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Germinal Center B Cells Constitute a Predominant Physiological Source of IL-4: Implication for Th2 Development In Vivo

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Protective immunity depends upon the capability of the immune system to properly adapt the response to the nature of an infectious agent. CD4⁺ Th cells are implicated in this orchestration by secreting a polarized pattern of cytokines. Although Th2 development in animal models and in human cells in vitro to a large extent depends on IL-4, the nature of the cells that provide the initial IL-4 in vivo is still elusive. In this report, we describe the anatomical localization as well as the identity of IL-4-producing cells in human tonsil, a representative secondary lymphoid organ. We demonstrate that IL-4 production is a normal and intrinsic feature of germinal center (GC) B cells. We also show that expression of IL-4 is highly confined to the GCs, in which the B cells constitute the prevalent cellular source. Furthermore, immunofluorescence analysis of colon mucosa reveals a strikingly similar pattern of IL-4-expressing cells compared with tonsils, demonstrating that IL-4 production from GC B cells is not a unique feature of the upper respiratory tract. Our results show that GCs provide the most appropriate microenvironment for IL-4-dependent Th2 polarization in vivo and imply a critical role for GC B cells in this differentiation process. The Journal of Immunology, 2002, 168: 3165–3172.

The initial signals that trigger the Th polarization process may be derived from the infectious agent itself and may be transferred to the lymphocyte compartment by innate cells of the immune system. For example, LPS triggers IL-12 secretion from dendritic cells (DCs) and thereby initiates Th1 polarization (10, 11). However, DCs do not necessarily produce IL-12 upon activation, but rather can secrete other cytokines, including IL-4 and IL-13, affecting Th2 development and B cell differentiation (12, 13). The concept of Th cell polarization therefore may be extended to other cells of the immune system, where several subsets of leukocytes in a joint effort undergo linked polarized differentiation in response to a defined Ag. Such physiological polarization, comprising both B and T cells, was recently shown to occur at the level of cytokines being produced in mice immunized with typical Th1- or Th2-inducing Ags, respectively (14).

Activation and differentiation of naive Th cells take place in secondary lymphoid organs (2, 6). The effects of cytokines are predominant among parameters known to have bearing on the polarization process, and Th1 development mainly depends on IL-12 (and in human also type 1 IFNs), whereas IL-4 most efficiently drives Th2 differentiation (7, 15–17). In this report, we show that production of IL-4 in human tonsils is highly restricted to GCs and that the predominant cellular source is the GC B cells themselves. IL-4-producing B cells are also present within GCs of colon mucosa, demonstrating that the phenomenon is not restricted to the tonsils. These findings indicate that IL-4-dependent Th2 differentiation in vivo relies on a strong B cell participation and the development of GCs. They also provide a possible explanation, at the molecular level, for previous findings demonstrating the necessity to recruit B cells into Ag presentation in vivo for the Th2 polarization to occur (18).

Materials and Methods

Histology

Crystall sections (8 μm) of human tonsil and colon mucosa were fixed in 4% paraformaldehyde, quenched for endogenous peroxidase activity with 0.3% H₂O₂, blocked with 5% goat serum, and incubated with primary mouse mAbs to CD3 (UCHT1, IgG1; DAKO, Glostrup, Denmark), IgD (IgD26, IgG1; DAKO), CD20 (2H7, IgG2b; BD PharMingen, San Diego,
CA), or, in the presence of 0.2% saponin, to Ki67 (Ki-67, IgG1; Boehringer Mannheim, Mannheim, Germany). Binding of primary mouse mAbs was revealed by Alexa 568-conjugated goat anti-mouse IgG (Molecular Probes, Eugene, OR). Remaining anti-mouse reactivity on sections was blocked with mouse serum, and endogenous biotin was blocked with a biotin blocking kit (DAKO) supplemented with 0.2% saponin. From this point, all incubations and washing steps were performed in the presence of 0.2% saponin. Sections were incubated overnight with preformed complexes of mouse anti-IL-4 (8D4-4, IgG1; BD PharMingen) and biotin-conjugated Fab′ anti-CD20 (Jackson ImmunoResearch Laboratories). For imaging, a laser-scanning confocal model (model MRC-1024; Bio-Rad, Hercules, CA) equipped with a 15 mW krypton/argon laser, and attached to an Eclipse E800 microscope (Nikon, Melville, NY) was used.

