APRIL secreted by neutrophils binds to heparan sulfate proteoglycans to create plasma cell niches in human mucosa

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The bone marrow constitutes a favorable environment for long-lived antibody-secreting plasma cells, providing blood-circulating antibody. Plasma cells are also present in mucosa-associated lymphoid tissue (MALT) to mediate local frontline immunity, but how plasma cell survival there is regulated is not known. Here we report that a proliferation-inducing ligand (APRIL) promoted survival of human upper and lower MALT plasma cells by upregulating expression of the antiapoptotic proteins bcl-2, bcl-xL, and mcl-1. The in situ localization of APRIL was consistent with such a prosurvival role in MALT. In upper MALT, tonsillar epithelium produced APRIL. Upon infection, APRIL production increased considerably when APRIL-secreting neutrophils recruited from the blood infiltrated the crypt epithelium. Heparan sulfate proteoglycans (HSPGs) retained secreted APRIL in the subepithelium of the infected zone to create APRIL-rich niches, wherein IgG-producing plasma cells accumulated. In lower MALT, neutrophils were the unique source of APRIL, giving rise to similar niches for IgA-producing plasmocytes in villi of lamina propria. Furthermore, we found that mucosal humoral immunity in APRIL-deficient mice is less persistent than in WT mice. Hence, production of APRIL by inflammation-induced neutrophils may create plasma cell niches in MALT to sustain a local antibody production.

Introduction

B cells encountering antigen in the T cell zone of secondary lymphoid organs migrate into primary follicles to divide extensively. After this initial proliferation, B cells have to process and present antigen to T cells to receive signals for Ig class switch recombination and somatic hypermutations, both mechanisms being under the tight control of a deaminase acting on cytidine present in Ig genes (1). The latter alters their affinity for the antigen. B cells harboring Igs with a too low affinity will not be able to compete for survival signals presented as trapped antigens in the light zone of germinal centers and undergo apoptosis. This selection process, facilitated by the open structure of germinal centers (2), ensures that only B cells expressing the highest affinity antibodies will receive sufficient survival signals and leave the germinal center as plasmablasts. After leaving the induction zone, plasmablasts must find an appropriate environment to terminate their differentiation and survive as plasma cells. The bone marrow is a favorable environment for plasma cells (3), because it expresses the cytokine IL-6, the ligand(s) for the adhesion molecule CD44, and the chemokine SDF-1 (4). Alternatively, plasmablasts home to a mucosa-associated lymphoid tissue (MALT), usually the site where they initially encountered the antigen (5). This occurs in particular when the MALT is inflamed (6). In contrast to the bone marrow, the factors that sustain plasma cell survival in MALT are not known.

A proliferation-inducing ligand (APRIL, also known as TNFSF13) and the B cell activation factor (BAFF, also known as TNFSF13B) are 2 closely related members of the TNF superfamily that share the transmembrane activator, calcium modulator, and cyclophilin ligand interactor (TACI, also known as TNFRSF13B) and the B cell maturation antigen (BCMA, also known as TNFRSF17) as canonical TNF-like receptors (7). Two recent reports suggested that these molecules contribute to survival of bone-marrow plasma cells. Blocking of APRIL/BAFF (8) as well as genetic deletion of BCMA (9) impairs the number of plasma cells recovered in the bone marrow after immunization. Although APRIL and BAFF share many characteristics, the differences with respect to receptor binding are considerable. BCMA binds APRIL with a 2-logs higher affinity than BAFF (10). BAFF but not APRIL binds to BAFF receptor (BAFF-R, also known as TNFRSF13C) (11), while only APRIL binds to heparan-sulfate proteoglycans (HSPGs) (8, 12). These differences are likely to explain the nonredundant functions of these 2 TNF ligands. BAFF (13) but not APRIL (14) is prerequisite for the generation and maintenance of a mature B cell compartment. The role of APRIL is more restricted to late stage of B cell differentiation, since APRIL-deficient mice have only impaired IgA and IgA/IgM antibody responses to T cell–dependent and –independent mucosal antigens, respectively (15).

