IL-17A and IL-17F Expression in B Lymphocytes

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Key Words

Autoimmunity · B cells · CD40 ligand · Cytokines · Interleukin-6 · Tonsil

Abstract

Background: Recent evidence suggests that cells other than Th-17 lymphocytes express interleukin (IL)-17A and IL-17F and contribute to the production of these cytokines in immunologically mediated diseases. B lymphocytes are known to be an important source of cytokines in chronic inflammatory diseases. We therefore investigated the potential of human B lymphocytes to produce IL-17A and IL-17F. **Methods:** Highly purified B cells were obtained using a multiple-step separation procedure which included rosette depletion, adherence depletion, CD3+ cell magnetic activated depletion and CD19+ magnetic activated positive cell selection. In these CD19+ B cell fractions, CD3+/CD4+ and CD14+ cells were negligible (<0.2%), and CD8 and CD161 mRNAs were undetectable. The CD19+/CD20+ B cells were stimulated with IL-4, interferon- γ , IL-6, IL-23 and transforming growth factor (TGF)- β , and the expression of IL-17A and IL-17F in response to stimulation was determined by quantitative reverse transcription (RT)-PCR, Western blot, immunocytochemistry and ELISA. **Results:** Evidence of expression of IL-17A and IL-17F in purified B cells was obtained using RT-PCR,

flow cytometry, immunofluorescence microscopy, Western immunoblotting and ELISA. Stimulation of B cells with IL-6, IL-23 or TGF- β upregulated the expression of both IL-17A and F cytokines. **Conclusions:** These novel findings provide evidence that cytokine-stimulated B lymphocytes could be a significant source of IL-17A and IL-17F and support the notion that these cells actively participate in immune responses via alternative mechanisms in addition to the classic release of antibodies.

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Introduction

Since the cloning and characterization of interleukin (IL)-17A (also known as IL-17) in 1995 [1], 5 additional members of the IL-17 family have been described [2–4]. Of these, the most recently discovered family member, IL-17F [5], is very closely related to IL-17A, with 50% amino acid sequence identity [6]. Both IL-17A and F induce the release of many proinflammatory cytokines and chemokines, such as IL-6, IL-8 and colony-stimulating factors, among others [7, 8]. Initial studies showed that the expression of IL-17A and F cytokines was mostly associated with a subset of activated peripheral blood CD4+ memory T cells [2, 9]. Recently, these IL-17-expressing T

cells were shown to be distinct from Th-1 and Th-2 cells by their particular production of IL-17A, IL-17F and IL-22 cytokines and were therefore dubbed Th-17 [10].

Although it is traditionally accepted that IL-17A and IL-17F are produced primarily by activated CD4⁺ Th-17 lymphocytes, many other cell types have also been shown to express them. CD8⁺ T cells produce significant amounts of IL-17A in vitro after being cultured in medium from *Klebsiella pneumoniae*-primed dendritic cells [11]. Human and murine natural killer (NK) T cells were also found to produce IL-17 after lipopolysaccharide stimulation or via T cell receptor and IL-23 receptor activation [12, 13]. In addition, invariant NK T cells producing IL-17 are the key effectors in the collagen-induced arthritis model in mice [14]. Furthermore, increasing evidence indicates that regulatory T cells and $\gamma\delta$ T cells are also a source of IL-17 cytokines [15–17]. Moreover, neutrophils could be the most important source of IL-17A in kidneys after ischemia-reperfusion injury [18]. Also, in atherosclerotic lesions, De Boer et al. [19] found that the cells expressing IL-17A and/or F were always mast cells and neutrophils, while Th-17 cells were not detected. Concerning IL-17F cytokine, it is also expressed in activated monocytes and inhibits the angiogenic response of endothelial cells [5].