**RT-PCR analysis**

Tonsils were minced and lymphocytes were enriched using density gradient centrifugation on Ficoll-Isopaque (Pharmacia Biotech, Uppsala, Sweden). Purification of B cells was done by rosetting T cells (and other CD2+ cells, including monocytes and DCs) with neuraminidase-treated sheep RBCs, rendering >98% B cells and <0.2% T cells as judged by FACS analysis of CD2+ vs CD3 expression. Depletion was performed with Pan Mouse IgG dynabeads (Dynabiotel, Oslo, Norway) coated with mouse anti-CD20 (BD Pharmingen), yielding >85% T cells and <4% B cells. The fractionated cells were lyzed in TRIZol (Life Technologies, Paisley, U.K.) either directly after isolation or after 3 h of culture in complete medium consisting of RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, nonessential amino acids, and 50 μg/ml gentamicin (all from Life Technologies, Gaithersburg, MD). For the ORIGEN analysis, sheep anti-mouse IgG-coated magnetic beads (Igen, Grove, PA) with unbound Fab blocked by mouse serum before applying a FACScan (BD Biosciences). Apoptosis was analyzed based on DNA fragmentation measured by TUNEL (Boehringer Mannheim) according to the manufacturer’s instructions. In some experiments, cells were concomitantly labeled for the expression of CD38 (BD PharMingen) and sorted into CD19+CD38− non-GC B cells and CD19+CD38+ GC B cells (>99% purity). GC B cells were further fractionated by incubating the cells with FITC-labeled annexin V (R&D Systems) in Ca2+−containing buffer and sorting them into cells exhibiting no binding of annexin V and cells being stained by annexin V. Sorted cells were restained and analyzed on a FACScan (BD Biosciences). This procedure did not result in well-separated populations but rather yielded one fraction lacking annexin V reactivity and one being enriched for cells exhibiting annexin V binding (Fig. 5b). Sorted cells were immediately lyzed in TRIZol (Life Technologies) and subjected to RT-PCR as described above.

**Cell cultures for determination of IL-4 secretion**

GC B cells were purified by negative selection as previously described (19, 20). Briefly, T cell-depleted tonsil sections were treated with 60% isotonic Percoll (Pharmacia Biotech) and, after centrifugation at 750 × g for 20 min, the buoyant fraction was further depleted using pan-mouse IgG magnetic beads (Dynabiotel) coated with mAbs to CD39 (AC2, IgG1; kindly provided by J. Gordon, Birmingham, U.K.) and IgD (TA 4.1, IgG3; BD Biosciences). This procedure efficiently enriched for GC B cells (95 ± 0.7% CD20+; 5 ± 5.2% CD20−, and 1 ± 0.7% CD3+ cells (mean ± SD). CD4+ and CD8+ T cells were depleted from 3 to 5 × 106/ml of complete medium only or with the addition of 10 ng/ml PMA and 1 μg/ml ionomycin (both from Sigma-Aldrich, St. Louis, MO). GC B cells were also stimulated with 2 μg/ml anti-CD40 mAb (2C6g, IgG1; kindly provided by S. Pauli, Stockholm University, Stockholm, Sweden). To block consumption of the IL-4 being released from cultured cells, all cultures contained 1 μg/ml of a blocking anti-IL-4 mAb (25463.1, IgG2a; R&D Systems). After 4 h of culture, supernatants were removed and stored at −20°C until further use. Corresponding cells were thoroughly recovered, washed in PBS, and then lysed in 0.5 ml of lysis buffer for 30 min at 4°C with gentle agitation. Lysis buffer contained PBS with 1% Triton X-100 (v/v), 1% BSA (w/v), 2 mM EDTA, 5 μg/ml aprotinin, 0.2 mM PMSF (Sigma-Aldrich), and 0.02% NaN3 (w/v). Lysates were centrifuged at 14,000 × g at 4°C for 15 min and supernatants were stored at −20°C until use.

