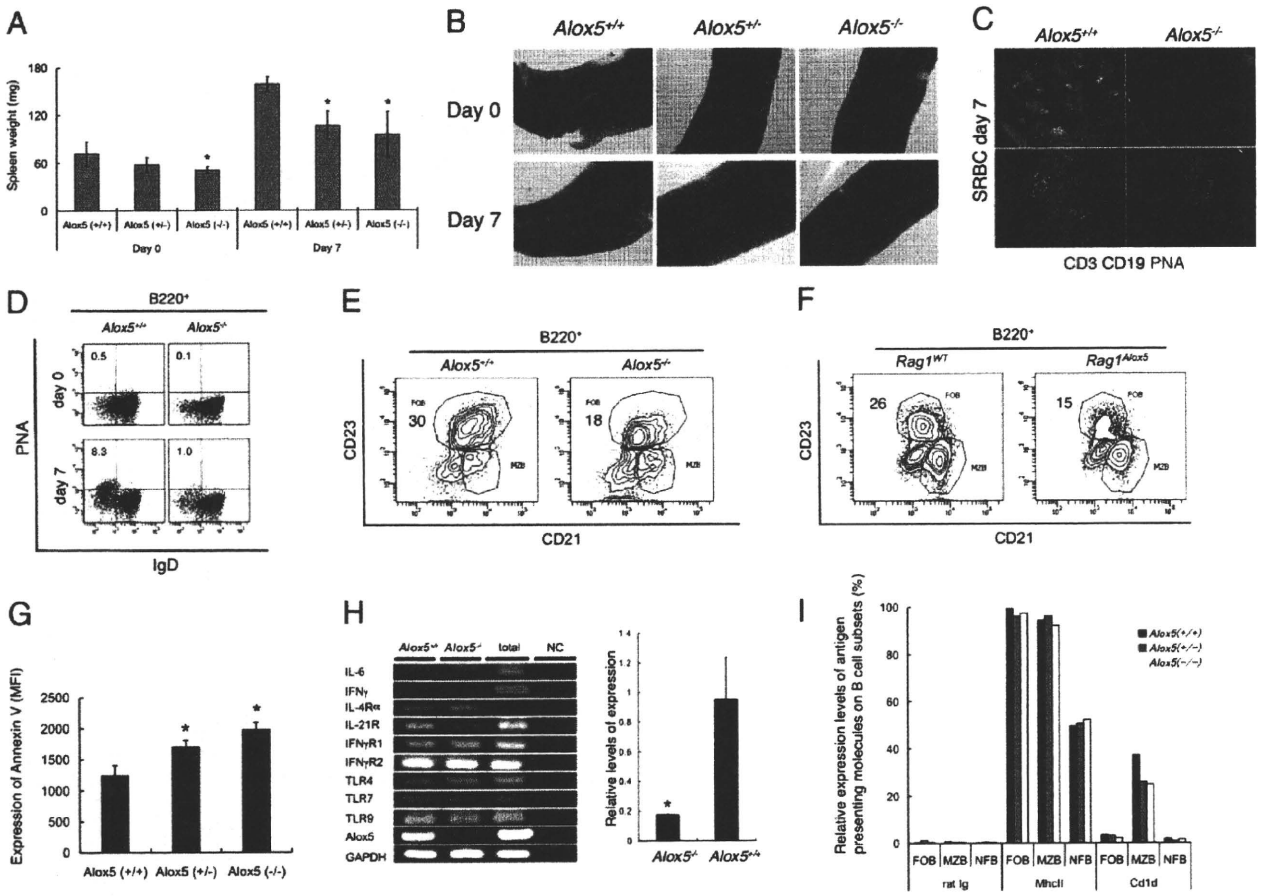

Figure 4. Analysis of antibody-producing cells in Alox5-deficient mice. **A:** Spleen weights of *Alox5*$^{+/+}$, *Alox5*$^{+/-}$, and *Alox5*$^{-/-}$ mice before (day 0) and after immunization with SRBCs (day 7). **B:** Features of the spleens of *Alox5*$^{+/+}$, *Alox5*$^{+/-}$, and *Alox5*$^{-/-}$ mice before (day 0) and after (day 7) immunization with SRBCs as examined using a stereomicroscope (SZX7, Olympus). Original magnification, ×15. **C:** Immunohistochemical analysis of the spleen of *Alox5*$^{+/+}$ and *Alox5*$^{-/-}$ mice after immunization with SRBCs (day 7). CD3$^+$ T cells (green), CD19$^+$ B cells (blue), and PNA$^+$ germinal center cells (red) are visualized. Original magnification: ×200 (upper panel) and ×400 (lower panel). **D:** FACS analysis of spleen cells of *Alox5*$^{+/+}$ and *Alox5*$^{-/-}$ mice before (day 0) and after (day 7) immunization with SRBCs. After immunization, the population of germinal center cells (B220$^+$IgD$^-$PNA$^+$) of *Alox5*$^{+/+}$ mice increased from 0.5% to 8.3% of the total B220$^+$ cells, whereas that of *Alox5*$^{-/-}$ mice increased from 0.1% to only 1.0%. **E, F:** A FACS analysis of follicular B cells (B220$^+$CD21^{int}CD23$^+$) of the spleen. **E:** Follicular B cells compose 30% and only 18% of the total B220$^+$ cell population in *Alox5*$^{+/+}$ and *Alox5*$^{-/-}$ mice, respectively. **F:** Follicular B cells compose 26% and 15% of the total B220$^+$ cell population in *Rag1*^{WT} and *Rag1*^{Alox5} mice, respectively. **G:** The expression levels of annexin V on follicular B cells of the spleen in *Alox5*$^{+/+}$, *Alox5*$^{+/-}$, and *Alox5*$^{-/-}$ mice after administration of SRBCs (day 7) as assessed by means of FACS analyzer. The mean fluorescence intensity (MFI) of annexin V on follicular