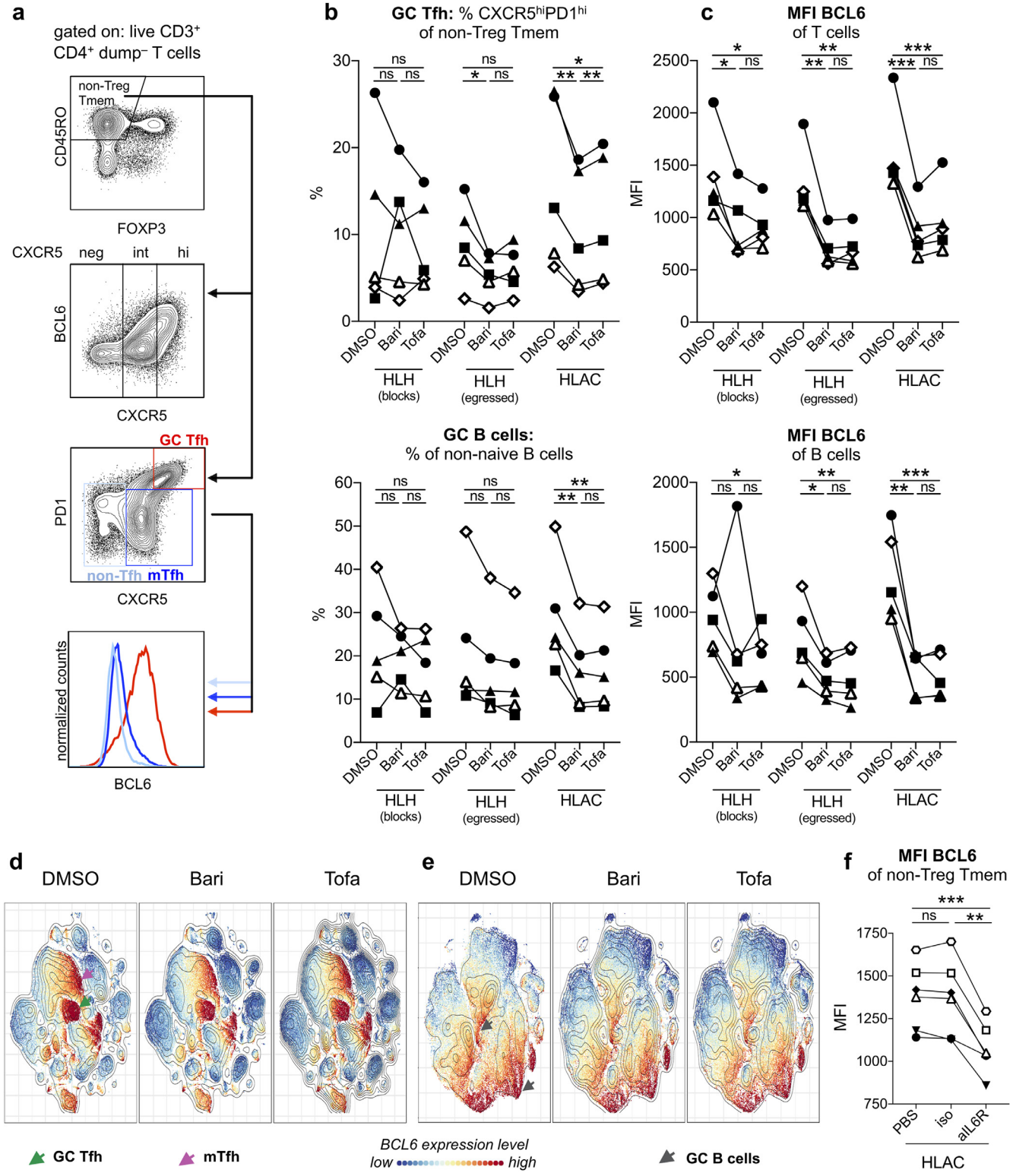

Fig. 4. JAK inhibition reduces BCL6 expression in T and B cells. (a) Gating strategy to define Tfh cell populations in adenoid tissue of a representative donor. Cells were pre-gated on live CD4$^+$ T cells (not shown; the "dump" channel included CD8, CD11c, CD19 and CD56). Naïve T cells and Tregs were then excluded and "non-Treg Tmem" were subsequently gated on CXCR5 negative ("neg"), intermediate ("int"), and high ("hi"). Alternatively, non-Tfh, mantle Tfh (mTfh) and GC Tfh cells were distinguished based on PD1 and CXCR5 expression. Histograms show BCL6 expression by the different Tfh and non-Tfh cell populations, as indicated by the colors. For full gating strategies see Supplemental Figs. 2 and 3. Plots show results from adenoid tissue of a representative donor. (b–e) Adenoid tissues were cultured as HLH and HLAC for 3 days in the presence of the JAK inhibitors Baricitinib ("Bari"), Tofacitinib ("Tofa") or "DMSO" solvent control, and analyzed by flow cytometry. 2–3 technical replicates were plated per donor and condition (a technical replicate equals 8 tissue blocks pooled for HLH and 2 × 96 U wells pooled for HLAC). (b-c) Mean values of technical replicates were calculated and are indicated by the symbols. Donors are indicated by individual symbols (shape and color, $n = 5$ donors in 4 independent experiments; same symbol shape = same experiment). Significance was calculated by two-tailed paired *t*-test (pairing within each donor as indicated by the connecting lines) and is labeled as described in Fig. 1. (b) shows frequencies of GC Tfh cells and GC B cells, while (c) shows median fluorescence intensity (MFI) of BCL6 in T and B cells, respectively. (d-e) Dimensionality reduction analysis by t-SNE was applied to gated live T cells (d) or live B cells (e). All 5 donors, culture conditions and drug treatments were analyzed together (per panel), and densities of cell populations are visualized individually for the three treatments. Selected cell populations, such as Tfh and GC B cells, are highlighted with arrowheads based on their marker profile. All individual markers are shown in Supplemental Figs. 9 and 10, and BCL6 is

Igκ (Supplemental Fig. 3). However, as also observed by others in tonsils [41,42] and as expected since plasma cells rapidly leave the lymphoid tissue, the frequencies of plasma cells were very low and did not show significant differences upon CD40L blocking (Supplemental Fig. 3 and data not shown).