**ECL-based detection of IL-4**

The amounts of IL-4 in culture supernatants and cell lysates were determined with a double-Ab method, using an ORIGEN Analyzer (Igen, Gaithersburg, MD), which is based on an ECL detection technique (21). For the ORIGEN analysis, sheep anti-mouse IgG-coated magnetic beads (Dynabeads M-280, final dilution of 1:80; Dynabiotel) were mixed with anti-IL-4 mAb (8D4-8, IgG1; BD PharMingen) at 50 ng/ml, biotinylated goat anti-IL-4 (polyclonal rabbit anti-IL-4; Jackson ImmunoResearch Laboratories) at 25 ng/ml, and sheep anti-mouse IgG-coated magnetic beads (Igen) according to the manufacturer’s recommendations. Dilution buffer used for these reagents was PBS supplemented with 1% Triton X-100 (v/v), 1% BSA (w/v), and 5% goat serum (v/v). After 30 min of incubation, 100 μl of this reaction mixture was added to 125 μl of each cell lysate or precleared culture supernatant (see below) in a 96-well microtiter plate. ECL signals were thereafter determined with the ORIGEN analyzer. ECL-based detection of IL-4 derived from cell culture supernatants and cell lysates was compared with ECL signals obtained with a serial dilution of a human IL-4 standard (Endogen, Woburn, MA) calibrated against World Health Organization IL-4 reference standard (lot no. 88/656). For quantification of IL-4 in culture supernatants and cell lysates, the IL-4 standard was diluted in complete medium and lysis buffer, respectively. The useful linear range spanned from 3 to >2000 pg/ml. Because it was determined in initial experiments that mAbs added to cultures to some degree interfered with the detection of IL-4, probably by competing with the anti-IL-4 mAb for binding to the sheep anti-mouse IgG-coated magnetic beads, supernatants were preincubated with 20 μl of the beads before being analyzed. This procedure completely prevented such interference.

**Results**

To identify the nature of the cell(s) that could provide IL-4 in Th2 development in human tonsil, cryostat sections were analyzed by...
immuno-fluorescence, using enzyme-catalyzed amplification of the IL-4-dependent signal. First, the anti-IL-4 mAb did not detect IL-4 bound to cognate receptors, as ruled out by the inability to detect rIL-4 loaded onto IL-4R-bearing THP-1 cells (a human monocyte-derived cell line), using flow cytometry. The stringency of this approach, was confirmed by using a polyclonal anti-IL-4 preparation instead, which indeed bound to the receptor-associated cytokine (data not shown). Second, the specificity of the IL-4 detection was confirmed by the complete absence of fluorescence when the mAb was preincubated with rIL-4 (Fig. 1, a and b). Double label-