In mucosa, the epithelium plays an important role in local antibody responses. Indeed, mucosal epithelial cells stimulated by pathogens via their TLRs produce plasma cell chemokines (5). This chemotraction explains the subepithelial homing for mucosal plasma cells.

Nonstandard abbreviations used: ACRP, adipocyte complement-related protein; APRIL, a proliferation-inducing ligand; BAFF, B cell activation factor; BAFF-R, BAFF receptor; BCMA, B cell maturation antigen; HSPG, heparan-sulfate proteoglycan; MALT, mucosa-associated lymphoid tissue; TACI, transmembrane activator, calcium modulator, and cyclophilin ligand interactor.

Conflict of interest: Olivier Donze is employed by Aptech Corporation, whose products were used in the present study.

Citation for this article: J Clin Invest. 118:2887–2895 (2008). doi:10.1172/JCI33760.
In addition to chemoattraction, the mucosal epithelium can fine-tune humoral responses by inducing extra–germinal center Ig class switch recombination (16, 17). Here we analyzed APRIL expression in human, upper oral and lower gut MALT. We found that MALT-infiltrating neutrophils and the tonsillar epithelium produced APRIL that accumulated on subepithelial HSPG, thereby creating APRIL-rich niches for plasma cells. The latter is consistent with the role observed in vitro for APRIL in the control of plasma cell survival.

**Results**

APRIL provides a survival signal to human MALT plasma cells. To investigate the role of APRIL in MALT plasma cells, we first analyzed expression of APRIL receptors by tonsillar plasma cells. Since no antibodies were available to date for staining endogenous TACI or BCMA in fixed tissues, we analyzed single cell suspensions isolated from tonsils by flow cytometry. Figure 1A shows that tonsillar plasma cells identified by a CD19^+CD38^high cytoplasmic-Ig^+ (Cyt-Ig^+) phenotype expressed BCMA but were negative for TACI. This was confirmed with 5 different mAbs against TACI that all stained the TACI^+ IM9 B cell line (18). Of note, BAFF-R was also not expressed, which was consistent with the absence of BAFF-R staining observed in situ on cyt-Ig^+ tonsillar plasma cells (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI33760DS1). We next assessed APRIL signaling in tonsillar CD19^+CD38^high plasma cells. Soluble oligomerized APRIL (adipocyte complement-related protein–APRIL [ACRP-APRIL]) sustained the in vitro survival of purified plasma cells, while no effect was seen on the CD19^+CD38^- population, consisting mainly of naive B cells (Figure 1B). Tonsillar plasma cells expressed high levels of intracellular bcl-2, bcl-xL, and mcl-1 was analyzed by flow cytometry on purified CD19^-CD38^ at the initiation of the culture and after 4 days of culture with ACRP-APRIL or control ACRP. Dotted lines correspond to isotype control staining. (D) Binding of APRIL_{AB} and APRIL_{H38} to plastic-coated HSPG (1 μg/ml) was revealed with the Aprily-5 mAb (1 μg/ml) (top panel). CD19^-CD38^ tonsillar plasma cells were incubated in plastic wells coated with HSPG- or HSPG-binding APRIL_{AB} for 4 days. Intracellular bcl-2 expression was analyzed as in C (bottom panel). Data are representative of 2 independent experiments performed with 2 different donors.

Sn, supernatant.
whether native trimeric APRIL could promote plasma cell survival after being oligomerized by binding to HSPG. We were not able to test this by binding of APRIL onto HSPG expressed by plasma cells, since we observed that the CD138 core protein carrying plasma cell HSPG is lost during cell isolation as previously reported by others (20, 21). Hence, we bound soluble trimeric APRIL onto plastic-coated HSPG. APRIL<sub>SS8</sub>, but not the mutant APRIL<sub>SS99</sub> lacking the HSPG interaction domain, readily bound to coated HSPG, as revealed with the anti-APRIL mAb, Aprily-5 (Figure 1D, top panel). Under these conditions, APRIL<sub>SS8</sub> sustained intracellular expression of bcl-2 in plasma cells (Figure 1D, bottom panel), showing that APRIL is functional when bound onto HSPG. Taken together, this analysis indicates that APRIL impacts humoral responses at a late stage by providing a survival signal to plasma cells.

