Targeting the humoral immune system of patients with rheumatoid arthritis

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Chapter 9

Sustained secretion of immunoglobulin by long-lived human tonsil plasma cells

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Abstract

Immunoglobulin-secreting cells comprise both short-lived proliferating plasmablasts and long-lived nonproliferating plasma cells. To determine the phenotype and functional activity of Ig-secreting cells in human lymphoid tissue, we used a tonsillar organ culture model. A significant proportion of IgA and IgG secretion was shown to be mediated by long-lived, nonproliferating plasma cells that coexpressed high levels of CD27 and CD38. The presence of such cells was further corroborated by the finding of enhanced expression in the CD19+ B-cell population of XBP-1, IRF-4, and particularly Blimp-1 genes involved in the differentiation of plasma cells. Intact tissue seemed to be necessary for optimal functional activity of plasma cells. A strong correlation was found between concentrations of interleukin-6 and IgA or IgG, but not IgM, in culture supernatants suggesting a role for interleukin-6 in the survival of long-lived plasma cells. Taken together, the present study demonstrates that human lymphoid tissue harbors a population of nonproliferating plasma cells that are dependent on an intact microenvironment for ongoing Ig secretion.

Introduction

Immunoglobulin secretion is the hallmark of terminal B-cell differentiation and is indispensable for an effective humoral immune response. The population of immunoglobulin-secreting cells (ISCs) comprises plasmablasts and plasma cells. Recent studies have shown that plasmablasts are proliferating, short-lived ISCs that are rapidly induced in an immune response, whereas plasma cells that develop after a germinal center reaction represent a long-lived ISC population that contributes to the production of persistent protective antibody of high affinity.[1-4] Plasma cells require the continued presence of the transcription factors Blimp-1 (B-lymphocyte-induced maturation protein 1), IRF-4 (interferon-regulatory factor 4), and XBP-1 (X-box-binding protein 1), which repress the B-cell gene expression program and induce the plasma cell gene program. Experimental studies in mice have shown that plasma cells from bone marrow and spleen can survive and secrete antibody for more than a year and that differentiation and survival of plasma cells in bone marrow depend on soluble factors, such as interleukin (IL)-6, and physical interactions with surrounding stroma including cell-cell contact via CD44, CXCL-12, and VLA-4.[5-8] *In vitro* data on interactions of isolated B-cell populations with transfected stromal cells, stromal cell lines, and splenic stromal cells suggest similar soluble factors and cellular interactions may be involved in humans, but the extent to which this holds true for function and survival of human ISCs *in vivo* is unclear.[9-20] Clearly, a representative *in vitro* model that mirrors the complexity of human ISCs interacting with their microenvironment could advance our understanding of human ISC biology. The traditional model of T-cell-dependent plasma cell differentiation suggests that these cells are generated in secondary lymphoid organs and then migrate to the bone marrow where they complete their maturation into long-lived nondividing high-rate Ig-producing plasma cells.[21] However, secondary lymphoid tissue such as spleen and tonsils are known to contain large numbers of plasma cells and in the murine spleen many of these are not dividing.[5,22] Although the human tonsil contains many plasma cells that differ phenotypically from bone marrow plasma cells,[22] the functional activity of these cells has not yet been fully characterized.

To identify and characterize functionally human tonsil plasma cells, we used a tonsillar organ culture model. Our data indicate that human secondary lymphoid

tissue contains a mixed population of long-lived IgA- and IgG-secreting plasma cells that depend on intact tissue architecture for survival and immunoglobulin secretion.

Materials & Methods

Organ culture model

Tonsils were obtained from patients undergoing tonsillectomy for chronic tonsillitis. The tonsillar tissue was cut into small pieces, ~2 to 3 mm in diameter, gently washed in phosphate-buffered saline (PBS) three times, and cultured on Gelfoam (Pharmacia & Upjohn, Kalamazoo, MI) in sixwell plates at a density of six to nine fragments/well in culture medium (RPMI 1640 medium with 15% fetal bovine serum, supplemented with ticarcillin and clavulanate potassium (Timentin; GlaxoSmithKline, Research Triangle Park, NC), amphotericin (Fungizone; Bristol-Myers Squibb Co., Princeton, NJ), sodium pyruvate, and nonessential amino acids)[23]. To reduce the potential contribution of passively shed antibody, supernatants were harvested the following day, centrifuged, and cryopreserved, and fresh medium was added to the cells with or without additional stimuli. Three to six wells per condition were tested and supernatants pooled to reduce variability of Ig production by individual tissue fragments. Two types of culture set-ups were used unless stated otherwise. The first included staggered cultures in which supernatants were removed and fresh medium was added at days 4, 7, and 10 when cultures were terminated to analyze B-cell populations. The second involved uninterrupted cultures that were terminated on day 8. The latter setup was used for comparison with parallel cultures of cell suspensions derived from the same tonsil. Cell suspensions were obtained by enzymatic digestion of tonsillar tissue fragments using collagenase type I (Invitrogen, Grand Island, NY) and DNase (Qiagen, Hilden, Germany). Afterwards, cell suspensions were washed with PBS, and mononuclear cells were isolated by Ficoll-Isopaque density centrifugation. In some experiments, cycloheximide (CHX) (10 μg/ml; Sigma-Aldrich, St. Louis, MO) or hydroxyurea (HU, 10^{-2} mol/L; Sigma-Aldrich) were added to the cultures to inhibit protein synthesis or DNA synthesis, respectively.

Cell staining and flow cytometric analysis

Tonsillar tissue fragments were harvested, ground with a glass plunger, and pressed through a wire mesh to obtain cell suspensions. Tonsillar mononuclear cells were counted, and viability was assessed using trypan blue staining. Cells [0.2 to 0.5 x 10^6 cells in 200 µl of PBS/1% bovine serum albumin (BSA)] were incubated with monoclonal antibody for 30 minutes in the dark at 4°C. After washing, cells were fixed with 1% paraformaldehyde overnight until flow cytometric analysis within 1 week after staining. Fluorescence-activated cell sorting analysis was performed on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). Analysis was performed with FlowJo software (Becton Dickinson). Forward scatter (FSC) was recorded in linear mode, side scatter (SSC) and fluorescence signals in logarithmic mode.