Altogether, these data present proof-of-principle that the adenoid culture systems established here can be utilized to observe effects of therapeutic experimental manipulation of molecules known to be important for GC cells.

3.4. JAK inhibition reduces BCL6 expression, Tfh and GC B cell frequencies

After demonstrating effects of therapeutic antibodies in HLAC above, we next strived to test small molecule compounds with expected strong impact on immune cells. Cytokines crucially direct and shape T and B cell differentiation, and JAK/STAT signaling is a major pathway downstream of most cytokine receptors [5,43]. We utilized the anti-inflammatory, small molecule JAK inhibitors (JAKi) Baricitinib and Tofacitinib, which are clinically approved for treatment of rheumatoid arthritis and in clinical trials for other autoimmune diseases such as SLE [44]. We observed a drastic reduction of GC B and Tfh cell frequencies when treating for three days with either of the JAK inhibitors (Fig. 4a and b; Supplemental Fig. 8a). Specifically, we observed significantly reduced expression levels of BCL6 in all culture conditions (Fig. 4c). At the same time, parent populations and viability were only marginally affected (Supplemental Fig. 8b and data not shown). Other subsets affected by JAKi were $OX40^+$ T cells and activated Tregs (Supplemental Fig. 8c-d). Unbiased analysis by dimensionality reduction confirmed that BCL6-expressing T cells were among the populations most strongly affected by JAK inhibition (Fig. 4d and e; Supplemental Figs. 9 and 10).

Since cytokine receptor signaling involves layers of feedback regulation, we also studied cytokines in the culture supernatants, and determined that concentrations of several cytokines were significantly affected by JAK inhibition (Supplemental Fig. 11).

Mechanistically, IL-4 signals through JAK1 and JAK3, with JAK1 being a target of both Tofacitinib and Baricitinib [44]. Since IL-4 as well as JAK inhibition influenced BCL6 in B cells (Figs. 3c, 4b and c), inhibition of IL-4 signaling by JAK inhibition may be responsible for the observed effects of JAK inhibitors on B cells. Next, we asked which cytokine receptor signals were involved in the effects of JAK inhibition on BCL6 expression in T cells, which did not seem affected by IL-4 (Supplemental Fig. 6d). Instead, inhibiting IL6R signaling by the clinically approved blocking antibody Tocilizumab led to rapid reduction of BCL6 expression in T cells (but not B cells) (Fig. 4f and data not shown). Thus, inhibiting the IL6R pathway, which signals *via* the Tofacitinib- and Baricitinib-targets JAK1 and JAK2 [44], can mirror the effects of JAK inhibition on T cells, suggesting that IL-6 contributes to BCL6 maintenance in human adenoid T cells *ex vivo*.

3.5. Antigen-responsive T cells can be measured in a rapid surface marker and cytokine secretion assay

Since the GC cell phenotype changed across three days in culture, we aimed at developing a shorter assay and, additionally, at enhancing the read-outs of immune cell activity by stimulation with a physiologically relevant antigen. Adenoid T and B cells did not spontaneously proliferate in culture, and polyclonal T cell stimulation with anti-CD3 and anti-CD28 antibodies triggered proliferation that needed 4–6 days to become well-detectable (data not shown). Hence, we modified a previously described [28] rapid activation-induced marker (AIM) assay that relies on the induction of CD25, OX40 and PD-L1 within 18 h, since commonly used read-outs for antigen-reactive peripheral blood T cells [40] are less suited for tonsil Tfh cells due to high background expression of CD40L paired with low intracellular cytokine expression [28,31]. The original [28,45] and follow-up papers [16,46,47] using the AIM assay have not excluded FOXP3-expressing Tregs although this has been suggested to be important [48]. Thus, we optimized the AIM assay gating strategy to exclude Tregs, which turned out to be especially important when using CD25 as AIM marker (Supplemental Fig. 12).

Since we used adenoid material from a cohort of young children of unknown infection history, we sought to establish stimulation with an antigen included in infant vaccine regimens with locally high vaccination coverage [49] that is, inactivated pertussis toxin (PT) protein. When stimulating HLAC cultures with an enzymatically inactive point mutant of PT, we observed highly reproducible, strong induction of above AIM markers in a dose-dependent manner in non-Treg memory T cells including GC Tfh cells (Fig. 5a and b). At the same time, the overall fraction of the gated parent T cell population was not changed (Fig. 5a). Other T helper cell subpopulations, as well as CD8 T cells, also responded to PT with upregulation of the three AIM markers, and B cells up-regulated CD25 and to a lower extent PD-L1 (Supplemental Fig. 12a and b and data not shown). Simultaneously, typical T cell cytokines (*e.g.* TNFα, IFN-γ, IL-4, IL-17A) were released in response to PT (Fig. 5c). While PT-induced AIM upregulation occurred to a lesser extent in HLH cultures, HLAC cultures provide additional advantages regarding the fact that cryopreserved cells can be used, with near-identical outcomes when fresh and cryopreserved cells from the same donor were compared (data not shown).