**FIGURE 1.** In situ IL-4 expression in human tonsil and lymphoid areas of colon mucosa is confined to GCs where B cells constitute the predominant cellular source. a, Anti-CD3 (red) and anti-IL-4 (green) immunofluorescent double labeling of human tonsil reveal a B cell follicle-restricted IL-4 expression. b, Preincubation of the anti-IL-4 mAb with rIL-4 completely abrogates IL-4-dependent fluorescence in an adjacent section, demonstrating the specific staining of IL-4-expressing cells. c, Higher magnification of B cell follicles reveals a few IL-4-expressing CD3+ T cells (arrows), whereas the vast majority of IL-4+ cells are non-T cells. d, Simultaneous anti-IgD (red) and anti-IL-4 (green) labeling establish that IL-4 is exclusively produced within the GCs of tonsil. e–g, Triple labeling with anti-CD3 (blue), anti-IL-4 (green), and anti-CD20 (red) demonstrates overlapping immunoreactivity of anti-CD20 and anti-IL-4 in GCs of tonsil (e) and, at higher magnification (f), the CD20+ B cell identity of IL-4-producing non-T cells (arrows), of which several are in close proximity to T cells (arrowheads). Identical expression pattern is visualized in GCs of colon mucosa (g). h–i, In some, often small-sized GCs, triple labeling with anti-CD3 (blue), anti-IL-4 (green), and anti-Ki67 (red) identifies a preferential localization of CD3+ IL-4+ cells in the dark zone defined by proliferating centroblasts exhibiting nuclear staining of Ki67 (h), whereas, at higher magnification (i), IL-4 is visualized in both Ki67+ cells (arrows) and cells lacking anti-Ki67 reactivity (arrowhead). DZ, dark zone; LZ, light zone. Tonsil stainings are representative of five different donors. Tissue samples of colon from two donors were analyzed and yielded similar results.
ing with mAbs to CD3 and IL-4 revealed a highly anatomically ordered expression pattern with almost all IL-4 localized to the follicles (Fig. 1a). Virtually no IL-4+/H11001 cells could be seen in the T cell areas or in the reticulated epithelium of the crypts, a tonsil area in some aspects resembling the marginal zone in spleen (2). Tonsillar GC T cells previously have been reported to express IL-4 (22) and CD3+/H11001 IL-4+/H11001 cells were readily identified within follicles, but the vast majority of IL-4+/H11001 cells did not express the T cell marker (Fig. 1c). Double labeling with anti-IgD and anti-IL-4 revealed a GC-restricted expression of the cytokine with no anti-IL-4 reactivity visualized in the mantle zone (Fig. 1d) or in primary follicles (data not shown). Three-color immunofluorescence using anti-CD20, anti-CD3, and anti-IL-4 identified CD20+/H11001 GC B cells as the major cellular source of IL-4 (Fig. 1, e–g). In some of the GCs being examined, IL-4-expressing B cells were predominantly localized in the dark zone, defined by staining of the proliferation-associated nuclear Ag Ki67 (Fig. 1h). However, IL-4 was visualized in both Ki67-positive and -negative cells (Fig. 1i), and most of the large GCs generally did not display such a clear boundary regarding IL-4 production. Finally, B cells expressing IL-4 were also identified in GCs in lymphoid areas of colon mucosa (Fig. 1g). Thus, at least in mucosa-associated lymphoid tissues, IL-4-expressing GC B cells appear to be a more general phenomenon.

To confirm IL-4 expression in B cells at the level of mRNA transcripts, freshly isolated tonsil cells were processed for RT-PCR analysis as a bulk population, as a B cell-depleted fraction (>85% T cells), or as purified B cells (>98% CD20+/H11001 cells, <0.2% CD3+/H11001 cells). Semiquantitative RT-PCR performed with RNA derived from these cell preparations demonstrated IL-4 transcripts in B cells, although at a lower level compared with T cells (Fig. 2). This was also true for cells cultured for 3 h, an in vitro procedure later used for IL-4 protein measurement at the single cell level (see below), and is in agreement with the relatively low level of secretion of IL-4 from murine B cells, which can be induced by polarized Th2 cells (14).

Next, we applied flow cytometry to characterize IL-4-expressing B cells at the single cell level. To this end, crude cell preparations from tonsils were cultured for 1 h to allow the cells to recover after the isolation procedure and for an additional 2 h in the presence of

**FIGURE 2.** B cells contain lower levels of IL-4 transcripts compared with T cells. The mRNA from total tissue-freed tonsil cells (top), T cell-enriched fraction (middle), or purified B cells (bottom) were prepared directly after isolation (0 h) and after 3 h of culture and were subjected to semiquantitative RT-PCR analysis using 32, 36, and 40 cycles of cDNA amplification with IL-4-specific primers. cDNA corresponding to equal amounts of total RNA was used in each reaction and quantities were backchecked by amplification of GAPDH target cDNA at 32 cycles. Flow cytometric analysis of CD20 vs CD3 expression for corresponding cell preparations is shown to the left.

