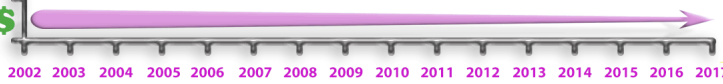




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## Long-Lived Plasma Cells from Human Small Intestine Biopsies Secrete Immunoglobulins for Many Weeks In Vitro

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# Long-Lived Plasma Cells from Human Small Intestine Biopsies Secrete Immunoglobulins for Many Weeks In Vitro

Luka Mesin,<sup>\*,†</sup> Roberto Di Niro,<sup>\*,†</sup> Keith M. Thompson,<sup>\*,†</sup> Knut E. A. Lundin,<sup>\*,‡</sup> and Ludvig M. Sollid<sup>\*,†</sup>

To understand the biology of Ab-secreting cells in the human small intestine, we examined Ab production of intestinal biopsies kept in culture. We found sustained IgA and IgM secretion as well as viable IgA- or IgM-secreting cells after >4 wk of culture. The Ab-secreting cells were nonproliferating and expressing CD27 and CD138, thus having a typical plasma cell phenotype. Culturing of biopsies without tissue disruption gave the highest Ab production and plasma cell survival suggesting that the environment regulates plasma cell longevity. Cytokine profiling of the biopsy cultures demonstrated a sustained presence of IL-6 and APRIL. Blocking of the activity of endogenous APRIL and IL-6 with BCMA-Fc and anti-human IL-6 Ab demonstrated that both these factors were essential for plasma cell survival and Ab secretion in the biopsy cultures. This study demonstrates that the human small intestine harbors a population of nonproliferating plasma cells that are instructed by the microenvironment for prolonged survival and Ab secretion. *The Journal of Immunology*, 2011, 187: 2867–2874.

The gut is the main site for production of Ab in humans. The majority of Ab-secreting cells in the human intestine produce IgA (80–90%), whereas most of the remaining cells produce IgM and almost none produce IgG (0–2%) (1–3). It is estimated that ~80% of IgA-secreting cells in the body reside in the gut mucosa (4). Ab-secreting cells of the gut, as at other mucosal sites, produce mainly J chain-containing IgA dimers and IgM polymers that are transported across the epithelial barrier to exert functions as secretory Abs on the mucosal surface (5, 6). Emerging evidence indicates that intestinal Abs affect both immune protection and immune exclusion of the mucosa in a non-inflammatory manner (7, 8).

Ab-secreting cells consist of both plasmablasts and plasma cells. Plasma cells are end-differentiated and nonproliferating cells, differentiated from B cells via an intermediate stage of proliferating plasmablasts. Further, plasma cells are often divided into short- and long-lived populations, and long-lived plasma cells can survive for years (9).

The population of plasma cells that exists in the bone marrow plays a pivotal role in maintaining systemic humoral immunity (10), and these cells are considered to be long-lived (11, 12). Even if relatively little is known about plasma cell biology (13), the survival of plasma cells seems to be regulated by a combination of both factors intrinsic to the plasma cell and extrinsic factors constituted by the microenvironment (14, 15). It has been dem-

onstrated that lymphoid tissue, particularly the bone marrow, can create a stromal microenvironment, a niche, where plasma cells can survive for extended periods of time (16–18).

Whether Ab-secreting cells resident at mucosal sites can be long-lived as well as provide long-term humoral immunity is a matter of debate (19, 15). Several observations have suggested that mucosal Ab-secreting cells are short-lived (20, 10, 12). However, sustained Ab production at mucosal sites has been shown in humans (21), and there are some indications of local long-term Ab production in nasal MALT of mice (22). Human tonsil tissue was also described as a site of sustained Ab secretion harboring long-lived plasma cells (23).

In the small intestine, plasma cells are located in the lamina propria, and this is the main GALT effector site (24, 25). In the small intestine of mice, the average half-life of lamina propria plasma cells was estimated to be 5 d and a maximum life span of the order 6 to 8 wk (26). In apparent contrast to these observations, evidence for a long-lived anticomensal IgA response was observed by reversible colonization of germ-free mice, suggesting the existence of long-lived IgA plasma cells in the gut (27).

Whether the lamina propria of the human small intestine contains a population of nonproliferating long-lived plasma cells remains an open question. In this study, we examined plasma cell longevity by culturing cells and biopsies from the human small intestine.

## Materials and Methods

### Small intestine tissue samples

Mucosal biopsy specimens of the duodenum were obtained by forceps sampling from individuals subjected to endoscopy because of abdominal pain, diarrhea, dysphagia, or abdominal distention. Only subjects that had macroscopically and microscopically normal intestinal mucosa were included in the study. The biopsies were transported on ice-cold RPMI 1640 medium to the laboratory for further processing within minutes after sampling. The participating subjects gave their informed consent for donating biopsy material. The study was approved by the Regional Ethics Committee of South-Eastern Norway.

### Tissue culture model

Intestinal biopsies were washed and cultured in 1 ml RPMI 1640 supplemented with 10% FCS and penicillin/streptomycin 50 µg/ml at 37°C and 5% CO<sub>2</sub> in 48-well trays. Two biopsy samples per well (size varying from 2 to 3 mm) were cultured for each condition in duplicate to minimize

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Abbreviations used in this article: AP, alkaline phosphatase; APRIL, a proliferation-inducing ligand; BCMA, B cell maturation Ag.

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discrepancies related to variations in sample size and handling. To reduce the passive shedding of Ab from tissue deposits, culture medium was harvested and replaced with fresh medium the following day. Tissue biopsies were kept in cultures for 3 or 4 wk with complete medium replacement every 2 d. Harvested culture supernatants were centrifuged and preserved at  $-20^{\circ}\text{C}$  for further analysis.

