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Review

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Immune senescence special issue. Free access sponsored by the National Institutes of Health

Mucosal immunosenescence: new developments and vaccines to control infectious diseases

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Infection of the aero-digestive tract represents a major disease burden of the elderly, and despite recent advances in our understanding of the mucosal immune system, its immunosenescence remains poorly defined. Age-associated alterations of the intestinal and respiratory immune systems occur at distinct times and in a distinct manner. A reduction in gut-associated lymphoreticular tissues, intestinal antigen-specific IgA antibody responses and lack of oral tolerance induction are all associated with aging. By contrast, nasopharyngeal-associated lymphoreticular tissue function remains intact during aging with notable signs of immunosenescence seen only in the elderly. The distinct timing of mucosal immunosenescence seen between the gut and respiratory system suggests the nasal route of vaccination might be preferable for effective mucosal vaccines in the elderly.

Introduction

The mucosal immune system consists of an integrated network of tissues, lymphoid and mucous membrane-associated cells and innate effector (e.g. mucins and defensins) and acquired (e.g. antibody; Ab) molecules. Along with cytokines, chemokines and their receptors, these effector Ab molecules, which are primarily of the IgA isotype, are key players in mucosal immunity and seem to function in synergy with the innate immune system [1,2]. Mucosal inductive sites include the Peyer's patches (PPs), one of the well-characterized gut-associated lymphoreticular tissues (GALTs), and the Waldeyer's ring of tonsils and adenoids that form the nasopharyngeal-associated lymphoreticular tissues (NALTs). Collectively, these comprise a mucosa-associated lymphoreticular tissue (MALT) network that continuously supplies antigen (Ag)-specific memory B and T cells to diffuse mucosal effector sites [1,2]. The migration of lymphocytes from inductive to mucosal effector tissues is the basis for the concept of the mucosal immune system, where either nasal or oral vaccination induces mucosal immunity in multiple distal effector sites [1,2].

Despite considerable progress in characterizing the mucosal immune system (Box 1) [3–5], we still do not have a clear picture of the age-associated changes that occur to mucosal immunity. This is a crucial gap in our understanding because infections of the respiratory and gastrointestinal (GI) tracts represent the leading cause of morbidity in the elderly. In this review, we will attempt to shed light on the changes occurring to the mucosal immune system during aging, and this knowledge should eventually lead to the development of effective mucosal vaccines for the elderly where it is most desperately needed.

The intestinal microbiota shapes up mucosal immunity: its role in immunosenescence

The mammalian lower intestine contains up to 10^{12} bacteria per gram of intestine [6,7]. The normal microbiota is essential to maintain appropriate homeostatic conditions, providing energy in the form of short-chain fatty acids and nutrients (vitamins K and B_{12}) and protection against colonization by pathogenic bacteria [7–9]. In addition to these functions, the intestinal microbiota plays a major role in maturation of the host immune system including intestinal secretory IgA (SIgA) Ab production and intraepithelial lymphocyte (IEL) development [6,7,10,11]. For example, germ-free (GF) mice have an immature mucosal immune system that includes hypoplastic PPs and diminished numbers of IgA-producing cells and $CD4^+$ T cells [6,12,13]. Adapting GF mice to conventional housing or mono-association of GF mice with *Escherichia coli* results

Glossary

AID: activation-induced cytidine deaminase
FAE: follicle associated epithelium
GALT: gut-associated lymphoreticular tissue
GC: germinal center
GI: gastrointestinal
M cell: microfold cell
MALT: mucosa-associated lymphoreticular tissue
MLN: mesenteric lymph node
NALT: nasopharyngeal-associated lymphoreticular tissue
nCT: native cholera toxin
PP: Peyer's patch
SIgA: secretory immunoglobulin A
TT: tetanus toxoid

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Box 1. Organization of the mucosa-associated lymphoreticular tissue

The surface of the mucosa-associated lymphoreticular tissue (MALT) is covered by the specialized follicle associated epithelium (FAE), 10–20% of which is M cells that exhibit a unique topical morphology (i.e. microfold or membranous) and form pockets for the inclusion of lymphoid cells, including B and T cells, dendritic cells (DCs) and macrophages [1,2]. M cells, which have short microvilli, small cytoplasmic vesicles and few lysosomes, are adept at uptake and transport of luminal Ags, including soluble proteins and particulates such as viruses, bacteria, small parasites and microspheres [1,2].

In general, murine MALT [mainly gut-associated lymphoreticular tissue (GALT) and nasopharyngeal-associated lymphoreticular tissue (NALT)] contains follicles [B-cell zones with germinal centers (GCs)] and parafollicular regions enriched in T cells. Distinct follicles (B-cell zones) are located beneath the domed area of the Peyer's patch (PP) or main body of the NALT and contain GCs where significant B-cell division is seen. These GCs, which contain the majority of surface IgA-positive ($sIgA^+$) B cells [1,2], are considered to be sites where frequent B-cell isotype switching to IgA and affinity maturation occur. However, unlike peripheral lymph nodes and the spleen in the systemic compartment, the efficiency of plasma cell development is low. All major T-cell subsets are also found in the T cell-dependent areas adjacent to follicles. The parafollicular T cells are mature cells, and >97% of these T cells use the αβ heterodimeric form of the T-cell receptor (TCR). Approximately two thirds of αβ TCR^+ T cells are $CD4^+$ and exhibit properties of Th1 and Th2 cells, including support for IgA Ab responses [1,2]. Furthermore, recent studies showed the presence of different subsets of regulatory T cells (Treg). Approximately one third of the αβ T cells in MALT are $CD8^+$; this T-cell subset contains precursors of cytotoxic T lymphocytes (CTLs) [1,2]. The immunohistology of murine PPs has shown that $CD11c^+$, $CD11b^+$ and $CD8^-$ immature DCs, which show high endocytic activity and low levels of MHC and costimulatory molecule expression, form a dense layer of cells in the subepithelial area [1,2]. Mature interdigitating $CD11c^+$, $CD11b^-$ and $CD8^+$ DCs with low endocytic activity and high numbers of major histocompatibility complex (MHC) class I and class II as well as B7 molecules have been identified in the interfollicular T-cell regions [1,2]. It has been shown that MALT DCs play essential roles in the induction of mucosal immunity, tolerance and inflammation.

in normal maturation of the mucosal immune system [14,15]. Furthermore, it was reported that bacterial stimulation of human intestinal epithelial cells induced IgA2 subclass switching, the subclass associated with lower digestive tract immunity [16]. Conversely, aberrant expansion of segmented filamentous bacteria was noted in activation-induced cytidine deaminase (AID)-deficient mice that lack an appropriate molecular environment for IgA class switching [17]. Taken together, these results clearly demonstrate a complex interplay between the gut microbiota and the mucosal IgA Ab response and development.

In humans, gut lavages taken from either aged or young subjects were shown to contain comparable IgA levels [18]. A study in mice provided similar results, showing that fecal extract samples from aged mice contained essentially the same levels of IgA as young adult mice [19]. Similar results have also been reported for total IgA responses in the serum of aged mice, rats and humans [19–24]. These results demonstrate a lack of any gross age-associated impairment in total IgA synthesis. Thus, one could predict that a normal quality and number of microbiota is maintained in both aged animals and humans. However, during aging, significant alterations occur in the species composition of intestinal microflora, with proteolytic bacteria including *Fusobacteria*, *Propionibacteria* and *Clostridia* increasing in the elderly, yet the total number of anaerobes is essentially unchanged (Figure 1) [8]. Further, the numbers and species diversity of beneficial or protective anaerobes including *Bacteroides* and *Bifidobacteria* were diminished in aged individuals [8]. These changes in the quality of the microbiota might result in increased putrefaction, a greater susceptibility to gastroenteritis and infections. Further, it is possible that the quality of the SIgA response could be altered in the elderly even though the absolute quantity of these Abs can be unchanged (Figure 1).

As of now, we still do not understand the molecular and cellular relationship between the microbiota and intestinal mucosal immunity in the elderly. Although these black boxes are currently being investigated, especially in the normal adult condition, much work will be required in the future to elucidate the roles of commensal bacteria in the induction, regulation and maintenance of Ag-specific immunity and tolerance in mucosal senescence.

The mucosal immune system in aging

Effect of aging on intestinal immune responses

As mentioned above, the GI tract in the elderly is particularly susceptible to infectious diseases, in part due to changes in their intestinal microbiota but probably also because of dramatic changes to mucosal immunity itself [25,26]. For example, aged rats (>24 months of age) given native cholera toxin (nCT) orally show significantly reduced levels of anti-CT IgA responses than identically immunized young rats (3–6 months of age) [27,28]. Similar results were observed in rhesus macaques given oral nCT [29]. When aged mice (16–24 months of age) were orally immunized with *Haemophilus influenzae* type b oligosaccharide (Hib) conjugated to diphtheria toxoid and nCT as mucosal adjuvant, Hib-specific IgA Ab responses were also reduced compared with those responses seen in young, adult mice (6–8 weeks of age) [30]. These results clearly indicate that orally induced Ag-specific mucosal IgA responses are diminished in aged animals (Figure 2).

Age-associated immune dysregulation occurs in both the mucosal and the systemic immune compartments as early as 12–14 months of age (so-called 'aging' mice as opposed to 'aged' mice) (Figure 2). Groups of mice at 1 (i.e. aging) or 2 years (i.e. aged) of age show reduced levels of Ag-specific mucosal and systemic immune responses when given oral ovalbumin (OVA) as antigen plus nCT (Figure 2) [19]. When Ag-induced cytokine responses were examined at both protein and mRNA levels, $CD4^+$ T cells from the spleen and PPs of young adult mice (8–12 weeks old) revealed elevated levels of interleukin 4 (IL-4) production. However, these cytokine responses were already and significantly diminished in aging mice, because antigen-induced IL-4 synthesis is required for the generation of effector memory cells from naïve $CD4^+$ T cells, a pathway that seems to be defective in aged mice (Figure 2) [19]. By contrast, it has been reported that anti-CD3 mAb stimulation, which provides activation signals for existing memory $CD4^+$ T cells, elicited higher levels of IL-4 synthesis even in senescent animals [31–33]. These findings suggest that, although an age-related reduction of naïve $CD4^+$ T

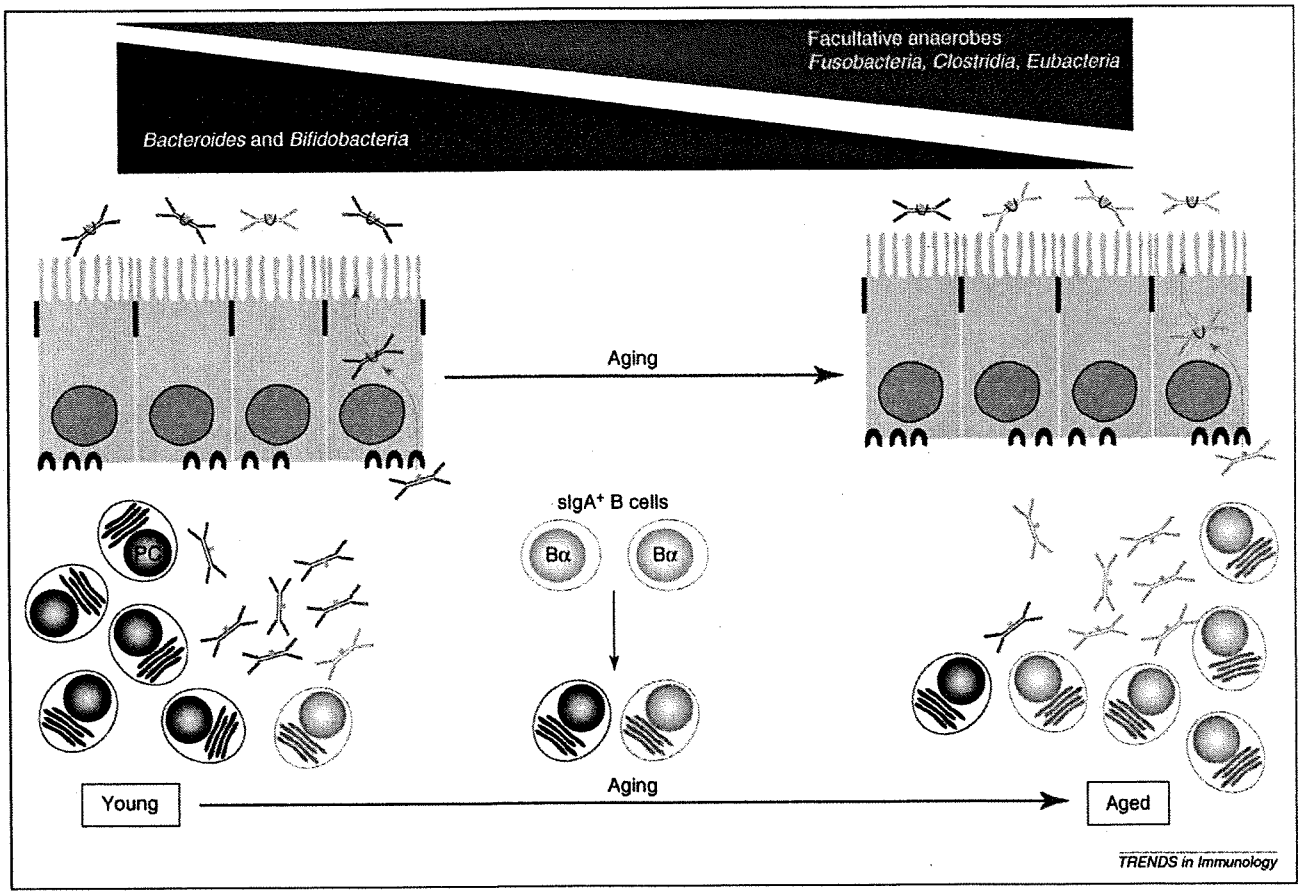

Figure 1. Age-associated changes in the intestinal microbiota of the elderly. The numbers and species diversity of beneficial or protective anaerobes including *Bacteroides* and *Bifidobacteria* diminish in the elderly. This age-related reduction is countered by a rise in *Fusobacteria, Clostridia* and *Eubacteria* species. As a direct result of these age-related changes in the microbiota, the quality of the secretory IgA (SIgA) response can be altered, although the absolute quantity of these Abs are generally unchanged. The alterations in the quality of SIgA Abs are indicated by the different colors of dimeric IgA (blue versus green) secreted by plasma cells. Bα cells indicate surface IgA$^+$ B cells that differentiate into plasma cells (sIgA$^+$).

cells is evident in the mucosa, a pool of memory T cells remain capable of responding to exogenous stimulation signals. Thus, one possible approach for the development of mucosal vaccines in the elderly might be accomplished by triggering of this memory CD4$^+$ T-cell pool.