B cells is shown. **H:** Expression levels of the transcripts of cytokines and receptors of follicular B cells sorted from the spleen of *Alox5*$^{+/+}$ and *Alox5*$^{-/-}$ mice as assessed by means of PCR. The **left panel** shows the RT-PCR results (25 cycles) investigating various molecules, including Alox5, and determines the down-regulation of the IL-21 receptor transcripts in the *Alox5*$^{-/-}$ cells. The **right panel** presents results from quantitative PCR of the IL-21 receptor of follicular B cells, indicating that the IL-21 receptor levels in these cells of *Alox5*$^{-/-}$ cells are approximately 20% of those in *Alox5*$^{+/+}$ cells. Total, total spleen cells; NC, no template control. **I:** The expression levels of major histocompatibility complex class II on spleen cells of *Alox5*$^{+/+}$, *Alox5*$^{+/-}$, and *Alox5*$^{-/-}$ mice. FOB, follicular B cells; MZB, marginal zone B cells (B220$^+$CD21^{hi}CD23$^-$); NFB, nonfollicular B cells (B220$^+$CD21^{lo}CD23$^-$). The results presented are representative of three to four independent experiments. In each experiment, 3 to 10 mice per group were used. $^*P < 0.05$ compared with the wild-type control.

whether humoral immunity regulated by Alox5 would work as a defense mechanism against microorganisms, an experimental colitis model was used in which DSS was orally administered to the mice in a conventional facility.[24] The results indicated that loss of Alox5 led to exaggerated enterocolitis (Figure 6A). Histologic examinations revealed severe erosion and an inflammatory reaction of the mucosa in *Alox5*$^{-/-}$ mice (Figure 6, B and C). Similar results were observed in *Rag1*^{Alox5} mice, implying that the Alox5 of B cells plays a pivotal role in establishing humoral immunity against pathogens under conventional conditions.

