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T cell-derived IL-3 plays key role in parasite infection-induced basophil production but is dispensable for *in vivo* basophil survival

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Abstract

Enhanced basophil production is often associated with T_h2-related conditions such as parasite infections or allergic inflammations. Our previous study demonstrated that T cell activation is necessary to promote basophil production in *Nippostrongylus brasiliensis* (Nb)-infected mice. Yet, mechanisms underlying how T cells aid infection-induced basophil production are not clear. In this report, we show that IL-3 produced by T cells activated by the infection enhances basophil production in Nb-infected mice. IL-3-deficient mice or Rag2−/− recipients of IL-3-deficient T cells but not of wild-type T cells failed to support basophil production following the Nb infection. Interestingly, although IL-3 was critical for preventing basophil apoptosis *in vitro*, IL-3 had little contribution to basophil survival and proliferation *in vivo*. Collectively, these results highlight a novel mechanism by which activation of adaptive immune components induces basophil production but not basophil survival via IL-3 production.

Introduction

Basophils are rare blood granulocytes that are generated in the bone marrow from the progenitors and enter the circulation as fully mature cells (1). Basophil numbers are particularly increased during parasite infections and allergic inflammations (1, 2). It has been implicated that basophils play important roles in the development of type 2 immunity (3, 4). We have previously reported that basophils, defined as $CD49b^+$ $Fc\varepsilon RI^+$ cells, promote differentiation of activated naive CD4 T cells into IL-4-producing T_h2 type effector cells by providing IL-4 necessary for T_h2 differentiation (5). *In vivo*, CD4 T cells primed in the presence of elevated numbers of basophils preferentially differentiate into IL-4-producing effector cells (5). Moreover, spontaneous T_h2 immunity develops in mice deficient in interferon regulatory factor 2 where basophil generation is elevated (6). Basophils were also shown to induce IgE-mediated chronic allergic dermatitis and allergen-induced T_h2 differentiation (7, 8). In support of this, depletion of basophils dramatically abolished the development of inflammatory lesions, reduced infiltration of other effector cells (9) and abrogated the T_h2 differentiation induced by allergen immunization (8). Thus, there is a considerable evidence supporting the hypothesis that basophils are important immunoregulatory cells particularly in T_h2 type immunity.

Mechanisms involved in basophil generation are poorly understood. An earlier study showed that incubating bone marrow cells with IL-3 *in vitro* promotes their differentiation into basophils (10). Similarly, *in vivo* IL-3 administration induces generation of basophils in mice (5). Treating *Nippostrongylus brasiliensis* (Nb)-infected mice with neutralizing anti-IL-3 antibody partially diminished basophil accumulation in the periphery (11). These results strongly suggest that IL-3 is an important hemopoietic cytokine that contributes to the basophil generation. However, studies from mice deficient in IL-3 demonstrated unexpected dispensability of IL-3 in basophil responses (12). In steady state, basophil production was not altered by the absence of IL-3, although parasite infection-induced basophil production was severely impaired (12). It has thus been proposed that IL-3 produced during the infection may be involved in promoting

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basophil activation and production (13). Alternatively, IL-3 may promote basophil survival (14).

The present study aims to test the roles of IL-3 in basophil biology both *in vitro* and *in vivo*. Consistent with previous reports, we found that IL-3-deficiency did not influence basal number of basophils. Upon Nb infection, however, T cell IL-3 production played critical roles in inducing basophil production and peripheral accumulation. Unexpectedly, although T cell IL-3 production was crucial in preventing basophil apoptosis *in vitro*, we did not find evidence supporting that such an anti-apoptotic function of IL-3 occurs *in vivo*. Our results provide direct evidence linking T cell activation and IL-3 production to basophil production and yet reveal unexpected *in vivo* dispensability of IL-3 in basophil survival during parasite infection.

Methods

Mice

OT-II TCR Tg mice [specific for ovalbumin peptide (OVA) 323-339 in the context of I-A^b] were obtained from William E. Paul (National Institutes of Health). BALB/c IL-3-deficient mice were previously reported (15). C57BL/6, OT-I TCR Tg, BALB/c and BALB/c Rag2−/− mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). All animals were maintained under pathogen-free conditions at the Lerner Research Institute. All experimental procedures were conducted according to the guidelines of the Cleveland Clinic Foundation Institutional Animal Care Committee.