When mice were immunized subcutaneously with OVA plus nCT as peripheral adjuvant, impaired OVA-specific, but intact CT-specific, immune responses were seen in the systemic immune compartment of 1-year-old mice [19]. Two-year-old mice showed the poorest priming, with responses to both OVA and CT being depressed. From these studies, one could suggest that the parenteral (i.e. nonoral) immune system in 1-year-old (or aging) mice might be in a transitional stage between a normal and age-associated deficiency (Figure 2). Thus, systemic Ab responses to the weak antigen OVA, which always requires an adjuvant for induction of specific immunity, was impaired in 1-year-old mice; however, nCT, a potent immunogen because of its innate cAMP-dependent adjuvant properties, induced normal plasma Ab responses in these mice. By contrast, mucosal immune responses including both OVA- and CT-specific Ab and cytokine responses, induced by oral OVA and nCT in 1-year-old mice (aging mice), were markedly reduced and were comparable to those seen in 2-year-old mice (aged mice) [19]. These results indicate that age-associated alterations might arise in the mucosal immune system of the GI tract earlier than in the parenteral immune compartment.

Oral tolerance in aging

The induction of mucosal and systemic immunity by oral Ag delivery is rather challenging and requires use of potent mucosal adjuvants, targeting vectors or other special delivery systems. The reason for this difficulty is that a major function of the mucosal immune system is in general to create a physiologically and immunologically quiescent state instead of an active condition [2,34]. Thus, tolerance (including both mucosal and systemic unresponsiveness) represents the most common response of the host to mucosally presented antigens. The continuous ingestion of several thousand different types of food protein is but one important example of oral tolerance, whereas tolerance to our indigenous microflora, which mostly colonizes the large intestine, represents another.

The nature, dose and frequency of Ag feeding, as well as host-related factors such as genetic background and the absence of a microflora in the GI tract, have been shown to influence the induction and maintenance of oral tolerance

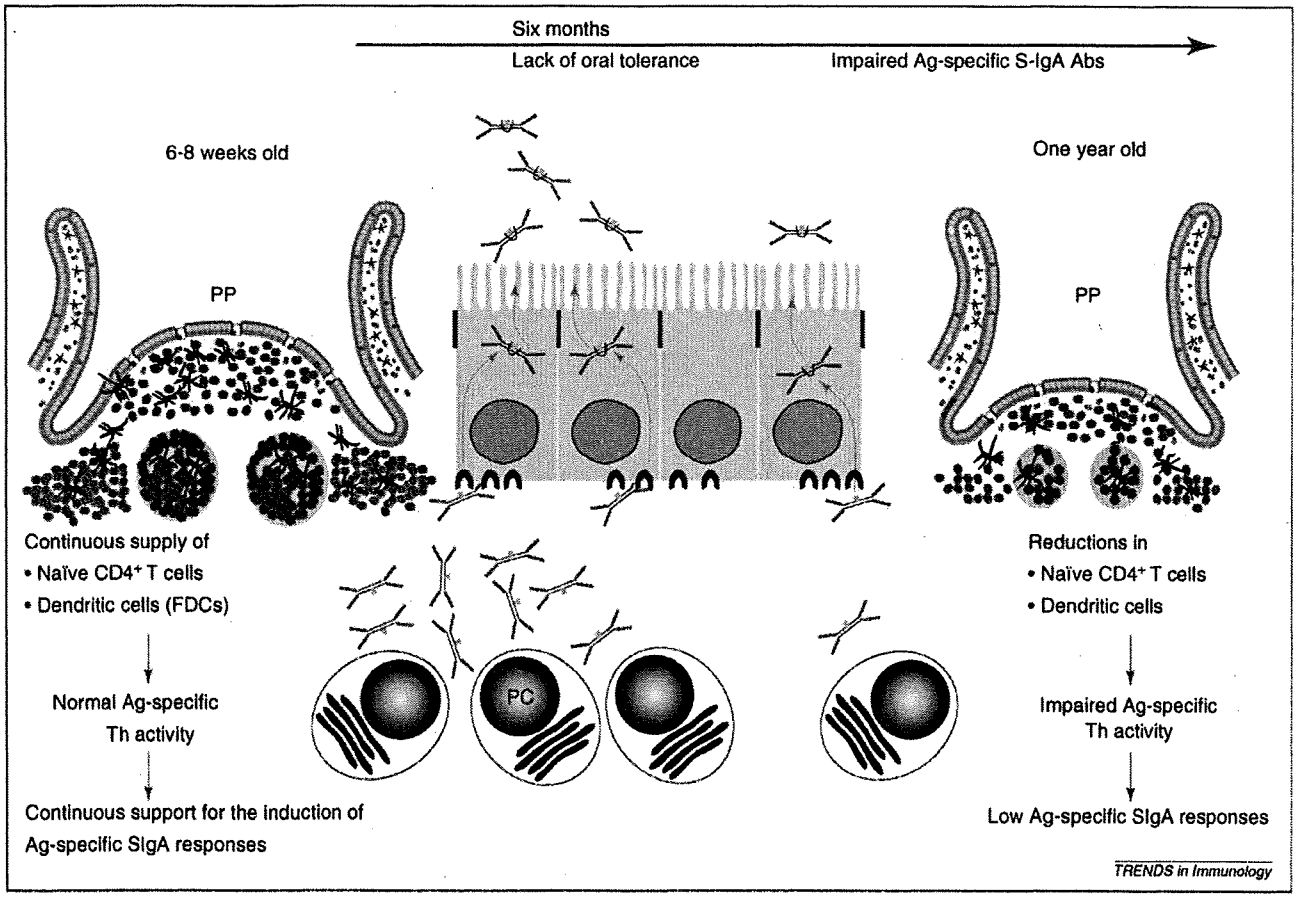

Figure 2. Evidence of early aging in the gastrointestinal (GI) tract. A reduction in Ag-specific intestinal secretory IgA (SIgA) responses can be seen in so-called 'aging' (1 year old) mice, and this is clearly manifested by a reduced Peyer's patch (PP). Reduced numbers of naïve $CD4^+$ T cells and follicular dendritic cells (FDCs) are also seen in the PP of aging mice. Similarly, an impaired ability to induce oral tolerance also occurs as early as 6 months of age.

[2,35–37]. For instance, the induction of oral tolerance in GF mice is particularly difficult [38]. By this token, it is reasonable to predict that age-related changes in the intestinal microflora could adversely affect the ability to induce oral tolerance in the elderly. Indeed, oral tolerance to OVA could be induced in all strains of young mice and in C3H/HeJ and $B6D2F_1$ mice at 20 and 38 weeks of age, respectively [39]; however, oral tolerance induction was completely abolished in A/J mice by 38 weeks of age [39]. Similarly, it has been shown that induction of oral tolerance to sheep red blood cells (SRBCs) or OVA is impaired with aging, and this occurs as early as 6–8 months of age (Figure 2) [40,41]. These results were due to dysregulation of both T- and B-cell responses in the PPs of aging mice, which in turn could stem from the senescence of both subepithelial dendritic cells (DCs) and follicular DCs, key antigen-presenting and immune-instructing cells for the initiation of balanced Ag-specific immune responses in PPs [41]. Interestingly, although $B6D2F_1$ mice become less susceptible to oral tolerance induction at 25 weeks and are totally refractory at 70 weeks of age (aged mice), mice given oral OVA at 8 weeks remain tolerant to OVA at 70 weeks of age [42]. This highlights the important finding that aging affects the inductive but not the effector phase of oral tolerance. Thus, oral tolerance established in early age can be maintained despite aging, whereas the induction of oral tolerance to new antigens is impaired in aged mice.

Alternative Ag-specific T-cell responses in the gut of aging mice

A substantial age-associated decline in the absolute numbers of lymphoid cells was found in the MALT, specifically in PPs and mesenteric lymph nodes (MLNs) [43]. Further, it was suggested that mucosa-associated, T cell–dependent but not T cell–independent B-cell responses decline in aging. *In vitro* Ab production by B cells from aged PPs and MLNs were depressed when T cell–dependent, but minimally affected when T cell–independent, B-cell mitogens were used for stimulation [43]. This finding suggests that T cells are more susceptible than B cells to immunosenescence in the mucosal compartment. It has been shown that PPs play key roles in the initiation of mucosal IgA immunity and oral tolerance [44,45]. As indicated above, a significant size reduction in PPs was seen in 1-year-old mice along with reduced Ag-specific mucosal Ab responses [19]. When the frequencies of T-cell subsets were examined, the ratio of $CD4^+$ and $CD8^+$ T cells and B cells were unchanged [19,41]. However, actual numbers of lymphocyte counts in PPs of 1-year-old mice were

significantly lower than those seen in young adult mice (6–8 weeks old) [19,41]. Further, it was reported that Ag-specific T-cell helper and regulatory functions in PPs were diminished by aging [19,41,46]. Others have shown that cytotoxic T-lymphocyte (CTL) activity against viral infections was significantly reduced in aged mice and in the elderly [47]. These findings clearly suggest that the development of effector T cells is influenced by senescence. Indeed, it has been shown that age-associated alterations closely parallel increases in memory type and loss of the naïve T-cell phenotype during aging [48–51]. In this regard, when the actual cell numbers of naïve $CD4^+$ T cells between young adult (6–8 weeks old) and aging (1 year old) mice were compared, the PPs of aging mice showed significant reductions in $CD4^+CD45RB^+$ naïve T-cell frequencies in addition to total cell numbers compared with young, adult mice [52].

Impaired mucosal DC function in aging

The mucosal DC subsets and their unique functions in various mucosal immune compartments have been well characterized [2]. Recently, studies have also examined age-related changes in mucosal DC function. For instance, mucosal DCs from aged mice exhibit less ability to trigger T and B cells [53–55]. Similarly, plasmacytoid DCs from aged mice show reduced interferon α (IFN-α) production in response to herpes simplex virus type 2 infection due to an impairment in IFN regulatory factor 7 activation [56]. By contrast, in humans, the antigen-presenting cell (APC) functions of aged DCs seem to be intact [57–60]. DCs from old individuals (>65 years old) have been shown to be as effective as DCs from young individuals (<30 years old) for T-cell stimulation when highly immunogenic proteins (e.g. *Mycobacterium* PPD, inactivated influenza virus and tetanus toxoid) were used [57,59,60]. Thus, how the quality of DC antigen presenting function changes with age remains a controversial and incompletely defined issue, with discrepancies between the human and mouse systems. However, few studies have assessed the effects of aging on murine DCs in mucosal immune compartments and their responses to either tolerogens or immunogens [41,55]. Our previous study indicated that $CD11c^+$ DCs in the PP subepithelial dome decrease in number in mice >1 year of age [41]. Furthermore, fewer follicular DCs (FDCs) and germinal center B cells were noted in the B-cell zone of the PP during aging [41]. These results suggest that the impaired Ag-specific SIgA responses and the lack of oral tolerance induction seen in aging mice [19,41,55] might be associated