The following monoclonal antibodies (Becton Dickinson) were used: CD3-FITC, CD27-PE or sIgD-PE, CD19-PerCpCy5.5, and CD38-APC. In each experiment, isotype- and concentration-matched controls were used. Data from 20,000 to 100,000 gated cells/sample were recorded, and the percent positive for each analyzed marker assessed using the isotype-matched negative control samples. Previous studies have shown that the majority of ISCs are phenotypically characterized as CD3- CD19+ $CD38^{br}$ cells[24,25].

Enzyme-linked immunosorbent assays (ELISAs)

Titers of total IgA, IgM, and IgG were determined in serially diluted culture supernatants. Total immunoglobulin levels were determined by a quantitative sandwich ELISA according to the protocol provided by the manufacturer (Bethyl Inc., Montgomery, TX). In brief, flat-bottom 96-well plates (Immulon-1H; Dynex, Chantilly, VA) were coated with capture antibody (goat anti-human IgM, IgA, or IgG; 10 µg/ml) in coating buffer (0.05 mol/L sodium carbonate, pH 9.6) for 18 hours, washed twice in TBS/0.05% Tween 20, blocked with 1% BSA in PBS for 1 hour, followed by incubation with standards and samples for 1 hour. After extensive washing, isotype-specific horseradish peroxidase-conjugated goat anti-human antibody was added for 1 hour. Plates were again washed, and color was developed with tetramethylbenzidine peroxidase substrate and peroxidase substrate solution (KPL, Gaithersburg, MD), which was stopped with 2 mol/L sulfuric acid. Absorbance was measured at 450 nm.

Human IL-6 was measured by ELISA according to the instructions provided by the manufacturer (Pierce, Rockford, IL). In brief, anti-cytokine precoated 96-well strip plates were incubated with biotinylated antibody reagent, human standard, and supernatants at serial dilutions, washed, and further developed with streptavidin-horseradish peroxidase conjugates. The sensitivity of this assay was 1 pg/ml. Other cytokines were measured using the Multiplex kit (Pierce).

Enzyme-linked immunospot assay for ISCs

Frequencies of ISCs were quantitated by ELISPOT. In brief, 96-well PolySorp plates (Nalge Nunc, Rochester, NY) were coated with goat anti-human Ig (10 μg/ml in coating buffer). After washing and blocking with 3% BSA (w/v) in PBS, serially diluted tonsillar mononuclear cells (100000 to 1600 cells/well) were added in culture medium and incubated overnight at 37°C. Each dilution of cells was assayed in duplicate or triplicate. Plates were then washed extensively with PBS containing 3% BSA and 0.05% Tween 20 and incubated for 1 hour at room temperature with biotinylated goat anti-human Ig. Plates were subsequently washed again and incubated with streptavidin-labeled alkaline phosphatase in PBS/3% BSA/0.05% Tween 20. After washing, plates were developed with 5-bromo-4-chloro-3-indolyl phosphate (Sigma) at 1 mg/ml in 2-AMP-buffer (2-amino-2-methyl-1-propanol, $MgCl_2$, Triton X-405; Sigma). Spots were counted to determine ISC frequency and perform spot size analysis with a CTL reader and Immunospot (version 3.2) software (CTL, Cleveland, OH) [26].

Quantitation of mRNA expression by real-time polymerase chain reaction (PCR) analysis

RNA expression was determined in CD19+ B-cells that were purified by negative selection of tonsillar mononuclear cells using the StemSep Method (Stem Cell Technologies Inc., Vancouver, BC, Canada). In brief, tonsillar mononuclear cells were incubated with a cocktail of tetrameric antibody complexes containing monoclonal antibodies to the following surface antigens: CD2, CD3, CD14, CD16, CD56, and glycophorin A. After 30 minutes of incubation at 4°C, magnetic colloid was added for another 30 minutes. Samples were then loaded in a magnetic column where antibody-bound cells were retained. The purity of the released population was ~90% as determined by staining of CD19 cells.

Total RNA was prepared using TRIzol (Invitrogen, Carlsbad, CA) and reverse-transcribed for 50 minutes at 42°C using random hexamer primers (Invitrogen) and SuperScript II reverse transcriptase (Invitrogen). Quantitative real-time PCR was performed with the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) and 3 pmol/μl gene-specific forward and reverse primers designed using the Primer Express software (Applied Biosystems) and listed in Table 1. The reaction conditions were as follows: stage 1: 10 minutes at 95°C (one cycle); stage 2, 15 seconds at 95°C and 1 minute at 60°C (40 cycles); stage 3, 15 seconds at 95°C (one cycle); stage 4, 15 seconds at 60°C (one cycle); and stage 5, 15 seconds at 95°C (one cycle). Gene-specific PCR products were continuously measured by means of an ABI Prism 7900 HT sequence detection system (Applied Biosystems). Samples were normalized using the housekeeping gene β-actin mRNA. Results are expressed as the relative fold increase over baseline.

Statistical analysis

Statistical analyses were performed using SPSS (Windows version 12; SPSS Inc., Chicago, IL). *P* values <0.05 were considered significant. Student's *t*-test and one-way analysis of variance were applied to analyze differences in immunoglobulin secretion and changes in B-cell subsets throughout time. χ^2 tests were used to analyze the fold increase of mRNA, proportion of B-cell subsets, and differences in spot numbers between different culture conditions. Linear regression analysis was done to determine the correlation between secretion of Ig and IL-6.