Alox5 Enhances the Growth of MCLs

Finally, we investigated the expression profiles of Alox5 in B-cell lymphomas composed of small lymphoid cells, including MCL, small lymphocytic lymphoma (SLL), and marginal zone lymphoma. Currently, naive B cells of the mantle zone, antigen-experienced B cells, and postgerminal center B cells are considered to be the postulated cell origins of MCL, SLL, and marginal zone lymphoma, respectively.[43] Immunohistochemical studies using L22 mAbs on frozen sections revealed the high expression of Alox5 in MCL and SLL but not in marginal zone lymphoma (Figure 7, A–I). These results seem to be in agreement with our observations that Alox5 was preferentially presented in naive and memory B cells. When the MCL cell lines were examined, three cell lines, including G519, MINO, and REC1, possessed Alox5 (Figure 7J). Interestingly, AA861, an Alox5 inhibitor, possessed the capacity to reduce the growth of MINO cells (Figure 7K). Moreover, AA861 combined with vincristine, an antitumor re-

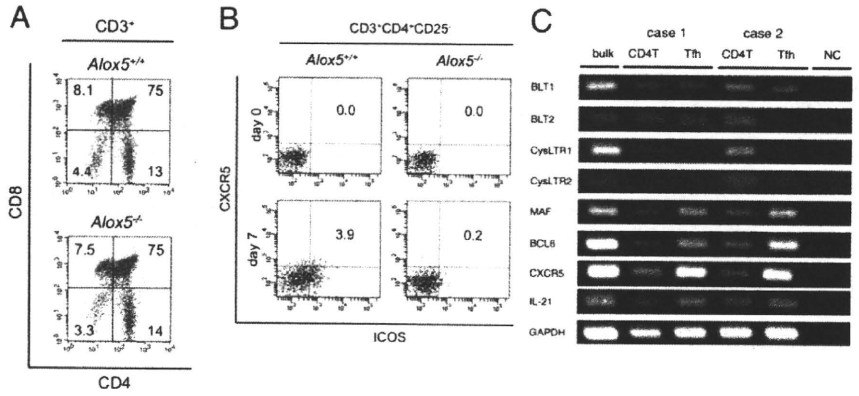

Figure 5. Developmental defects of Tfh cells in Alox5-deficient mice. **A:** FACS analysis of thymocytes in $Alox5^{+/+}$ and $Alox5^{-/-}$ mice. The population recognized by the expression of CD4 and CD8 are shown. **B:** FACS analysis of spleen cells of $Alox5^{+/+}$ and $Alox5^{-/-}$ mice before (day 0) and after (day 7) immunization with SRBCs. After immunization, the population of Tfh cells ($CD3^+CD4^+CD25^-ICOS^+CXCR5^+$) in $Alox5^{+/+}$ mice increased from 0.0% to 3.9% of the total $CD3^+CD4^+$ cells, and that of $Alox5^{-/-}$ mice increased from 0.0% to 0.2%. **C:** Expression levels of transcripts of leukotriene receptors in Tfh cells sorted from human tonsils. Results of two cases assessed by means of RT-PCR (28 cycles) are shown. Glyceraldehyde-3-phosphate dehydrogenase was used as the positive control. Bulk, total lymphocytes; NC, no template control. The results presented in (**A**) and (**B**) are representative of three to four independent experiments. In each experiment, three to six mice per group were tested.

agent for MCL, resulted in dramatic reduction of the growth activities of MINO cells. Therefore, Alox5 may be associated with the cellular integrities of not only primary B cells of the mantle zone, from which they originate, but also of MCL cells.

Discussion

In this study, we followed comprehensive immunoprecipitation and proteomics methods to identify an L22 Ag as Alox5. As implicated by the expression of Alox5 in mantle zone B cells ($CD23^+$ and $CD23^-$) around germinal centers, Alox5 plays a pivotal role in specific immunity as a regulator of cell fate and responsiveness to IL-21 of naive follicular B cells.[37,38,44] Alox5 defects also impinge on the generation of memory B cells, suggesting that it would completely support the preservation of B-cell repertories maintained by naive and memory B cells. The Alox5 pathway is essentially associated with a variety of inflammatory diseases, including asthma, atherosclerosis, rheumatoid arthritis, liver cirrhosis, and cancer, which are caused by an underlying anomaly of acquired immunity.[15,31] Therefore, primary B cells may act as a modulator in these pathologic situations. As a major source of leukotrienes in lymphoid tissues, primary B cells may contribute to establishment of the histologic features, such as lymphadenitis lesions.