### Single-cell suspension

Intestinal biopsy specimens were incubated twice in 2 mM EDTA, 2% FCS in PBS with constant rotation for 30 min to remove the epithelium and intraepithelial lymphocytes. The remaining tissue fragments were enzymatically digested with 1 mg/ml blend collagenase (Sigma-Aldrich) in 3% FCS in PBS at  $37^{\circ}\text{C}$  for 30 min. This step was repeated twice. Afterward, cell suspensions were filtered through a 40- $\mu\text{m}$  cell strainer (BD Biosciences) and washed in PBS.

### Cell suspension culture model

Viable cells in single-cell suspensions of human intestinal lamina propria were counted by trypan blue exclusion. Viable lamina propria cells were seeded at  $5 \times 10^4$ ,  $10^4$ , or  $5 \times 10^3$  cells per well in 96-well microtiter plates and cocultured with or without autologous irradiated PBMCs,  $5 \times 10^4$  cells/well, for 3 or 4 wk at  $37^{\circ}\text{C}$  and 5%  $\text{CO}_2$  in 150  $\mu\text{l}$  RPMI 1640 10% FCS with penicillin/streptomycin 50  $\mu\text{g}/\text{ml}$  supplemented with or without the following reagents: human recombinant IL-6 (R&D Systems), 10 ng/ml; anti-human IL-6 Ab (R&D Systems), 1  $\mu\text{g}/\text{ml}$ ; recombinant human BCMA-Fc (Enzo Life Sciences), 1  $\mu\text{g}/\text{ml}$ ; recombinant human a proliferation-inducing ligand (APRIL) (Enzo Life Sciences) 1  $\mu\text{g}/\text{ml}$  or control mouse IgG1 (R&D Systems) 1  $\mu\text{g}/\text{ml}$ . Two thirds of culture supernatants were removed and replenished weekly with fresh complete culture medium. To control for the contribution of Ab that had already been synthesized in cultures and the decay, one third of weekly replenished medium was cultured in parallel with or without feeder cells but with no biopsy-derived cells. This set of experiments was done by plating  $10^4$  biopsy-derived cells/well.

### Quantitative sandwich ELISA

Sandwich ELISA was performed to measure total IgA, IgG, and IgM in culture supernatants. ELISA plates (Nunc) were coated overnight at  $4^{\circ}\text{C}$  with anti-IgA (I-2261), anti-IgG (I-2136), or anti-IgM (I-2386; Sigma-Aldrich) capture Ab and blocked with 2% skimmed milk in PBS for 1 h at room temperature. Supernatants from cell cultures and human reference serum (Bethyl Laboratories) containing a known amount of the different Ig isotypes were diluted in 2% skimmed milk in PBS, added to the wells, and incubated for 1 h at room temperature. After extensive washing, alkaline phosphatase (AP)-conjugated anti-IgA (A-9669), anti-IgG (A-3437), or anti-IgM detection Ab (A-9544; Sigma-Aldrich) diluted 1:2000 in 2% skimmed milk in PBS was added and the plates incubated for 1 h at room temperature. Plates were washed and developed with phosphatase substrate (Sigma-Aldrich). Absorbance was measured at 405 nm. Data were analyzed using GraphPad Prism software.

### ELISPOT assay

Frequencies of Ab-secreting cells were quantified by ELISPOT assay. MultiScreen ELISPOT plates (Millipore) were coated overnight at  $4^{\circ}\text{C}$  with anti-IgA or anti-IgM capture Ab (Sigma-Aldrich) and then blocked with RPMI 1640, supplemented with 10% FCS at  $37^{\circ}\text{C}$  for 2 h. Subsequently, cells were added to plates at different cell concentrations and at several dilutions in duplicate starting at  $2 \times 10^5$  cells per well and were incubated overnight at  $37^{\circ}\text{C}$ , 5%  $\text{CO}_2$ . The following day, the cells were removed, and the plates were washed six times with PBS-0.01% Tween and three times with PBS. Next, AP-conjugated anti-IgA and anti-IgM (Sigma-Aldrich) were added, and the plates were incubated for 1 h at room temperature. Spots were developed using the AP Conjugated Substrate kit (Bio-Rad) and counted manually with a Carl Zeiss Axioplan 2 microscope.

### Flow cytometry

Small intestine lamina propria cells were analyzed by flow cytometry using the following Abs for staining; anti-IgA- or anti-IgM-FITC (Southern Biotech), anti-CD138-FITC (eBioscience), anti-Ki-67-FITC and isotype control (BD Biosciences), anti-CD138-PE (eBioscience), anti-CD38-PE (BD Biosciences), anti-CD14-PerCP (BD Biosciences), anti-CD20-PerCP (Exbio), anti-DR-PerCP (BD Biosciences), and anti-CD27-allophycocyanin (BD Biosciences). Briefly, cells were incubated with an appropriate combination of labeled Ab diluted in PBS 3% FCS for 45 min on ice and washed in PBS with 3% FCS. After washing, cells were resuspended in PBS with propidium iodide and analyzed by FACSCalibur with CellQuest

software (BD Biosciences). For intracellular staining, cells were fixed for 30 min with 1% paraformaldehyde and permeabilized with 0.2% saponin in PBS followed by Ab staining.

### Cytokine assay

The level of cytokines in tissue supernatants was measured by human inflammation Cytometric Bead Array kit (BD Biosciences) or by ELISA (eBioscience, R&D Systems) following the instructions of the manufacturers.