Results

Histological and flow cytometric characteristics of B-cell populations from tonsillar organ cultures

We used a tonsillar organ culture model to study human plasma cell biology on the basis of the assumption that intact tissue would provide optimal survival conditions for ISCs[7]. To validate the potential usefulness of tonsillar organ cultures for studying human plasma cells, we monitored their presence in organ culture by histology and flow cytometric analysis. First, we performed histological analysis of fresh tonsillar tissue and of tissue fragments harvested at

Table 1 Overview primers used for Real-Time PCR

Primer to	Forward Sequence	Reverse Sequence
B2M	5′-TGACTTTGTCACAGCCCAAGATA-3′	5′-AATCCAAATGCGGCATCTTC-3′
B-actin (A)	5′-CGTGGACATCCGCAAAGAC-3′	5′-TGCATCCTGTCGGCAATG-3′
Bcl-6	5′-TCCGTGCCCATGTGCTTA-3′	5′-GAGTCTGAAGGTGCCGGAAA-3′
BLIMP-1	5′-AGCTTTCATCCCCTCGTACAAC-3′	5′-CGCTCAGGCCATTACAATTCAT-3′
BSAP/PAX-5	5′-CAGGACATGGAGGAGTGAATCA-3′	5′-ATGTCGCAGGGCCTGACA-3′
c-Myc	5′-AGACAGATCAGCAACAACCGAAA-3′	5′-AGCTCCGTTTTAGCTCGTTCCT-3′
IL-6	5′-TACCCCCAGGAGAAGATTCCA-3′	5′-CGAGGATGTACCGAATTTGTTTG-3′
IRF-4	5′-ACCCGCAGATGTCCATGAG-3′	5′-TGGCATCATGTAGTTGTGAACCT-3′
XBP-1	5′-CCGGAGCTGGGTATCTCAAAT-3′	5′-ATCCACAGTCACTGTAAGCATCCA-3′
AID	5′CTTCAAAGATTATTTTTACTGCTGGAATAC-3′	5′-GATGCGCCGAAGCTGTCT-3′
IgJ	5′-CCCGGATTACTTCCAGGATCA-3′	5′-TCGGATGTTTCTCTCCACAATG-3′

consecutive time points in culture. This revealed persistence of clusters of plasma cells at all time points, dissolution of germinal centers, and slow disintegration of tissue fragments (Figure 1, A and B), paralleled by a drop in the number of live mononuclear cells isolated from the tissue fragments throughout a 2-week culture period (Figure 1C). Flow cytometric analysis showed that individual subsets within the B-cell population remained present at constant percentages, with the exception of germinal center cells, and to a lesser extent ISC: in one experiment the percentage of CD19+ cells among mononuclear cells gradually dropped from 65.7% at baseline to 54.8% at day 4, 43.5% at day 7, 45.3% at day 10, and 41.5% at day 13, whereas the percentage of CD19+ CD27br CD38br cells (phenotypically corresponding to ISC) dropped from 2.5% at baseline to 0.6% at day 13 (Figure 2), the changes being representative of 15 experiments (Table 2). These flow cytometric analyses of pre- and post-culture B-cell populations confirmed that the decrease in the percentage of CD19+ cells in organ cultures reflected the loss of IgD- CD27+ germinal center cells and that a proportion of plasma cells survives in tonsillar organ cultures.

Table 2 Changes in tonsillar B-cell subsets after organ culture

Marker	Baseline	SD	Cultured	SD	*P* value
CD19	62.4	9.7	39.5	14.1	<0.0001
CD38br	1.9	0.8	1.7	1.6	0.55
CD27+	51.1	7.1	38.6	11.5	0.0001
CD27-	45.7	7.2	58	12.7	0.0005

Data indicate the percentage of B-cell subsets in freshly obtained tonsillar mononuclear cells (baseline) and after 13 days of organ culture from 15 separate experiments. *P* values indicate changes in percentages between fresh and cultured tonsillar mononuclear cells (*t*-test)

Persistent secretion of immunoglobulin by predominantly nonproliferating ISCs in tonsillar organ cultures

To investigate whether plasma cells were functionally active, we measured immunoglobulin secretion during three consecutive blocks of time of 3 days each, starting at day 1. IgG was the predominant isotype secreted, followed by IgA and IgM (Figure 3A). The amounts of Ig measured in three consecutive blocks of 3 days remained stable, reflecting persistent and constant secretion of Ig.