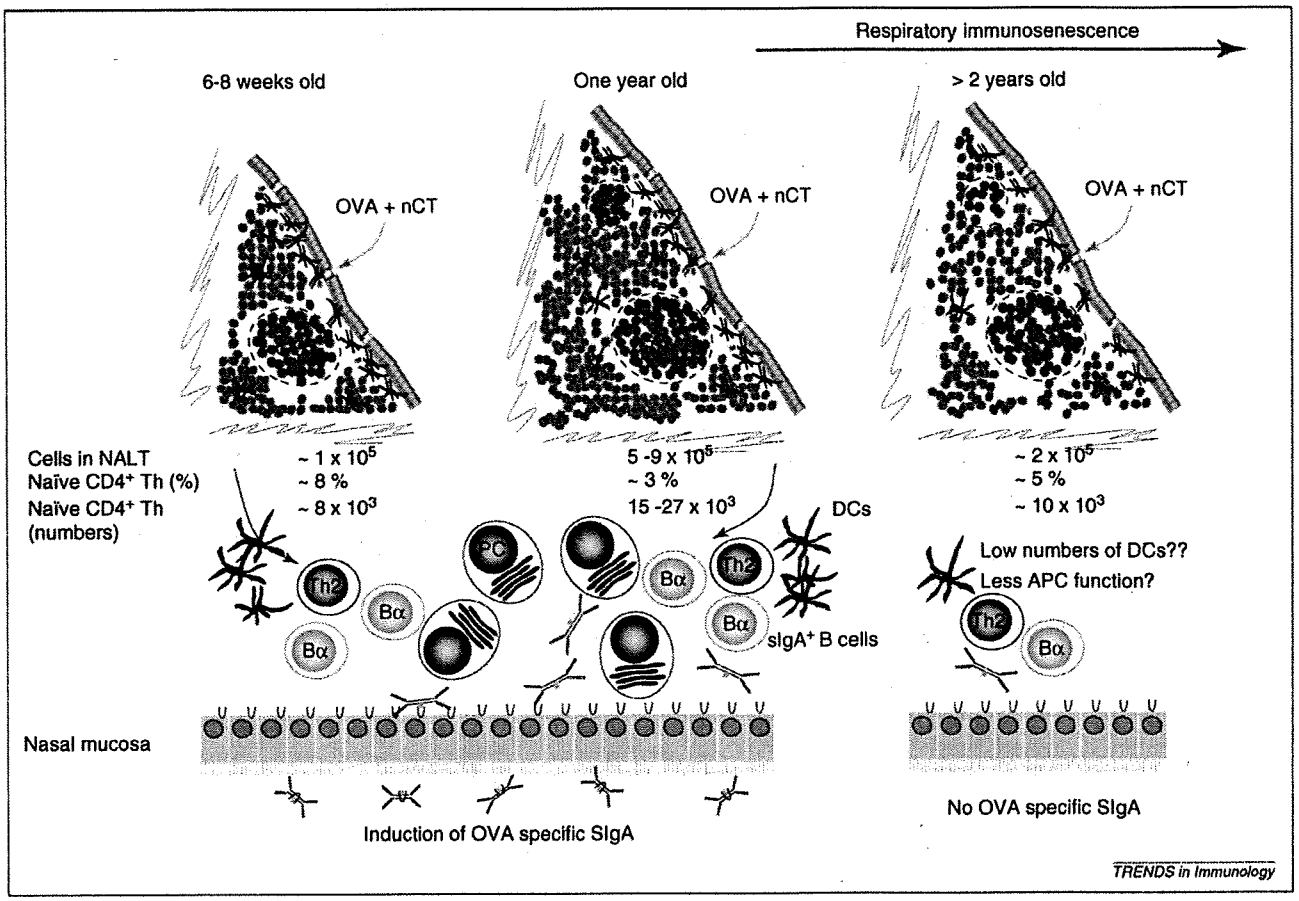

Figure 3. Nasal associated lymphoid tissue (NALT) exhibits a slower rate of immunosenescence. Nasal immunization with ovalbumin (OVA) plus native cholera toxin (nCT) induces OVA-specific secretory IgA (SIgA) and systemic IgG Ab responses in 1-year-old (i.e aging) mice which are identical to those in young adult (6–8 week-old) mice. By contrast, the signs of mucosal immunosenescence are already evident in the gut associated lymphoid tissue (GALT) of 1-year-old mice (see Figure 2). However, by 2 years of age, mice given OVA plus nCT nasally fail to induce OVA-specific SIgA but retain systemic IgG Ab responses. Therefore, NALT shows a relatively slower decline in immune responses with senescence becoming apparent only in aged mice. The absolute numbers of $CD4^+$ naïve T cells in the NALT are maintained in aging and aged mice.

with decreased DC and FDC functions in PPs in addition to impaired T-cell responses.

A recent study from another group indicated that DCs from the MLNs of aged mice showed less ability to stimulate Ag-specific T-cell immunity against infection by the protozoan *Encephalitozoon cuniculi* when compared with DCs from young adult mice [55]. Interestingly, IL-15 treatment restored this impaired DC function in aged mice [55] This finding could have important implications for intervention in the age-related decline of immune function.

Nasal immunity: a slower decline in function with age

Relatively little work has investigated the age-associated alterations in the upper respiratory tract. In this regard, Ag-specific mucosal immune responses were examined in aged mice nasally immunized with OVA plus nCT. We described above that oral administration of OVA along with nCT showed an impairment in mucosal and systemic responses in 1-year-old mice [19], the same age of mice have also been used to examine whether similar impairments could be seen when this antigen-adjuvant combination was administered nasally. In contrast to oral immunization, nasal immunization induced normal Ag-specific mucosal and systemic immune responses in 1-year-old (i.e. aging) mice [19,52]. Thus, equivalent levels of OVA-specific Ab responses in plasma and external secretions and Ag-specific Ab-forming cells (AFCs) in the nasal cavity were seen in both young and old mice, clearly showing nasal immunization could induce both mucosal and systemic immunity in aged mice (Figure 3) [52]. Furthermore, 1-year-old mice given nasal tetanus toxoid (TT) and nCT vaccine were protected from tetanus toxin challenge as were nasally vaccinated young adult mice [52]. These results suggest that the GALTs and NALTs are characterized by distinct rates of age-related functional decline.

To support this notion further, organogenesis of NALT has been shown to begin during the postnatal period, whereas GALT development initially occurs during gestation [61,62]. These studies clearly point to significant differences in the developmental timing of the two major mucosal inductive tissues. Considering these developmental differences, one would predict that age-associated alterations might also occur independently of each other. The findings that nasal but not oral immunization induced normal levels of Ag-specific immune responses in 1-year-old mice strongly supports the contention that aging occurs more slowly in NALT than GALT immune systems. Given these differences, the nasal route might therefore be the preferred and more effective means of administering vaccines to induce both Ag-specific mucosal and systemic immune responses. That said, one has to always take consideration of the safety issues related to nasal vaccines, especially the possibility of vaccine antigen deposition in the central nervous system (CNS). This is a genuine concern because it has been shown that nasal administration of mice with TT plus nCT resulted in the transient accumulation of these antigens in the CNS [63]. Although the results generated by the murine study must be carefully considered in any future development of nasal vaccines, we should also appreciate the fact that the anatomy of the nasal cavity is different between mice and humans.

It has been shown that increased numbers of memory-type and decreased numbers of naïve $CD4^+$ T cells are associated with aging [50,64–66]. When the frequencies of naïve $CD4^+$ T cells in NALT and GALT (i.e. the PPs) were compared in young adult and 1-year-old mice, reduced frequencies of $CD4^+$, $CD45RB^+$ T cells were seen in aging mice [52]. However, the size and the total lymphocyte count in the NALT increases approximately five- to ninefold during the aging process through the first year (Figure 3). Although the total lymphocyte count is ultimately reduced by 2 years of age, the NALT contains approximately twice the number of lymphocytes (Figure 3). These results suggest that the continuous generation of a naïve T-cell population in the NALT plays a pivotal role in maintaining young adult mouse levels for the induction of both systemic and mucosal immune responses to nasally administered antigens in aging (i.e. 1 year old) mice.

When examining immunosenescence, it is often suggested that experimental mice should be at least 2 years old before they can be considered equivalent to elderly humans [41,48,52]. When 2-year-old (or aged) mice were immunized nasally with OVA and nCT as adjuvant, they failed to undergo induction of Ag-specific SIgA responses in their external secretions (Figure 3) [52,67]. However, these same mice showed OVA-specific systemic immune responses (i.e. T-cell proliferation, Th1 and Th2

Box 2. Novel mucosal vaccine targeting strategies

The major mucosal inductive tissues like Peyer's patches (PPs) and nasopharyngeal-associated lymphoreticular tissue (NALT) are covered by follicle associated epithelium (FAE) containing M cells (see Box 1) [1,2]. Reoviruses initiate infection via M cells, an ability that has been associated with the protein sigma 1 (pσ1). Nasal M-cell targeting protocols have exploiting this phenomenon by using plasmid DNA (the vaccine) and the covalently attached reovirus pσ1 to poly-L-lysine (PL). This approach has effectively induced Ag-specific mucosal IgA Ab responses (Figure 4) [74]. Further, a novel M cell–specific mAb (NKM 16-2-4) has been used as a carrier for M-cell targeting with a mucosal vaccine. Oral administration of chimeric vaccine consisting of NKM 16-2-4 and tetanus toxoid or botulinum neurotoxin type A toxoid, together with native cholera toxin (nCT) as mucosal adjuvant, can induce high levels of Ag-specific plasma IgG and mucosal IgA Ab responses and protection against the toxin challenge (Figure 4) [75]. These studies clearly show that an M cell–targeting delivery system for the development of effective mucosal vaccines might also be effective in the elderly.

FluMist, a trivalent nasal vaccine consisting of type A (H1N1 and H3N2) and type B live attenuated influenza virus, is an example of a potent NALT immune system–targeting vaccine already in the current market. FluMist can successfully reduce the chances of influenza illness among children (age 15–85 months) by 92% compared with a placebo. Further, the effectiveness of FluMist was also demonstrated in adults 18–49 years of age; however, this efficacy was not seen in individuals 50–64 years of age. The safety studies resulted in a higher rate of sore throats in a group >65 years of age. Therefore, FluMist is approved for use in healthy non-pregnant people 2–49 years of age. Although nasal application of FluMist is not yet approved in the elderly, it would be possible that an adaptation of this strategy for specific mucosal targeting (M cells or mucosal dendritic cells) with adjuvant could be applied the elderly. Together, the evidence accumulated by the FluMist vaccine and current ongoing studies in the development of safe and potent mucosal targeting vehicles and adjuvants should lead to the generation of mucosal vaccines for the control of infectious diseases of the aged.

cytokines), which were essentially identical to the responses seen in young adult mice [52,67]. These results further reinforce the findings that immunosenescence occurs earlier in the mucosa than in the systemic immune system [19], even though the process of NALT immunosenescence is milder than that of GALT in 2-year-old mice.

Mucosal vaccines designed for the elderly

The elderly are in general much more susceptible to infections. In fact, the severity and mortality caused by the infectious pathogens invading mucosal surfaces such as influenza virus and the bacterial pathogen *Streptococcus pneumoniae* (pneumococcus) are sharply increased in humans of advanced age [68–70]. In the United States, influenza virus infection annually caused at least 36 000 deaths during 1990–1999 and 226 000 hospitalizations during 1979–2001 [71,72]. Pneumococcus is similarly a major human bacterial pathogen and a significant cause of morbidity and results in >40 000 deaths in the United States each year [73]. The highest incidence of influenza and pneumococcal diseases occurs among persons >65 years of age. The development of effective vaccines for the elderly remains a largely unmet need, so to provide effective protection against influenza and *S. pneumoniae* in the elderly, one should strongly consider developing a new generation of vaccines that could induce pathogen-specific immunity in the respiratory tract. Although it has been shown that effective protection can be provided by pathogen-specific systemic IgG without mucosal IgA responses [74], pathogen-specific SIgA responses are a necessary component for providing the first line of effective immunity against these respiratory pathogens at their entry site in the elderly. Pathogen-specific SIgA at the surface of the nasal mucosa and upper respiratory tract reduce invasion, dissemination and/or growth of pathogenic bacteria or viruses. Indeed, it was reported that influenza hemagglutinin (HA)-specific SIgA responses play a key role in protection against influenza in the upper respiratory tract and provide cross-protection against infection with a variant virus within the same subtype when compared with those of serum IgG Abs [75,76].

To explore new avenues for effective mucosal vaccine development, investigators have begun to target mucosal tissues and immune cells for vaccine delivery. To this end,