**FIGURE 3.** IL-4 expression in tonsil cells in vitro is more frequent among B cells than T cells and is restricted to B cells exhibiting a GC B cell phenotype. Tissue-freed cells from tonsil were cultured for 3 h in complete medium with brefeldin A (10 μg/ml) added during the last 2 h and were subsequently processed for three-color FACS analysis of intracellular IL-4 content in relation to marker protein expression. a. Majority of IL-4-producing cells do not express the T cell marker CD3. b. Preincubation of the anti-IL-4 mAb with rIL-4 (20 μg/ml) abrogates IL-4 staining. c. Phenotypic analysis of IL-4+/H11001 CD3− cell with FITC-conjugated mAbs to indicated proteins. CD3− cells have been excluded from data by electronic gating.
brefeldin A, imposing intracellular retention and accumulation of otherwise secreted proteins. This period of time in culture did not dramatically alter the levels of IL-4 transcripts in B and T cells, respectively (Fig. 2). In agreement with in situ observations, IL-4 production was more frequent among CD3⁺ cells than CD3⁻ T cells (20 ± 7.2% vs 3.1 ± 1.1%; mean value ± SD, n = 7) (Fig. 3, a and b). Three-color FACs analysis allowed CD3⁻ cells to be electronically gated and further analyzed for marker expression in relation to intracellular IL-4 content (Fig. 3c). CD3⁻ IL-4⁺ cells displayed a characteristic GC B cell phenotype (1, 23) expressing /H11006
cells (20) was also confirmed by analysis of DNA fragmentation, using the TUNEL method (25) (Fig. 4). Freshly isolated cells, however, did not exhibit any detectable sign of nuclear fragmentation, and IL-4-positive cells in situ also were not preferentially TUNEL positive (Fig. 5, a and b). We also sorted GC B cells by flow cytometry for their ability to bind annexin V (Fig. 5, c and d) because this property identifies apoptotic cells at an early stage of the apoptotic process (26). No major difference in mRNA content for IL-4 could be detected in the two fractions of GC B cells, which were divided on the basis of their annexin V binding capacity (Fig. 5e). Different amounts of IL-4 transcripts instead could be visualized if GC B cells were compared with the sorted fraction of non-GC B cells. Based on these experiments, we conclude that GC B cells with an IL-4-producing phenotype are not preferentially apoptotic in vivo or directly after isolation, but upon culture they initiate the apoptotic process, a feature which is indeed an important hallmark for GC B cells (20).

GC B cells producing IL-4 thus rapidly die in culture. In an attempt to detect secreted IL-4, we therefore measured the amounts being spontaneously produced from purified GC B cells during a short period of only 4 h in culture. To compensate for this brief period of secretion, cells were cultured at a relatively high density of 5 × 10⁶ cells/ml. Low amounts of the cytokine could indeed be detected in supernatants from such short-term cultures, and an increase in concentration was observed if an antagonizing mAb to the IL-4R was included at the beginning of cultivation (Fig. 6a). Next, we compared purified GC B cells and the tonsillar CD4⁺CD45RO⁺ T cells (memory Th cells) for their ability to release IL-4. Fig. 6b summarizes the levels being released by GC B cells from three separate donors and by memory Th cells from two of these donors, respectively. Whereas GC B cells and memory Th cells spontaneously secreted similar amounts, polyclonal activation with PMA and ionomycin did not have any significant effect on the IL-4 production from any of the cell types during this short period of stimulation. Importantly, although released levels of the cytokine were measured for cells cultured for 4 h only, even this short period of time rendered a large fraction of the GC B cells apoptotic, whereas the T cells remained viable (Fig. 6c). As shown in Fig. 6c, despite the fact that PMA and ionomycin and, to some degree, anti-CD40 stimulation could prevent the apoptotic cell death of cultured B cells, the increase in viability was not accompanied with a sustained production of IL-4 (see Fig. 6b). These reagents also failed to increase the concentration of IL-4 in supernatants from GC B cell cultured for 16 h, in which the levels of IL-4 actually remained unchanged compared with supernatants taken after 4 h (data not shown). Furthermore, cell lysates from cultured GC B cell did not contain more IL-4 than lysates derived from isolated and T cell-depleted tonsil cells.