**APRIL-rich niches are created in MALT.** We next studied APRIL expression in MALT with a pair of APRIL-specific antibodies recognizing either APRIL-producing cells (Stalk-1 Ab) or secreted APRIL (Aprily-2 mAb). We already established specificity of this pair of Abs with inhibition of the in situ reactivity by the corresponding antigens and size identification of the detected products by western blot analysis of tissue lysates (22). Figure 2A shows that crypt tonsillar epithelium contained APRIL-producing (Stalk-1 staining) cells. This is specific to tonsil epithelium, since normal human skin does not contain such cells (23). Some cells (Figure 2A, arrow) stained brighter with Stalk-1 than others. High-power microscopy revealed the trilobular nucleus of these cells (Figure 2B), which together with the uniform expression of elastase (Figure 2C) and CD15 (data not shown) identified them as neutrophils. The other Stalk-1–stained cells had a morphology of epithelial cells. Keratin 8<sup>+</sup> cells that characterized the tonsillar crypt (24) were mixed with other pan-keratin<sup>+</sup> (Figure 2D) and syndecan-1<sup>+</sup> (Figure 2E) epithelial cells. Keratin 8<sup>+</sup> and keratin 8<sup>-</sup> crypt epithelial cells produced APRIL (Figure 2F). In these crypts, we observed secreted APRIL (Aprily-2 staining) in the subepithelial zone on pan-keratin<sup>+</sup> epithelial cells (Figure 2G), bound to epithelial cells expressing syndecan-1, an APRIL-binding HSPG (8, 12) (Figure 2H). However, cells that produced APRIL did not stain for secreted APRIL. As a result, this created zones rich in APRIL in the subepithelium, separated from the main production site that located toward the apical lumen (Figure 2I). We observed a similar production and retention of APRIL at the level of the surface epithelium (data not shown). Hence, APRIL secreted by epithelial cells was retained in the subepithelial zone by binding to HSPG, forming APRIL-rich niches.

**Plasma cells accumulate in subepithelial APRIL-rich niches of MALT.** Plasma cells home close to the epithelium in MALT (25, 26). In tonsils, we observed that plasma cells homed to the APRIL-rich niches (Figure 3A) beneath pan-keratin<sup>+</sup> cells (Figure 3B). The yellow color observed at the epithelium level is due to depots of secreted Ig. This homing was specific to plasma cells, since less differentiated CD20<sup>+</sup> B cells (27) were excluded from these zones (Figure 3C). Confocal microscopy demonstrated that secreted APRIL was either retained in the vicinity of or directly on plasma cells (Figure 3D). Accumulating plasma cells expressed syndecan-1 (Figure 3E), allowing them to bind APRIL (Figure 3F, arrow). These plasma cells produced IgG and to a lesser extent IgA, but no IgM (Figure 3G). We next studied expression of prosurvival factors in these plasma cells. We could not perform 2-color immunofluorescence for bcl-x<sub>L</sub>, mcl-1, and bcl-2 due to their low expression level. However, using an enhanced enzymatic detection assay and staining of serial sections, we observed that subepithelial IgG<sup>+</sup> plasma cells expressed bcl-x<sub>L</sub>, mcl-1, and bcl-2 (Supplemental Figure 2). This in situ analysis confirmed the in vitro sustained expression of these 3 survival factors observed in the presence of exogenous APRIL. It
should be noted that a majority of epithelial cells were also positive for mcl-1. Furthermore, bcl-2 was also expressed strongly by naive B cells underneath subepithelial plasma cells and weakly by basal undifferentiated epithelial cells as previously reported (19, 28). Cells brightly stained by the anti–bcl-xL present in the epithelium did not show the same localization as plasma cells. Serial sections of the subepithelial zone showed that epithelial cells formed an ordered structure, surrounding plasma cells, thereby creating niche-like structures with a diameter that could be up to 500 μm, with many plasma cells inside (Figure 4). This circular build up of epithelium and plasma cells invaginated deeply into the crypt lumen, with a concomitant decrease in diameter until the structure stopped. These APRIL-rich invaginations could be close to one another so that a single crypt contained numerous niches.