For many years, B cells were traditionally associated mostly with the production of antibodies. However, recent findings have underlined their great importance as modulators of the immune response in a number of chronic immunological disorders including allergic and autoimmune diseases, through the release of cytokines and other inflammatory mediators. We and others have shown that B cells can be an important source of a large number of regulatory cytokines, including tumor necrosis factor- α , lymphotoxin, IL-2, IL-6, IL-10, IL-13 and IL-17E, among others [19–25]. Furthermore, an earlier report indicated that a human myeloma B cell line expressed detectable amounts of IL-17 mRNA under standard culture conditions [26]. However, so far there has been no comprehensive analysis of factors regulating the expression of IL-17 cytokine in the B cell line or in other B cells. In the present study, we investigated the possibility that normal B lymphocytes could be a source of IL-17A and/or IL-17F cytokines. The cytokines IL-6, IL-23 and transforming growth factor (TGF)- β are a few of several well known factors required for IL-17A and F cytokine expression in T lymphocytes, splenocytes and other cells [27–29]. IL-17A is also induced by IL-23 in the experimental autoimmune encephalomyelitis model [30, 31]. We demonstrate that highly purified B cells (CD19+/

CD20⁺) from tonsils express mRNA and immunoreactivity for IL-17A and F, which can be regulated by TGF- β , IL-23 and IL-6.

Materials and Methods

Purification of CD19⁺ B Lymphocytes from Tonsils

Highly purified B lymphocytes were obtained from human pediatric tonsils (3–7 years old) discarded after tonsillectomy. The isolation method consisted of a multiple-step purification procedure according to methods described elsewhere [32, 33]. The last step consists of a positive selection using magnetic beads coated with anti-CD19 antibodies followed by confirmation with CD20 antibodies. Both CD19 and CD20 are well-known B cell markers used in both selection and confirmation of B cell lineage [34, 35]. Compared to surface immunoglobulin, these two surface receptors have a wider pattern of expression throughout B cell development and differentiation, with the convenience that they are also expressed in both resting and activated B cells [36, 37]. Even though positive cell isolation with anti-CD19-coated magnetic beads may induce some cell stimulation, it does not interfere with postisolation processes, such as activation or proliferation/differentiation of cells [38, 39]. Briefly, the tonsillar tissue was dissected and finely minced into a cell suspension. The mononuclear cells were obtained by Ficoll-Paque (GE Healthcare) gradient centrifugation (2,200 rpm for 30 min). After washing, the T lymphocytes were removed by E rosette depletion: 200 million cells per tube (6–8 tubes) are mixed with 10 ml of neuraminidase-treated sheep red blood cells and incubated at 37°C (20 min) and then on ice (30 min); the cells were separated by Ficoll-Paque density centrifugation, and the enriched B cells were collected at the interface (approximately 50–60% of the input cells are recovered). Monocytes/macrophages and NK cells are then removed by adherence depletion: the washed cells depleted of T cells were resuspended in complete culture medium (2 million/ml) and incubated in plastic culture flasks (37°C, 2 h). After the incubation, the supernatants were carefully recovered (approximately 50% of the input cells are recovered). Residual CD3⁺ T lymphocytes are then depleted from these enriched B cells by positive selection and magnetic cell sorting using anti-CD3 antibody-coated magnetic beads: the cells were mixed with anti-CD3 antibody-coated magnetic activated cell sorter (MACS) microbeads for 15 min at 4°C and passed through MACS LD or MS columns, according to the manufacturer's instructions (Miltenyi Biotec). The eluted unbound cell fraction (CD3 negative) was then labeled with anti-CD19 antibody-coated magnetic microbeads and purified through an MS column. After washing, the CD19⁺ B cells were eluted from the column. All of the CD19-positive cells were tested for the CD20, CD3 and CD14 markers by flow cytometry. The cells were also tested for CD3, CD4, CD8 and CD161 mRNA expression by quantitative reverse transcription (RT)-PCR.

Flow Cytometry Analysis

To verify the purity, aliquots (5×10^5 cells) of freshly isolated CD19⁺ cells were immediately surface stained (30 min at 4°C) with mouse anti-CD20 conjugated to peridinin-chlorophyll-protein (BD Biosciences) or Alexa 488 (AbD Serotec); the cells were also stained with mouse anti-CD3-phycoerythrin-, anti-CD4-al-

lophycocyanin- or anti-CD14-FITC-conjugated antibodies (BD Biosciences), either alone or in combination. Isotype staining was performed with mouse IgG-conjugated antibodies, following the recommendations of the vendor (AbD Serotec).