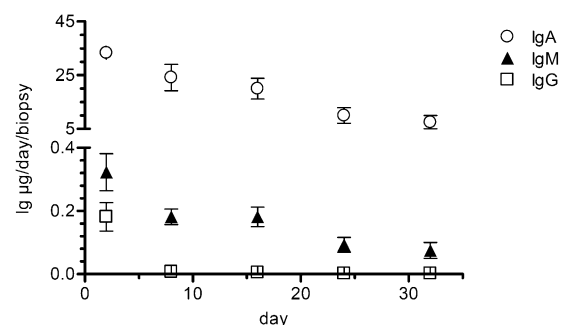
## Results

### Sustained Ab secretion *in vitro* by small intestine Ab-secreting cells

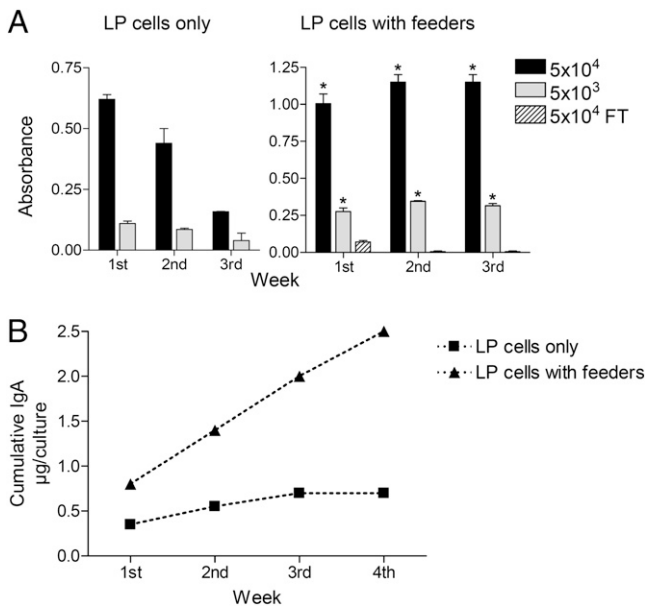
Duodenal biopsies of adult individuals were collected and cultured *in vitro*. Ab secretion was measured by ELISA to assess whether plasma cells in the samples were viable and functionally active (Fig. 1). We found that IgA was the predominant Ig isotype produced, in the range 10–35  $\mu\text{g}/\text{day}/\text{biopsy}$ , followed by IgM and IgG. Notably, IgA and IgM were persistently produced during the culture period of 1 mo, although IgA was produced in much higher concentrations. The production of IgG was not detectable after 2 d.

To analyze in more detail the dynamics of Ab secretion at the cellular level, we used single-cell suspension cultures. We observed that the secretion of IgA from small intestine cells was dependent on the culturing conditions (Fig. 2A). When  $5 \times 10^3$  small intestine lamina propria cells were cultured alone, Ab secretion was almost at an undetectable level already in the first week. By increasing the number of cells in culture to  $5 \times 10^4$ , higher levels of Ab were observed, as expected, but the production rapidly dropped over time. By contrast, when cells were cocultured with autologous irradiated PBMCs, stable and high levels of Ab were still measured 3 wk after culture initiation. No Ab production was detected when cells were frozen and thawed prior to culture to provoke cell death by disruption. This confirmed that Abs in the cultures were actively produced, excluding the possibility of passive release from either intracellular or extracellular deposits.

We attempted to measure the amount of Ab secreted during each week of culture. We needed to take into account that complete replacement of the medium was not possible and that there is decay of Ab in the cultures either mediated by proteases or cellular mechanisms. To overcome this, we split the culture supernatant in three; one third was kept in culture in the absence of biopsy-derived cells, one third was kept in culture with biopsy-derived cells, and one third was frozen for subsequent analysis. The difference between the two samples kept in culture 1 wk later would represent



**FIGURE 1.** IgA is the predominant isotype secreted by small intestine biopsy cultures. Intact tissue biopsies were cultured for 32 d. Culture supernatants were completely removed every 2 d and replenished with fresh medium. Ab concentration was measured by sandwich ELISA on days 2, 8, 16, and 32. Data shown are the average amounts of Ab secreted per day  $\pm$  SD from six independent experiments.



**FIGURE 2.** IgA in small intestine cell cultures is actively secreted over time. *A*, Small intestine tissue biopsies prepared as lamina propria (LP) single-cell suspensions were seeded at a density of  $5 \times 10^4$  or  $5 \times 10^3$  cells/well in 96-well microtiter plates, with or without autologous irradiated PBMCs as feeders ( $5 \times 10^4$  cells/well), and cultured for 3 wk. Samples that underwent freezing and thawing treatment (FT) were cultured in parallel. Two thirds of the culture supernatants were removed and replenished with fresh culture medium weekly and assayed for the presence of Ab by sandwich ELISA. The bars represent the average  $\pm$  SD of eight independent experiments of four different donors. Data were analyzed for statistical significance using Student *t* test. \* $p < 0.005$  (compared with LP cells only). *B*, Single-cell suspensions of small intestine tissue biopsies were seeded at a density of  $10^4$  cells/well in 96-well microtiter plates, with or without feeder cells, and cultured for 4 wk. Culture supernatants were removed weekly and cultured in parallel with the original culture to assess the Ab decay and accumulation rate over time. The data presented are the mean cumulative IgA titers from eight independent experiments of four different donors measured by sandwich ELISA.

the amount of Ab that was neo-synthesized over a week. PBMCs alone when cultured gave no significant Ab production (data not shown). We observed that the neo-production of Ab was stable for  $>4$  wk when the cells were cocultured with autologous PBMCs and was estimated  $\sim 60$  ng/day/ $10^4$  lamina propria cells (Fig. 2*B*). This is in contrast to what was observed in cultures without feeder cells where neo-secretion in week 2 and 3 was in the range 20–30 ng/day/ $10^4$  lamina propria cells and undetectable in week 4. We conclude from these experiments that intestinal Ab-secreting cells produce Ab over several weeks in culture and that this secretion can likely be enhanced by factors derived from PBMCs. Clearly, however, these culturing conditions do not represent the true small intestine microenvironment and were not further investigated.