3.6. Inhibition of Pertussis Toxin mutant-induced immune responses by anti-inflammatory drug treatment

The response to PT led to a high fraction of AIM-positive cells in all T cell subpopulations including some naïve cells (Fig. 5a; Supplemental Fig. 12a and b), and hence it was plausible that bystander activation of non-PT-specific cells was involved. Notably, similarly high fractions of AIM-positive tonsil cells were observed by others using streptococcus protein antigen (SpeA) as stimulus [16], although originally the authors concluded that bystander activation in the AIM assay was negligible [28]. First, we confirmed that high fractions of AIM-positive cells were not due to activation by non-human FBS-derived proteins, since performing the AIM assay with human AB serum instead of FBS-containing medium led to similarly high fractions of responding cells (Supplemental Fig. 13). We also confirmed that the response was PT-dose-dependent and occurred across different cell densities (Supplemental Fig. 14a). Upon longer culture (4–6 days), PT also induced proliferation of T cells but not B cells (Supplemental Fig. 14b). We furthermore excluded direct effects of PT on isolated T cells by stimulating high-purity sorted naïve $CD4^+$ T cells, total $CD4^+$ T cells, memory $CD4^+$ T cells or cocultures of $CD4^+$ T and B cells, which revealed that B and T cells were necessary and sufficient to mediate the AIM response to PT, while T cells alone did not react (Supplemental Fig. 15). The AIM response in whole HLAC cultures was stronger than in density-matched isolated T cell:B cell cocultures (Supplemental Fig. 15), which may suggest not only mutual interaction and activation loops of T and B cells but also the contribution of non-lymphocyte subsets to the AIM response. Although we cannot exclude the latter, the combined total fraction of non-lymphocyte populations in adenoid HLAC cell suspensions was below 5% (Supplemental Fig. 16).

shown as an example here with low (blue) to high (red) expression according to the color scale. (f) Adenoid cells were cultured as HLAC for 20 h in the presence of 100 nM IL6R blocking antibody Tocilizumab ("aIL6R"), equal amounts of hIgG1 isotype control ("iso"), or equivalent dilution of solvent control ("PBS"; 1:40). Donors are indicated by individual symbols (shape and color; $n = 6$ donors in 2 independent experiments with symbol color indicating experimental batch). Significance was calculated by two-tailed paired *t*-test (pairing within each donor) and is labeled as in Fig. 1.