Infection of mice with RNA or DNA viruses induces an antiviral antibody response, which is largely restricted to IgG2a. This also provides the functional importance of Alox5 as a coordinator of host defense. The regulation of IgG2a, which is an IL-4–independent Ig isotype, and the mechanism of interferon γ derived from type I helper T cells remain investigative priorities. Although we could not fully elucidate the precise mechanism of IgG2a production by Alox5, it has been previously reported that Alox5 deficiency may lead to an imbalance of type I and II helper T cells.[45] Thus far, we considered the possible involvement of Alox5 in the development of helper T-cell subpopulations. $CD4^+$ T cells possess leukotriene receptors, which can instructively work for the mobilization of effector T cells to inflammatory foci; however, little is known about the role of leukotrienes in the development of Tfh cells.[14,40–42,46] Leukotriene receptors encoded in germlines form a receptor spanning seven membranes coupled with G-proteins in the cytoplasm, similar to the chemokine receptors of Tfh cells, such as CXCR5, CCR6, and CXCR3. Leukotriene receptors seem to be down-regulated (or at least not up-regulated) in Tfh cells; therefore, such lipid mediators might act on the initial process of differentiation of naive helper T cells. The prerequisite for the differentiation of naive helper T cells is the interaction of the T-cell receptor on naive $CD4^+$ helper cells with major histocompatibility complex class II molecules on professional APCs. Therefore, professional APCs, which express Alox5, profoundly affect the initial steps of the differentiation of naive helper T cells. $Rag1^{Alox5}$ mice exhibited defects of specific antibody responses to foreign antigens, although Tfh cells could be recognized in response to the administration of foreign antigens in these

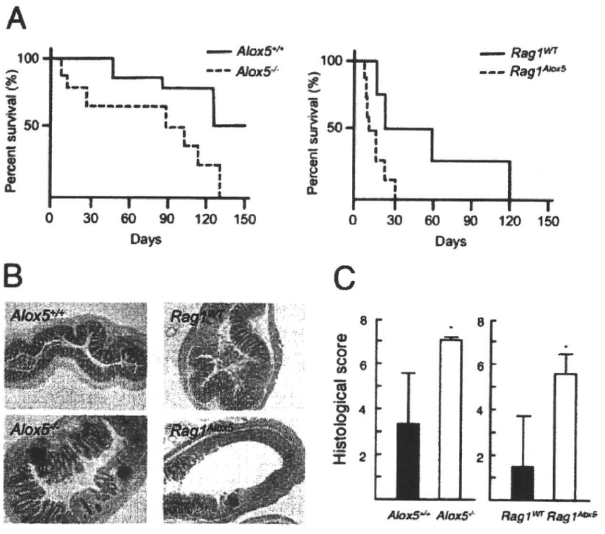

Figure 6. Alox5 defects attenuate intestinal tissue damage, with high mortality in experimentally induced chronic enterocolitis models. **A:** Survival curves of DSS-induced colitis models. Mice were maintained in a conventional facility and were allowed to freely drink water containing 1.5% DSS. The **left** and **right panels** illustrate the percentage survivals of $Alox5^{+/+}$ and $Alox5^{-/-}$ mice and $Rag1^{WT}$ and $Rag1^{Alox5}$ mice, respectively. $n = 7$ to 8 per group. **B:** Histologic findings of mice intestines after DSS administration on day 7. The **left** and **right panels** demonstrate the intestines of $Alox5^{+/+}$ or $Alox5^{-/-}$ mice and $Rag1^{WT}$ or $Rag1^{Alox5}$ mice, respectively. The intestines of $Alox5^{-/-}$ and $Rag1^{Alox5}$ mice manifested severe enterocolitis. Original magnification, ×100. **C:** The total histologic score is presented as mean with SEM ($n = 3$ to 4 mice per group). $^*P < 0.05$ compared with the wild-type control.