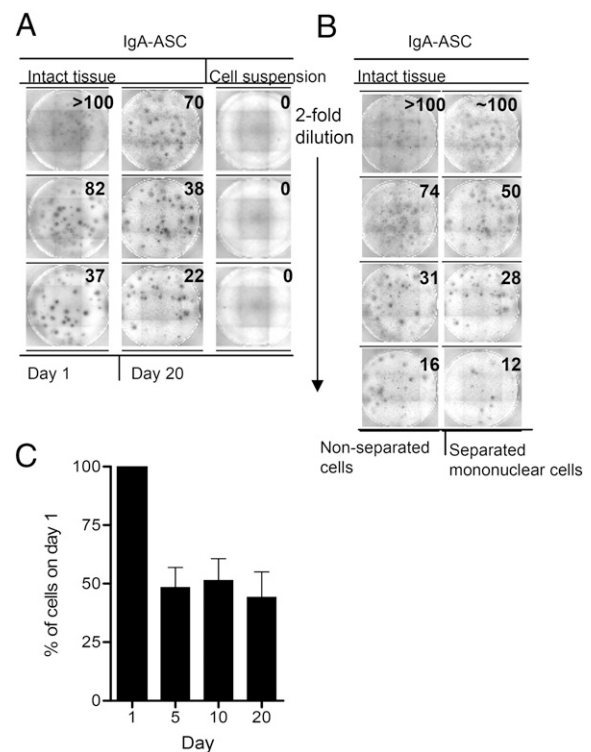
#### Dynamics of Ab production by Ab-secreting cells

Next, we aimed to estimate the number of functionally active Ab-producing cells in culture at various time points. To this end, small intestine tissue fragments were cultured for various periods of time and then prepared as single-cell suspensions by collagenase treatment before ELISPOT testing. Even if the culturing conditions were fairly simple and provided no additional survival factors other than those derived from tissue cells and serum-supplemented culturing medium, the cells appeared to survive for an extended time as IgA-secreting cells were detected on day 20 of culturing

(Fig. 3*A*). The intact tissue was a prerequisite for prolonged cell survival. When small intestine tissue biopsies were cultured that had been processed into a single-cell suspension immediately upon sampling, no IgA spots were detected after 20 d (Fig. 3*A*). To remove tissue debris and dead cells, mononuclear cell separation was performed before the IgA ELISPOT assay. This gave an improved secretion profile of spot-forming cells. Spots were uncountable in undiluted samples if mononuclear cells were not isolated, and notably, equal numbers of spots in diluted samples were enumerated (Fig. 3*B*). The dynamics of Ab-secreting cell survival and secretion was assessed by enumerating cells from intact tissue cultures at different time points (Fig. 3*C*). When compared with day 1, the number of spots dropped to 50% at day 5 and then remained stable up to day 20. This may be due simply to adaptation to the culture conditions or alternatively suggests the presence of a short-lived population, disappearing before day 5, and a longer-lived one with an extended half-life of  $>20$  d.

#### Characterization of small intestine plasma cells surviving in culture

We used flow cytometry to identify and characterize plasma cells of the small intestine and to enumerate the absolute number surviving in culture at extended time points. Small intestine lamina propria cells isolated from biopsies after different days of culturing were



**FIGURE 3.** Life span of small intestine Ab-secreting cells (ASC) in vitro. Small intestine tissue fragments or cell suspensions were cultured for 20 d, with replenishment of culture medium at days 1, 5, 10, and 15. At different time points, the tissue fragments were harvested, enzymatically digested, and assayed by ELISPOT. *A*, Viable ASC actively secreting IgA were detected at day 20 only when tissue fragments and not cell suspensions were cultured. *B*, The mononuclear cell fraction obtained by additional density gradient centrifugation was assayed in parallel with the cell suspension on day 20 by ELISPOT. *C*, ASC numbers in intact small intestine tissue cultures at days 1, 5, 10, and 20 were compared. The survival of ASC as a function of time is defined as the percentage of spots compared with day 1. In *A* and *B*, one of several independent experiments from five donors is shown. In *C*, the average values  $\pm$  SD from four independent experiments per time point from different donors are shown.

stained with Ab to CD14, CD20, CD27, CD38, CD138, Ki-67, IgA, IgG, and IgM. Ab-secreting cells were identified by forward and side scatter gating of viable (propidium iodide-negative) leukocytes and the expression of CD138 and CD27 (Fig. 4A) (3); monocytes and macrophages, representing another frequent population of large mononuclear cells in the lamina propria, were excluded by gating on CD14<sup>-</sup> cells (data not shown). We have recently shown that plasma cells of the human small intestine express detectable amounts of surface Ig (3) as demonstrated previously in mice (28). In fact, isotype distribution gave similar results when we performed staining for either intracellular or surface IgA. IgA<sup>+</sup> cells accounted for 80–90% of the CD27<sup>+</sup> CD138<sup>+</sup> cells when examined on fresh biopsies (day 0). No surface IgA<sup>+</sup> Ab-secreting cells were detected when an IgA-deficient subject was analyzed as a control (Fig. 4B). Almost all CD138<sup>+</sup> IgA<sup>+</sup> cells were negative for CD20, which is expressed on all B cells including plasmablasts but not plasma cells and expressed high levels of CD38, which is known to be strongly expressed on plasma cells (Fig. 4C). This confirmed the specificity of CD138 as a marker for human intestinal lamina propria Ab-secreting cells. Importantly, CD27<sup>+</sup> CD138<sup>+</sup> small intestine lamina propria cells were nonproliferating as indicated by lack of intracellular Ki-67 expression, consistent with a plasma cell phenotype (Fig. 4D).