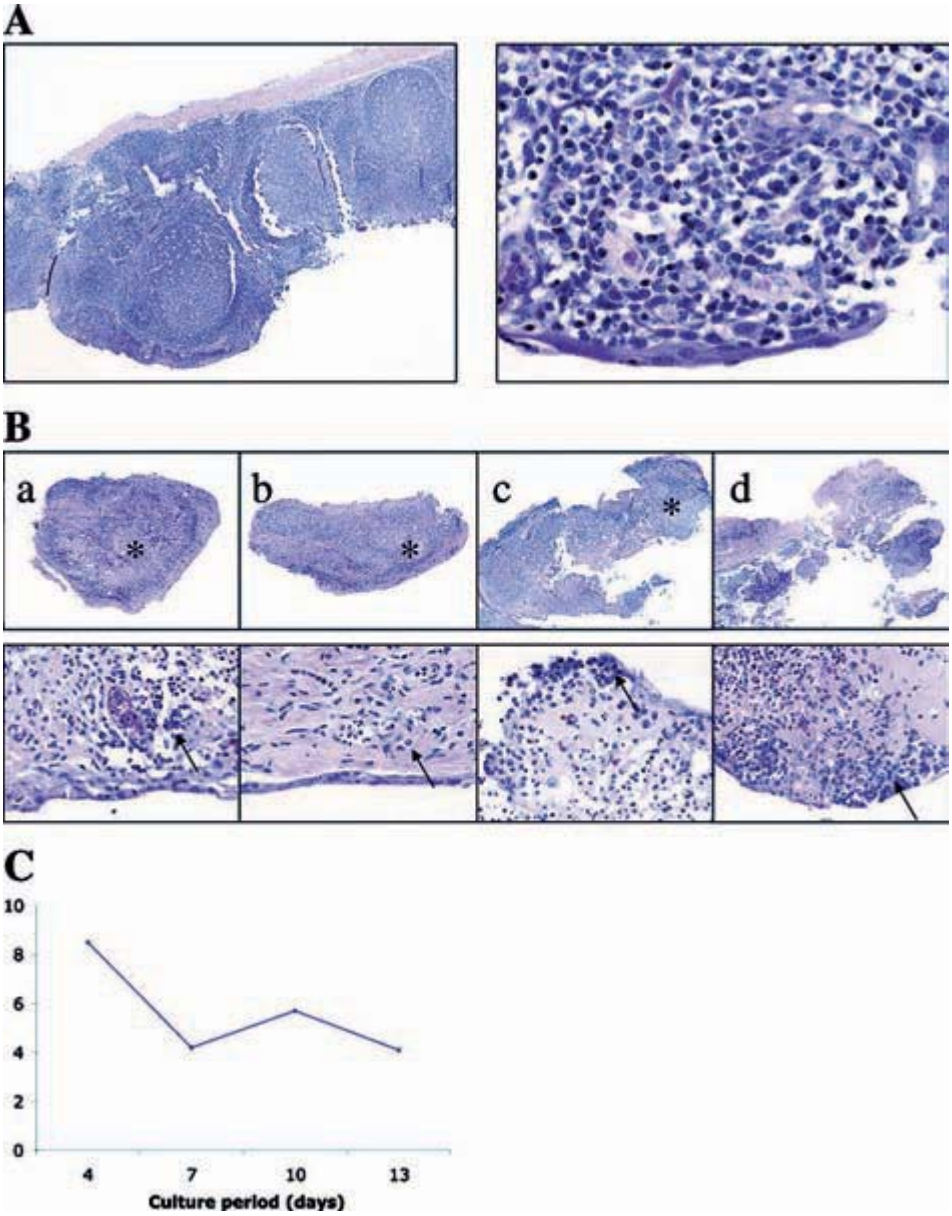


Figure 1 Hematoxylin-stained sections of formalin-fixed freshly isolated (**A)** and cultured **(B).** tonsillar tissue fragments, serially harvested at different days after initiation of culture (**a.** day 4; **b.** day 7; **c.** day 10; **d.** day 13). Tonsillar organ cultures were replenished with fresh culture medium at days 1, 4, 7, and 10. **Asteriks** refer to remnants of germinal centers; **arrows**, to clusters of plasma cells. **C:** The number of live mononuclear cells ($x10^6$/well) retrieve from tonsillar tissue fragments at consecutive time points as determined by trypan blue staining.

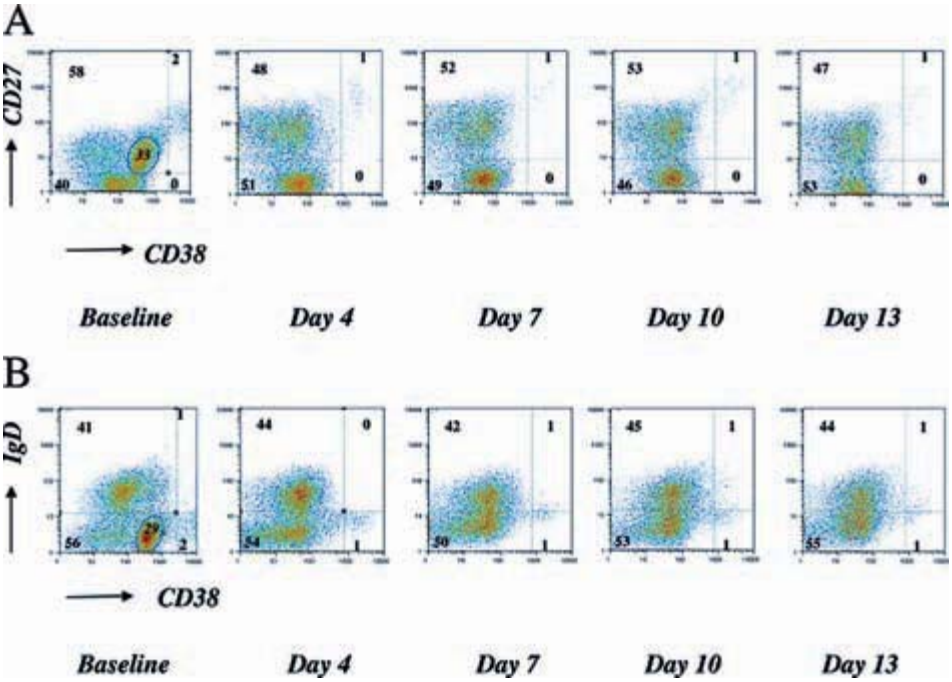
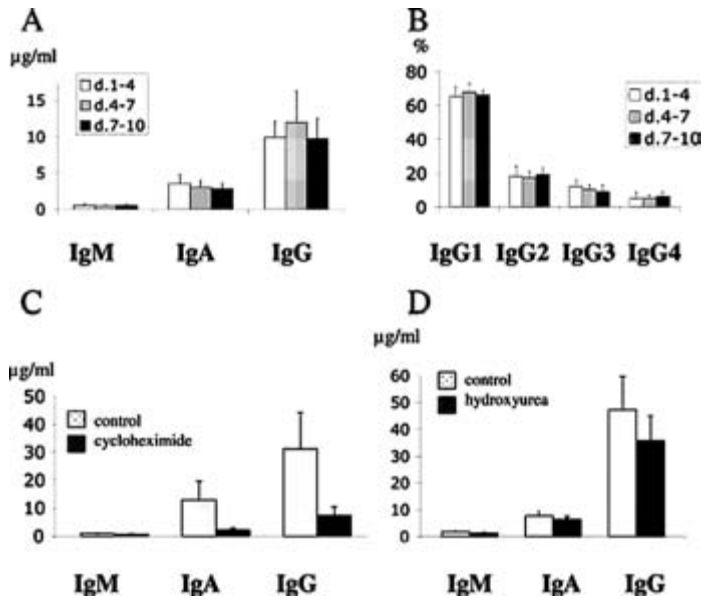


Figure 2 Flow cytometric analysis of mononuclear cells isolated from tonsillar tissue fragments, serially harvested at different days after initiation of culture. Tonsillar organ cultures were replenished with fresh culture medium at days 1, 4, 7, and 10. Mononuclear cells were obtained by enzymatic digestion of tissue fragments and stained with panels of antibodies to characterize different CD19+ B-cell subsets by coexpression of CD27 (*y* axis) and CD38 (*x* axis) (**A),** or IgD (*y* axis) and CD38 (*x* axis) (**B**), including the population of plasma cells ($CD27^{bright}$ $CD38^{bright}$ in **A**, $CD38^{br}$ in **B**). The plots show an early contraction from baseline to day 4 of the $CD27^{bright}$ $CD38^{bright}$ population containing plasma cells, which then remains stable, and a more pronounced reduction of germinal center cells (gated with ovals).