To verify the expression of IL-17A and F, cell aliquots (2×10^6 in 2 ml) were cultured for 48 h in complete medium and stimulated with IL-23 (10 ng/ml); brefeldin A (10 μ g/ml; Sigma) was added to the medium for the last 4 h of incubation. The cells were washed and fixed-permeabilized with BD Cytotfix/Cytoperm solution (BD Biosciences) and then stained with conjugated antibodies, i.e. mouse anti-IL-17A-Alexa Fluor 647 (BD Biosciences) or mouse anti-IL-17F-carboxyfluorescein (R&D Systems; 30 min at 4°C). Alternatively, mouse anti-IL-17A and mouse anti-IL-17F antibodies (BD Biosciences) conjugated with Alexa 488 were used. The cells were then washed with BD Perm/Wash solution (BD Biosciences) and then with PBS; isotype staining was performed with mouse IgG-conjugated antibodies, following the recommendations of the vendor. Data were collected (20,000 events each time) with a FACSCalibur flow cytometry system (BD Biosciences) and analyzed with the CellQuest Software (BD Biosciences). Typically, after a 48-hour culture, two morphologically distinct cell subpopulations formed and were gated into regions R1 and R2 in the dot plot profile forward scatter (FSC) and side scatter (SSC); region R2 comprises a subpopulation with a lower FSC and a high SSC profile, which is characteristic of apoptotic cells (stained with annexin V), as described elsewhere [40].

Cell Culture and Treatments

Aliquots from the MACS-purified CD19+ B cell population were used for total RNA extraction (basal). The remaining cells were cultured in complete medium at a density of 1×10^6 cells/ml. The composition of the B cell culture medium was as described previously [22], i.e. RPMI 1640 supplemented with 10% FBS, 2 mM glutamine, 1 mM sodium pyruvate, 15 mM HEPES buffer (pH 7.0) and 100 U/ml penicillin and streptomycin. To verify the functionality of the B cells, they were stimulated with antihuman CD40 antibody (AbD Serotec), alone or in combination with human recombinant IL-4 cytokine; after 72 h of stimulation, the mRNA expression of activation-induced cytidine deaminase (AICD) was examined by quantitative RT-PCR. In another set of experiments, the cells were stimulated with phosphorothioate-modified CpG oligonucleotide (ODN2006) or negative control oligonucleotide (InvivoGen) and agonistic anti-CD40 antibody (AbD Serotec), either alone or in combination; after 48 h, the cells were harvested and total RNA was extracted for quantitative RT-PCR analysis of IL-10 mRNA. In another set of experiments, the cells were also stimulated with either IL-23, TGF- β or IL-6; after 24, 48 and 72 h, the cells were harvested and the total RNA was extracted for quantitative RT-PCR analysis of IL-17A and F mRNA expression.

RNA Extraction and Real-Time RT-PCR

Total RNA was extracted using the RNeasy Mini kit as directed by the manufacturer (Qiagen). DNase I digestion was performed on the column before eluting the RNA. Reverse transcription and quantitative RT-PCR were performed as described elsewhere [41]. Real-time PCR amplification of cDNA targets was performed using a StepOnePlus Real Time thermal cycler and Power SYBR Green PCR Kit (both from Applied Biosystems), which contains the dye Rox, a passive reference used to normalize