We also studied MALT of the lower gut. We observed APRIL-producing cells (Figure 5A) as well as secreted APRIL in the apical zone of lamina propria villi. Similarly to the tonsillar epithelium, secreted APRIL dissociated from APRIL-producing cells and was dispersed throughout the subepithelial zone (Figure 5A, inset). All APRIL-producing cells were elastase+CD15+ neutrophils (data not shown). Plasma cells accumulated in these APRIL-rich zones. They also expressed syndecan-1 (data not shown), allowing them to bind secreted APRIL (Figure 5B). Some secreted APRIL bound also to non–plasma cells present in this zone, most likely via an interaction with HSPG, characterized by depot-like structures. The accumulating plasma cells produced IgA and to a lesser extent IgM, but no IgG (Figure 5B). Taken together, our in situ analysis shows that plasma cells accumulate in APRIL-rich niches in MALT subepithelium from upper tonsil and lower gut in a similar manner.

Regulation of APRIL production and plasma cell accumulation in MALT. We next compared APRIL production and plasma cell accumulation in tonsils with different degrees of infection. Epithelial keratinocytes from a noninfected crypt produced a basal level of APRIL that was similar to that produced by keratinocytes from chronically and acutely infected crypts (Figure 6A). We confirmed APRIL production by epithelial cells with the detection of APRIL mRNA in cultures of primary keratinocytes derived from human skin (HEK cells) and of immortalized keratinocytes from the HACAT cell line (Figure 6B, left panel). In the former cells, quantitative RT-PCR for APRIL showed that agonists for TLR2 (peptidoglycan [PGN]), TLR4 (LPS), TLR7 (loxoribine), and TLR9 (CpG) upregulate APRIL transcription (Figure 6B, right panel), indicating that pathogens upregulate APRIL expression in keratinocytes. The number of APRIL-producing Stalk-1bright neutrophils infiltrating these different epithelia varied markedly. While they were barely detectable in crypt epithelium from noninfected tonsils, their number significantly increased in chronically infected cases to reach a maximum in acutely infected specimens (Figure 6C). In infected tonsils, we also observed APRIL-producing neutrophils in blood vessels draining the organ, from where they extravasated into the extrafollicular area (Supplemental Figure 3), indicating that APRIL-producing blood neutrophils migrated toward the infected crypt epithelium after extravasation. The difference in the number of APRIL-producing neutrophils made that depots of secreted APRIL were small in control tonsils, were more pronounced during chronic infection, and were at least 10 times more prominent in acutely infected tonsils (Figure 6D). In the latter, APRIL depots covered the whole basal epithelium of the infected crypt (Supplemental Figure 4). Plasma cell accumulation varied according to the size of APRIL depots in the subepithelial zone. The number of plasma cells present in control tonsils doubled during a chronic infection and was at its maximum in acutely infected tonsils (Figure 6E). In the latter case, the plasma cells completely filled the invaginations described in the previous paragraph (Supplemental Figure 4), secreting high amounts of Ig that not only covered the pathogen present in the crypt lumen but also bound to the surrounding epithelium. Hence, APRIL-rich niches are formed in the subepithelial zone of an infected crypt. These niches sustain plasma cell survival close to the pathogen, thereby enabling a lasting local humoral response.
APRIL prolongs the mucosal humoral immune response. We next studied to what extent APRIL modulates humoral mucosal immunity in vivo in a mouse model. For this purpose, we measured Ig production at different times after oral vaccination with OVA in WT and APRIL-deficient C57BL/6 mice. Although at the peak of the response, 3 weeks after the last boost, the amount of OVA-specific Ig secreted by gut plasma cells was similar in WT and APRIL-deficient C57BL/6 mice (Figure 7), clear differences appeared 3 weeks later. At that moment, gut plasma cells still produced detectable antibodies in WT mice, while production had ceased in APRIL-deficient mice. This experiment shows that humoral immunity to mucosal antigens is shortened in APRIL-deficient mice, highlighting the in vivo role of APRIL for MALT plasma cell survival.