FIGURE 4. IL-4 synthesis and apoptotic progression in GC B cells are concurrent events in vitro. Tissue-freed cells from tonsil were cultured for 3 h in complete medium with brefeldin A (10 μg/ml) added during the last 2 h and were analyzed by FACs for apoptosis by TUNEL (FITC) concomitantly to anti-CD3 PEcy5 and anti-CD10 PE or anti-IL-4 PE staining, as indicated. Only CD3-negative events are shown.

FIGURE 5. Induction of apoptosis is not a prerequisite for IL-4 expression in GC B cells. a and b, Simultaneous in situ detection of IL-4 (red) and apoptosis by the TUNEL method (green) demonstrate the presence of IL-4 expression in both apoptotic (arrows) and nonapoptotic (arrowheads) cells within a GC of tonsil. The separate image of TUNEL-mediated fluorescence is shown (a) to facilitate interpretation of the merged image (b). c, Isolated and T cell-depleted tonsil cells were incubated with anti-CD19 PEcy5 and anti-CD38 PE mAbs and sorted by FACS into CD19⁺/CD38⁻ GC B cells and CD19⁻/CD38⁺ non-GC B cells. d, GC B cells were stained with annexin V-FITC and subjected to further fractionation by FACS into an annexin V⁺ fraction (black histogram) and into a fraction being enriched for annexin V binding cells (red histogram). e, mRNA was prepared from the sorted cell populations in c and d and was analyzed by RT-PCR with specific primers for IL-4. cDNA corresponding to equal amounts of total RNA were used in each PCR, and quantities were back-checked by amplification of GAPDH target cDNA.
from the memory T cells, irrespective of under which conditions cells were cultured (Fig. 6d). The significance of this finding is that B cells spontaneously release IL-4 and hence that concentrations in culture supernatants most likely reflect actual synthesis of the cytokine. Although GC B cells and memory T cells accordingly produce similar amounts of IL-4 in these cultures, it is important to emphasize that secretion of IL-4 was measured using an equal number of B cells and T cells, respectively. In vivo, the tonsil generally contains more GC B cells than memory Th cells (Fig. 6e). In addition, the high incidence of cell death among cultured GC B cells clearly makes it difficult to compare levels of IL-4 being released in vitro to the in vivo situation. Although PMA/ ionomycin or the anti-CD40 mAb increased the viability of cultured cells, these entities may not be representative for the entire panel of survival and growth factors delivered, for example, by follicular DCs and activated T cells in vivo (3, 4, 27), which perhaps also can sustain the production of IL-4.

**Discussion**

Although it appears that Th2 differentiation may proceed in an IL-4-independent manner (28), the efficiency and magnitude of polarization are severely reduced in IL-4 knockout mice or in mice rendered deficient in IL-4R signaling (15, 16). Therefore, to delineate mechanisms that drive Th2 development in vivo, identification of cells capable of providing an initial burst of IL-4 still remains an important issue. This led us to investigate the cellular sources of IL-4 in the human tonsil, and in this report we describe the identification of GC B cells as a novel cellular source of IL-4 in vivo.