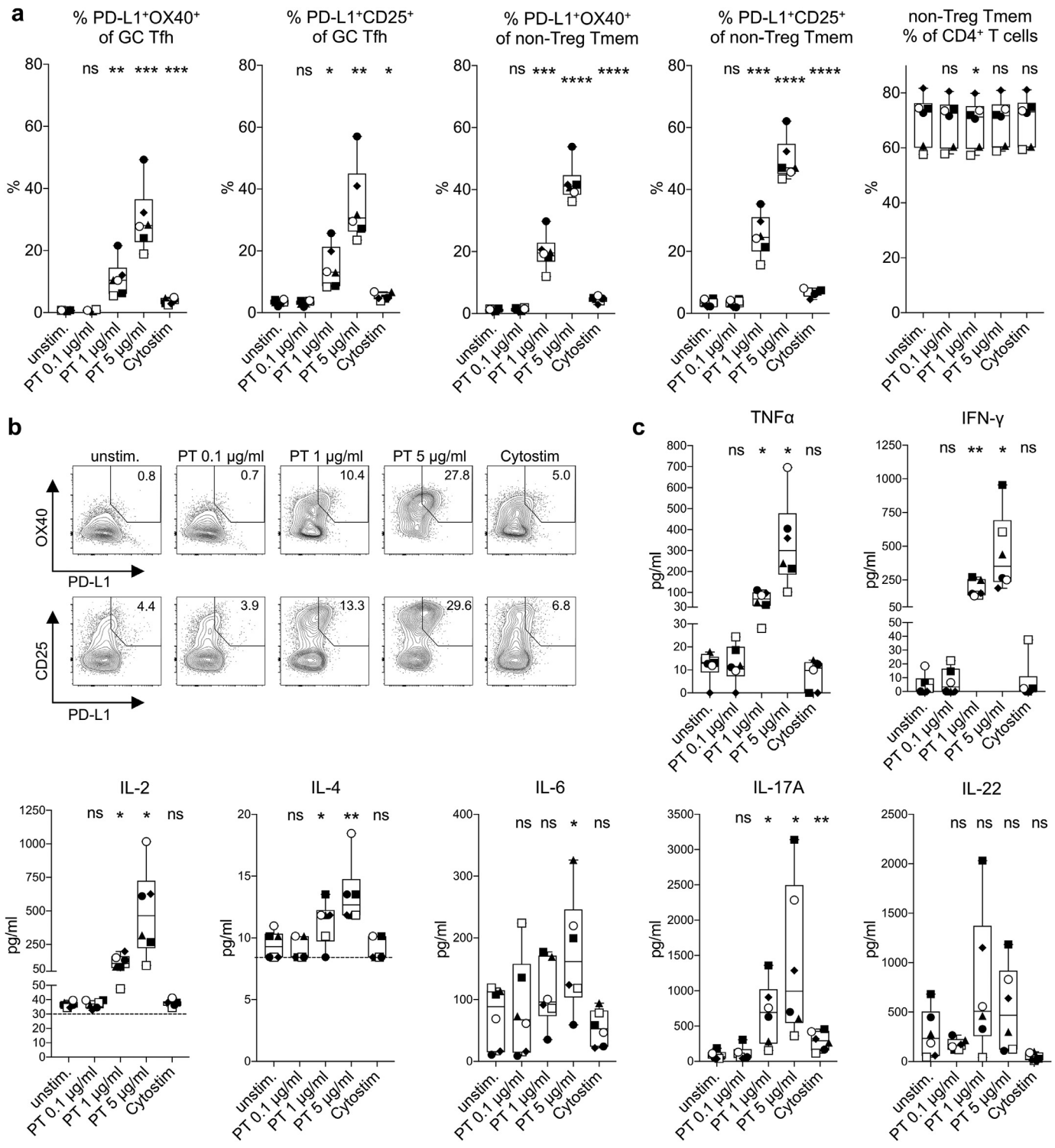

Fig. 5. Antigen-responsive cells can be measured in a surface marker assay and release a multitude of cytokines. HLAC cells were stimulated for 18 h in an AIM assay with Pertussis toxin mutant (PT), Cytostim as a control, or left unstimulated ("unstim."). Data are displayed as box plots with whiskers indicating minimum to maximum values, and $n = 6$ donors represented by individual symbols. One representative experiment of 3 (with 5–6 donors each) is shown. Significance was calculated by two-tailed paired *t*-test (pairing within each donor). p values of indicated stimuli *versus* "unstim." culture in medium only are indicated as follows: ns: not significant; *: $p<0.05$; **: $p<0.01$, ***: $p<0.001$; ****: $p<0.0001$. (a) Frequencies of various T helper cell populations were determined by flow cytometry. All samples were pre-gated on live singlet CD4$^+$ T cells; see Supplemental Fig. 12a-b for gating strategy. (b) Contour plots quantifying AIM-double-positive cells within GC Tfh cells from one representative donor by flow cytometry as in (a). (c) Cytokines in supernatants of AIM cultures were measured by Luminex. Cytokine values below detection limit were set to "0"; cytokines in medium only (without cells) were undetectable except for IL-2 and IL-4 where values detected in medium alone are indicated by the dashed line. IL-21 (not shown) was undetectable in all samples.

Together with reports of direct stimulation of TCR signaling pathways in T cells by PT given that costimulation is present [50–52], these data suggest that B cells provide costimulation that is necessary to trigger an antigen specificity-independent AIM response of T cells to PT stimulation of the TCR complex. Consistent with antigen-independent activation by PT-induced cytokines being involved in induction of AIMs, we observed that neither tetanus toxoid protein (that is often included in vaccine formulations together with PT) nor

Pertussis- or Tetanus-derived peptide megapools could trigger a strong AIM response in human adenoid cells (Supplemental Fig. 17 and Supplemental methods). Instead, peptide pools only marginally increased the fraction of activated cells (Supplemental Fig. 17), consistent with an expected small fraction of antigen-specific cells.

We reasoned that after initial activation of PT-reactive and/or antigen-specific T and B cells, bystander activation may be triggered by cytokine release and hence may be suppressible by JAKi. Thus, we combined PT stimulation with Baricitinib treatment, and JAKi inhibited basal and activation-induced CD25 and PD-L1 expression, while interestingly OX40 upregulation was not prevented (Fig. 6a and b; Supplemental Fig. 18). Instead, OX40 was significantly up-regulated upon Baricitinib treatment (Fig. 6b), confirming our results in unstimulated three-day cultures (Supplemental Fig. 8c) and suggesting different mechanisms for induction of the different AIMs. Besides, release of some but not all PT-induced cytokines could be prevented by JAK inhibition (Fig. 6c, Supplemental Fig. 19a). Notably, we confirmed BCL6 downregulation upon JAK inhibition as described above for three-day cultures after only 20 h of HLAC treatment (Supplemental Fig. 19b). Strikingly, PT stimulation abrogated the JAKi-mediated down-regulation of BCL6 while marginally affecting viability (Supplemental Fig. 19b). While IL6R blocking led to reduced BCL6 expression in T cells (Fig. 4f), it did not affect the induction of AIMs (data not shown), consistent with the fact that PT did not induce much IL-6 release (Supplemental Fig. 19a). Because PT strongly induced TNFα release (Fig. 5c), we asked whether blocking TNF signaling – which does not involve JAK/STAT pathways [53] – could, similarly to the effect of JAK inhibition on the AIM response, abrogate AIM induction by PT. Indeed, blocking TNF signaling with two different clinically approved anti-TNF reagents [53], Etanercept and Adalimumab, led to dose-dependent inhibition of the AIM response affecting upregulation of all three AIMs (Fig. 6d and data not shown) along with decreased levels of TNFα and IFN-γ in the supernatant (Supplemental Fig. 20). This suggests that TNFα, in addition to other cytokines that signal through JAK/STAT pathways, contribute to bystander activation in the PT-induced AIM response.

Altogether, we present a versatile, robust and highly reproducible *ex vivo* human lymphoid tissue response assay triggered by stimulation of T cells with mutant PT protein in the presence of costimulation by B cells, involving cytokine-mediated activation likely similar to *in vivo* settings and suppressible by approved anti-inflammatory drugs.

4. Discussion

We optimized and systematically compared human tonsil-based *ex vivo* culture systems combined with comprehensive flow cytometric phenotyping of T and B cell subsets. Although our phenotyping was limited to a predefined set of known markers, it enabled the detection of certain phenotypic changes in CXCR5$^+$ Tfh-like cells during culture. These changes may reflect a rather physiological acquisition of phenotypes of circulating blood memory Tfh cells, which compared to GC Tfh cells lack BCL6 protein expression, largely lack PD1 and ICOS expression and express CCR7 [1,15]. These changes occurring in the absence of proliferation and despite high viability in HLAC cultures suggest actual phenotypic changes rather than preferential outgrowth of certain populations, and supporting this hypothesis, a similar change in CXCR5 and PD1 expression was recently documented after one-day cultures of murine flow-sorted Tfh cells [47]. During culture, GC B cells were largely lost, which may be due to rapid cell death concurring with overall reduction in B cell frequencies and their pro-apoptotic phenotype and constant need of survival signals [1]. Supporting this scenario, providing the survival signal CD40L maintained GC B cell frequencies and BCL6 expression, in accordance with the described rescue of GC B cells from apoptosis by CD40L, possibly involving sustained BCL6 expression [1,35,54]. Nevertheless, future studies should monitor apoptotic or other cell death phenotypes in more detail and, although difficult to assess in HLH due to differing amounts of input material in tissue blocks, absolute cell numbers could be determined to further dissect phenotypic changes *versus* death of B cells and other cells in the cultures. Such studies could further illuminate the contributions of BCL6 induction *versus* rescue from GC B cell death when CD40L signals are provided, or blocked. Surprisingly, we did not observe significant changes in IgA secretion upon CD40L blocking despite reduced GC B cell frequencies. However, this may be different in longer-term cultures (5 to 14 days) that suffice for B cell to plasma cell differentiation, as performed by others [31,34,55–58]. Furthermore, plasma cell frequencies in our experiments were low, as also observed by others for tonsils [41] and perhaps reflecting that *in vivo* plasma cells should leave the secondary lymphoid organs rapidly or may die *ex vivo*. Instead of *de novo* B cell differentiation, our study likely reflects the effects that anti-inflammatory drugs have on *in vivo* generated, pre-existing GC Tfh and GC B cells, which may be a therapeutically relevant setup for testing drugs that may be used for the treatment of patients with ongoing autoimmune disease.