the signal from SYBR Green/double-stranded DNA and to correct for well-to-well fluorescence fluctuations. The following primers were used: IL-17A forward, 5'-CAT CCA TAA CCG GAA TAC CAA TA-3', IL-17A reverse, 5'-TAG TCC ACG TTC CCA TCA GC-3'; IL-17F forward, 5'-GTG CCA GGA GGT AGT ATG AAG C-3', IL-17F reverse, 5'-ATG TCT TCC TTT CCT TGA GCA TT-3'; GAPDH forward, 5'-AGT CAA CGG ATT TGG TCG TAT T-3', GAPDH reverse, 5'-ATG GGT GGA ATC ATA TTG GAA C-3'; IL-10 forward, 5'-CTG AGAA CCA AGA CCC AGA CAT-3', and IL-10 reverse, 5'-TCA CTC ATG GCT TTG TAG ATG C-3'. The cycling conditions were as follows: 95°C for 10 min followed by 40 cycles of 95°C for 20 s, 60°C for 20 s and 72°C for 30 s. The sizes of the RT-PCR products (174 bp for IL-17A; 200 bp for IL-17F; 139 bp for GAPDH; 187 bp for IL-10) were confirmed by agarose electrophoresis. At the end of the amplification, a melting temperature analysis of the amplified gene products was performed routinely for all cases; the PCR products were melted by gradually increasing the temperature from 60 to 95°C in 0.3°C steps, and the dissociation curves were generated with the Melting Curve analysis tool of the StepOne software (Applied Biosystems). We confirmed that only one product was consistently amplified in all PCR reactions. The negative water and RT controls showed no amplification. The relative expression of the genes of interest normalized to GAPDH was determined by the delta-delta Ct method [41, 42].

Western Blotting and Immunofluorescence Microscopy of IL-17A and IL-17F

The CD19+ B cells were cultured in the presence or absence of recombinant human IL-23 cytokine (10 ng/ml) for 48 h. Brefeldin A (10 μ g/ml; Sigma) was added for the last 4 h of culture. Whole protein lysates were prepared in RIPA buffer (50 mM Tris, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100) containing a cocktail of protease inhibitors (Roche). Protein concentrations were determined with the BCA Protein Assay Kit (Thermo Scientific). Forty micrograms of total protein extracts were separated on denaturing 15% SDS-polyacrylamide gels, electroblotted into nitrocellulose membranes (Bio-Rad) and probed with nonconjugated primary antibodies, i.e. goat antihuman IL-17A or goat antihuman IL-17F antibodies (both from R&D Systems) diluted at 0.5 μ g/ml in Odyssey Blocking buffer (overnight at 4°C) according to the manufacturer's recommendations. The membrane was then washed and probed with the secondary antibody, i.e. donkey antigoat conjugated to Dylight800 (Thermo Scientific); incubation was performed for 1 h at room temperature. The membrane was washed and scanned with the Odyssey Infra Red Imager, and the bands were quantified using the software Odyssey version 3.0 (Thermo Scientific). For immunofluorescence microscopy, the CD19+ B cells were cultured on sterile glass cover slides coated with poly-L-lysine (for cell attachment) and stimulated as described above. Brefeldin A (10 μ g/ml) was added for the last 4 h. After fixation (4% paraformaldehyde), CD20 surface staining was performed with conjugated mouse antihuman CD20-Alexa 555 antibody (Invitrogen). After permeabilization (PBS containing 0.1% Triton X-100), intracellular staining of IL-17A and F was performed with nonconjugated primary goat antihuman IL-17A or goat antihuman IL-17F antibodies (R&D Systems). Antigoat IgG-Alexa 488 secondary antibody was added according to the manufacturer's instructions (Invitrogen). Images were obtained with a CoolSnap-Pro color camera (Media Cybernetics) attached to an Olympus RX51 fluorescent microscope.

Fig. 1. Flow cytometry analysis of tonsillar CD19+ B cells. Freshly purified B cells were immediately verified for the absence of T lymphocytes and macrophages/monocytes. A representative example is shown. Gating was performed according to the FSC and SSC of the cells. **a** The FSC/SSC profile showing the granularity of the events (20,000) used to set the region indicated. **b** Histogram of immunolabeled cells with antihuman CD20-Alexa 488 (A488) (white area) and isotype control antibody (gray area). **c** Double-stained B cells with antihuman CD20-peridinin-chlorophyll-protein (PerCP) and antihuman CD14-FITC antibodies. **d** Distribution of immunolabeled cells with antihuman CD3-

phycoerythrin (PE) and antihuman CD4-allophycocyanin (APC) conjugated antibodies; the double-positive CD3+/CD4+ cell fraction (T lymphocytes) was less than 0.2% of the total gated events. CD14+ cells (macrophages/monocytes) accounted for less than 0.2% of the total gated events. **e** Real-time RT-PCR analysis of expression of AICD mRNA by B cells costimulated with anti-CD40 antibody (ab) and IL-4 cytokine for 72 h. **f** Expression of IL-10 mRNA in B cells costimulated with CpG oligonucleotide and anti-CD40 antibody (ab) as determined by real-time RT-PCR. * $p < 0.05$, significant to the medium control.