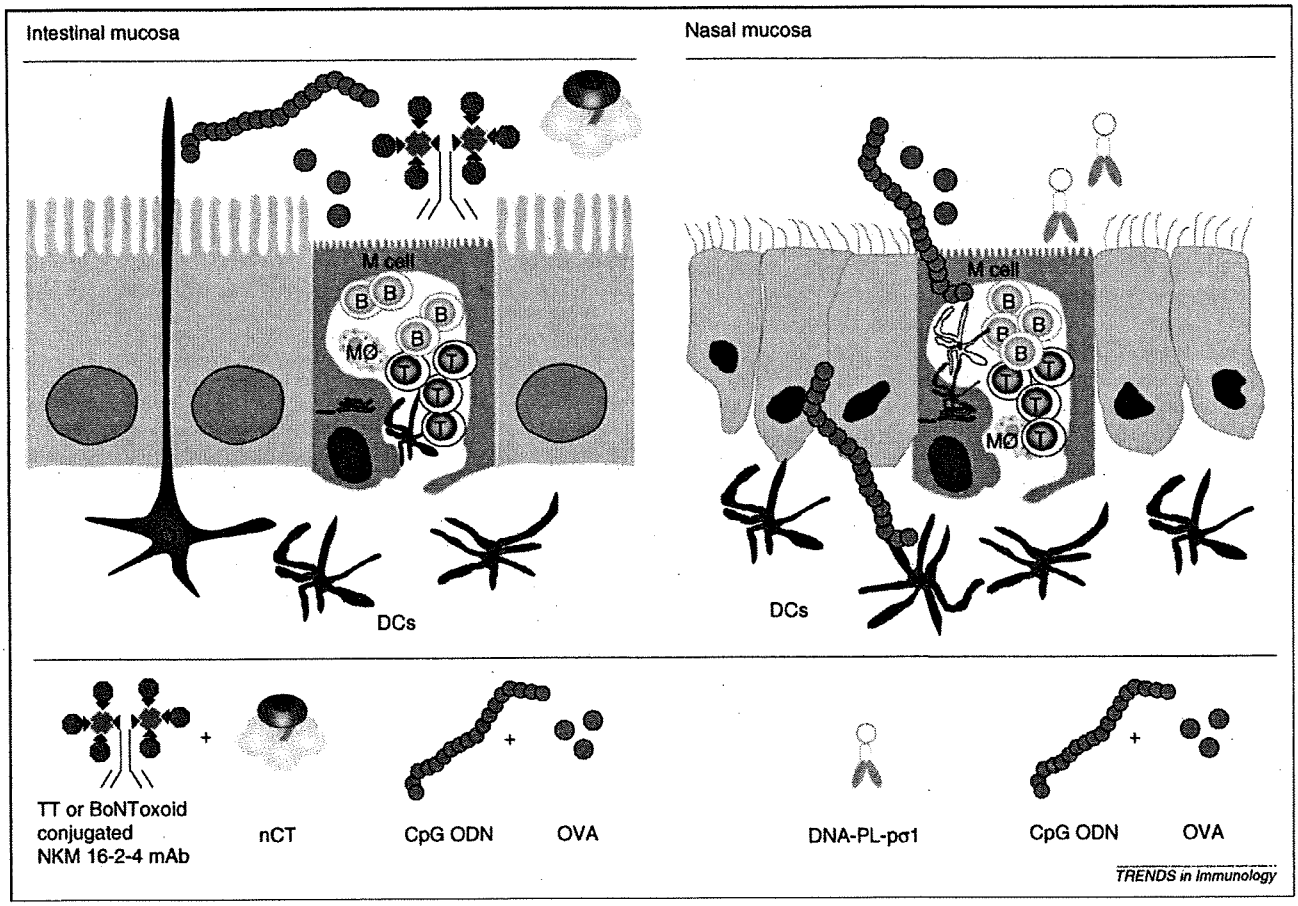

Figure 4. Mucosal targeting approaches for vaccination of the elderly. Mucosal M-cell and/or dendritic cell (DC) targeting can facilitate Ag-specific secretory IgA (SIgA) Ab responses to provide effective immunity at the entry site of pathogens. Specific M-cell targeting can be achieved by using protein σ1 of reovirus or the M cell–specific mAb NKM 16-2-4. Nasal immunization with the chimeric DNA vaccine (DNA-PL-pσ1) induces Ag-specific immune responses. Oral immunization with the chimeric vaccine (NKM 16-2-4 conjugated to BoNToxoid or TT) together with nCT results in the induction of protective immunity. The Toll-like receptor (TLR) 9 ligand CpG ODN is currently the most promising adjuvant used in vaccines for the elderly. In murine models, CpG ODN can be used as an effective mucosal (intestinal or respiratory) adjuvant to target mucosal plasmacytoid DCs for the induction of Ag-specific immune response. Thus, oral or nasal immunization with nominal antigen OVA plus CpG ODN results in the induction of Ag-specific immune responses in both the mucosal and systemic compartments of aged mice. BoNToxoid, botulinum neurotoxin type A; DC, dendritic cell; Mϕ, macrophage; nCT, native cholera toxin; OVA, ovalbumin; TT, tetanus toxoid.

mucosal M cell– or DC- targeting Ag delivery systems have been shown to induce Ag-specific SIgA responses (Box 2; Figure 4) [77–80]. CpG ODN as vaccine adjuvant has been shown to restore Ag-specific immune responses to OVA, diphtheria toxoid, hepatitis B, polysaccharide of *S. pnuemoniae,* amyloid β and tumor cells in aged mice and rats [81–87]. When 3-month-old (or young adult) and 18-month-old (or aged) mice were orally immunized with OVA plus CpG ODN as adjuvant, both groups of mice showed high and equivalent levels of OVA-specific systemic IgG and mucosal IgA Ab responses [88]. Furthermore, recent work showed that when a nasal adjuvant was constructed with a plasmid encoding the Flt3 ligand cDNA (pFL) coupled to CpG ODN and then given with OVA to 2-year-old mice, significant levels of OVA-specific IgA responses were induced in external secretions including nasal and vaginal washes and saliva [67]. By contrast, nCT as nasal adjuvant in 2-year-old (or aged) mice failed to induce mucosal immunity, although significant systemic immune responses were noted [52,67]. These findings demonstrate that mucosal delivery of CpG ODN as adjuvant offers an attractive possibility for the development of an effective mucosal vaccine for the elderly.

In addition to the above mucosal targeting system or adjuvants, direct mucosal application of cytokines, chemokines or costimulatory molecules possessing mucosal adjuvanticity [2] are also considered to be attractive candidates for the induction of protective immunity in the elderly. Indeed, it has been shown that the effect of aging on IL-2 production was abrogated by exogenous IL-2 delivery [48]. Thus, mucosal IL-2 treatment can overcome age-impaired mucosal immune responses by enhancing mucosal immunity or abrogating unresponsiveness in aged mice [89]. Furthermore, recent studies showed that keratinocyte growth factor (KGF) or IL-7 treatment prevented and/or reversed thymic atrophy [90,91] (also see the article by Lynch *et al.* in this issue). Because KGF and IL-7 have been shown to be involved in the development and regulation of the mucosal immune system [1,2], mucosal delivery of the growth factor cytokine family (e.g. IL-2, IL-7, IL-15 and KGF) could help improve mucosal vaccines for the elderly.

Are we there yet? Yes, we can...

Despite its relatively rapid immunosenescence, mucosal vaccination is still a particularly attractive route for inducing protective immunity against pathogens in the elderly. Because immunosenescence occurs earlier in the mucosa than in the systemic compartments, our efforts in the design of mucosal modulators including novel mucosal adjuvants that can overcome mucosal immunosenescence in the elderly are one of major priorities for aging research. Thus, one should continue to study not only CpG ODN but also seek other mucosal adjuvant candidates for supporting the induction of protective immunity in the elderly, especially against influenza and *S. pneumoniae* infections. The development of mucosal vaccines for the elderly has only just begun and offers both promise as well as significant hurdles. For instance, we must fill the gaps between mouse and human studies. A significant concern is that humans are exposed to environmental Ags, vaccines and antibiotic treatments, whereas mice stay in a relatively clean environment. Further, genetic and anatomic differences need to be considered. Especially, for nasal vaccine development, one must discover the human counterparts for mouse NALT, in addition to tonsils and adenoids. Translational research such as the use of nonhuman primates and *in vitro* human cell systems might be partial solutions. Furthermore, recent progress in the development of a new generation of humanized mice should offer a powerful new tool for understanding immunosenescence in humans. Differences in the immune responses of the elderly populations of various countries are influenced by commensal flora, nutritional uptake, genetic background, life style, and so forth. Thus, this complexity must also be taken into account when studying human mucosal immunosenescence, because the mucosal immune system is directly and continuously exposed to these micro- and macro-environments. The development of universal, safe and effective mucosal vaccines for both young and aged populations will require global enthusiasm and international cooperation at the bench-top and the clinic, as well as pharmaceutical and government support.

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Expression of IL-5Rα on B-1 cell progenitors in mouse fetal liver and involvement of Bruton's tyrosine kinase in their development

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ABSTRACT

B-1 cells are a subset of B cells responsible for the production of natural antibodies. Although the amount of natural antibody is tightly regulated, how this regulation occurs remains unknown. We examined the expression of IL-5 receptor, a cytokine receptor critical for homeostatic proliferation of B-1 cells, on B-1 cell progenitors in the fetal liver. We identified B-1 progenitors expressing low levels of IL-5 receptor α chain (IL-5Rα) and eosinophil progenitors expressing higher levels of IL-5Rα in the fetal liver. Moreover, the number of these B-1 progenitors were significantly reduced in the fetuses of mice deficient in Bruton's tyrosine kinase (Btk), even though IL-5 and thymic stroma lymphopoietin signaling are intact in early B lineage cells in Btk-deficient mice. These data suggest that IL-5 is possibly involved in B-1 cell development and an uncharacterized, Btk-dependent regulatory signaling pathway is involved in unexpectedly early stages of B-1 cell differentiation.

1. Introduction

Vertebrates are protected from pathogens by two arms of the immune system: innate and acquired immunity. The innate immune system relies primarily on various pattern-recognition receptors such as the family of Toll-like receptors. Since this type of receptors is readily expressed and can sense a wide variety of pathogens, the innate immune system is activated immediately upon pathogen entry. In contrast, activation of the acquired immune system requires time to select and expand antigen-specific T and B cells. With its high specificity for antigen and long-lasting memory, activation of the acquired immune system is important for complete elimination of pathogen and immunity to subsequent infections by the same pathogen. A certain level of immunoglobulins exist in the body independent of infection, and these are designated natural antibodies. These natural antibodies are produced by a special subset of B lymphocytes called B-1 cells [1]. B-1 cells are distinguished from conventional B cells (B-2 cells) by their high expression of IgM, low expression of IgD, expression of CD43, and lack of CD23 and CD21 expression. These cells can be further sub-divided into $CD5^+$ B-1a cells and $CD5^-$ B-1b cells. By their preformed reactivity to various pathogens, natural antibodies can be included in the innate immune system. In fact, X-linked immunodeficiency (XID) mice deficient in B-1 cells and natural antibodies are more susceptible to *Streptococcus pneumoniae* infection than wild-type mice, and the wild-type susceptibility can be restored to XID mice by administration of normal mouse serum [2]. In addition, recent studies have shown that B-1 cells and natural antibodies are indispensable for effective induction of certain acquired humoral immune responses [3] and cellular immune responses [4]. Despite their importance in innate and acquired immunity, the differentiation pathway of B-1 cells is poorly understood. One hypothesis is that B-1 cells are an activated form of B-2 cells, and that this activation occurs through B cell receptors that have certain specificity like to phosphatidylcholine. This hypothesis is supported by the observation that in the trasngenic mice of B cell receptor cloned from B-1 cell, all the B cells show a B-1 phenotype [5]. Another hypothesis is that B-1 cells arise from progenitors other than progenitors for B-2 cells. This idea stems from studies showing that transfer of adult bone marrow into irradiated recipient mice resulted in repopulation of B-2 but not B-1 cells, whereas fetal liver cells gave rise to both B-1 and B-2 cells in the same situation [6,7]. Consistent with the latter hypothesis, B-1 lineage-committed progenitors were recently identified as $CD19^+$ $B220^-$ cells in fetal and juvenile mouse bone marrow [8]. These "B-1 progenitors" display a phenotype of proB cells and are responsive to thymic stroma lymphopoietin (TSLP).

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However, regulatory mechanisms controlling their differentiation or origin of these B-1 cell progenitors are still unknown.

Interleukin-5 (IL-5) was originally identified as a soluble factor secreted from activated T cells that can promote antibody secretion from B cells [9]. Its receptor consists of a ligand-specific α chain (IL-5Rα/CD125) and signal-transducing β chain (βc/CD131) that is shared among IL-3 receptor and GM-CSF receptor [10]. While expression of IL-5Rα on B-2 cells is limited to activated cells, most B-1a and B-1b cells constitutively express IL-5Rα [11]. Furthermore, IL-5 knock-out mice, IL-5Rα knock-out mice, and mice injected with anti-IL-5 neutralizing mAb show a reduction in number and size of B-1 cells [12–14]. These data suggest that IL-5 signaling is closely related to the differentiation and maintenance of B-1 cells. Although many IL-5-dependent proB or preB cell lines have been established, no *in vivo* counterpart of such cells have been described, nor has a role(s) for IL-5 in early B cell development been elucidated [15,16]. In this study, we examined the B-1 cell progenitor activity of fetal liver cells expressing IL-5Rα and the signaling requirements for B-1 cell progenitors.

2. Materials and methods

2.1. Mice

C57BL/6J, BALB/cA, BALB/cByJ, and C.B-17/Icr-scid/scid (SCID) mice were purchased from the Japan CLEA company (Shizuoka, Japan). IL-5Rα knock-out mice were previously described [13]. Btk knock-out mice were provided by Dr. F.W. Alt (Howard Hughes Medical Institute, The Children's Hospital, Boston, MA) [17]. C57BL/6.*xid* mice were provided by Dr. A. Singer (National Institute of Health, Bethesda, MD). All mice were housed and maintained in the Laboratory Animal Research Center, the Institute of Medical Science, the University of Tokyo or Research Animal Facility of National Institute of Biomedical Innovation. Fetuses were obtained from pregnant mice 15 days after vaginal plugs were observed. Due to the genetic background of IL-5Rα knock-out mice (C57BL/6), Btk knock-out mice (C57BL/6) Xid mice (C57BL/6) and SCID mice (BALB/c congenic), either C57BL/6 or BALB/c mice were selected in each experiment. $CD19^+$ $B220^-$ cells were similarly observed in both strains, although BALB/c strain tends to have higher number of these cells (data not shown). All experiments were operated under the guidelines of animal use at University of Tokyo and National Institute of Biomedical Innovation.