Consistently with ELISPOT experiments, the number of plasma cells markedly dropped from day 0 to day 5 but remained stable from day 5 to day 30 (Fig. 5A). The frequency of plasma cells among gated cells (viable leukocytes, CD14<sup>-</sup>) was estimated to be 5–10% on day 0, 2–5% on day 5, 5–10% on day 10, 10–15% on day 20, and up to 50–60% on day 30. The enrichment of plasma cells at late culture times reflects preferential survival of plasma cells compared with other cell populations, suggesting that survival of these cells is also an intrinsic capacity.

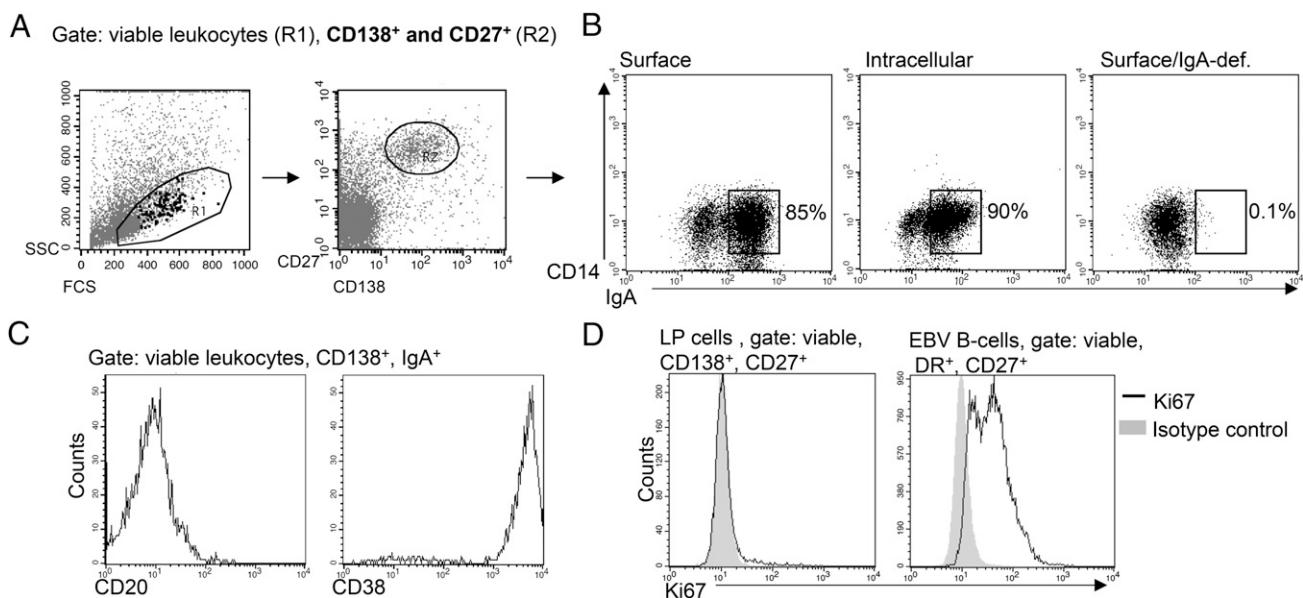
The plasma cell population of the small intestine was further divided into distinct subpopulations based on surface Ig expres-

sion. The Ig isotype distribution of cells detected on day 0 or day 1 was as previously described (2), IgA 80% and IgM 20%, whereas on day 5 and after it appeared to be slightly altered. The surviving plasma cell population detected on days 5, 10, or 20 was still predominantly of IgA isotype, but ~55–60%, whereas IgM was stable ~10–15%. On day 30, plasma cells expressing and those not expressing surface IgA became hardly distinguishable and appeared as one homogeneous population. Nonetheless, we still observed an IgA/IgM ratio of 8:2 when the same samples were assayed by ELISPOT at day 20 (Fig. 5B). This suggests that upon culture, some of the IgA<sup>+</sup> plasma cells lost surface Ig expression.