IgG1 was the predominant subclass, followed by IgG2, IgG3, and IgG4 (Figure 3B). CHX (10 µg/ml) blocked IgG secretion by 72% (mean, range 42 to 87%; *n*=4), implying most Ig in organ cultures was actively synthesized (Figure 3C). HU (10^{-2} mol/L) had a modest effect on Ig secretion, indicating most Ig was produced by nonproliferating ISCs (Figure 3D). The ELISA data were corroborated by ELISPOT analyses of mononuclear cells retrieved from tonsillar organ cultures. The relative amounts of secreted immunoglobulin mirrored the frequency of ISCs as assessed by ELISPOT, i.e., IgG > IgA > IgM ISCs (Figure 4, A and B). The finding that the preponderance of Ig secreted could be blocked with CHX in both ELISA and ELISPOT (as shown by the shift in spot size; Figure 4C) demonstrated that continued Ig secretion was the result of *de novo*

protein synthesis. Together, these data confirmed the persistence of functional nondividing, predominantly IgA- and IgG-secreting terminally differentiated plasma cells in tonsillar tissue culture.

To investigate further whether the ISCs that formed large spots in ELISPOT (arbitrary threshold defined as 0.1 mm^2 for IgG and 0.016 mm^2 for IgA) were nonproliferating cells, we used HU to block proliferation and examine its effects on Ig secretion by individual ISCs. In cultures with HU the percentage of CD19+ CD27+ CD38br cells was 0.53% compared with 1.55% in control cultures, whereas concentrations of IgA and IgG as determined by ELISA, and numbers of IgG and IgA ISCs as determined by ELISPOT were similar (Figure 4, D and E). Of note, large-sized spots did not disappear with HU, confirming these represent nonproliferating plasma cells.



Analysis of factors involved in plasma cell differentiation

Additional evidence for the persistence of plasma cells in tonsillar tissue was obtained from RT-PCR analysis of mRNA extracted from CD19+ cells isolated from cultured tonsillar tissue fragments as compared with mRNA from freshly isolated tonsillar CD19+ cells obtained at baseline (Figure 5). This revealed a marked up-regulation of plasma cell genes, in particular XBP-1, IRF-4, and Blimp-1, suggesting a relative survival advantage of long-lived plasma cells within the B-cell population and/or further maturation of plasma cells *in vitro*. Of note, AID mRNA was significantly lower, consistent with loss of germinal center cells, and in keeping with the aforementioned results from flow cytometry.

Figure 3 Concentrations of immunoglobulins and IgG subclasses detected in supernatants of tonsillar organ cultures. Tonsillar tissue fragments immobilized on Gelfoam were cultured for 10 days without additional stimulus. Culture supernatants were removed at days 1, 4, 7, and 10, and replenished with fresh culture medium. Concentrations of immunoglobulins (IgM, IgA and IgG) **(A)** and IgG subclasses **(B)** were measured by sandwich ELISA. The bars represent the mean (± SEM) of 11 experiments for IgM, IgA and IgG, and five experiments for IgG subclasses. The amounts of the various Igs produced during each time frame were not significantly different (one-way analysis of variance). The addition of CHX (10 μg/ml, **(C)** blocked Ig secretion significantly (P=0.03 for IgA, P=0.02 for IgG; paired t-test, log-transformed data; n=4). The addition of HU **(D)** had a modest but statistically significant effect on IgG secretion (P=0.005; paired t-test, log-transformed data; n=6).