Irrespective of which method is used for detection, the B cells consistently display a comparatively high production of IL-4 protein, in terms of both fraction of cells producing the cytokine and total quantity being produced. In contrast, the B cells clearly contain less mRNA for IL-4 compared with the T cells. This imbalance suggests separate mechanisms for regulation of IL-4 synthesis in B and T cells, respectively. Our results support the interpretation that IL-4 is produced in B cells only during their Ag-dependent differentiation within GCs because 1) in situ, IL-4 can be visualized virtually only within GCs, 2) ex vivo, IL-4 transcripts are enriched in the GC fraction of sorted B cells, 3) in vitro, IL-4-producing non-T cells homogeneously display an appropriate GC B cell phenotype, and 4) the intrinsic nature of GC B cells to rapidly undergo apoptosis when being cultured (20) applies to the majority of IL-4-producing B
cells. Furthermore, CD40 ligation, most likely accounting for a decisive role in the final differentiation stage of centrocytes in vivo (30, 31), appears to reduce the synthesis of IL-4 in cultured GC B cells because the CD40-mediated increase in viability is not accompanied by an increase in IL-4 production. This may lend evidence to the idea that Th cells abort IL-4 production in GC B cells when these differentiate into memory cells. Clearly, production of IL-4 is most frequent among the CD77+ centroblasts as judged by FACS analysis, indicating that light zone-associated events indeed may be responsible for turning off the synthesis of IL-4 in their progeny. However, it is difficult to assess the precise function of CD40 signaling in this context because of the property of the CD40 pathway to both interfere with the apoptotic progression and simultaneously induce further B cell differentiation beyond the GC state. That IL-4 is produced in GC B cells as a consequence of apoptotic events, however, is unlikely because most IL-4-producing cells do not exhibit nuclear fragmentation in situ, nor are IL-4 transcripts enriched in the annexin V-positive fraction of ex vivo-analyzed GC B cells.

Because priming of naive Th cells appears to necessarily take place on interdigitating DCs (IDCs) in the T cell zone (2), we expected IL-4 to be produced in this area. Murine DCs have now been identified as a potential source of IL-4 (12), and secretion of IL-4 from IDCs would indeed provide a route for Th2 development in vivo. Detectable IL-4 production, however, was highly restricted to the GCs in all donors analyzed by in situ immunofluorescence (Fig. 1). Very few IL-4-producing cells were observed in T cell areas, and this limited number of cells apparently did not include IDCs (defined as CD40+ and/or CD83+ cells intervening within the dense T cell accumulations; data not shown), although we cannot exclude that expression of IL-4 below the detection limit takes place in cells within the T cell zone. However, taking this into consideration, it is clear that GCs account for the absolute predominant production of IL-4 in the tonsils, and a similar distribution of IL-4-producing cells was also observed at inductive sites of colon lamina propria. Therefore, we propose that GCs provide the most favorable microenvironment for Th2 development in these mucosa-associated lymphoid tissues. Considering the prominent role IL-4 seems to play in mucosa-associated immunity compared with systemic immunity (32), it would be interesting to also investigate IL-4 production in the human spleen and lymph nodes.

Except for T and B cells, additional subsets of leukocytes are indeed capable of producing IL-4. Eosinophils and basophils can produce IL-4 and, under certain circumstances, perhaps support a peripheral outgrowth of Th2 cells (9). An essential role for these cells in Th2 polarization, however, is unlikely, mainly because of an inappropriate anatomical localization. Rather than being present in secondary lymphoid organs during early inductive events of immunity, they are recruited into inflamed target tissues, partially by the action of preexisting Th2 cells (7). On the contrary, physical interaction between Ag-specific T and B cells take place within the developing GC only a few days after primary immunization (33) and, at this time point, a mutual agonistic outcome of such cellular collaboration is evident by a shift from extra- to intrafollicular T cell proliferation (34). Although it has generally been believed that GC formation depends on Th2 cells, the initial T and B cell encounter takes place within a time period that probably is not sufficiently long to allow development of fully committed Th2 cells (35, 36). Thus, the kinetics of these in vivo cellular events emphasize that the formation of GCs may precede Th2 development in the primary immune response. A nonobligatory role for Th2 cells in the GC reactions is compatible with the incidence of immune responses characterized by an exclusive production of IFN-γ-dependent Ig isotypes, such as IgG2a, in the mouse. These responses are predominated by Th1 cells, and the development of GCs is probably driven by T cells being strongly influenced by, e.g., IL-12 during their initial priming within the T cell zone (7, 37). Furthermore, using an adoptive transfer system, it was recently demonstrated by Smith et al. (38) that Ag-specific Th1 and Th2 cells are equally capable of supporting B cell clonal expansion and Ab production in vivo.