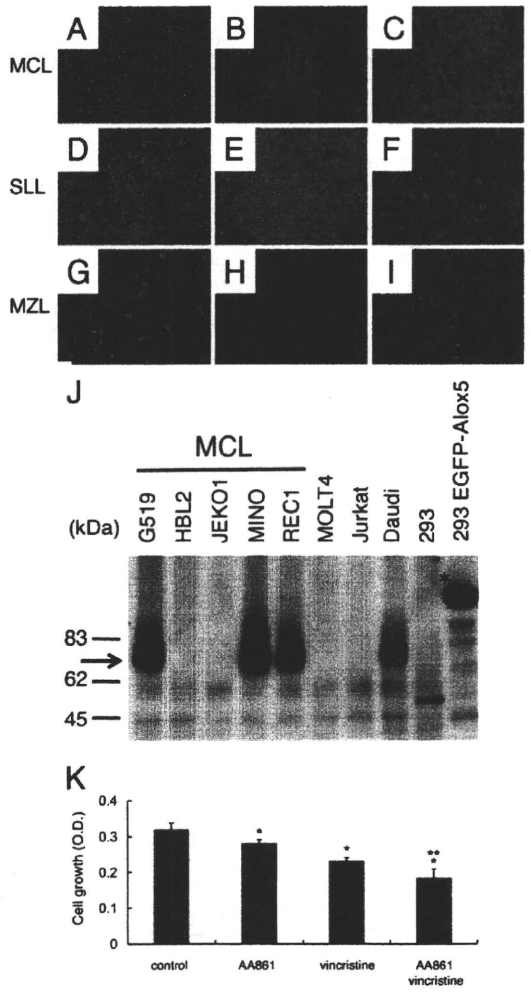

Figure 7. Alox5 defines cell growth of mantle cell–derived B-cell malignancies. **A–I:** Alox5 expression in B-cell lymphomas composed of small lymphoid cells. Immunohistochemical analysis using L22 mAbs was performed on frozen sections of independent tumor tissues: MCL (**A–C**), SLL (**D–F**), and marginal zone lymphoma (MZL) (**G–I**). Original magnification, ×200. **J:** Alox5 expression in cells derived from MCL at the protein level (**arrow**). Immunoblot analysis with anti-Alox5 pAbs was performed on human cell lines. HEK 293 cells with or without EGFP-Alox5 (**asterisk**) were used as the control. **K:** Cell growth of MINO under 10 μmol/L AA861 or 50 μg/ml of vincristine assessed by using WST1. A 96-well plate containing 1×10^5 cells per well was incubated with reagents for 24 hours. Results are representative of four independent experiments. $^*P < 0.01$ compared with the control; $^{**}P < 0.05$ compared with vincristine alone.

mice (data not shown). Thus, the lipid mediators derived from macrophages or dendritic cells but not from B cells would affect the process of differentiation of Tfh cells.

In this study, we could not fully elucidate a mechanism of the production of leukotrienes from primary B cells. Macrophages or dendritic cells produce Alox5-related leukotrienes by activating cytosolic phospholipase A2. It may be supposed that B-cell receptor signaling leads to the mobilization of calcium ions through inositol phosphate, leading to the activation of cytosolic phospholipase A2 and the subsequent liberation of arachidonate from membrane glycerolipids as a substrate of Alox5. We observed that *Alox5*$^{-/-}$ mice can produce B1 B cells ($B220^+CD5^+IgM^+$), related to producing natural antibodies, in the spleen and peritoneal cavity (data not shown). In this context, leukotrienes of primary B2 B cells may regulate the adaptive humoral immune response.[47] Indeed, we found transcripts of Alox5 in mouse follicular B cells, and we are trying to detect Alox5 in these cells at protein levels.

Maintenance of the B-cell repertoire is one of the most important elements in achieving adaptive humoral responses and protecting the host from pathogens. Perhaps primary naive and memory B cells express Alox5 and preserve their integrity to maintain the B-cell repertoire. According to this story, it was of interest to note that MCL, one of the most refractory tumors against conventional therapies, might depend at least in part on the function of Alox5 in terms of cellular growth. The evidence that primary B cells and MCL, which originates from primary B cells, rely on Alox5 for their cellular integrity can provide insights for understanding the unique tumor biology of MCLs. Further investigations should elucidate the mechanism of the additive effects of Alox5 inhibitors as chemotherapeutic reagents to treat MCL.[48,49]