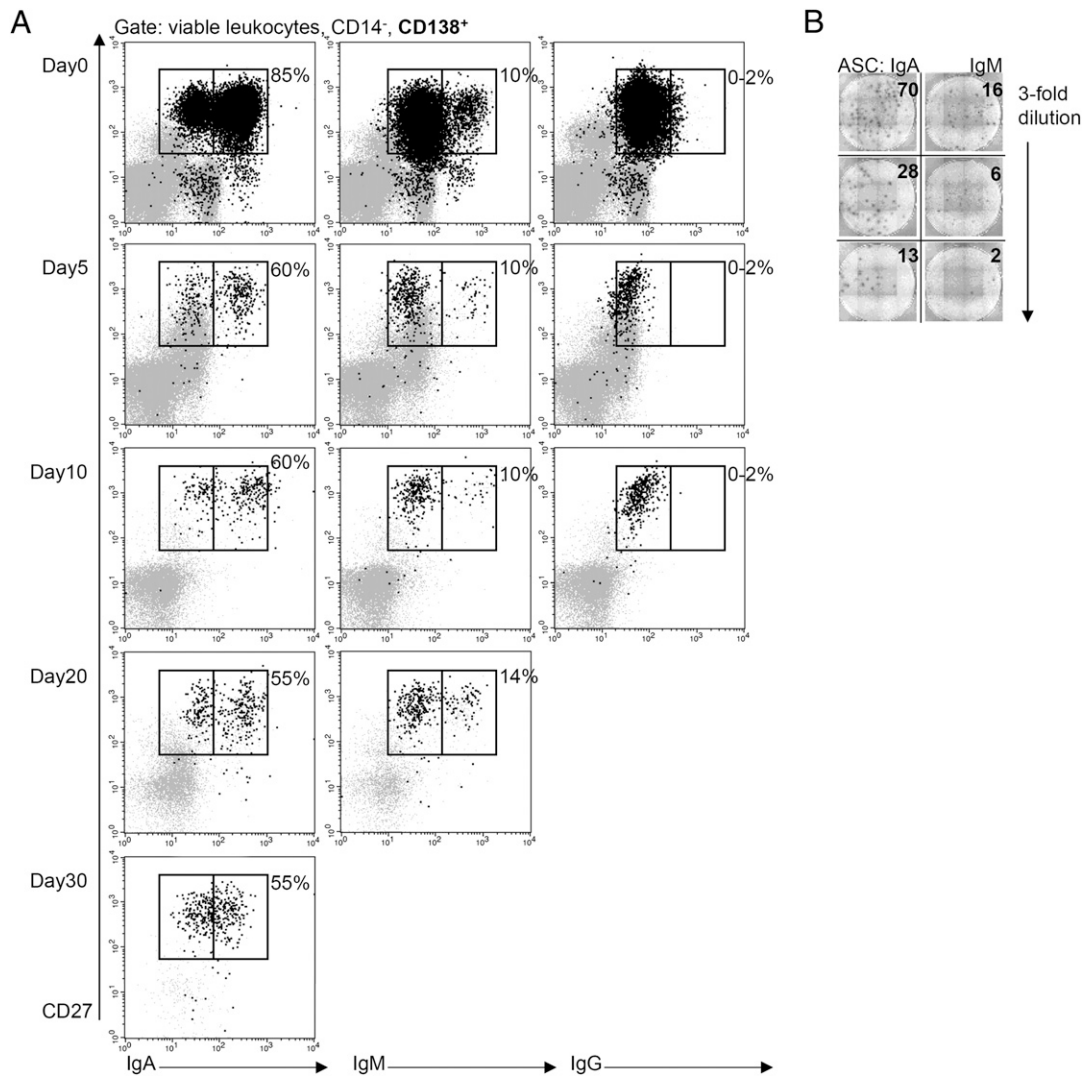
#### *Small intestine tissue provides soluble survival factors for plasma cells*

To determine whether small intestine tissue provides essential factors in sustaining the long-term survival of plasma cells, we measured a number of cytokines in the supernatants of small intestine biopsy cultures. The cytokines were quantified in culture supernatants by an ELISA or by a cytometric bead array assay as well as directly in lamina propria tissue by immunohistochemistry (data not shown). Notably, IL-6 and APRIL, two cytokines known to be essential for the long-term survival of plasma cells, were released in the culture supernatants for at least 20 d (Fig. 6). Neither BAFF (data not shown) or IL-21, known to be involved in plasma cell differentiation and survival, nor inflammatory cytokines such as IL-1 $\beta$ , TNF, IL-12p70, or IL-10 were detected in the small intestine tissue cultures at any time points. In contrast, the proinflammatory chemokine IL-8 was present in elevated concentrations, above 10,000 pg/ml, during the time course of the cultures.

To analyze further the lamina propria microenvironment, we assessed the role of IL-6 and APRIL in plasma cell survival in small intestine cell suspension cultures. We found that human recombinant IL-6 and soluble oligomerized APRIL when added to



**FIGURE 4.** Phenotypic analysis of human small intestine plasma cells by flow cytometry. Lamina propria cells were obtained by enzymatic digestion of small intestine tissue fragments cultured for 1 d, stained extracellularly, then permeabilized for intracellular staining and analyzed by flow cytometry. Dead cells and debris were excluded by FSC/SSC gating and propidium iodide staining. *A*, Viable leukocytes were gated using FSC/SSC (R1). A second dot plot was used to gate (R2) on plasma cells, highlighted in the FSC/SSC plot, by CD138 and CD27 expression. *B*, Flow cytometry plots of small intestine lamina propria plasma cells, gated as in *A*, stained for surface or intracellular IgA; an IgA-deficient patient was used as a negative control. *C*, Representative flow cytometry histograms demonstrating expression of CD20 and CD38 on small intestine IgA plasma cells. *D*, Flow cytometry histograms of lamina propria single-cell suspension (*left*) and a proliferating EBV-B cell line used as a positive control (*right*) analyzed for intracellular Ki-67 expression and with an isotype control Ab used to indicate background are shown. FSC/SSC, forward and side scatter.