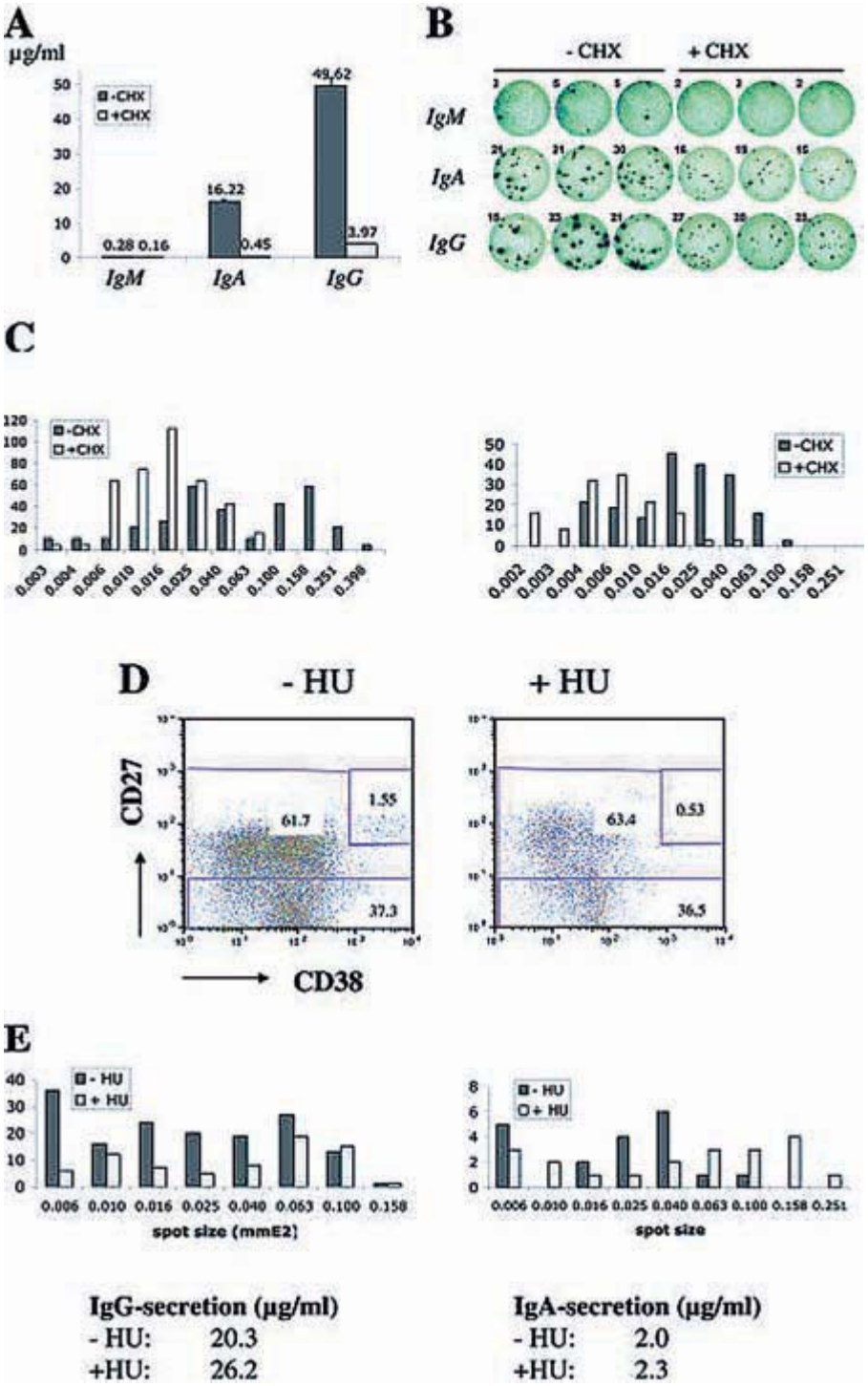

A
µg/ml
-CHX
+CHX
0.28 0.16
16.22
0.45
49.62
3.97
IgM
IgA
IgG
B
- CHX
+ CHX
IgM
IgA
IgG
C
-CHX
+CHX
D
- HU
+ HU
CD27
CD38
61.7
1.55
37.3
63.4
0.53
36.5
E
- HU
+ HU
spot size (mmE2)
spot size
IgG-secretion (µg/ml)
- HU: 20.3
+HU: 26.2
IgA-secretion (µg/ml)
- HU: 2.0
+HU: 2.3

Figure 4 Immunoglobulins predominantly IgA and IgG, in tonsillar tissue cultures are actively secreted by plasma cells with different secretion rates. Tonsillar tissue fragments immobilized on Gelfoam were cultured for 12 days with replenishment of culture medium at days 1, 4, and 8. At day 8, the tissue fragments from one plate were harvested, enzymatically digested, and mononuclear cells isolated by Ficoll-Isopaque. A: Culture of tissue fragments in another plate was continued for an additional 4 days, with or without CHX (10 µg/ml), for measurements of immunoglobulin in supernatants by sandwich ELISA. B: A sample of mononuclear cells from day 8 were used for ELISPOT to determine the frequency of ISC, with or without CHX. Numbers refer to the number of spots per well, containing 50,000 (IgM), 25,000 (IgA), 12,500 (IgG) mononuclear cells. B and C: Note that the addition of CHX did not eliminate spots but merely resulted in a shift towards smaller spotsizes for both IgG and IgA spots. To confirm that secretion of IgA and IgG in tonsillar organ cultures is primarily independent of proliferation, tonsillar tissue fragments immobilized on Gelfoam were cultured for 8 days. Culture supernatants were removed at day 1 and fresh culture medium added, with or without HU (10^{-2} mol/L) as indicated. At the end of the culture period, tissue fragments were harvested, enzymatically digested, and mononuclear cells isolated by Ficoll-Isopaque. A proportion of the mononuclear cells were stained with panels of antibodies, including CD19, CD27 and CD38, and analyzed by flow cytometry. The top graph depicts expression of CD27 (*y* axis) versus CD38 (*x* axis) of CD19+ cells (D), whereas the bottom graph shows the effect of HU on number of IgA- and IgG-secreting cells by ELISPOT (E). The results shown in A-C are representative of two experiments, whereas those shown in D and E represented another experiment.

Intact tissue is a prerequisite to maintain Ig production

The persistence of functional plasma cells in clusters surrounded by stromal cells suggested intact tissue was required for the maintenance of Ig secretion by plasma cells. Support for this concept was obtained in experiments in which mRNA expression of B-cell populations and Ig secretion from organ culture was compared with that by whole cell and mononuclear cell preparations obtained by enzyme digestion with or without subsequent Ficoll-Isopaque isolation of mononuclear cells. Importantly, comparable numbers of cells were cultured in each of these conditions, being derived from a pool of randomly selected, similar sized tissue fragments. Figure 6A shows stronger expression of Blimp-1 and lower expression of BSAP in the B-cell population that was isolated from tissue fragments versus that from cultured cell suspension. Figure 6B shows high Ig concentrations in supernatants of cultured organ fragments, as compared with concentrations in supernatants of cultured whole cell suspensions, and mononuclear cell suspensions. In addition, cell suspensions were prepared from the tissue fragments harvested at the end of these cultures, and Ig

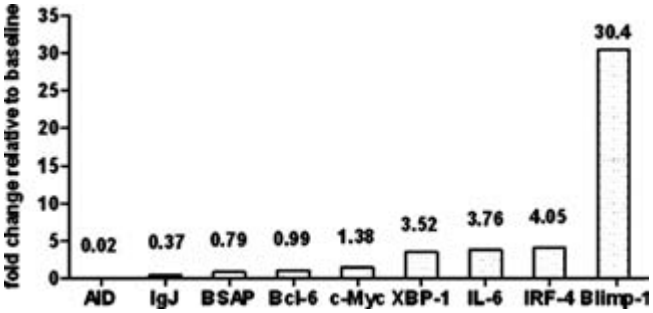


Figure 5 Enhanced transcription of genes involved in survival of long-lived plasma cells. Tonsillar tissue fragments immobilized on Gelfoam were cultured for 8 days. Culture supernatant was removed at days 1 and 4 and replaced with fresh culture medium. At the end of the culture period, tissue fragments were harvested, enzymatically digested, and mononuclear cells isolated by Ficoll-Isopaque. mRNA was isolated from negatively sorted B cells (see Material and Methods) and analyzed by semiquantitative RT-PCR for fold increase in expression of varied transcripts as compared with the baseline sample. The bars represent the mean of two experiments.

secretion measured in culture supernatants obtained after 4 additional days in culture. Notably, Ig concentrations again were lower in supernatants from the cultured whole cell suspension than from the parallel organ culture (IgG, 49.6 versus 5.2 μg/ml), indicating that plasma cells cannot maintain Ig secretion when tissue is disrupted and cells are dispersed. The relatively low Ig secretion in cultures of dispersed cells could not be attributed to absence of phenotypically defined plasma cells, as determined by flow cytometric analysis (Figure 6C). The data are compatible with a model in which tissue context promotes Ig secretion but not necessarily maintenance of long-lived plasma cells.