The generally low viability in HLH blocks is in agreement with other reports [26,59], even though viability data has not always been reported, and advanced tissue deterioration has been described after more than three days of culture [26]. Consequently, HLH cultures have been previously used only for basic cellular phenotyping and for studying of virus production by *ex vivo* infected cells or cytokine release [24,26,27,59]. Our optimized method including live cell gating enabled us to confidently study cells in HLH blocks along with egressed cells. Testing different media (RPMI or X-VIVO15 medium), FBS sources or human serum, media volumes and medium change, culture without amphotericin B, addition of IL-7, culture under "physiological" oxygen levels (5% O_2), different collagen sponge providers, or different sizes of tissue blocks did not lead to improved viability (data not shown).

In general, we found that lower technical variability, reduced amounts of required input material, and higher viability favored HLAC over HLH. Thus, it remains to be shown whether tissue culture systems provide key advantages over suspension cultures. Nevertheless, it is possible that HLH might be more similar to the *in vivo* situation. Accordingly, in settings where stromal cells and/or 3D structure play an important role, HLH could be more suitable than HLAC, as shown for HIV spread and bystander killing [22,23]. We did not observe differences in the phenotypic changes occurring during culture when comparing standard HLAC cultures to HLAC cultures preceded by enzymatic digestion of tissue (unpublished observation). Together with the fact that these changes also occurred in HLH tissue explants, stromal cells that may not be recovered from the tonsillar tissue during processing for HLAC cultures did not seem to play a significant role in the read-outs we studied. However, it should be noted that our read-outs were focused on T and B lymphocytes and flow cytometric analysis, hence the determined composition of immune cells in our study might exclude "sticky" or very fragile cells. Furthermore, other tissues which are less "soft" than adenoid tissue may behave differently regarding cell recovery with different extraction methods. To study cells such as follicular dendritic cells or other stromal cells, microscopy may be more suitable than flow cytometry, and different methods of cell extraction may be required [23,60,61]. As noted above, it cannot be excluded that for certain immune cell reactions the presence of stromal cells is required and hence such reactions may not be accurately reflected in our culture system. Therefore, it will be important in the future to devise *ex vivo* tissue cultures that can model the natural structure and composition of human lymphoid tissue and maintain viability and cellular phenotypes for several weeks, especially in order to enable modeling of long-term cell differentiation events such as *de novo* antigen-specific GC responses. However, others have shown that co-cultures of sorted