In summary, we demonstrated the fundamental role of Alox5 in establishing specific antibody responses. Alox5 regulates not only primary resting B cells of the naive and memory B-cell phenotypes but also Tfh cell generation, thereby preserving specific antibody production. It has not yet been determined whether Alox5-related lipid mediators take part in the plasticity of the generation of helper T cells. However, studies of the expression profiles of leukotriene receptors on helper T-cell species would enable us to recognize the biological significance of lipid mediators in the differentiation of helper T cells. Lipid metabolism of arachidonic acid, affected by amounts from the oral intake or function of related enzymes, may affect the primary B-cell response by altering IL-21– and Tfh-mediated stimulation.

Acknowledgments

We thank Dr. Jose A. Martinez-Climent (University of Navarra, Pamplona, Spain) for providing MCL cell lines.

References

1. Watt V, Ronchese F, Ritchie D: Resting B cells suppress tumor immunity via an MHC class-II dependent mechanism. J Immunother 2007, 30:323–332
2. Vinuesa CG, Sanz I, Cook MC: Dysregulation of germinal centres in autoimmune disease. Nat Rev Immunol 2009, 9:845–857
3. Rodríguez-Pinto D: B cells as antigen presenting cells. Cell Immunol 2005, 238:67–75
4. Kurosaki T, Shinohara H, Baba Y: B cell signaling and fate decision. Annu Rev Immunol 2010, 28:21–55
5. McHeyzer-Williams LJ, McHeyzer-Williams MG: Antigen-specific memory B cell development. Annu Rev Immunol 2005, 23:487–513
6. Schmidlin H, Diehl SA, Blom B: New insights into the regulation of human B-cell differentiation. Trends Immunol 2009, 30:277–285
7. Ishii Y, Takami T, Yuasa H, Takei T, Kikuchi K: Two distinct antigen systems in human B lymphocytes: identification of cell surface and intracellular antigens using monoclonal antibodies. Clin Exp Immunol 1984, 58:183–192