Tonsillar tissue secretes soluble factors including high concentrations of IL-6 that may contribute to Ig secretion by plasma cells

To determine whether tonsillar tissue provides essential factors in the maintenance of long-lived plasma cells we measured a number of cytokines in supernatants of tonsillar organ culture. Notably, IL-6 was released in high quantities (Table 3). The role of IL-6 in survival of long-lived plasma cells was further supported by the observation that IL-6 concentrations in supernatants correlated highly with secretion of IgA and IgG but not IgM (Figure 7).

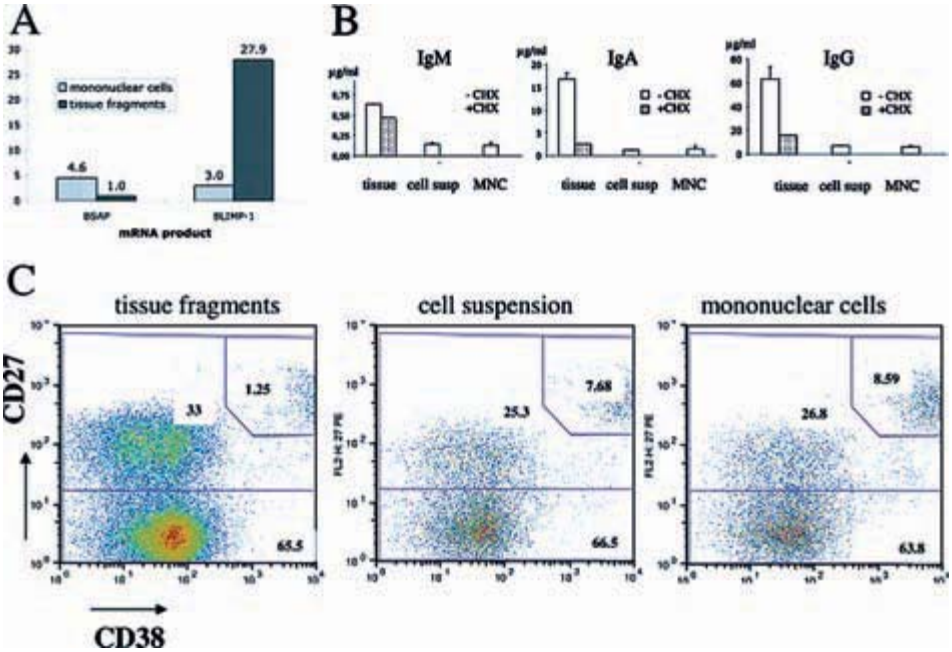


Figure 6 Integrity of tissue fragments is a prerequisite for continued high Ig secretion. Tonsillar tissue fragments and whole cell or mononuclear cell suspensions were cultured for 8 days as described in Material and Methods. Cell suspensions were procured by enzyme digestion of tonsillar tissue; the mononuclear cell fraction by additional Ficoll-Isopaque isolation. Expression of mRNA was determined by RT-PCR (**A**), immunoglobulin secretion was measured by ELISA (**B**), and the number of plasma cells was assessed by flow cytometric analysis (**C**). The data are representative of two experiments on cultures of tissue fragments, whole cell suspension, and mononuclear cell suspension, and of seven experiments with tissue fragments and mononuclear cell suspensions.

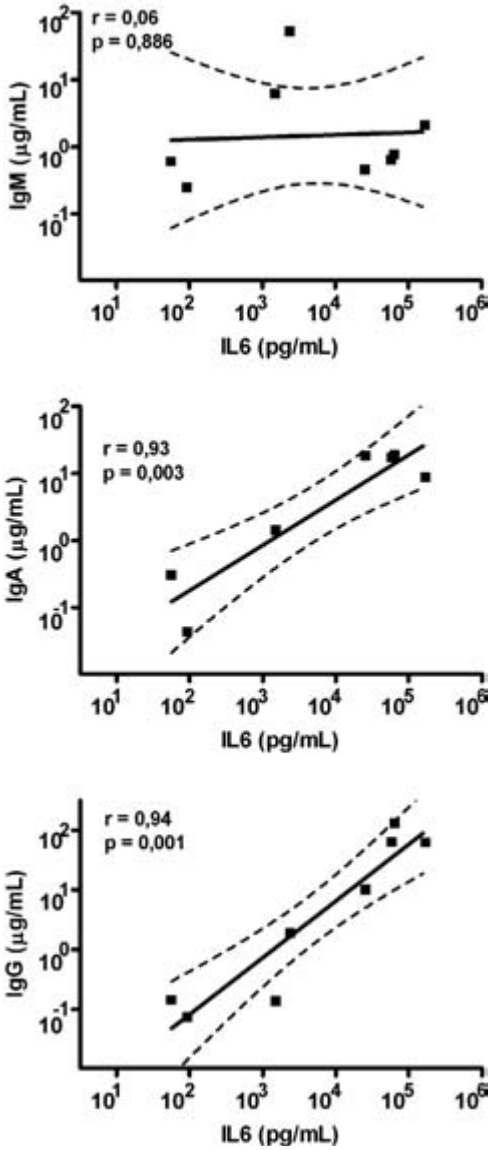


Figure 7 Secretion of IgA and IgG, but not IgM, by tonsillar plasma cells is correlated with IL-6 secretion. Tonsillar tissue fragments or whole cell suspensions were cultured for 8 days as described in Material and Methods. Concentrations of immunoglobulins and IL-6 were measured in supernatants obtained at the end of culture by ELISA. IgA and IgG, but not IgM, were highly correlated with IL-6 levels (log-transformed data, six experiments; *P*=0.003, 0.001, and 0.886, respectively) as determined by linear regression analysis. The figure shows the linear fit and the borders of the 95% confidence interval.