Cells

Purification of CD4 T cells, splenic dendritic cells (DCs) and basophils were previously reported (5). In brief, CD4 T cells were negatively isolated by depleting non-T cells using FITC-conjugated antibodies (CD8, B220, NK1.1, CD24, MHC II and FcγR), followed by anti-FITC microbeads (Miltenyi Biotec, Auburn, CA, USA). For isolation of splenic DCs, spleen cells were incubated with collagenase D (Invitrogen, Carlsbad, CA, USA) and DNase I (Roche, Indianapolis, IN, USA), followed by centrifugation using a Nycodenz (Sigma, St Louis, MO, USA) gradient. $CD11c^+$ DCs were further positively isolated using a CD11c microbeads (Miltenyi Biotec). For isolation of basophils, bone marrow and liver cells from mice implanted with a miniosmotic pump (Alzet, Cupertino, CA, USA) containing 5 μg IL-3 were harvested and incubated with FITC–anti-FcεRI plus anti-FITC microbeads. All magnetic separation was performed using an MS or LS column. Purity of the CD4 T cells, splenic DCs and basophils was usually >95% for CD4 T cells and >90% for splenic DCs and basophils. Basophils were subsequently labeled with carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes, Eugene, OR, USA) and used in the co-culture experiments. To measure blood basophils, anti-FcγR (CD16/32), anti-FcεRI (MAR-1) and anti-CD45 (30-F11) antibodies were used and analyzed by FACS. In some experiments, basophils were labeled with CFSE and adoptively transferred into wild-type (WT) or knockout (KO) mice to examine *in vivo* survival.

In vitro co-culture experiment and Annexin-V staining

T cells were stimulated with peptide and splenic DCs in the presence of CFSE-labeled basophils. Culture was harvested 48 h after stimulation, and CFSE-labeled basophils were counted. Cells were immediately incubated with PE-labeled Annexin-V (PharMingen, San Diego, CA, USA) according to the manufacturer's instruction. In some experiments, a Transwell culture was used. Annexin-V expression was assessed using a FACSCalibur (Becton Dickinson, San Jose, CA, USA).

Nb infection

Five hundred Nb L3 were subcutaneously injected into mice. Mice were sacrificed 10 days post-infection. Basophil accumulation as well as Annexin-V expression in various tissues were determined by FACS analysis. In some experiments, 4×10^6 T cells isolated from WT or IL-3-deficient mice were transferred into Rag2−/− mice. The recipient mice were then infected with Nb.

Intracellular staining

To measure cytokine production, cells were *ex vivo* stimulated with phorbol myristate acetate (PMA) (10 ng ml^{-1}) plus ionomycin (1 μM) for 4 h. Monensin (2 μM) was added during the last 2 h of stimulation. Harvested cells were fixed with 4% PFA, permeabilized with PBS containing 0.1% BSA plus 0.1% saponin and stained with PE-labeled anti-IL-3 antibody (PharMingen) and allophycocyanin-labeled anti-CD4 antibody (eBioscience, San Diego, CA, USA). In some experiments, intracellular bcl-2 expression was measured using FITC-anti-bcl-2 antibody (clone 3F11, PharMingen) according to the manufacturer's instruction.

Results

Nb infection-mediated basophil production is impaired in IL-3-deficient animals

In order to test the contribution of IL-3 to Nb infection-induced basophil responses, groups of WT and IL-3-deficient mice were infected with Nb and basophil production was examined 4 and 10 days after infection. As shown in Fig. 1(A), basophils defined as FcεRI/CD49b-expressing cells dramatically increased in the bone marrow as well as in the spleen, lung and liver following Nb infection. The increases in basophil numbers did not occur during early infection (day 4 post-infection), but reached a peak response 10 days post-infection, after which the level gradually decreased (11). By contrast, mice deficient in IL-3 did not show any signs of increase in basophil numbers. We then determined basophils in the circulation to see if blood basophils increase during the infection. We utilized an alternative staining strategy reported by Mack *et al.* (16) by staining $CD45^{intermediate}$ $FcγR^{high}$ cells. As shown in Fig. 1(B), FcεRI-expressing basophils were predominantly $CD45^{intermediate}$ $FcγR^{high}$ cells. Gr-1 negative gate originally used by Mack *et al.* was not necessary to gate basophils. This became a particularly useful tool to measure blood basophils because we found that FcεRI on blood basophils as measured by anti-FcεRI antibody appears to be down-modulated during the peak response, making basophil gating strategy based on FcεRI/CD49b less reliable (Fig. 1C). However, it should be noted that down-regulation of FcεRI expression was not observed on basophils from other tissues including