**FIGURE 5.** Flow cytometric analysis of plasma cells of the human small intestine at different time points of cultures. Tissue fragments were cultured over time and replenished with fresh culture medium at days 1, 5, 10, 15, 20, and 25. Lamina propria cells were obtained by enzymatic digestion of tissue fragments, stained, and analyzed by flow cytometry. FCS/SSC gating and propidium iodide staining were used for exclusion of dead cells and debris. *A*, Flow cytometry plots of small intestine cells at days 0, 5, 10, and 20. CD138<sup>+</sup> cells are highlighted and shown in black. Numbers refer to the percentage of surface IgA-, IgM-, or IgG-expressing cells in the CD138<sup>+</sup> CD27<sup>+</sup> population. *B*, The same sample as in *A* at day 20 was analyzed for IgA and IgM Ab-secreting cells (ASC) by ELISPOT assay. Each result is from one representative experiment. In all experiments, from five donors at least two biopsies were cultured independently and pooled to be tested. FCS/SSC, forward and side scatter.

the cultures increased the level of Ig production during 3 wk. This effect was observed in the second week of exogenous IL-6 or APRIL stimulation. To confirm the relevance of endogenous APRIL and IL-6, we undertook experiments to block the activity of endogenous APRIL and IL-6. Small intestine cells were cultured with the APRIL decoy receptor-Fc fusion protein, BCMA-Fc, or an anti-human IL-6 Ab. Both BCMA-Fc and anti-IL-6 significantly diminished Ab production, whereas the addition of control Ab had no effect (Fig. 7).

## Discussion

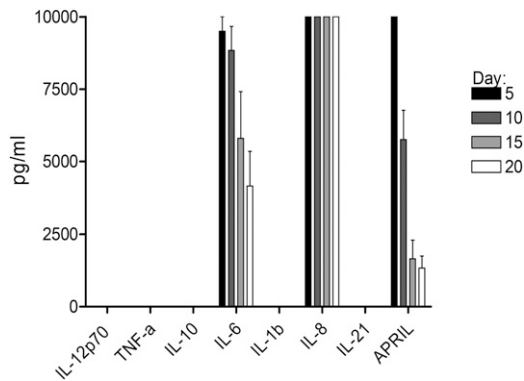
In this study, we have identified IgA- and IgM-secreting plasma cells from the small intestine that survive in culture for several weeks. Importantly, this suggests that such plasma cells have the potential to be long-lived in vivo.

Ab-secreting cells in the lamina propria, as characterized by us and others, express CD138, CD38, and CD27 but not CD20 (3, 29, 30). We have recently found that the majority of these Ab-secreting cells express membrane IgA and IgM (3). It has also

been observed that plasma cells in the lamina propria of the intestine do not proliferate (3, 25, 29). Thus, these cells have primarily a plasma cell-like phenotype.

Upon culture of human intestinal biopsies, either as a single-cell suspension or intact tissue, we observed that a significant proportion of the cells survived for at least 4 wk in vitro. The surviving cells coexpressed CD138 and CD27 and were Ki-67 negative, thus nonproliferating, which is consistent with the plasma cell phenotype. We observed both IgA- and IgM-secreting cells that survived in culture for weeks, hence this plasma cell longevity is not unique to IgA cells.

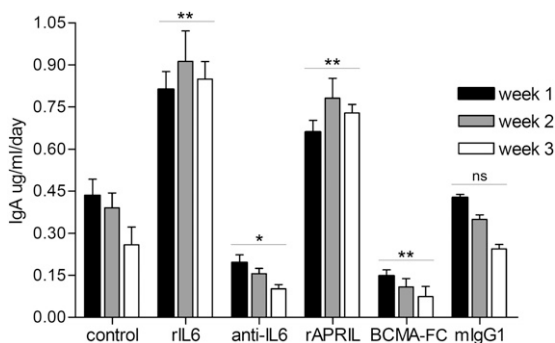
Mucosal Ab responses, particularly those related to infections of the gastrointestinal tract, have been described to be short-lived (20, 12). In keeping with this, most of the plasma cells in the gut lamina propria are thought to have a particularly short life span (31, 2). The half-life in vivo of mouse intestinal IgA plasma cell has been determined to be 5 d and the maximum lifetime from 6 to 8 wk, whereas all IgM plasma cells were found to be short-lived (9, 26). It was, therefore, surprising that a persistent intestinal IgA



**FIGURE 6.** Cytokine secretion from small intestine tissue in vitro. Small intestine tissue fragments were cultured for 20 d. Supernatants from the cultures were collected on days 5, 10, 15, and 20 and stored to be assayed simultaneously for the presence of IL-12p70, TNF- $\alpha$ , IL-10, IL-6, IL-1 $\beta$ , and IL-8 by a cytometric bead array assay or of IL-21 and APRIL by ELISA. Data shown are the average  $\pm$  SD of six independent experiments.

plasma cell response specific for commensal bacteria, similar to the long plasma cell half-lives measured in the bone marrow, was recently described (27). A prolonged specific IgA response was observed in mice, in a reversible germ-free colonization system, and was attenuated in mice colonized with other species of commensal bacteria. This finding strongly supports the notion that gut IgA plasma cells can be long-lived, that the IgA response is regulated by attrition and competition for limiting survival niches, and that the IgA repertoire, at least to commensals, is dominated by reactivity to Ags currently being present in the intestine.

The observation we have made with survival of plasma cells from the small intestine in culture is remarkably similar to what was previously described for culturing of long-lived plasma cells from the bone marrow or lymph nodes of mice (16, 17). Most of the plasma cells died rapidly when plated in media; however, a sub-population of plasma cells secreted high levels of Ab for up to 2 or 4 wk when cocultured with stromal cells. Further, mucosal plasma



**FIGURE 7.** In vitro effect of APRIL and IL-6 on Ab production by small intestine lamina propria cells. Intestinal tissue biopsies prepared as single-cell suspensions were seeded at a density of  $10^4$  cells/well in 96-well microtiter plates and incubated for 3 wk with either of the following reagents: medium only (control), recombinant IL-6 (rIL-6), anti-IL-6, BCMA-Fc (BCMA-Fc), recombinant APRIL (rAPRIL), or control Ab mouse IgG1 (mIgG1). Two thirds of the volume of culture supernatant was removed and replenished with fresh culture medium at the end of each week. Supernatants were assayed for the presence of IgA by sandwich ELISA. Data are presented as mean  $\pm$  SD of six independent experiments. Differences between treatment versus control (medium only) were analyzed for statistical significance using Student *t* test. \* $p < 0.01$ , \*\* $p < 0.005$ ; ns, not significant.

cells in human tonsil organ cultures maintained persistent secretion of Ab for 10 d if cultured without additional stimuli (23).