Discussion
In this report, we present evidence that APRIL-rich niches are created in mucosa to sustain local plasma cell survival. In infected mucosa, the major source of APRIL were neutrophils. This is consistent with previous reports on tumors (22, 23, 29). Infection in the oral cavity and presence of pathogen in the tonsillar crypt resulted in a massive influx of neutrophils. We observed these neutrophils in blood vessels draining the organ, extravasated into the parenchyma, and infiltrating the tonsillar epithelium, close to the invading pathogen. Such neutrophil migration toward the tonsillar epithelium has already been documented (30) and is driven by several chemokines (CXCL-1, -5 and -8) produced by TLR-stimulated epithelial cells (31). In the absence of detectable infection, APRIL-producing neutrophils were barely present in tonsils, but keratinocytes from the tonsillar epithelium produced APRIL in this condition. Pathogen-associated products stimulating TLR, known to be expressed by tonsillar epithelial cells (17, 32), upregulated this epithelial production. The basal APRIL expression by epithelial cells observed in the absence of detectable infection may reflect a constant exposure of these cells to pathogen products, since epithelial cells of tissues protected from pathogens by a physical barrier, such as the skin, do not produce APRIL unless an inflammatory reaction is ongoing (23). By contrast, epithelial cells of the small intestine did not produce APRIL. The absence of APRIL production by small intestinal epithelium warrants further investigations, since it also expresses TLR (33), and epithelial cells from colon further down in the gastrointestinal tract produce APRIL (16). Hence, APRIL was uniquely produced in small intestine by neutrophils that homed into villi of lamina propria, also under the influence of the chemokine CXCL-8, produced by TLR-stimulated intestinal epithelium (34). The chemotraction of APRIL-producing neutrophils and the upregulation of APRIL production by tonsillar epithelial cells, both under the control of TLR stimulation, demonstrate that APRIL production in MALT is regulated by inflammation.

Both types of APRIL-producing cells, neutrophils and keratinocytes, secreted the molecule and the soluble product concentrated in the subepithelial zone bound to HSPG, as previously observed in tumor lesions (22, 23, 29), retained by epithelial cells by virtue of syndecan-1 expression. These syndecan-1–expressing epithelial cells formed invaginations into the crypt lumen, wherein plasma cells accumulated. There, APRIL bound to epithelial cells could stimulate plasma cell survival in trans, as observed in our in vitro assay, with plastic-coated HSPG. APRIL also bound directly to syndecan-1 expressed by plasma cells, providing in this case a cis stimulation. These APRIL-rich niches constitute, therefore, a proper environment for plasma cell survival. Hence, mucosa epithelium has pleiotropic functions in humoral immunity. It not only induces Ig switch and retains memory B cells (35) but also promotes survival of plasma cells.

Figure 4 APRIL-rich niches invaginate deeply into the crypt lumen. Serial sections, at a distance of 100 μm apart, of crypt epithelium starting in the tonsillar parenchyma and ending in the crypt lumen (images A–D) were stained with an anti-Ig (5 μg/ml). Two separate invaginations (plain and dotted lines) containing cytoplasmic Ig+ plasma cells are shown. Epithelium shows depots of secreted Ig. Original magnification, ×10. Figures shown are representative of 5 tonsils from patients with acute tonsillitis.