Quantification of Released IL-17A and F by ELISA

IL-17A and F cytokines released by B cells in the culture medium were determined by means of the Duo Set ELISA kits (R&D Systems) according to the manufacturer's instructions. Optical densities were determined with the Infinite M1000 plate reader and i-Control software (Tecan). Data were expressed as picograms of released cytokine per milliliter. The lower limits of detection of IL-17A and IL-17F in this setting were 5 and 300 pg/ml, respectively.

Results

Purity of the CD19+ B Lymphocytes

We isolated CD19+ B lymphocytes from tonsils using a multiple-step purification procedure which included E rosette depletion of T cells from the mononuclear cell fraction, followed by adherence depletion of macro-

phages/monocytes and other adherent cells (e.g. NK cells and dendritic cells); after further depletion of CD3+ cells by magnetic activated cell sorting, the CD19+ B cells were selected with magnetic microbeads coated with anti-CD19 antibodies. These CD19+ B lymphocytes were confirmed by staining with anti-CD20 antibodies and flow cytometry analysis (fig. 1a–d). In these cell fractions, the percentage of double-labeled CD3+/CD4+ cells (T lymphocytes) was negligible (0.1%); the percentage of CD14+ cells (macrophages/monocytes) was also very low (<0.2%). Using real-time RT-PCR, we confirmed that the relative amount of CD3 and CD4 mRNA in these CD19+ cell fractions was comparatively much lower (1,925- and 2,935-fold, respectively) than that of a CD3+ cell fraction; CD8 (a cytotoxic T cell marker) and CD161 (an NK cell marker) mRNAs were undetectable in these CD19+ B cell

Fig. 2. a, b Expression of IL-17A and IL-17F mRNA in purified CD19+CD20+ B cells from tonsils. Increased expression of IL-17A (**a**) and IL-17F (**b**) mRNA in tonsillar CD19+CD20+ B lymphocytes stimulated by IL-6, IL-23 and TGF-β for 24, 48 and 72 h as indicated. The basal expression level in freshly isolated B cells was

used as a reference. Total RNA was extracted and reverse transcribed. IL-17A and IL-17F were amplified by real-time PCR and normalized to GAPDH. * Significantly different ($p < 0.05$) to the basal (freshly isolated cells) and medium controls, as determined by Tukey's multiple comparison test.

fractions. When these cells were tested for possible evidence of functional class switch activity, the expression of mRNA of the key enzyme AICD was potently induced by anti-CD40 antibody and IL-4 (stimulation for 4 days) in combination but not by each alone (fig. 1e). IL-17A and F cytokines were not induced by anti-CD40 and IL-4 stimulation, either alone or combined (result not shown). When double stimulated with phosphorothioate-modified CpG oligonucleotide and agonist CD40 antibody, these B cells responded by expressing significantly higher levels of IL-10 mRNA than those with single stimulation (fig. 1f).

B Cells Express IL-17A and IL-17F mRNA and Protein

To find out if B cells express IL-17A and F cytokines and if their expression is induced by factors known to do so in T cells, we stimulated CD19+ B cells with recombinant IL-6, IL-23 or TGF-β for different periods of time (fig. 2a, b). IL-17A mRNA expression in B cells increased significantly after 24–78 h of stimulation with TGF-β, compared to the basal and nonstimulated (medium) conditions. Also, TGF-β significantly increased IL-17F expression after 72 h of stimulation, compared to the basal and nonstimulated controls. Stimulation with IL-23 induced IL-17A mRNA expression significantly only after 48 h; this factor also stimulated IL-17F expression significantly after 24–48 h of stimulation, but the relative mRNA levels were lower than those of IL-17A. IL-6 significantly

induced IL-17F after 48–72 h of stimulation, while IL-17A was also increased, albeit not significantly (fig. 2a, b).