8. Takami T, Ishii Y, Yuasa H, Kikuchi K: Three distinct antigen systems on human B cell subpopulations as defined by monoclonal antibodies. J Immunol 1985. 134:828–834
9. Kokai Y, Ishii Y, Kikuchi K: Characterization of two distinct antigens expressed on either resting or activated human B cells as defined by monoclonal antibodies. Clin Exp Immunol 1986, 64:382–391
10. Murakami YI, Yatabe Y, Sakaguchi T, Sasaki E, Yamashita Y, Morito N, Yoh K, Fujioka Y, Matsuno F, Hata H, Mitsuya H, Imagawa S, Suzuki A, Esumi H, Sakai M, Takahashi S, Mori N: c-Maf expression in angioimmunoblastic T-cell lymphoma. Am J Surg Pathol 2007, 31: 1695–1702
11. Rådmark O, Werz O, Steinhilber D, Samuelsson B: 5-Lipoxygenase: regulation of expression and enzyme activity. Trends Biochem Sci 2007, 32:332–341
12. Mahshid Y, Lisy MR, Wang X, Spanbroek R, Flyqare J, Christensson B, Björkholm M, Sander B, Habenicht AJ, Claesson HE: High expression of 5-lipoxygenase in normal and malignant mantle zone B lymphocytes. BMC Immunol 2009, 10:1–12
13. Toyama H, Okada S, Hatano M, Takahashi Y, Takeda N, Ichii H, Takemori T, Kuroda Y, Tokuhisa T: Memory B cells without somatic hypermutation are generated from Bcl6-deficient B cells. Immunity 2002, 17:329–339
14. King C, Tangye SG, Mackay CR: T follicular helper (TFH) cells in normal and dysregulated immune responses. Annu Rev Immunol 2008, 26:741–766
15. Fazilleau N, Mark L, McHeyzer-Williams LJ, McHeyzer-Williams MG: Follicular helper T cells: lineage and location. Immunity 2009, 30: 324–335
16. Amirav I, Luder AS, Kruger N, Borovitch Y, Babai I, Miron D, Zuker M, Tal G, Mandelberg A: A double-blind, placebo-controlled, randomized trial of montelukast for acute bronchiolitis. Pediatrics 2008, 122: 1249–1255
17. Medeiros AI, Sá-Nunes A, Turato WM, Secatto A, Frantz FG, Sorgi CA, Serezani CH, Deepe DS Jr., Faccioli LH: Leukotrienes are potent adjuvant during fungal infection: effects on memory T cells. J Immunol 2008, 181:8544–8551
18. Boyd RS, Jukes-Jones R, Walewska R, Brown D, Dyer MJ, Cain K: Protein profiling of plasma membranes defines aberrant signaling pathways in mantle cell lymphoma. Mol Cell Proteomics 2009, 8:1501–1515
19. Chen XS, Sheller JR, Johnson EN, Funk CD: Role of leukotrienes revealed by targeted disruption of the 5-lipoxygenase gene. Nature 1994, 372:179–182
20. Koshiba S, Ichimiya S, Nagashima T, Tonooka A, Kubo T, Kikuchi T, Himi T, Sato N: Tonsillar crypt epithelium of palmoplantar pustulosis secretes interleukin-6 to support B-cell development via p63/p73 transcription factors. J Pathol 2008, 214:75–84
21. Tonooka A, Kubo T, Ichimiya S, Tamura Y, Ilmarinen T, Ulmanen I, Kimura S, Yokoyama S, Takano Y, Kikuchi T, Sato N: Wild-type AIRE cooperates with p63 in HLA class II expression of medullary thymic stromal cells. Biochem Biophys Res Commun 2009, 379:765–770
22. Hanaka H, Shimizu T, Izumi T: Stress-induced nuclear export of 5-lipoxygenase. Biochem Biophys Res Commun 2005, 338:111–116
23. Muramatsu M, Kinoshita K, Fagarasan S, Yamada S, Shinkai Y, Honjo T: Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. Cell 2000, 102:553–563
24. Obermeier F, Dunger N, Deml L, Herfarth H, Schomerich J, Falk W: CpG motifs of bacterial DNA exacerbate colitis of dextran sulfate sodium-treated mice. Eur J Immunol 2002, 32:2084–2092
25. Shen Y, Iqbal J, Huang JZ, Zhou G, Chan WC: BCL2 protein expression parallels its mRNA level in normal and malignant B cells. Blood 2004, 104:2936–2939
26. Radwanska M, Guirnalda P, De Trez C, Ryffel B, Black S, Magez S: Trypanosomiasis-induced B cell apoptosis results in loss of protective anti-parasite antibody responses and abolishment of vaccine-induced memory responses. PLoS Pathog 2008, 4:e1000078
27. Sanz I, Wei C, Lee FE, Anolik J: Phenotypic and functional heterogeneity of human memory B cells. Semin Immunol 2008, 20:67–82
28. Sukumar S, Conrad DH, Szakal AK, Tew JG: Differential T cell-mediated regulation of CD23 (Fc epsilonRII) in B cells and follicular dendritic cells. J Immunol 2006, 176:4811–4817