Figure 5 APRIL-rich niches are also present in human small intestine. (A) APRIL-producing cells (green) and depots of secreted APRIL (red) are present in intestinal villi. Inset shows secreted APRIL (red) dissociating from APRIL-producing cells (green). (B) Ig isotype (5 μg/ml, green) produced by plasma cells accumulating in APRIL-rich niches (red). Original magnification, ×20 (A); ×40 (A, inset, and B). Figures shown are representative of at least 5 intestines analyzed.
The preferential production of IgA and IgG in gut and tonsil, respectively, has been known for decades (36, 37). In situ specific switch factors are involved in this process (16, 17, 38). Here we observed that plasma cells accumulate in niches underneath an epithelium that allows the full effector functions of their secreted Ig. Indeed, IgA-producing cells accumulate in APRIL-rich niches preferentially localized underneath a contiguous epithelium, so that transcytosis is required for Ig molecules to reach the intestinal lumen. Notably, intestinal epithelial cells transcytose IgA but not IgG (39). In contrast, IgG-producing cells accumulated in a zone lined by a noncontiguous crypt epithelium, allowing passive diffusion of IgG molecules to reach the crypt lumen (40).

**APRIL-null mice produce less IgA and IgM against T cell–independent antigens given orally as well as less IgA to T cell–dependent antigens.** Although the role of APRIL in the Ig switch process (16, 41–43) explains the IgA deficiency, it does not explain the IgM deficiency. Here we show that APRIL also induces a survival signal, late in the B cell differentiation process, at the plasma cell stage. This survival signal has consequences in vivo, since humoral immunity to mucosal antigens is shortened in APRIL-deficient mice. Furthermore, the deficient production of selective Ig isotypes (IgA and IgM) in APRIL-null mice upon oral challenge is fully consistent with the accumulation of plasma cells producing IgA and IgM in APRIL-rich niches that we observed in human gut. Hence, APRIL regulates plasma cell survival in MALT, as it does in the bone marrow (44). This MALT plasma cell survival is promoted by 3 related, antiapoptotic factors, bcl-2, bcl-xL, and mcl-1, each known, albeit not simultaneously, to be active in plasma cells (9, 19, 44). This discrepancy could be due to a species (mouse versus human) or site (bone marrow versus MALT) differences. In this study, we confirmed that BCMA is the unique canonical TNF-R expressed by tonsilar plasma cells as previously reported (45). Therefore, APRIL signaling in MALT

**Figure 6** Inflammation regulates APRIL production in tonsils. (A) A representative example of Stalk-1 staining for crypt epithelium from control and acutely infected tonsils (left and middle panels). Quantification of the staining obtained in 5 control, 5 chronically, and 5 acutely infected tonsils is shown in the right panel. (B) APRIL transcription was analyzed by 40 cycles of RT-PCR in the indicated cells. Actin was used as housekeeping gene (left panel). Quantitative RT-PCR for APRIL mRNA was performed on epithelial HEK cells treated for 48 hours with PGN (10 μg/ml), LPS (100 ng/ml), Loxoribine (250 μg/ml), or CpG (10 μg/ml) (right panel). (C–E) A representative example of Stalk-1 (C), Aprily-2 (D), and anti-Ig (E) stainings is shown as in A (left and middle panels). Quantification of the number of Stalk-1–stained neutrophils infiltrating the crypt epithelium (C), of the Aprily-2 staining (D), and of the number of plasma cells (PCs) in the subepithelial crypt area is also shown in right panels. Original magnification, ×40.
plasma cells is likely to be mediated by BCMA, rendering this receptor not only crucial for the longevity of bone marrow (9) but also of MALT plasma cells. BCMA expression also indicates that BAFF may also be implicated in MALT plasma cell survival, similar to the role of BAFF reported in the bone marrow (9, 46).

New modulatory functions in humoral immunity from the mucosal epithelium have recently been uncovered (17). We further show here that epithelial cells are also implicated in the control of mucosal plasma cell survival, either directly, by producing APRIL, or indirectly, by recruiting APRIL-producing neutrophils. By contrast, a role for neutrophils in the regulation of humoral immunity has not been established to date. The only indirect observation is that neutropenia can be linked to a decrease in serum Ig (47–49). It is known that plasma cell longevity is not defined by intrinsic characteristics but rather by environment (50). APRIL is part of this environment. In bone marrow, granulocytes produce APRIL, indicating that APRIL supply is constitutive under normal conditions (22). This may explain, at least partly, the longevity of bone marrow plasma cells (51). In contrast, the present report shows that APRIL supply in MALT is not constitutive but highly dependent on inflammation. As a result, half-life of plasma cells in MALT is more restrained as previously suggested (52).