The production of IL-17A and F proteins was analyzed by ELISA on the supernatants from cells stimulated with cytokines. The quantities of IL-17A released by nonstimulated B cells (medium) were very low or undetectable (table 1). When the B cells were stimulated with IL-6, IL-23 and TGF-β, the levels of IL-17A cytokine released reached 30.3, 16.2 and 97.8 pg/ml, respectively; TGF-β also induced the highest release of IL-17F after 72 h (1.3 ng/ml).

Expression of IL-17A and F proteins was also determined by flow cytometry using purified cells from tonsils or peripheral blood that were stimulated (48 h) and treated with brefeldin A for the last 4 h to enhance the detection of intracellular cytokine immunoreactivity. By flow cytometry (fig. 3a–h), >99% of gated tonsillar B cells were CD20 positive (fig. 3b). Dual staining with anti-IL-17A and anti-CD20 antibodies revealed that about 18% of the cells were positive for both proteins (fig. 3c). A higher proportion of cells (25%) was positive for both CD20 and IL-17 proteins (fig. 3d). Compared to the tonsillar B cells, the percentage of B cells from peripheral blood double stained with anti-CD20 and anti-IL-17A antibodies was much lower (6%; fig. 3g). A similar result (about 6%) was observed with the anti-IL-17F antibody (fig. 3h).

To provide further evidence of cytokine protein expression, the tonsillar B cells were attached to glass cover slides and stimulated with cytokines and brefeldin A

Fig. 3. Expression of IL-17A and IL-17F proteins in purified CD19+CD20+ B cells from tonsils and peripheral blood. The cells were stimulated with IL-23 for 48 h, and brefeldin A was added for the last 4 h of culture. The cells were then stained with anti-IL-17A and anti-IL-17F conjugated antibodies and analyzed by flow cytometry. **a** The FSC/SSC profile of tonsillar B cells used to verify the granularity and to set the gate indicated. **b** CD20 immunostaining of gated cells (white area) and cells stained with isotype control (gray area). **c** Distribution of double-immunostained cells with anti-IL-17A and anti-CD20 conjugated antibodies.

d Double-immunostained cells with anti-IL-17F and anti-CD20 conjugated antibodies. **e** FSC/SSC profile of B cells purified from peripheral blood of a healthy adult subject and stimulated as described above. **f** Typical profile of purified CD19+ peripheral B cells stained with isotype control antibodies. **g** Distribution of double-immunostained peripheral B cells with anti-IL-17A and anti-CD20 conjugated antibodies. **h** Immunostaining of peripheral B cells with anti-IL-17F antibody (white area) and the isotype control antibody (gray area). PerCP = Peridinin-chlorophyll-protein.

Table 1. IL-17A and IL-17F cytokines released by tonsillar B cells

Stimulant	IL-17A, pg/ml			IL-17F, pg/ml		
	24 h	48 h	72 h	24 h	48 h	72 h
Medium	BLD	8.3 ± 6.2	BLD	BLD	377 ± 28	BLD
IL-6	BLD	7.1 ± 5.2	30.3 ± 6.1	BLD	1,055 ± 79	1,246 ± 133
IL-23	15.9 ± 7.1	16.2 ± 8.0	8.8 ± 2.8	1,072 ± 114	795 ± 95	BLD
TGF-β	BLD	BLD	97.8 ± 12.2	600 ± 54	946 ± 42	1,350 ± 56

Purified B cells were cultured in medium alone or containing recombinant IL-6, IL-23 or TGF-β cytokines for the periods indicated. IL-17A and F cytokines released in the medium were quantified by ELISA.

Results are means ± SD. BLD = Below the limits of detection; these were 5 and 300 pg/ml for IL-17A and IL-17F, respectively.