The T cells residing within GCs of human tonsil have recently regained attention (39). This separate population of memory Th cells, which appears to be specialized in providing help for the GC B cells, differs from other memory Th cells by its expression of CD57 as originally demonstrated by Poppema et al. (40) and recently confirmed by Kim et al. (39). Even though they strongly support Ig secretion from cultured GC B cells (39), they are, in contrast to their CD57+CD45RO+ counterparts, poor in eliciting responses from peripheral B cells (41). Accordingly, counteracting apoptotic cell death may be involved as an important mechanism for the helper activity of the GC T cells (20). In addition, in contrast to the extrafollicular subset of CD45RO+ Th cells, the GC Th cells produce large amounts of IL-10 (39), a cytokine that in the presence of follicular DCs comprehensively promotes terminal differentiation of GC B cells into plasma cells (4, 42). IL-4 together with CD40 ligand instead favors the continuous differentiation from centroblasts via centrocytes to memory B cells (3, 4, 43). Therefore, the comparable levels of IL-4 being released ex vivo by the GC B cells themselves and by the tonsillar memory Th cells suggest that memory development may constitute more of a default pathway for GC B cells in vivo, whereas plasma cell differentiation also requires IL-10 provided by the T cells.

In this study, we focused on the predominant source of IL-4 in tonsils and, therefore, have not in greater detail studied the cytokine profile of the different subsets of T cells. Similar to B cells, IL-4-producing T cells appear to be mostly contained within GCs, as we demonstrated in situ using immunofluorescence (Fig. 1). An enrichment of IL-4-producing T cells within GCs is also in line with the results published by Butch et al. (22) and by Toellner et al. (44), but was not clearly evident from the work by Kim et al. (39). However, in the latter report, the investigators used PMA/ionomycin stimulation to induce secretion of IL-4 from T cells. Extended in vitro polyclonal activation may induce IL-4 expression in memory Th cells, which in vivo are relatively quiescent. Furthermore, we feel that, in the chronically infected tonsil, it may not be appropriate to consider the cytokine profile of the GC T cell subset as a criteria for how the cytokine microenvironment of the follicles may influence the T cell polarization process. First, extrafollicular priming of T cells, preceding the migration toward follicles (45, 46), may have influenced the acquisition of a certain cytokine-secreting phenotype of T cells within GCs. Second, although the CD57+ GC T cells appear to be a resident population, other Th cells may enter the B cell follicles and then migrate toward chemokines produced in inflamed tissues (47–49). After an initial expansion, a decrease in the number of T cells within the growing GCs has indeed been reported to occur (45). Nevertheless, production of high levels of IL-10 from the CD57+ GC T cells is not in conflict with our suggestion of a GC B cell-mediated Th2 polarization (7).

In conclusion, our findings and supporting results provided by Harris et al. (14) of IL-4-producing B cells indeed being capable of eliciting Th2 polarization shed new light on investigations performed in vivo, delineating the inherent property of B cells to support their own Ag-dependent differentiation by inducing Th2 polarization (18). Besides supporting switching to IgE and IgG4 (7, 9, 50), production of IL-4 from GC B cells may be important