2.2. Antibodies and reagents

Monoclonal antibody (mAb) against IL-5Rα (clone H7) was purified from ascitic fluid of BALB/c nu/nu mice that had been injected with a corresponding hybridoma. Purified mAb was biotinylated using NHS-biotin (Pierce/Thermo Fisher Scientific, Rockford, IL) or labeled with Alexa Fluor 647 using a monoclonal antibody labeling kit (Molecular Probes/Invitrogen, Carlsbad, CA). Anti-mouse IgM mAb (clone M41) was purified and conjugated to Fluorescein Isothiocyanate (FITC) in our laboratory. The following mAbs were purchased from BD Biosciences (San Jose, CA): FITC-conjugated CD3, CD5, Gr-1, CD43, CD45R/B220; Phycoerythrin (PE)-conjugated CD19 (1D3), CD23, CD43, CD127/IL-7Rα; biotinylated anti-IgM^a; Peridinin chlorophyll protein (PerCP)-conjugated CD45R/B220; and allophycocyanin (APC)-conjugated CD45R/B220, CD117/c-kit, and CD11b/Mac-1. FITC-conjugated TER-119, PE-conjugated CD19 (6D5) and PE-Cy7-conjugated CD45R/B220 were purchased from eBioscience (San Diego, CA). FITC-conjugated CD11b/Mac-1 was purchased from CALTAG/Invitrogen (Carlsbad, CA). Anti-JAK2 antiserum, anti-SHC antiserum, and anti-phosphotyrosine mAb were purchased from Upstate Biotechnology/Millipore (Billerica, MA). Anti-stat5 antiserum was a gift from Dr. Hiroshi Wakao (DNAX Research Institute, Palo Alto, CA). Mouse IL-5 was purified from culture supernatant of CHO cells stably transfected with an IL-5 expression vector using an affinity column to which anti-IL-5 mAb (NC17) had been immobilized [18]. Stem cell factor and IL-7 were purchased from Peprotech (Rocky Hill, NJ). Flt3-L and TSLP were purchased from R&D Systems (Minneapolis, MN).

2.3. Cell preparation, staining, and sorting

A single-cell suspension was prepared by mincing fetal livers on nylon mesh filters or flushing femurs and tibias of adult mice using a 27G needle attached to a syringe. Fetal liver cells were then depleted of erythroid cells using anti-TER119 microbeads and magnetic separation columns (LD column) with a Quadro MACS magnet according to the manufacturer's instructions (Miltenyi Biotech, Bergisch Gladbach, Germany). In some experiments, fetal liver cell suspensions were just depleted of erythrocytes by ACK lysing buffer (0.15 M NH_4Cl, 1.0 mM $KHCO_3$, 0.1 mM EDTA, pH 7.3). In the case of adult bone marrow, cells were first incubated with purified monoclonal antibodies against Gr-1, CD3, and TER-119, and labeled cells were depleted with anti-Rat IgG microbeads using LD columns with a Quadro MACS magnet. For detection of $IL\text{-}5R\alpha^+$ cells in the fetal liver, erythroid-depleted cells were stained with FITC anti-IgM, biotinylated anti-IL-5Rα, PerCP CD45R/B220, and APC CD19 antibodies. For the detection of $IL\text{-}5R\alpha^+$ cells in the bone marrow, lineage-depleted cells were stained with a cocktail of FITC-conjugated lineage marker antibodies, biotinylated anti-IL-5Rα and allophycocyanin-conjugated anti-c-kit. In either case, biotinylated anti-IL-5Rα andtibody was detected with PE-conjugated streptavidin. For detection of prolymphocytes (ProL), cells were stained with a FITC-conjugated lineage marker antibody cocktail, as well as with PE-anti-IL-7Rα and APC-anti-c-kit. For the enumeration of B-1 progenitors, fetal liver cells were stained with FITC-labeled lineage marker antibody cocktail (Gr-1, TER-119, CD3, DX5, anti-IgM), PE-anti-CD19 and PE-Cy7-CD45R/B220. Stained cells were analyzed by FACSCalibur or LSR II flow cytometer (BD Bioscience), or sorted with FACSAria cell sorter (BD Bioscience).

2.4. Cell cultures

OP9 stromal cells were maintained in α-MEM supplemented with 15% FCS and antibiotics [19]. A single-cell layer of OP9 cells was prepared in 24-well culture plates and irradiated with 30 Gy. Sorted cells were cultured for seven days on irradiated OP9 cell layers in OPTI-MEM I medium supplemented with 2% FCS, 50 μM 2-mercaptethanol (2-ME), antibiotics, and the indicated cytokines. At the end of the culture period, non-adherent cells were harvested, counted, and stained with fluorescence-labeled mAbs as noted.

2.5. Establishment of IL-5-dependent early B lineage clones

Long-term bone marrow cultures were initiated from C57BL/6J mice or C57BL/6.*xid* mice according to the protocol established by Whitlock and Witte [20]. Briefly, bone marrow cells were isolated from each femur and cultured in 7 mL of RPMI1640 medium supplemented with 5% FCS, 50 μM 2-ME, and antibiotics in a 25-cm^2 culture flask (day 0). On day 3, 3 mL of fresh medium were added to each flask. On day 7, 7 mL of medium were removed from each flask and 3 mL of fresh medium were added. On day 10, 4 mL of fresh medium were added. The same cycle of medium change was then repeated. Five weeks later, growing non-adherent cells were collected and cloned on an ST2 cell layer in 96-well, flat-bottom culture plates in the presence of 50 U/mL of IL-5 [21]. In order to generate stroma-independent clones, the resulting clones were re-cloned in the presence of IL-5 without ST2. Cloned cell lines were

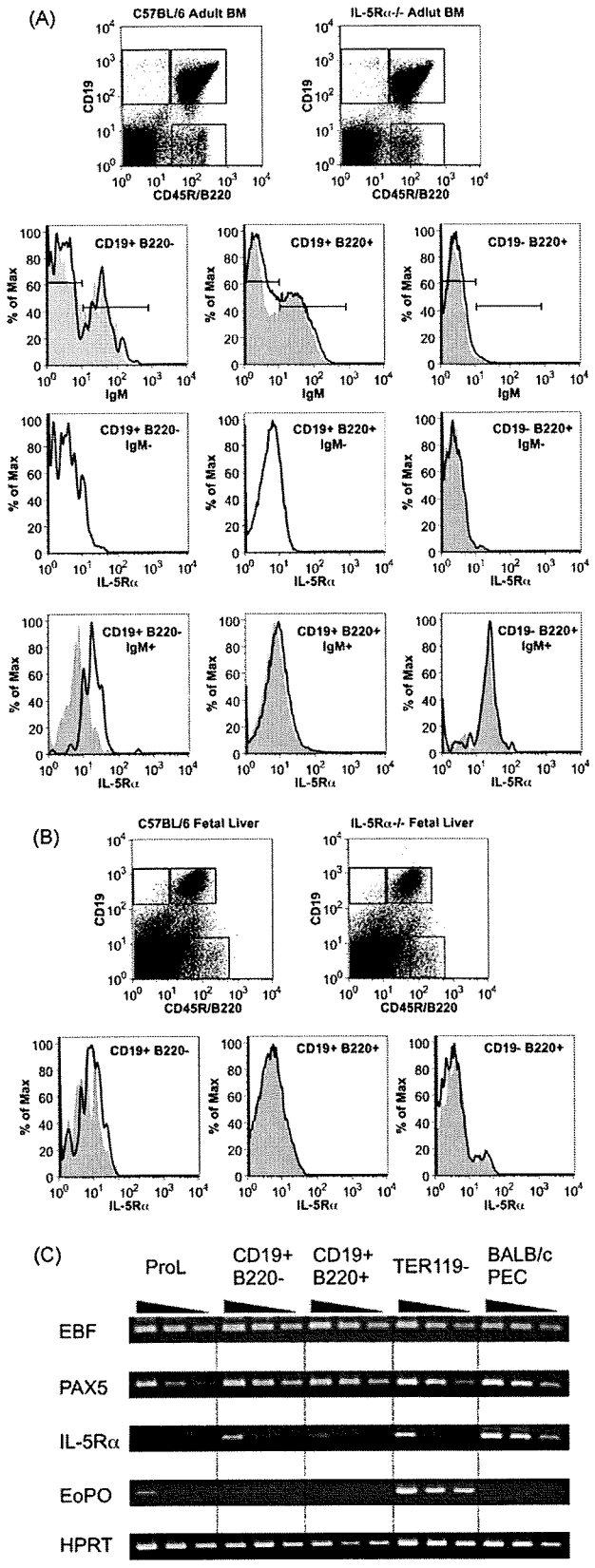

Fig. 1. CD19$^+$ B220$^-$ fetal liver cells show weak expression of IL-5Rα, which is detectable only by RT-PCR. (A) Bone marrow cells from C57BL/6J mice and negative control IL-5Rα knock-out mice were stained with antibodies against CD19, CD45R/B220, IgM, and IL-5Rα, then analyzed on a FACSCalibur flow cytometer. B lineage cells were sub-fractionated based on CD19 and B220 expression (top row), and each subset was further divided based on expression of surface IgM (second row). Staining of IL-5Rα is shown in the bottom two rows. Solid lines and filled histograms represent C57BL/6J and IL-5Rα knock-out mice, respectively. (B) Cells from day 15 C57BL/6J and IL-5Rα knock-out fetal liver were analyzed as in (A) but omitting anti-IgM staining and sub-fractionation. (C) Total RNA was extracted from sorted fetal liver cells and the expression of indicated genes was analyzed by semi-quantitative RT-PCR. ProL were purified from TER119$^-$ fetal liver cells as Lin$^-$ c-kit^{Lo} IL-7Rα$^+$ cells. CD19$^+$ B220$^-$ cells and CD19$^+$ B220$^+$ cells were sorted from TER119$^-$ fetal liver cells. TER119$^-$ cells were prepared by depleting TER119$^+$ cells from fetal liver cells using MACS beads. Total peritoneal cells from BALB/cA mice were used as a control (BALB/c PEC).

maintained in RPMI1640 supplemented with 5% FCS, 50 μM 2-ME, and 50 U/mL of IL-5.

2.6. Proliferation assay

Bone marrow-derived, stroma-independent cell lines were plated in a 96-well flat-bottom culture plate (10^4 cells per well) with 200 μL of culture medium containing various concentrations of IL-5 and then cultured for 48 h. The cells were pulse-labeled with 0.2 μCi of [^{3}H]thymidine during the last 12 h of the culture period and incorporated [^{3}H] thymidine was measured using a MATRIX 96 Direct Beta Counter (Packard Instruments, Meridien, CT).

2.7. Flow cytometric analysis of STAT5 phosphorylation

TER119$^-$ fetal liver cells or whole bone marrow cells were stimulated with 10 ng/mL IL-7 or 100 ng/mL thymic stroma lymphopoietin (TSLP) at 37 °C for 5 min in RPMI1640 medium supplemented with 8% fetal calf serum (FCS) and 50 μM 2-ME. At the end of the incubation period, cells were fixed by adding five volumes of 1% formaldehyde in 1.25x PBS at 37 °C for 10 min. Fixed cells were washed and permeabilized on ice for 30 min with Perm III solution (BD Bioscience). Then cells were stained with FITC-conjugated CD43, PE-conjugated CD19 (6D5), PerCP-conjugated CD45R/B220, and Alexa Fluor 647-conjugated anti-phospho-STAT5 (Y694) mAbs. Cells were analyzed on a FACSCalibur flow cytometer.

2.8. PCR detection of immunoglobulin gene rearrangement

Rarranged immunoglobulin genes were detected by PCR [22]. Sorted cells were lysed in PCR lysis buffer (10 mM Tris–HCl pH 8.4, 50 mM KCl, 2 mM $MgCl_2$, 0.5% Nonidet P40, 0.5% Polyoxyethylene Sorbitan Monolaurate, and 40 μg/mL proteinase K) at 500 cells/μL overnight at 50 °C. After treatment at 95 °C for 10 min and serial 5-fold dilution, 2 μL of samples were used as template for PCR. Primers and their sequence used in each reaction were as follows: D–J rearranged IgH; DHL-5′ (GGAATTCG(A/C)TTTTTGT(C/G)AAGGGATCTACTACTGTG) and J3-3′ (GTCTAGATTCTCACAAGAGTCCGATAGACCCTGG), germline IgH; Mu0-5′ (CCGCATGCCAAGGCTAGCCTGAAAGATTACC) and J3-3, α-actin; actin-S (GGTGTCATGGTAGGTATGGGT) and actin-AS (CGCACAATCTCACGTTCAG).

2.9. RT-PCR

Total RNA was extracted from sorted fetal liver cells or non-adherent cells from cultures using the RNeasy mini RNA isolation kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. cDNA was synthesized from extracted RNA using oligo dT primers and Superscript III reverse transcriptase (Invitrogen). The amount of cDNA was normalized according to the amount of cDNA of hypoxanthine-guaninephosphoribosyltransferase (HPRT) and used as template in each reaction. Sequences of primers were as follows: EBF-5′, GCCTTCTAACCTGCGGAAATC; EBF-3′, GGCGCACATAGAAATCCTGTT; Pax5-5′, CTACAGGCTCCGTGACGCAG; Pax5-3′, TCTCGGCCTGTGACAATAGG; IL-5Rα-5′, ACATTTATCAACAGCAAAGGGTTT; IL-5Rα-3′, AGTTAAAGCAAT-

GATCTGGAAAGG; EPO-5′, CCTACTTCAAACAGCCAGTA; EPO-3′, ATCATTGCGTGGGATCTTGA; HPRT-5′, AGTCCCAGCGTCGTGATTAG; HPRT-3′, CGAGAGGTCCTTTTCACCAG.