Fig. 4. Detection of IL-17A and F cytokine expression by immunofluorescence microscopy and Western immunoblotting. **a, b** CD19⁺ tonsillar cells were cultured on sterile glass coverslips coated with poly-L-lysine and stimulated with IL-23 for 48 h, and brefeldin A was added for the last 4 h. CD20 immunofluorescence staining was performed with conjugated primary anti-CD20-Alexa 555 (red-orange) antibody. IL-17A or F immunostaining was performed with nonconjugated primary anti-IL-17A or anti-IL-17F antibodies, followed by secondary antibody conjugated with Alexa 488 (green). The merged images show yellow cells indicating expression of both CD20 and IL-17 proteins. **c** Western immunoblot to show IL-17A and F proteins. Lanes 1–4

correspond to IL-17A proteins; lanes 5–8 correspond to IL-17F proteins. Lanes 1 and 5: freshly isolated cells (basal); lanes 2 and 6: cells cultured in complete medium; lanes 3 and 7: cells stimulated with IL-23 for 48 h; lanes 4 and 8: similar to lanes 3 and 7 but with brefeldin A added for the last 4 h. The image shows 2 immunoreactive bands (green) of approximately 15 and 17 kDa. The protein molecular weight markers (S) of 20 and 15 kDa (Kaleidoscope, Bio-Rad) are also shown in red (autofluorescence). **d** Densitometry of Western immunoblot showing increased expression of both IL-17A and F in response to IL-23 stimulation and further enhancement with brefeldin A (Bref A).

added at the end of the period. Immunostaining was performed using a different set of nonconjugated antibodies, and data were obtained by fluorescence microscopy. These B cells were positively stained with both IL-17A and IL-17F antibodies, and this expression corresponded to that detected with anti-CD20 antibody, as observed by the yellow color in the merged images (fig. 4a, b).

Furthermore, Western immunoblots made with whole cell lysates from cells stimulated with IL-23 for 48 h confirmed the expression of immunoreactive bands of expected sizes (about 15 and 17 kDa) for IL-17A and IL-17F; these immunoreactive signals were increased 2-fold by IL-23 stimulation compared to basal (freshly isolated) and medium controls and were greatly enhanced by brefeldin A treatment (fig. 4c, d). Taking these results together, we provide multiple evidence that B lymphocytes express both IL-17A and F cytokines and that this expression can be regulated.

Discussion

Th-17 cells and their characteristic IL-17A and F cytokines are regarded as promoters of autoimmune conditions such as inflammatory bowel disease, rheumatoid arthritis and multiple sclerosis [43, 44]. This has led to interest in using IL-17A and IL-17F as therapeutic targets [45]. However, this approach may be simplistic as it has now become clear that Th-17 cells are not unique in expressing these proinflammatory cytokines [11, 12, 16]. Moreover, the role of IL-17 cytokines may be complex. For example, IL-17A can have a beneficial effect during the early stages of inflammatory bowel disease [46]. Further work is needed to understand which cells produce these cytokines and in which tissues and context.

In view of this, we examined the intriguing possibility that B lymphocytes could express IL-17A and IL-17F. Evidence showing that B cells can express IL-17 cytokine has been published previously. Zhou et al. [26] demonstrated positively that the human myeloma B cell line AF10 expresses IL-17A mRNA; these authors amplified and cloned a cDNA encoding the IL-17 protein from the AF10 B cell line, which could stimulate the release of IL-6 in cultured mouse fibroblasts.

In our work, an important issue in regard to the validity of our findings was the purity of the B cells. We purified B cells using a multiple-step procedure to ensure the removal of T cells and other cells known to express these cytokines. These CD19⁺ B cells were confirmed to express the CD20 B cell-specific marker (>98% of the gated

population). In addition, the presence of CD3⁺/CD4⁺ and CD14⁺ cells was negligible in these fractions (<0.2%). These results were confirmed by quantitative RT-PCR, showing that the expression of CD3, CD4, CD8 and CD161 mRNAs was very low or undetectable. We provided evidence that these cells responded functionally according to the stimulus provided; these B cells responded vigorously to dual stimulation with IL-4 and CD40 antibody to induce AICD, the hallmark enzyme required for class switch recombination in B lymphocytes. Also, these B cells can release IL-10 when stimulated with Toll-like receptor 9 and CD40 receptor ligands.