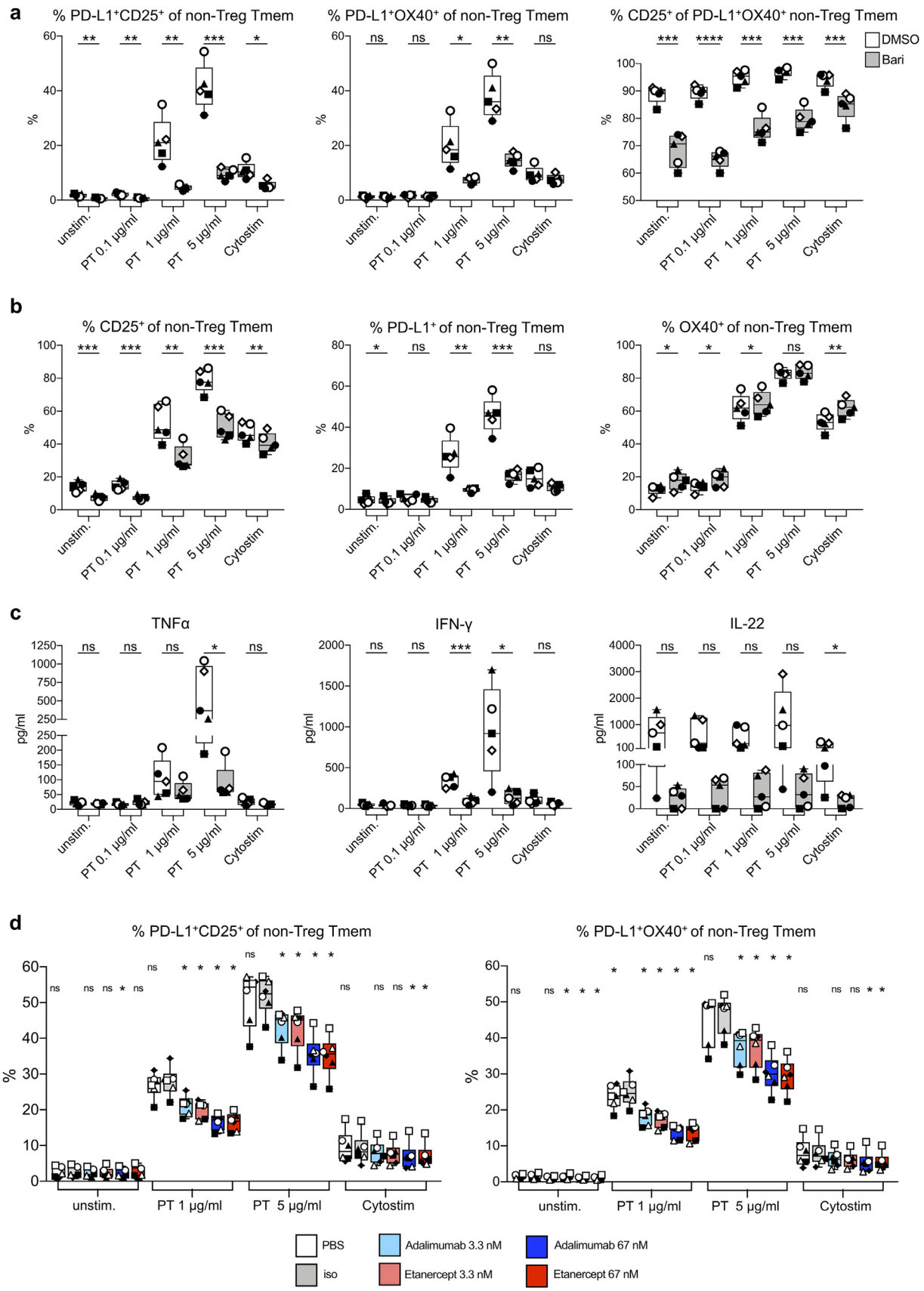

Fig. 6. The AIM response can be blocked by JAK/STAT inhibition and TNF also contributes to PT-induced bystander activation. (a–c) HLAC cells were pre-incubated for 2 h with the JAK inhibitor Baricitinib (Bari; gray boxes) or equivalent dilution of DMSO (white boxes) as solvent control. Subsequently, cells were stimulated for 18 h in the AIM assay with Pertussis toxin mutant (PT), Cytostim as a control, or left unstimulated ("unstim."). Data are displayed as box plots with whiskers indicating minimum to maximum values, and $n = 5$ donors represented by individual symbols. Significance was calculated by two-tailed paired *t*-test (pairing within each donor) and p values of DMSO *versus* Bari treatment are indicated as follows: ns: not significant; *: $p<0.05$; **: $p<0.01$, ***: $p<0.001$; ****: $p<0.0001$. (a,b) Frequencies of various T helper cell populations were determined by flow cytometry,

CD4 T cells and B cells can be used to observe T cell help for B cell antibody production after 5–10 days [31,55], although culture systems maintaining or mimicking the complex *in vivo* 3D structure and cell composition may still be desirable given the importance of spatial organization in initiation and regulation of the GC response [2,62]. Such systems representing the *in vivo* 3D structure of cells in the GC *ex vivo* would rely on tissue explant models, and current set-ups of HLH appear insufficient for long-term assays as it has been described previously that HLH tissue structure decomposes within a few days of culture [26], and this is also supported by our data and the rapid egress of cells from the tissue blocks into the medium. The method described here is suitable for short-term cultures (18 h to 3 days) and rapid screening-like approaches to study drug effects on *in vivo* generated GC cells, while mimicking *de novo* antigen-specific GC responses *in vitro* would take approximately 1–2 weeks of culture. Future efforts should, thus, be made to enhance viability and phenotype stability in HLH. Alternatively, immune-organoids are an exciting avenue as alternative 3D-models, which may be inspired by developments of organoids in other fields [63,64]. For immune organs, such models need further improvement in the future, going beyond recent approaches for murine immune-organoid models that rely on artificially engineered CD40L-expressing feeder cells [57,65] or related cultures of human B cells with feeder cells as CD40L source [34,66–68]. It cannot be excluded that addition of non-physiological feeder cells, or other additional factors such as cytokines, may lead to artificial effects not occurring *in vivo*. Hence, we manipulated our culture system as little as possible within the short-term read-outs used to decipher effects of anti-inflammatory drugs, but future efforts should be made to further improve mirroring the *in vivo* situation while carefully inspecting potential side effects of added exogenous factors. It remains to be determined whether the best strategy to mimic human lymphoid organs *in vitro* will be (i) the *ex vivo* tissue block culture in improved HLH systems, (ii) the development of alternative culture explants for example similar to thymic slice cultures [69] under optimization of the physical and biochemical environment for GC cells, (iii) designer synthetic immune tissues as recently described for murine GC reactions [70], or (iv) *de novo* generated organoids from human dissociated GC cells or precursor cells. It is also possible that, depending on the research question and read-out of interest, suspension cultures similar to HLAC may be equally suitable.

Vaccination studies can serve as a model system for human *in vivo* GC reactions. Peripheral circulating blood Tfh cells, antigen-specific T cells (where tetramers are available) as well as specific antibodies in the serum can serve as a proxy of the ongoing adaptive immune response to viral infection or vaccination, often peaking after about 2 weeks or more [71–73]. However, blood and tissue Tfh cells are divergent [1,4,15], and fine needle aspirates may be a promising new approach that could enable minimally invasive sampling from human lymph nodes [74]. Nevertheless, explorative and mechanistic studies using preclinical drug candidates will continue to require human tissue culture systems that model the *in vivo* situation as much as possible.