2.10. Immunoprecipitation and western blotting

Bone marrow-derived, stroma-independent cell lines were washed with Hanks' balanced salt solution (HBSS) and cultured for 12 h without IL-5. Cells were collected and stimulated with 2000 U/mL of IL-5 or with medium alone for 5 min at 37 °C. Stimulated cells were pelleted, resuspended to a concentration of 10^8 cells/mL in lysis buffer (150 mM NaCl, 20 mM Tris–HCl, pH 7.4, containing 1% Triton X-100, 10% glycerol, 5 mM EDTA, 10 mM NaF, 1 mM sodium orthovanadate, 100 U/mL aprotinin, 10 mM iodoacetamide, and 25 μg/mL p-nitrophenyl-p′-guanidinobenzoate) and allowed to sit on ice for 15 min. After centrifugation at 15,000 × *g* for 20 min, supernatants (cell lysates) were collected. JAK2, STAT5, or SHC was immunoprecipitated from 400 μL of each cell lysate by adding 5 μL of corresponding antiserum and 20 μL of protein G sepharose 4 Fast Flow (GE Healthcare, Buckinghamshire, UK). The immunoprecipitated proteins were subjected to SDS-PAGE (8%) and transferred to PVDF membranes (Millipore, Billerica, MA), blocked with 5% BSA, and incubated with anti-phosphotyrosine mAb (4G10). Membranes were developed with horseradish peroxidase-conjugated anti-mouse IgG antibody and ECL reagent (GE Healthcare), followed by exposure to X-ray imaging film (Fuji Film Inc., Tokyo, Japan). Membranes were incubated with stripping buffer (62.5 mM Tris–HCl, pH 6.7, 10% SDS, 100 mM 2-ME) at 50 °C for 40 min, blocked again, and then incubated with antiserum against JAK2, STAT5, or SHC. Signal was developed using ECL to confirm the existence of each protein in the immunoprecipitation reactions.

3. Results

3.1. Expression of IL-5Rα on CD19⁺ B lineage progenitors in the bone marrow

Since mice deficient in IL-5 or its receptor component IL-5Rα show reductions in the number and activity of B-1 cells, it was reasonable to expect that B-1 cell progenitors would express IL-5Rα during their development. Committed B-1 cell progenitors have been reported to be Lin⁻ CD19⁺ B220⁻ cells in mouse bone marrow. Thus, we first examined expression of IL-5Rα on CD19⁺ B220⁻ cells in the bone marrow and in the fetal liver. As shown in Fig. 1A, weak but uniform expression of IL-5Rα was detected in the IgM⁺ subfraction of CD19⁺ B220⁻ bone marrow cells, but not in IgM⁻ CD19⁺ B220⁻ B-1 progenitors or in any other cells with B cell markers. Day 15 fetal liver does not contain IgM⁺ cells [23], and all CD19⁺ B220⁻ cells were negative for IL-5Rα staining (Fig. 1B). Since IgM⁺ CD19⁺ B220⁻ cells are considered to be mature B-1 cells, IL-5Rα was considered to be negative in the early stages of B cell development at least based on the flow cytometric analysis in both adult and fetus. We then further examined expression of IL-5Rα in fetal liver B lineage cells using an RT-PCR assay, which is more sensitive than flowcytometer. IL-5Rα mRNA was detected

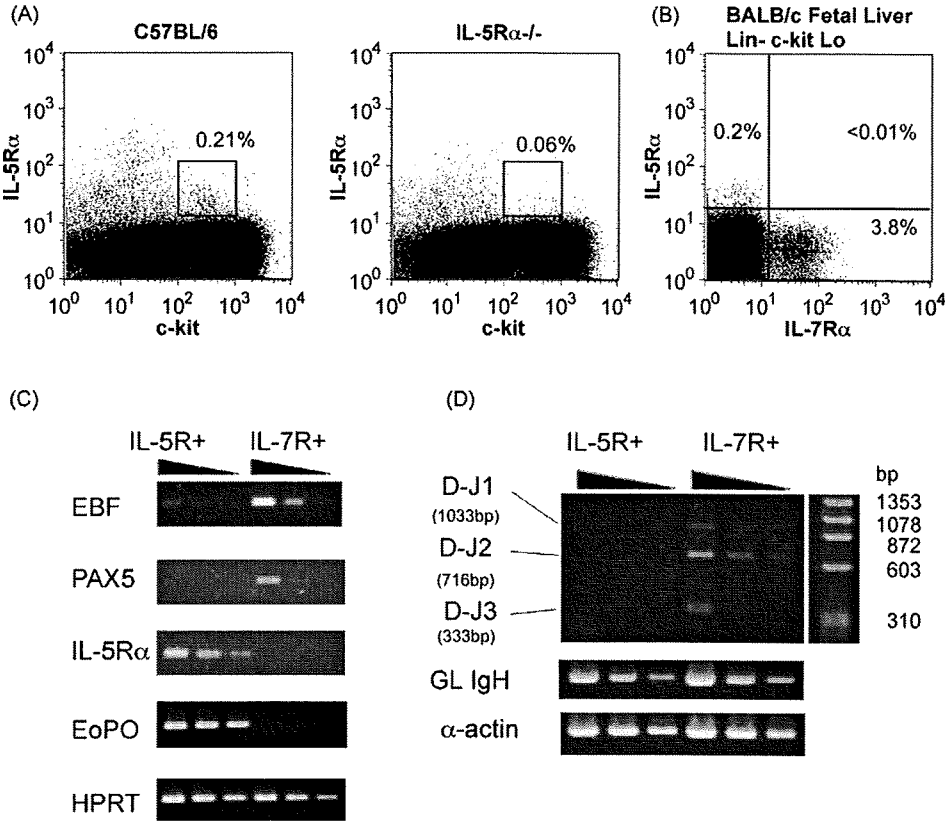

Fig. 2. Lin⁻ IL-5Rα⁺ fetal liver cells do not overlap with or share properties with ProL. TER119⁻ cells from day 15 C57BL/6J, IL-5Rα knock-out (A), or BALB/c (B) fetal liver were stained with an anti-lineage marker antibody cocktail, anti-c-kit and anti-IL-5Rα antibodies (A), or with anti-IL-5Rα and anti-IL-7Rα antibodies (B). A. Each dot plot shows the Lin⁻ gated population. Gating for c-kit^{Lo} IL-5Rα⁺ cells and their frequency within the Lin⁻ population are shown. B. Frequencies of IL-5Rα⁺ IL-7Rα⁻ cells, IL-5Rα⁻ IL-7Rα⁺, and IL-5Rα⁺ IL-7Rα⁺ cells are shown within the gate. (C) Total RNA was prepared from FACS-isolated Lin⁻ c-kit^{Lo} IL-5Rα⁺ cells (IL-5R+) and Lin⁻ c-kit^{Lo} IL-7Rα⁺ cells (IL-7R+). Expression of the indicated genes was analyzed by semi-quantitative RT-PCR. (D) Genomic DNA was extracted from FACS-isolated Lin⁻ c-kit^{Lo} IL-5Rα⁺ cells (IL-5R+) and Lin⁻ c-kit^{Lo} IL-7Rα⁺ cells (IL-7R+), and gene recombination between immunoglobulin heavy chain D and J regions were detected by PCR. The expected sizes of the bands are indicated. Germline immunoglobulin heavy chain and α-actin genes were also amplified from the same samples.

in the CD19$^+$ B220$^-$ and CD19$^+$ B220$^+$ cells but not in the ProL [24] (also called as common lymphoid progenitors (CLP) [25]) in the fetal liver cells (Fig. 1C). Since IL-5Rα message was much higher in the CD19$^+$ B220$^-$ cells than in the CD19$^+$ B220$^+$ cells, we concluded that CD19$^+$ B220$^-$ fetal liver cells are the major B cell progenitor that expresses IL-5Rα. Notably, much higher IL-5Rα signal was detected in the TER119$^+$ cell-depleted fetal liver, suggesting that cells other than CD19$^+$ B220$^-$ cells also express IL-5Rα in the fetal liver.

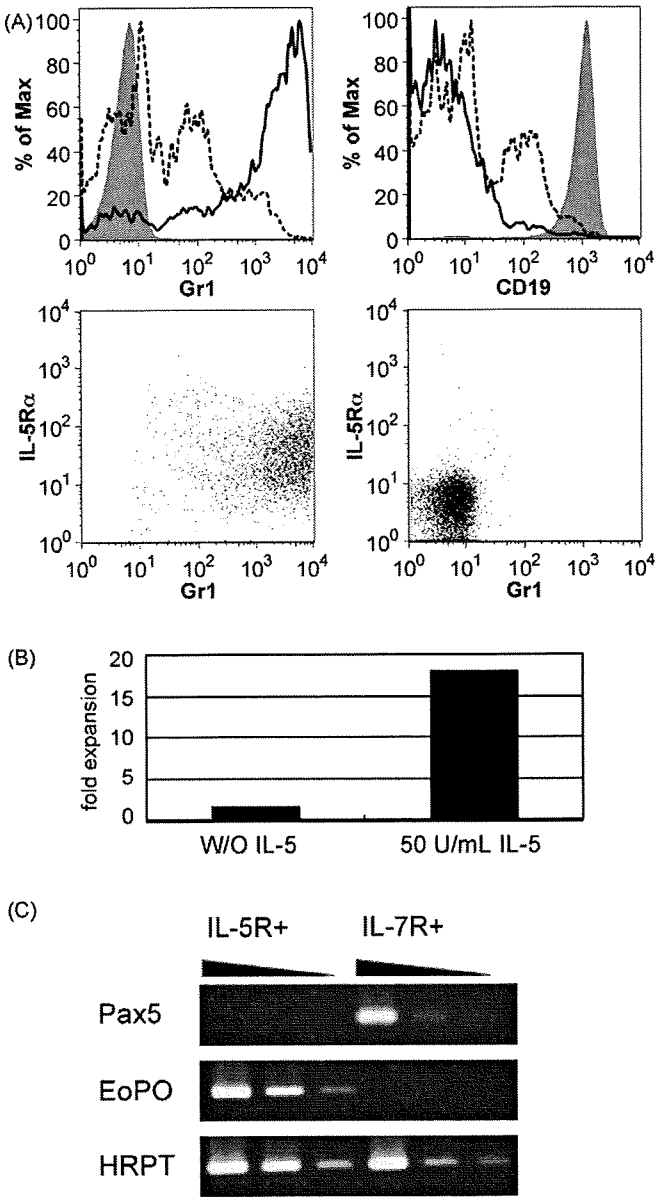

Fig. 3. Lin$^-$ IL-5Rα$^+$ cells differentiate into eosinophils in culture. (A) Sorted Lin$^-$ c-kit^{Lo} IL-5Rα$^+$ cells or Lin$^-$ c-kit^{Lo} IL-7Rα$^+$ cells from day 15 BALB/cByJ fetal livers were cultured on a single cell layer of OP9 stromal cells with 1 ng/mL IL-7 alone or 1 ng/mL IL-7 + 50 U/mL IL-5 for five days. Non-adherent cells were harvested and stained for Gr-1 (upper left) or CD19 (upper right). Each histogram indicates cultures initiated from Lin$^-$ c-kit^{Lo} IL-7Rα$^+$ cells (filled), Lin$^-$ c-kit^{Lo} IL-5Rα$^+$ cells (dotted line), or Lin$^-$ c-kit^{Lo} IL-5Rα$^+$ cells with IL-5 (solid line). Expression of Gr-1 and IL-5Rα in the Lin$^-$ c-kit^{Lo} IL-5Rα$^+$ cell culture induced by IL-7 and IL-5 (lower left) and Lin$^-$ c-kit^{Lo} IL-7Rα$^+$ cell culture induced by IL-7 alone (lower right) are shown. (B) Proliferation of Lin$^-$ c-kit^{Lo} IL-5Rα$^+$ cells with or without IL-5 in the culture is shown as harvested cell number divided by input cell number (fold expansion). (C) Expression of Pax5 and EoPO in cells harvested from Lin$^-$ c-kit^{Lo} IL-5Rα$^+$ cell culture supplemented with IL-7 and IL-5 (IL-5R+) and from Lin$^-$ c-kit^{Lo} IL-7Rα$^+$ cell culture supplemented with IL-7 (IL-7R+) were analyzed by semi-quantitative RT-PCR.