In this study, we provided several lines of evidence demonstrating that CD19⁺/CD20⁺ B cells from tonsils express mRNA and proteins of the IL-17A and F cytokines. This expression can be further upregulated by stimulation with recombinant IL-6, IL-23 or TGF- β . These factors are known to stimulate the release of IL-17 family cytokines in T lineage lymphocytes, splenocytes and other cells [27–31]. Although the levels of IL-17A released by B cells were about 4–10 times lower than those produced by CD4⁺ cells stimulated with CD3, CD28 and IL-23 for 72 h, the stimulated B cells released comparable IL-17F levels to those of CD4⁺ T cells stimulated with IL-6 and TGF- β for 72 h [47–49]. Some studies have been unable to detect IL-17 in B cells. Fossiez et al. [9] failed to detect IL-17A mRNA in B cells isolated from peripheral blood or tonsils; these authors stimulated the B cells with PMA and ionomycin for 4 h and tested the expression by Northern hybridization. Although we did not test these mitogens, we observed that B cells stimulated with cytokines required at least 24 h to obtain a detectable signal and reached a maximum at about 48–72 h of stimulation. We speculate that the negative result of Fossiez et al. [9] was perhaps because their stimulation period was too short or that these mitogens could not induce these cytokines in B cells.

The precise role or physiological relevance of IL-17 produced by stimulated B cells in vivo is not yet known. The fact that IL-17 plays roles either in inflammatory or regulatory immune responses complicates this topic [50]. Recent evidence strongly links IL-17 cytokine to the pathogenesis of several autoimmune diseases, including systemic lupus erythematosus and rheumatoid arthritis [51, 52]. In fact, IL-17 was shown to induce AICD expression in arthritic mice and also enhanced the production of autoantibodies in the BXD2 mouse model of rheumatoid arthritis and promoted the development of germinal centers [53–55]. Moreover, pre-germinal center B cells and plasma cell precursors become the predominant type

of blood B cells in systemic lupus erythematosus patients [56]. Although Th-17 lymphocytes were previously considered the main source of IL-17, other cell types can become significant sources of these cytokines, such as mast cells [29]. Nothing is yet known about the potential role of B cells as a source of IL-17 cytokines in these disorders. However, a hypothetical role of these cytokines produced by B cells in the regulation of the adaptive immune response could be speculated. This possibility is suggested by the observation that $\gamma\delta$ T cells exert a beneficial regulatory effect during acute allergic airway disease, and this mechanism is mediated by IL-17 cytokine production [50]. On the other hand, therapeutical depletion of B cells using rituximab (anti-CD20 antibody) is generally beneficial and effective in a wide range of autoimmune diseases, thus confirming that a particular B cell subset is driving the disease [57]. However, B cell depletion therapy can induce exacerbation of symptoms and even provoke serious clinical complications in some patients with autoimmune disorders, including ulcerative colitis and systemic lupus erythematosus. Such consequences have been attributed to the unwanted deletion of a regulatory B cell subset, which presumably helps to control inflammation, mediated by the production of IL-10 [58–60].

Although speculation, we hypothesize that upon B cell stimulation (e.g. by IL-23 cytokine), released IL-17 could function in an autocrine manner, with the knowledge that B cells express high levels of IL-17 receptors (IL-17RA) [61]. Toll-like receptor activation could contribute to the overstimulation of this subset of B cells, triggering the release of IL-10. Thus, the overstimulated B cells could play a critical role in regulating germinal center B cell responses and the production of pathogenic autoantibodies.

In summary, we demonstrated that human CD19+/CD20+ B lymphocytes originating from tonsils can express IL-17A and F when these cells are stimulated with TGF- β , IL-23 or IL-6. The relevance or physiological importance of IL-17 released from B cells in autoimmune disorders requires further investigation.

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