29. Huggins J, Pellegrin T, Felgar RE, Wei C, Brown M, Zheng B, Milner EC, Bernstein SH, Sanz I, Zand MS: CpG DNA activation and plasma-cell differentiation of CD27-naive human B cells. Blood 2007, 109: 1611–1619
30. Richards S, Watanabe C, Santos L, Craxton A, Clark EA: Regulation of B-cell entry into the cell cycle. Immunol Rev 2008, 224:183–200
31. Shimizu T: Lipid mediators in health and disease: enzymes and receptors as therapeutic targets for the regulation of immunity and inflammation. Annu Rev Pharmacol Toxicol 2009, 49:123–150
32. Finkelman FD, Katona IM, Mosmann TR, Coffman RL: IFN-gamma regulates the isotypes of Ig secreted during in vivo humoral immune responses. J Immunol 1988, 140:1022–1027
33. Qazi KR, Gehrmann U, Domange Jordö E, Karlsson MC, Gabrielsson S: Antigen-loaded exosomes alone induce Th1-type memory through a B-cell-dependent mechanism. Blood 2009, 113:2673–2683
34. Pillai S, Cariappa A: The follicular versus marginal zone B lymphocyte cell fate decision. Nat Rev Immunol 2009, 9:767–777
35. Bialecki E, Paget C, Fontaine J, Capron M, Trottein F, Faveeuw C: Role of marginal zone B lymphocytes in invariant NKT cell activation. J Immunol 2009, 182:6105–6113
36. Casciola-Rosen L, Rosen A, Petri M, Schlissel M: Surface blebs on apoptotic cells are sites of enhanced procoagulant activity: implications for coagulation events and antigenic spread in systemic lupus erythematosus. Proc Natl Acad Sci U S A 1996, 93:1624–1629
37. Ozaki K, Spolski R, Feng CG, Qi CF, Cheng J, Sher A, Morse HC III, Liu C, Schwartzberg PL, Leonard WJ: A critical role for IL-21 in regulating immunoglobulin production. Science 2002, 298:1630–1634
38. De Totero D, Meazza R, Zupo S, Cutrona G, Matis S, Colombo M, Balleari E, Pierri I, Fabbi M, Capaia M, Azzarone B, Gobbi M, Ferrarini M, Ferrini S: Interleukin-21 receptor (IL-21R) is up-regulated by CD40 triggering and mediates proapoptotic signals in chronic lymphocytic leukemia B cells. Blood 2006, 107:3708–3715
39. Spanbroek R, Hildner M, Steinhilber D, Fusenig N, Yoneda K, Rådmark O, Samuelsson B, Habenicht AJ: 5-lipoxygenase expression in dendritic cells generated from CD34(+) hematopoietic progenitors and in lymphoid organs. Blood 2000, 96:3857–3865
40. Vogelzang A, McGuire HM, Yu D, Sprent J, Mackay CR, King C: A fundamental role for interleukin-21 in the generation of T follicular helper cells. Immunity 2008, 29:127–137
41. Bauquet AT, Jin H, Paterson AM, Mitsdoerffer M, Ho IC, Sharpe AH, Kuchroo VK: The costimulatory molecule ICOS regulates the expression of c-Maf and IL-21 in the development of follicular T helper cells and TH-17 cells. Nat Immunol 2009, 10:167–175
42. Nurieva RI, Chung Y, Martinez GJ, Yang XO, Tanaka S, Matskevitch TD, Wang YH, Dong C: Bcl6 mediates the development of T follicular helper cells. Science 2009, 325:1001–1005
43. Swerdlow SH, Campo E, Harris NL, Jaffe ES, Pileri SA Stein H, Thiele J, Vardiman JW: WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues, ed 4. IARC Press, Lyon, France, 2008
44. Ettinger R, Kuchen S, Lipsky PE: The role of IL-21 in regulating B-cell function in health and disease. Immunol Rev 2008, 223:60–86
45. DiMeo D, Tian J, Zhang J, Narushima S, Berg DJ: Increased interleukin-10 production and Th2 skewing in the absence of 5-lipoxygenase. Immunology 2008, 123:250–262
46. Tager AM, Bromley SK, Medoff BD, Islam SA, Bercury SD, Friedrich EB, Carafone AD, Gerszten RE, Luster AD: Leukotriene B4 receptor BLT1 mediates early effector T cell recruitment. Nat Immunol 2003, 4:982–990
47. Bafica A, Scanga CA, Serhan C, Machado F, White S, Sher A, Aliberti J: Host control of Mycobacterium tuberculosis is regulated by 5-lipoxygenase-dependent lipoxin production. J Clin Invest 2005, 115: 1601–1606
48. Runarsson G, Liu A, Mahshid Y, Feltenmark S, Pettersson A, Klein E, Björkholm M, Claesson HE: Leukotriene B4 plays a pivotal role in CD40-dependent activation of chronic B lymphocytic leukemia cells. Blood 2005, 105:1274–1279
49. Ihara A, Wada K, Yoneda M, Fujisawa N, Takahashi H, Nakajima A: Blockade of leukotriene B4 signaling pathway induces apoptosis and suppresses cell proliferation in colon cancer. J Pharmacol Sci 2007, 103:24–32