We found that the sustained in vitro Ab production depended on the culturing conditions. In single-cell suspension cultures, the most prolonged Ab secretion was observed when autologous irradiated PBMCs were used as feeder cells, implying that additional secreted factors may enhance plasma cell survival and Ab secretion. When  $10^4$  lamina propria cells/well were plated on autologous irradiated PBMCs, the amount of secreted IgA was 60 ng/day in the 4th week of culture. We estimated that IgA plasma cells represent  $\sim 5\%$  of human intestinal lamina propria cells based on this and our previous study (3). Therefore, the average secretion of a single IgA plasma cell was calculated to be  $\sim 100$  pg/cell/day.

To constitute conditions similar to those in vivo, we investigated the plasma cell survival by using intact small intestine tissue biopsies. In these cultures, both the tissue architecture and stromal or stromal-like cells, which may provide survival factors, are reasonably well preserved. A number of Ab-secreting cells died within the first days of culture, which could be due to culturing conditions leading to the disruption of plasma cell niches. Alternatively, this may indicate that a proportion of lamina propria plasma cells is short-lived. This would be in keeping with data from in vivo mouse studies showing that small intestine plasma cells belong predominantly to a short-lived population (9, 26).

Even if the total number of plasma cells dropped extensively in the first 5 d of culture, we observed that the number of plasma cells remained constant over the following 25 d. In our interpretation, this survival kinetics provides evidence that survival niches exist in the mucosa where long-lived plasma cells stay. Plasma cells can by contact induce bone marrow stromal cells to produce survival factors and create a special microenvironment known as a "niche." Moreover, cell-cell interactions with surrounding stroma, that is, via CD44 (32) and CXCL-12 (11), were described as crucial survival factors maintaining plasma cells in such niches. Similarly, our findings underscore the importance of intact tissue, which not only provides soluble factors but also acts as a structural scaffold of the small intestine microenvironment. It is worth emphasizing that our estimated half-life of plasma cells or Ab secretion from in vitro cultures of small intestine biopsies is mainly dependent on plasma cells located in lamina propria niches of a single biopsy. Newly generated plasma cells are unlikely to displace plasma cells already situated in niches, as the lamina propria of the small intestine contains very few B cells and as we and others (29, 30) have found no evidence for local plasma cell proliferation. We are unable to rule out a small contribution from newly generated plasma cells that may be proliferating locally at a low rate (33). We did not try to sustain plasma cell survival in vitro for longer than 30 d, as an obvious limitation of the biopsy culture assay is that tissue-like structures are not well preserved and that the resemblance with the in vivo situation is impaired over time.

Bone marrow stromal cells, basophils, and eosinophils produce IL-6 and APRIL, factors that are crucial for the long-term survival of plasma cells (17, 32, 34, 35). Both intestinal epithelial and smooth muscle cells have the ability to release significant amounts of IL-6 (36, 37). We demonstrated that small intestine tissue provides IL-6 detectable up to day 20 of culturing. APRIL, produced by macrophages, dendritic cells, and neutrophils in the lamina propria of the small intestine (38, 39), was also detected in intact tissue culture supernatants. When human recombinant IL-6 or APRIL was added to cell suspension cultures, Ig secretion was drastically increased, whereas the opposite effect was obtained with anti-human IL-6 neutralizing Ab or BCMA-Fc, an antagonist of APRIL. This is in line with the observations that the B cell maturation ligand BAFF and APRIL in conjunction with IL-6 can

sustain bone marrow plasma cell survival in vitro and in vivo (40). Notably, we did not detect BAFF in the small intestine lamina propria. This is consistent with the notion that BAFF is predominantly involved in IgA class switching particularly to the IgA<sub>2</sub> subclass in the distal intestine of mice (41), and the BAFF receptor is not expressed on human mucosal plasma cells (39). Taken together, these observations strongly indicate that the lamina propria may create bone marrow-like survival niches for plasma cells resident in the small intestine by providing APRIL and IL-6. We also found large quantities of IL-8 in the culture supernatants, which is produced by gut macrophages and enterocytes (42, 43) as well as by other lamina propria cells of non-inflamed tissue (44). However, it is not known whether IL-8 is relevant for plasma cell survival. These data need to be interpreted with caution, as the survival phenotype is clearly the result of individual and synergistic effects of cytokines on both plasma cells as well as other cell types.

If there exist two populations of plasma cells in the gut, one short-lived and one long-lived, it would be important to understand the basis for this. Recent data on other tissues suggest that longevity of plasma cells is primarily extrinsically regulated by the formation of survival niches in the stromal environment (32, 16, 17). Only some plasma cells may have accessed such survival niches. In fact, it has been argued that longevity is conditional and governed by both intrinsic and extrinsic factors (15) and that the ability to access such niches, "competence to compete," is the determining factor for plasma cells to become long-lived (45).

In conclusion, this study demonstrates that the human small intestine lamina propria harbors a nonproliferative Ab-secreting cell population surviving for >4 wk in culture. In addition, the small intestine tissue culture model we describe offers a simple experimental system to explore the biology of human intestinal plasma cells.

## Disclosures

The authors have no financial conflicts of interest.

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