Methods

Mouse experiments. All animal care and procedures were approved by the veterinary office of the Canton of Geneva. APRIL−/− mice on a C57BL/6 background were kindly provided by A. Ashkenazi (Genentech Inc., San Francisco, California, USA). WT C57BL/6 mice were bred in house. Age- and sex-matched mice were vaccinated orally 3 times with 0.2 ml PBS, containing 1 mg OVA (Sigma-Aldrich) and 10 µg Cholera Toxin (List Biological Laboratories Inc.) 10 days apart. Ig from fecal extracts were obtained by adding 100 µl PBS with 0.2% NaN3 to 100 µg aqueous feces. After overnight vortexing and 1,500-g centrifugation for 10 minutes at 4°C, total anti-OVA Ig in the supernatant was assessed in a standard ELISA with 96-well plates coated with OVA at 1 µg/ml and an anti-mouse Ig-conjugated to HRP as detection reagent.

Cells and reagents. HEK cells were isolated from human epidermis and maintained in KGM medium (Biorea), according to Viard-Leveuge et al. (53). The following antibodies, anti-TACI (clone 1A1, rat IgG2a), anti-BCMA (clone Vicky1, rat IgG2a), anti–BAFF-R (clone 11c1, IgG1), and anti-

Figure 7
Mucosal humoral immunity is short lasting in APRIL-deficient mice. Groups of APRIL−/− and WT mice were vaccinated orally with OVA. Secretion of OVA-specific Ig by gut plasma cells was tested 3 and 6 weeks after the last vaccination in fecal extracts by ELISA.
with signs of an ongoing humoral response (presence of Peyer’s patches) after deparaffinization, sections were incubated for 10 minutes in methanol plus 0.6% hydrogen peroxide at room temperature, followed by washing with phosphate-buffered saline solution. Antigen retrieval was performed by boiling slides once in 0.01 M citrate buffer, pH 6.0, for 3 minutes. Incubation with antibodies was carried out for 1 hour at room temperature. For immunoperoxidase staining, secondary reagents were goat anti-rabbit or anti-mouse IgG sera conjugated to biotin (Jackson Immunoresearch Laboratories Inc.), followed by streptABComplex/HRP (Dako) and 3-amin-9-ethylcarbazole substrate (Sigma-Aldrich). For anti-bcl-2, bcl-xL, and mcl-1, the EnVision plus HRP (Dako) was used for revelation with 3 periods of boiling in EDTA buffer, pH 8.6, for antigen retrieval. For 2-color immunofluorescence stainings, fluorochrome-conjugated isotype-specific goat anti-mouse sera (Jackson Immunoresearch Laboratories Inc.) and goat Ig anti-rabbit IgG (Molecular Probes) conjugated to phycoerythrin were used. Images were visualized under low or fluorescent microscopy with Axioptot 1 (Carl Zeiss AG), acquired with an Axioscan (Carl Zeiss AG) color CCD camera, and treated on a Pentium III computer with Axiosvision software (Carl Zeiss AG). In some experiments, confocal analyses with an LSM 510 (Carl Zeiss AG) were performed. For quantification of neutrophils and plasma cells, cell acquisition was done with the x40 objective, and stained cells were counted in the entire field, corresponding to a tissue area of 30 mm². For the Stalk-1 and Apripy-2 signal quantification, images acquired with the x40 objective were processed in MetaMorph Image Analysis software (Molecular Devices). Color thresholds were selected in the Hue, Saturation, Intensity (HSI) space. Saturation values obtained in the thresholded field were added, logged to a spreadsheet, and expressed in arbitrary units.

Acknowledgments
We thank S. Izu for critical reading of the manuscript. The expertise of Sergei Startchik in signal quantification is greatly acknowledged. This work was supported by the Dr Henri Dubois-Ferrière Dinu Lipatti Foundation and the Swiss National Science Foundation.

Received for publication August 28, 2007, and accepted in revised form May 28, 2008.

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