3.2. Fetal liver Lin$^-$ c-kit^{Lo} cells also express IL-5Rα

To identify additional IL-5Rα$^+$ populations in the fetal liver, Lin$^-$ fetal liver cells were stained with both anti-IL-5Rα and anti-c-kit antibody. As seen in Fig. 2A, low but significant IL-5Rα staining was detected in a small proportion of the Lin$^-$ c-kit^{Lo} fraction. Although surface phenotype of these cells, i.e. the absence of lineage markers and low-level expression of c-kit, is identical to that of ProL, none of the Lin$^-$ c-kit^{Lo} fetal liver cells co-expressed IL-5Rα or IL-7Rα (Fig. 2B), indicating that IL-5Rα$^+$ fetal liver cells are a distinct cell type from ProL, which is IL-7Rα$^+$. To determine the nature of the relationship between Lin$^-$ c-kit^{Lo} IL-5Rα$^+$ cells and ProL, we compared the gene expression and immunoglobulin gene configuration. In contrast to fetal liver ProL cells that express both early B cell factor (EBF) and Pax5, Lin$^-$ c-kit^{Lo} IL-5Rα$^+$ cells do not express Pax5 and express only small amounts of EBF (Fig. 2C). Remarkably, Lin$^-$ c-kit^{Lo} IL-5Rα$^+$ fetal liver cells express

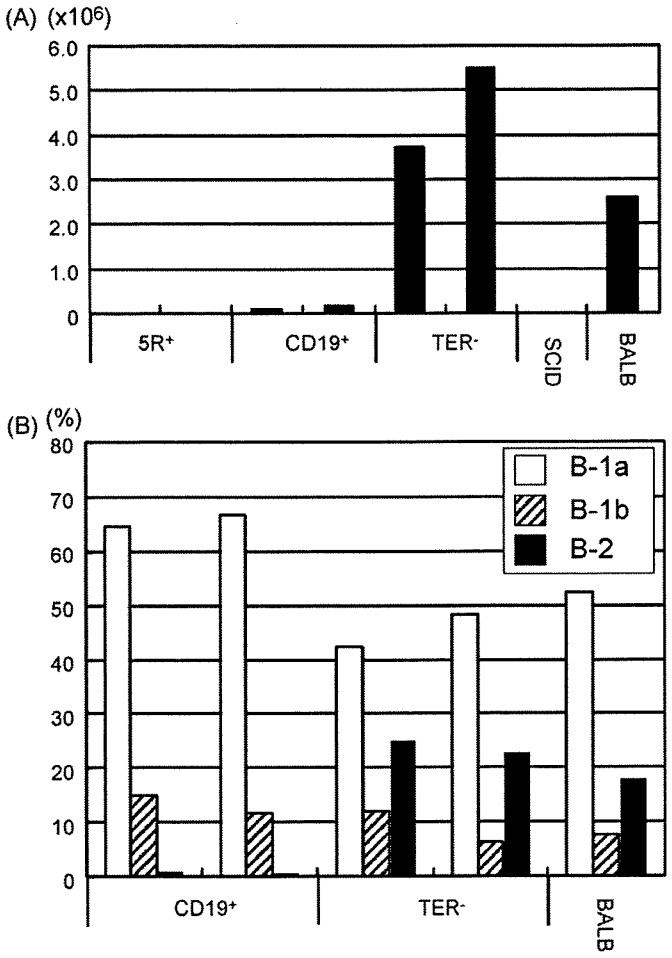

Fig. 4. CD19$^+$ B220$^-$ fetal liver cells differentiated only into B-1 cells in SCID mice. Sorted Lin$^-$ c-kit^{Lo} IL-5Rα$^+$ cells, CD19$^+$ B220$^-$ cells, or MACS-purified TER119$^-$ cells from day 15 BALB/cA fetal liver were intravenously injected into SCID mice, which had been pre-conditioned by 1 Gy irradiation (4000 cells/each mouse, except for 2×10^5 cells/mouse for TER119$^-$ fetal liver cells). Eight weeks later, peritoneal cells were collected, counted, and stained for CD5, CD23, IgM^a, and B220. (A) The number of B220$^+$ or IgM^{a+} B lineage cells is shown in the chart. 5R$^+$, mice injected with Lin$^-$ c-kit^{Lo} IL-5Rα$^+$ cells; CD19$^+$, mice injected with CD19$^+$ B220$^-$ cells; TER$^-$, mice injected with TER119$^-$ cells; SCID, SCID mouse with irradiation only; BALB, BALB/cA mouse without treatment. (B) Proportion of B cell subsets in peritoneal B cells. Open bars, shaded bars, and solid bars represent CD23$^-$ CD5$^+$ B-1a cells, CD23$^-$ CD5$^-$ B-1b cells, and CD23$^+$ CD5$^-$ B-2 cells, respectively. CD19$^+$, mice injected with CD19$^+$ B220$^-$ cells; TER$^-$, mice injected with TER119$^-$ cells; BALB, BALB/cA mouse without treatment. The figures are representative of two separate experiments with similar results and each bar represents data from individual mouse.

a significant amount of eosinophil peroxidase (EoPO), which is a marker for eosinophils and their progenitors. We used a PCR method that detects D–J rearranged alleles in the Lin$^-$ IL-5Rα^{Lo} IL-7Rα^+ fetal liver cells, but were unable to detect any D–J rearrangement (Fig. 2D). These observations suggest two possibilities regarding the nature of Lin$^-$ IL-5Rα^+ fetal liver cells: these cells are the most primitive B cell progenitors or they are eosinophil progenitors.

3.3. Lin$^-$ IL-5Rα^+ fetal liver cells are eosinophil progenitors

At this point in our study, we had two candidates for B-1 cell progenitors: CD19$^+$ B220$^-$ IL-5Rα^{Lo} fetal liver cells and Lin$^-$ IL-5Rα^+ fetal liver cells. To test their differentiation potential, Lin$^-$ IL-5Rα^+ fetal liver cells and ProL were compared using a culture system that supports B cell differentiation (Fig. 3A). Co-culturing these progenitors with the OP9 stromal cell line in the presence of IL-7 caused fetal ProL to differentiate into CD19$^+$ B cells. In contrast, IL-5Rα^+ fetal liver cells differentiated into CD19$^-$ Gr-1$^+$ myeloid cells. Notably, these cells also expressed IL-5Rα and their proliferation was dramatically increased when IL-5 was added to the culture (Fig. 3A and B). Expression of eosinophil peroxidase was detected in cells harvested from these cultures, but not from cultures initiated with ProL. Thus, IL-5Rα^+ fetal liver cells gave rise to eosinophils (Fig. 3C).

3.4. CD19$^+$ B220$^-$ IL-5Rα^{Lo} fetal liver cells are B-1 cell progenitors

In the presence of IL-7, B cell differentiation is arrested at the proB stage [24]. Since B-1 cells and B-2 cells cannot be distinguished

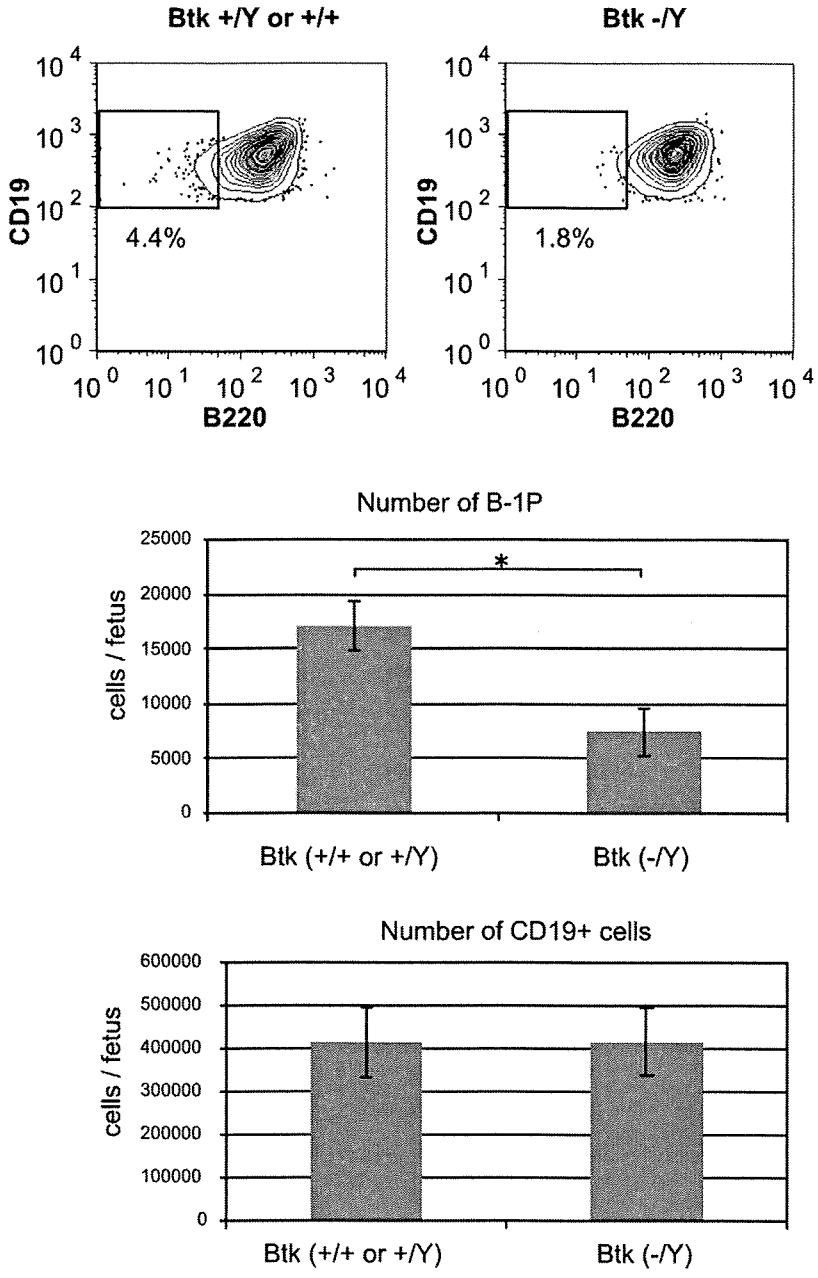

Fig. 5. Btk knock-out mouse fetuses have significantly fewer B-1 progenitors than wild-type mice. Day 15 fetuses were collected from Btk+/− females, which had been mated with Btk−/Y males. Fetal liver cells were separately stained for CD19 and B220 while each fetus was genotyped by PCR. Representative FACS analysis plot (upper) and average number of B-1 progenitors (middle) and CD19+ cells (bottom) from seven Btk intact (+/+ or +/Y) and seven Btk deficient (−/Y) fetuses are shown. ($^*P < 0.005$) Btk (+/−) fetuses were omitted.

at this stage, the potential of CD19$^+$ B220$^-$ IL-5Rα^{Lo} fetal liver cells to differentiate into B-1 cells was assessed by a transfer experiment using lymphocyte-deficient SCID mice. TER-119$^-$ fetal liver reconstituted all B cell subsets in the peritoneal cavity, and numbers of peritoneal B cell subsets were comparable to those in BALB/cA mouse. CD19$^+$ B220$^-$ IL-5Rα^{Lo} fetal liver cells also reconstituted peritoneal B cell of SCID mice though the number was much lower than TER119$^-$ fetal liver cell did. Notably, majority of repopulated B cells were B-1a and B-1b cells and few B-2 cells were observed. In contrast, no B lymphocytes were observed after transfer of Lin$^-$ c-kit^{Lo} IL-5Rα^+ fetal liver cells (Fig. 4). These results strongly suggest that fetal liver CD19$^+$ B220$^-$ cells, but not Lin$^-$ c-kit^{Lo} IL-5Rα^+ cells, are committed to B-1 cell differentiation.

3.5. Btk positively regulates generation of B-1 progenitors in the fetal liver

B-1 cells are absent from X-linked immunodeficient (XID) mice, which have a point mutation in Bruton's tyrosine kinase (Btk), and from Btk knock-out mice. To determine whether Btk is required for differentiation of B-1 cells at the progenitor stage, we examined fetal liver cells in the Btk knock-out mouse. Strikingly, the number of CD19$^+$ B220$^-$ B-1 progenitors was significantly lower in the liver of Btk knock-out fetuses (Fig. 5), suggesting that the generation or maintenance of B-1 cell progenitors depends on Btk-mediated signaling. Phosphorylation and activation of Btk occurs upon activation of BCR and pre-BCR signaling, as well as in response to signaling involving cytokines such as IL-5 and IL-6 [26-29]. As shown in a previous report, CD19$^+$ B220$^-$ B-1 cell progenitors do not have a rearranged immunoglobulin heavy chain V gene, meaning that they do not express BCR or pre-BCR [8]. Thus, Btk-dependent B-1 cell differentiation signals must rely on pathways other than pre-BCR or BCR signaling. Since B-1 cell progenitors express IL-5Rα, we first tested the requirement of Btk in IL-5R signaling in early B lineage cells by establishing IL-5-dependent preB cell clones from XID mice. In the presence of IL-5, two stromal cell-independent preB cell clones, Xid 5-4A6G and Xid 5-4G12A were subcloned from stroma-dependent cell lines from long-term Whitlock–Witte bone marrow cultures [20] initiated with XID mice. In the parallel experiment, single clone, B6 5-4G5B was established from control C57BL/6J cell lines. Clones Xid 5-4G12A and B6 5-4G5B were analyzed further. Clone Xid 5-4G12A proliferated in the presence of IL-5 in a dose-dependent manner, similar to the clone B6 5-4G5B, which was derived from a Btk-sufficient mouse (Fig. 6A). In support of IL-5-dependent proliferation of Xid 5-4G12A cells, three known signaling molecules downstream of IL-5R, namely JAK2, STAT5, and SHC, were phosphorylated upon IL-5 stimulation equally in clone Xid 5-4G12A and clone B6 5-4G5B (Fig. 6B). Together with the observation that the number of B-1 progenitors in IL-5Rα knock-out fetal liver is comparable to that in the control (Fig. 1B), these results suggest that IL-5 signaling is not responsible for Btk-dependent generation of B-1 progenitors.