The strong effect that Baricitinib and Tofacitinib had on BCL6 expression in T and B cells conforms with the fact that both of them inhibit the kinase JAK1 (among other JAKs), which is a central interface of signaling downstream of multiple cytokine receptors affecting Tfh and GC B cell differentiation that is, IL-4, IL-6, and IL-21, through signaling *via* different STATs such as STAT3 and STAT5 [5,7,43,44,75]. Recent data have also shown that the balance of IL-2 induced STAT5 and IL-6 induced STAT3 activity modulates murine Tfh cells; interestingly, IL-6 ensured hyporesponsiveness to IL-2 and hence IL6R signaling was needed for BCL6 and Tfh maintenance in an *in vivo* infection model [76]. These results strengthen the *in vivo* relevance of our data in human cells, where IL-6 was needed for BCL6 maintenance in T cells *ex vivo*, and PT induced slight BCL6 downregulation along with IL-2 secretion. Furthermore, GWAS studies determined STAT4 as a risk locus for human autoimmune diseases such as SLE and rheumatoid arthritis, and IL-12 signaling promotes human Tfh cells *via* STAT4 and JAK2 [4,5,44,77]. Our phenotypic and cytokine data provide novel insights into the regulation of BCL6 in primary human tissue. Indeed, our data also suggest that BCL6 downregulation by JAK inhibitors may be caused by blocking signaling through different cytokine receptors, such as IL6R signaling in T cells and IL-4 signaling in B cells. Mechanistically, STATs have been shown to directly regulate BCL6, yet here is a complex interplay and overlap of diverse STATs and JAKs used downstream of different cytokine receptors and their usage can differ by activation state of the T cell [5,44,75,78,79]. It is intriguing to speculate that BCL6 – like other lineage-specifying loci in T cells – may be regulated *via* super-enhancers, and combined evidence from the literature links super-enhancers to Tfh cells and BCL6 [80–82].

We optimized a previously published [16,28] AIM assay by specifically excluding Treg/Tfr cells in our analyses and by introducing a stimulant (PT mutant) that can be used to robustly stimulate adenoid cells from pediatric patients. Notably, in early studies PT has been used to stimulate HLH cells, although the read-out was different [83]. T and B cells together were necessary and sufficient for the AIM response, suggesting that costimulation by B cells in addition to TCR-stimulating effects of PT on T cells [50–52] initiates the response. The strong response to PT that we observed may also encompass adjuvant effects that have been described for PT independent of enzymatic activity [84–87]. The high fractions of PT-reactive AIM-positive cells are in a similar range as observed by others for MHC class II-dependent stimulation with streptococcal SpeA protein [16], yet suggesting involvement of bystander activation and superantigen-like effects. Supporting the hypothesis of bystander activation of non-PT-specific cells through cytokines released by PT-responsive cells, PT stimulation induced massive cytokine release, and blocking of cytokine signaling (by JAK inhibition or blocking TNF signaling) inhibited AIM induction by PT. Interestingly, OX40 upregulation was not prevented by JAK inhibition, suggesting OX40 to be a marker for antigen responses that is less responsive to bystander activation by cytokines than PD-L1 and CD25. TNF blocking, however, negatively affected all three AIMs (CD25, OX40 and PD-L1). OX40 (together with CD25) has been suggested as marker for antigen-reactive cells before [88], and especially in tonsil cells, where background activation seemed to be higher than in PBMCs [89], high fractions of cells responded to PT or SpeA stimulation with OX40 upregulation [16,59]. Although it remains to be shown whether the here discovered mechanisms of AIM regulation play a role *in vivo*, others have shown that the AIM markers on T cells can be an indicator of *in vivo* generated antigen-specific cells, at least in human PBMCs [28,46,48,90] or even *in vivo* in mouse models [47].

pre-gated on live singlet non-Treg memory $CD4^+$ T cells ("non-Treg Tmem"). AIM double-positive cells are quantified in (a) and cells positive for individual AIM markers are quantified in (b). (c) Cytokines in supernatants of AIM cultures were measured by Luminex. Cytokines in medium only (without cells) were undetectable (except for IFN-γ: extrapolated value in medium alone x dilution factor was 86 pg/ml). IL-22 cytokine values below detection limit were set to "0". (d) HLAC cells were pre-incubated for 1–1.5 h with TNF blocking agents Adalimumab or Etanercept at 1.11x of indicated final concentrations, or isotype control (high concentration), or solvent control PBS. Subsequently, cells were stimulated with indicated reagents (PT or Cytostim) for 18 h in the AIM assay as in (a–c), or cultured in medium as control ("unstim."). Box plots are shown with whiskers indicating minimum to maximum values, and $n = 6$ donors represented by individual symbols (2 independent experiments/batches with symbol color indicating experimental batch). Significance was calculated by two-tailed paired *t*-test (pairing within each donor) and p values of treatment *versus* isotype control treatment within stimulation conditions are symbolized as follows: ns: not significant ($p \geq 0.05$); *: $p < 0.05$ (for better legibility, further levels of lower p values are not symbolized by additional stars here). AIM positive cells are shown for the pre-gated live "non-Treg Tmem" population gated as in Fig. 3.