Table 3 Concentration of cytokines (pg/ml) in supernatant from one tonsillar organ culture

IL-1α	49.1
IL-1β	10.5
IL-2	6.7
IL-4	3.6
IL-6	58,246.3
IL-7	2.5
IL-10	7.5
IL-12 p40	3.6
IL-12 p70	10.4
IL-15	25.2
IL-18	46.8
IFN-α	8.5
IFN-γ	0.4
TNF	40.9
SDF-1β	10.5
GM-CSF	394.2

IFN: interferon; TNF: tumor necrosis factor; GM-CSF: granulocyte macrophage-colony-stimulating factor

Discussion

The present study was conducted to determine whether long-lived nondividing plasma cells resided within human tonsil and to determine features that would promote ongoing Ig secretion. These findings were prompted by a phenotype analysis of plasma cells from various tissue sites suggesting that tonsil plasma cells were mostly composed of recently generated immature Ig-secreting cells[22]. The current findings indicate that, although they have a different phenotype than bone marrow plasma cells, they are primarily composed of nondividing long-lived plasma cells. We used a tonsillar organ culture model as a means to preserve tissue architecture and maintain function of plasma cells. Tonsillar tissue fragments were cultured for up to 2 weeks, without the need for exogenous cytokines or extra growth factors. Our study demonstrates that tonsillar tissue harbors a population of nonproliferating, long-lived plasma cells. Within the

context of intact lymphoid tissue, viability of a proportion of resident plasma cells was preserved. Plasma cells survived in clusters surrounded by stroma, facilitating cell-cell contact and optimal use of autocrine and paracrine factors. A significant proportion of IgA and IgG appeared to be secreted by such non-proliferating, long-lived ISCs, some producing large amounts of Ig as shown by ELISPOT. A previous study in immunized rats has shown that greater spot size correlates with a more mature plasma cell population[27]. The notion that mature plasma cells comprise an important part of the tonsillar B-cell population was further corroborated by the finding of high levels of mRNA for Blimp-1, a key transcriptional repressor in the formation and maintenance of (long-lived) plasma cells[28,29]. Blimp-1 affects numerous aspects of plasma cell maturation, ranging from migration, adhesion, and homeostasis to antibody secretion. Interestingly, a previous analysis of *in vivo* ISCs induced by immunization revealed a developmental pathway in which increasing levels of Blimp-1 expression define developmental stages of plasma cell differentiation[30]. Two other key transcriptional factors of plasma cells were also strongly expressed: IRF-4 and XBP-1. XBP-1, downstream of Blimp-1, has been shown by gene expression profiling in mice, to coordinate multiple changes in cellular structure and function resulting in the characteristic phenotype of professional secretory cells, whereas IRF-4 is essential for plasma cell differentiation, independent of Blimp-1 and upstream of active XBP-1[31-33].

Our results are in keeping with recent data from mouse studies on bone marrow, suggesting that plasma cells depend on specific survival niches for viability and function. However, the data suggest that the niche may be more important in promoting Ig secretion by plasma cells rather than merely their survival. Our data indicate human tonsils must contain such a survival niche. In mice IL-6, tumor necrosis factor, and interactions through CD44 were shown to play a pivotal role in maintenance of plasma cell homeostasis. Interestingly, the effect of IL-6 was optimal at the highest concentrations tested[7]. In humans, survival of ISCs generated through interaction of peripheral blood B-cells and activated T-cells similarly depended on (exogenous) IL-6[34]. The strong correlation between high secretion of IL-6 and IgA and IgG secretion found in our study suggests homeostasis of human plasma cells depends on similar mechanisms. Studies of human tonsils have shown IL-6 to be mainly produced by extrafollicular cells and stromal cells[35,36]. Furthermore, co-culture of human B-cells with stromal

cells has revealed cell-cell contact to be critical for the optimal production of IL-6, in line with data from murine systems[20,37,38]. Preliminary data in our group have indicated freshly isolated tonsillar plasma cells to be the predominant B-cell subset expressing IL-6 mRNA (Slota R, Lipsky PE, Grammer AC, unpublished data), which if secreted could act as autocrine factor. Other soluble factors have also been shown to be necessary for B-cell survival/maturation, such as BAFF produced by stromal cells[8].

Our study underscores the importance of close cell-cell contact to ensure the optimal release of autocrine and/or paracrine factors, necessary for Blimp-1 expression and Ig secretion by plasma cells, but not necessarily their survival. Indeed, under conditions of microgravity in a rotating-wall vessel, Ig secretion in tonsillar organ cultures is strongly reduced (Margolis L, personal communication)[39]. Taken together, these findings suggest that Ig secretion by plasma cells is influenced by physical, antigen-independent, interaction with stromal cells. To what extent pre-existent inflammation contributed to our data remains to be determined[40], but a recent study in a mouse nephritis model did show that nonproliferating, long-lived plasma cells persist in inflamed tissue[41]. A recent study by our group showed that T lymphocytes also play a pivotal role in maintaining survival of tonsillar plasma cells. Most of the latter cells were nonproliferating, as indicated by lack of Ki-67 expression, and long-lived when implanted in immunodeficient mice[42]. As discussed in this article, CD138 expression does not reliably identify (long-lived) plasma cells isolated from tonsils using collagenase, as this cleaves CD138 from the surface of tonsil plasma cells[42].

In conclusion, our study demonstrates that in humans a small population of nonproliferating plasma cells, secreting large amounts of IgA and IgG, can be readily isolated from tonsillar organ cultures even at extended culture periods of 2 weeks. Furthermore, an intact microenvironment seems critical for continued Ig production by the plasma cell population. The tonsillar culture model thus seems to be a valid surrogate experimental system, providing a simple, valuable tool to evaluate the effects of new therapies directed at plasma cells.

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