3.6. Btk is dispensable for TSLP signaling in the B linage cells

TSLP was originally cloned from a thymic stroma cell line and reported to promote dendritic cell activation, as well as B cell differentiation [30,31]. The receptor for TSLP consists of TSLP-specific receptor and IL-7Rα [32]. IL-7Rα knock-out mice show a complete loss of B cells, while IL-7 knock-out mice have a significant number of B-1 cells [33,34]. This difference can be explained by TSLP, whose signal is disrupted in IL-7Rα knock-out mice, but not in IL-7 knock-out mice. Thus, TSLP can be expected to play certain role(s) in B-1 cell development. Furthermore, while JAK1 and JAK3 are activated during IL-7R signaling, there is no report of JAK family kinase activation during TSLP signaling, even though STAT5 is

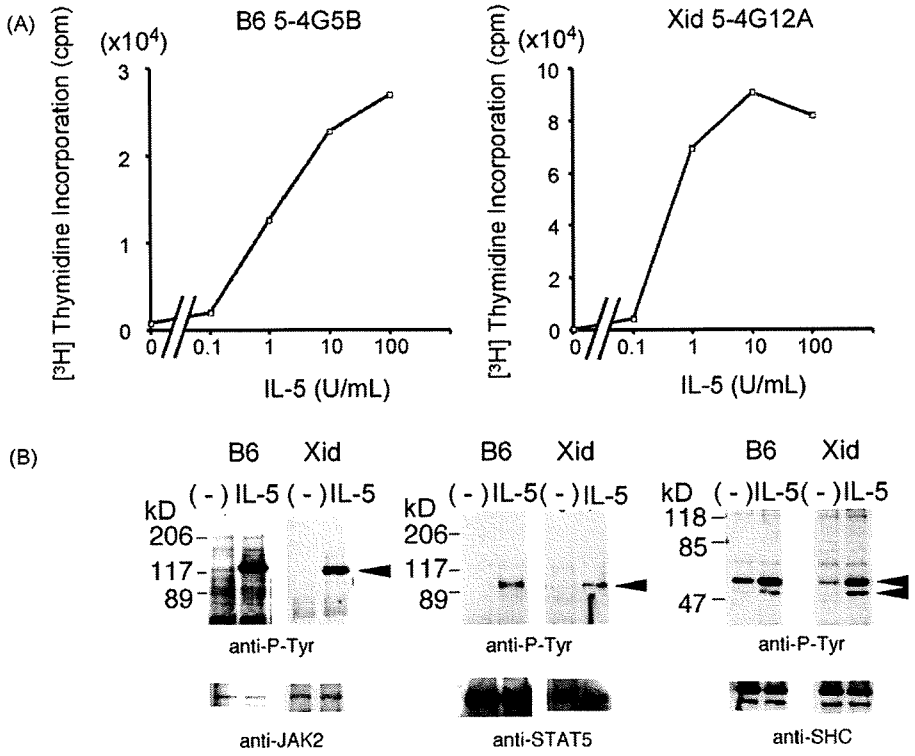

Fig. 6. An early B lineage cell clone from XID mice shows intact response to IL-5 stimulation. (A) An XID mice-derived early B lineage cell clone, Xid 5-4G12A, proliferated in the presence of IL-5 in a dose-dependent manner (right). Proliferation response of C57BL/6J-derived control clone (B6 5-4G5B) is shown on the left. (B) JAK2 (left), STAT5 (center), and SHC (right) was immunoprecipitated from lysates from B6 5-4G5B (B6) and Xid 5-4G12A (Xid) cells that have been stimulated for five minutes with medium ((−)) or 2000 U/mL of IL-5 (IL-5) and subjected to western blotting using anti-phosphotyrosine mAb. The position of each protein is shown with triangles. The total amount of each protein in the precipitates was confirmed by re-probing each membrane with antiserum for the corresponding protein (lower panels).

a downstream target of both IL-7 and TSLP signaling [35]. Instead, a dominant-negative form of Tec kinase, which belongs to the Tec kinase family along with Btk, inhibits TSLP-induced tyrosine phosphorylation of STAT5 [36]. Taken together, these findings suggest the possibility that Btk phosphorylates STAT5 in TSLP signaling and thereby contributes to the development of B-1 cell progenitors. To test whether Btk is involved in TSLP-mediated tyrosine phosphorylation of STAT5, TER119$^-$ fetal liver cells were stimulated with TSLP and phosphorylation of STAT5 was analyzed by flow cytometry. Although a significant fraction of STAT5 in fetal liver cells, particularly in the non-B cell population, is phosphorylated prior to stimulation, an increase in the proportion of cells positive for phospho-STAT5 was observed upon stimulation with TSLP or IL-7. IL-7- or TSLP-induced phospho-STAT5 positive cells were observed mostly among the CD19$^+$ B220$^+$ proB and preB cells. These changes were also observed in cells from Btk-deficient fetuses upon not only upon IL-7 stimulation but also upon TSLP stimulation, suggesting that at least TSLP-induced tyrosine phosphorylation of STAT5 is Btk-independent (Fig. 7). The same result was obtained when bone marrow cells from Btk knock-out mice were analyzed (data not shown).

4. Discussion

In this study, we identified two classes of IL-5Rα-expressing populations in the fetal liver: a subset of Lin$^-$ c-kit^{Lo} cells and CD19$^+$ B220$^-$ cells. In the CD19$^+$ B220$^-$ population, expression of IL-5Rα is low and detectable only by RT-PCR, but this population differentiated into only B-1 cells in irradiated SCID mice. This is consistent with a previous report showing that fetal Lin$^-$ CD19$^+$ B220$^-$ cells represent B-1 committed progenitors, although progenitors were taken from fetal bone marrow in their experiments [8]. Since expression of IL-5Rα is one of the markers that distinguish both B-1a and B-1b cells from B-2 cells [11], observation of IL-5Rα on fetal liver B-1 progenitors raises the possibility that IL-5Rα is expressed on B-1 cells throughout the stages of their differentiation. Furthermore, roles of IL-5R signaling in early B-1 cell differentiation is of interest. Although IL-5Rα expression is below detectable levels by FACS on early B lineage cell lines derived from long-term bone marrow cultures, these cells are still dependent on IL-5, suggesting that very low levels of IL-5Rα expression are sufficient for signal transmission (T. Kouro, unpublished observations, 1996). In IL-5-dependent proB/preB cell lines, replacing IL-5 in the culture medium with GM-CSF induces conversion of these cell lines to macrophage-like cells [15]. Since IL-5 and GM-CSF share a receptor component, namely the β chain, one possible role for IL-5 is to prevent B-1 cells from differentiating into macrophages. Therefore, IL-5 is an important candidate regulator of B-1 cell differentiation during the early stages of B-1 cell development. To reveal the effect of IL-5 on B-1 cell differentiation, novel cell culture systems that support differentiation of CD19$^+$ B220$^-$ IL-5Rα^{Lo} B-1 progenitors into mature B-1 cells need to be developed. In our analysis, we detected only small amount of IL-5Rα mRNA in CD19$^+$ B220$^+$ ProB/PreB cells in the fetal liver or adult bone marrow, even when RT-PCR was used (Fig. 1C). If IL-5Rα is expressed throughout B-1 cell differentiation, it is more likely that differentiation of B-1 cells into preB and later stages takes place outside of the fetal liver or bone marrow. To identify the steps of differentiation from B-1 progenitors to B-1 cells and the site of differentiation, expression of IL-5Rα may be used as a B-1 lineage marker. In addition, analysis of transcription factors that control the *il-5ra* gene promoter in B-1 cells or B-1 progenitors may reveal a "master regulator gene" that determines B-1 and B-2 cell fate decisions.

A requirement for Btk in order to fully generate B-1 progenitors is another important observation in this study. Btk-deficient

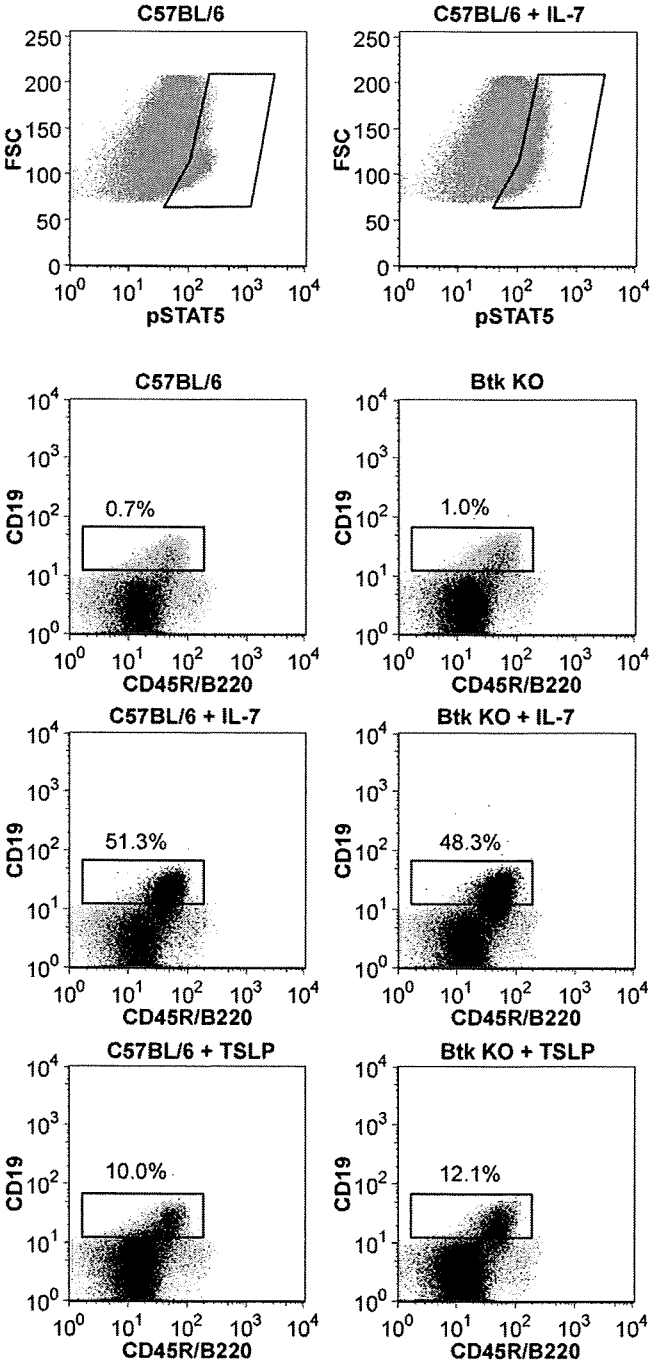

Fig. 7. Btk is dispensable for TSLP-induced tyrosine phosphorylation of STAT5. TER119$^-$ fetal liver cells from day 15 C57BL/6J or Btk knock-out fetuses were incubated with 10 ng/mL of IL-7, 100 ng/mL of TSLP, or medium alone for five minutes at 37 °C. Then cells were fixed, permeabilized, stained for CD19, B220, and phosphorylated STAT5, and analyzed by FACS. Cells with phosphorylated STAT5 were gated as indicated in the top row. Phospho-STAT5$^+$ cells were overlaid on total cells in B220 vs. CD43 dot plot. Percentages of phospho-STAT5$^+$ cells in the CD19$^+$ B lineage cells are indicated within each plot (second row and below).

mice have a defect in B-1 cell development and this has been attributed to an impairment in B cell receptor signaling, since many gene targeting experiments that affect B cell receptor signaling lead to reductions or absence of B-1 cells [37-39]. Btk is phosphorylated during preB receptor signaling, and this phosphorylation can be mimicked by cross-linking CD79b on proB cells of RAG2 knock-out mice. In this mouse model, in fact Btk is required for the CD79b-cross-linking-induced preB-like cells [27]. However, the

$CD19^+$ $B220^-$ IL-$5R\alpha^{Lo}$ B-1 progenitors in the current study show an absence of rearranged IgH V genes, suggesting that the requirement for Btk-dependent signaling stems from molecule(s) other than BCR or pre-BCR. Since Btk is activated upon IL-5 stimulation in a cell line [28] and B-1 progenitors express IL-5Rα (Fig. 1C), we first suspected IL-5R signaling to be a factor responsible for Btk-dependent B-1 progenitor generation. However, functional Btk was dispensable at least for IL-5-induced proliferation of early B lineage cells (Fig. 6). It has been reported that IL-5 signaling in XID mice is impaired in IL-$5R\alpha^+$ B cells, but intact in eosinophils [40]. Our observations demonstrate the existence of another Btk-independent IL-5R signaling pathway in early B lineage cells. Preserved number of B-1 progenitors in IL-5Rα knock-out fetal liver also supports the IL-5R signal-independent generation of B-1 progenitors (Fig. 1B). Despite several pieces of evidence suggesting involvement of Btk in TSLPR signaling, Btk was found to be dispensable in TSLPR signaling at least for STAT5 phosphorylation. Consistent with this conclusion, TSLP was recently shown to be dispensable for IL-7-independent B cell differentiation [41]. Thus, other cytokine receptor signaling involved in IL-7-independent B cell differentiation, such as Flt3 [42], should be examined to see whether it depends on Btk. In addition, calnexin and MHC II are known to associate with CD79a and CD79b, which are signaling components of the B cell receptor [43,44]. Since cross-linking of CD79b results in phosphorylation of Btk [27], these BCR-independent CD79 signaling pathways are another candidate for the signaling that triggers Btk-dependent B-1 differentiation.

Another IL-$5R\alpha^+$ population in the lineage marker-negative fetal liver cells showed eosinophil progenitor activity. In adult mouse bone marrow, Lin^- c-kit^{Lo} $CD34^+$ IL-$5R\alpha^+$ cells are reported to be eosinophil progenitors and to expand upon helminth infection [45]. Lin^- c-kit^{Lo} IL-$5R\alpha^+$ fetal liver cells may be the fetal counterparts of adult eosinophil progenitors. Since eosinophilopoiesis during the fetal stage is largely unknown, detailed analysis of these cells for their timing of differentiation, cytokine requirements, and regulatory mechanisms of differentiation and expansion will reveal currently unknown aspects of eosinophilopoiesis.

In summary, we have demonstrated that IL-5Rα is expressed on B-1 cell progenitors in the fetal liver and that Btk positively regulates generation of these cells. Although the roles of IL-5R signaling in B-1 cell differentiation or mechanisms of Btk-dependent B-1 cell progenitor generation remain unknown, our observations open the door to studies into previously unknown regulatory mechanisms of B-1 cell differentiation.

Note added in proof

An article about B-1 cell progenitors in the adult bone marrow was published during the processing of this article, in which negative role of Btk for B-1 progenitor development is shown [46]. Opposite role of Btk in fetal and adult progenitors is noteworthy since it may explain different property of fetal and adult lymphopoiesis.

Conflict of interest

The authors have no financial conflicts of interest.

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