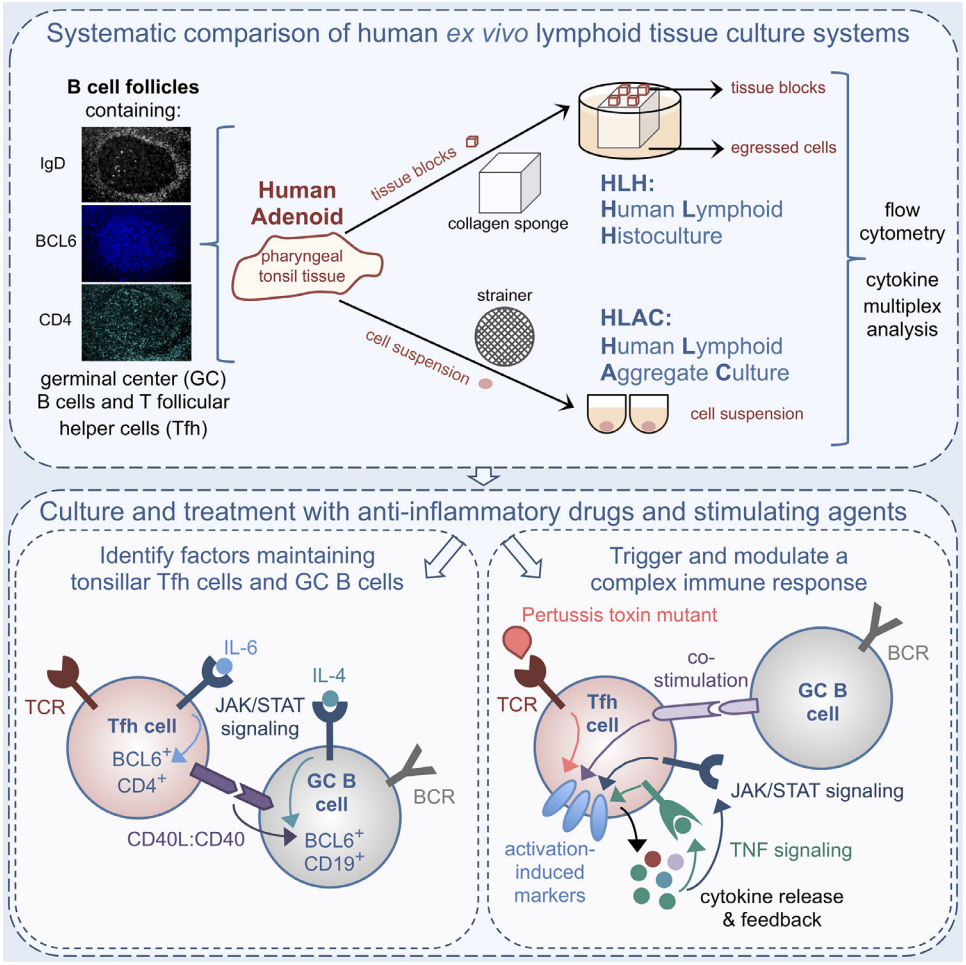

Fig. 7. Summary of the culture systems and results. The upper panel shows a schematic view of the adenoid culture systems employed to study lymphocytes in human secondary lymphoid tissue *ex vivo* (see also Supplemental Fig. 1). Lower left: JAK/STAT signaling contributes to BCL6 maintenance in Tfh and GC B cells, with a role for IL-4 signaling and CD40L in B cells as opposed to IL6R signaling in T cells. Lower right: An optimized activation-induced marker assay triggers a complex T cell immune response using enzyme-dead PT protein plus B cell costimulation, leading to upregulation of the AIMs OX40, PD-L1 and CD25 along with cytokine release. Released TNF as well as JAK-dependent cytokine signals contribute to bystander activation and amplification of the response.

We chose to measure several cytokines in the supernatants by multiplex analysis as part of a method that was suitable for a screening approach. Nevertheless, it shall be noted that these measurements do not provide information on the cell types producing these cytokines, which besides CD4 and CD8 T cells could be B cells or other cells, although the total fraction of non-lymphocyte populations was below 5% in our assays. Potential follow-up studies should take these limitations into account and study cytokines of interest and the cells producing them in more detail. In regards to GC Tfh cells, however, it is well-established in the literature [16,28] and confirmed by our unpublished observations that these cells produce low levels of the measured cytokines as opposed to other T cell subsets. Although cell populations cultured in isolation might behave differently than cells in bulk, analysis of secreted cytokines in the supernatants of cultured sorted adenoid-derived B or T cells revealed that IL-6 was produced by B cells and not by CD4 T cells (unpublished observation) and hence may reflect a prime example of how mutual interaction between B cells and GC Tfh cells contributes to BCL6 expression in Tfh cells.

Bystander activation by cytokine release likely plays a role in complex immune responses *in vivo*, and our PT-induced AIM assay provides a robust and strong response that is highly reproducible between donors and can be used to study modulation of an immune response by anti-inflammatory drugs. Although being used as an antigen in most childhood vaccines against whooping cough, PT in the AIM assay appears to have additional superantigen-like functions that we exploited in our assay. The commercially available PT point mutant that we used has advantages compared to other superantigens in that it is non-toxic (as opposed to the highly toxic superantigen SEB), supports T cell proliferation and does not mask staining with anti-CD3 antibodies (as opposed to the commercial superantigen Cytostim), thus being an optimal reagent to trigger an immune response in complex cellular systems with flow cytometry and cytokine release read-outs.

In conclusion, our data and optimized culture systems verified by proof-of-concept experiments, as well as the AIM assay, provide a framework for future studies on activation and drug-based manipulation of lymphoid tissue GC cells *ex vivo* (Fig. 7). These assays can be useful for rapid screening approaches to dissect drug effects and mechanisms on the phenotype and maintenance of *in vivo* generated GC cells. In addition, we provide new mechanistic insights into factors regulating BCL6 in human primary T and B cells in an *ex vivo* human lymphoid tissue culture system.

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Declaration of Competing Interest

TL, ÖSA, and MH are employees of Sanofi-Aventis Deutschland GmbH. DB and AS received speaker compensation from Sanofi-Aventis Deutschland GmbH. All other authors declare no competing financial interests.

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Author Contributions

AS designed experiments with input from DB, JH, ÖSA, TL, and OTK; AS performed all experiments except Luminex measurements; AS analyzed all data, interpreted results, prepared figures, and wrote the manuscript; JH performed preliminary HLH experiments and established parts of the T/B cell antibody panels; ÖSA performed Luminex measurements and Luminex raw data analyses; CAR and RG provided clinical samples; OTK, TL, and MH contributed essential tools and reagents; DB conceived of the project, interpreted results, wrote the manuscript, and provided overall direction of the study; all authors approved the final version of the manuscript.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ebiom.2020.102684.

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