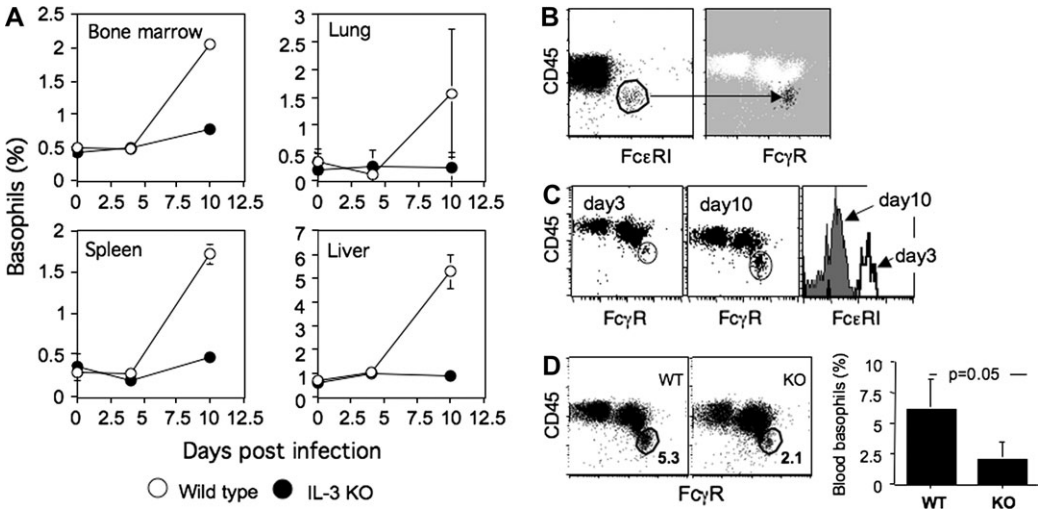

Fig. 1. Impaired basophil production in Nb-infected IL-3-deficient mice. (A) Groups (n = 3) of WT (open circles) and IL-3-deficient (closed circles) mice were infected with Nb. Proportions of basophils in the indicated tissues (liver, lung, bone marrow and spleen) were determined 4 and 10 days after infection. Uninfected mice are also shown as day 0. Shown are mean ± SD of individually tested mouse. (B) Gating strategy using FcγR and CD45 is shown from blood sample from uninfected mice. Note that FcεRI-expressing basophils belong to $FcγR^{high}$ $CD45^{intermediate}$ cells. (C) FcεRI expression of blood basophils from mice infected with Nb (day 3 and day 10) is shown. Histogram represents FcεRI expression of gated basophils (shown as circles) based on $FcγR^{high}$ $CD45^{intermediate}$ expression. (D) Blood cells were stained for FcεRI, FcγR and CD45. $FcεRI^{+}$ basophils are shown as $FcγR^{high}$ $CD45^{intermediate}$ cells. Blood basophils, defined as $FcγR^{high}$ $CD45^{intermediate}$ cells, were measured 10 days post-infection. Mean ± SD from five individually tested mice are shown.

liver, lung, bone marrow and spleen throughout the course of infection, suggesting that the down-regulation seems predominantly to occur in the circulating basophils. Circulating blood basophils increased at the peak of the responses in WT mice infected with Nb (Fig. 1C and D), suggesting that enhanced production of basophils in the bone marrow might be responsible for the increased basophil accumulation in the peripheral tissues. In contrast, circulating basophils were significantly lower in IL-3-deficient mice infected with Nb (Fig. 1D). Consistent with previous report (12), however, IL-3-deficiency did not completely abolish basophil production at basal level. The level was constant throughout the Nb infection, indicating that IL-3 is important principally for the infection-induced basophil production but not for the basal maintenance.

Activated T cell-derived IL-3 is responsible for infection-induced basophil production in vivo

We have previously shown that Nb infection-induced basophil production requires the presence of T cells (11). T cell activation appears to be important during this process since T cells that are not responsive to Nb infection, such as 5C.C7 TCR transgenic CD4 T cells, failed to induce basophil production (11). These results suggest that factors produced by activated T cells might be involved in basophil production. It was previously proposed that IL-3 produced by activated CD4 T cells triggers splenic non-B non-T cells to produce IL-4 in *Schistosoma*-infected mice (13). However, whether T cell-derived IL-3 induces basophil production during Nb infection has not been tested. To test this possibility, we adoptively transferred T cells isolated from either WT or IL-3-deficient mice into Nb-infected BALB/c Rag2−/− recipients. Basophil accumulation and production were determined in the liver and in the bone marrow 10 days post-infection, respectively. As demonstrated previously (11), Nb infection of Rag2−/− recipients of WT T cells resulted in dramatic increase of basophils in the liver (data not shown) and in the bone marrow (Fig. 2A). The level of basophils in these tissues dramatically increased between 7 and 10 days post-infection (data not shown). By contrast, such increases in basophils did not occur in Nb-infected Rag2−/− recipients of IL-3-deficient T cells. Given that only transferred T cells are deficient in IL-3, these results directly indicate that T cell-derived IL-3 induces basophil production following the Nb infection *in vivo*. Basal level of basophils was maintained in the recipients irrespective of IL-3, supporting earlier finding of IL-3-independent nature of basophil maintenance (12). Blood basophil levels were also significantly higher in Rag2−/− recipients of WT T cells (Fig. 2B), consistent with the results shown in Fig. 1(D).

It is important to note that increased basophil production was not found in Rag2−/− recipients of WT T cells without Nb infection (Fig. 2A). T cells transferred into Rag2−/− recipients undergo homeostatic proliferation and acquire capacity to produce several cytokines including IL-2 and IFNγ (17, 18). Thus, whether the failure of homeostatically proliferating T cells within Rag2−/− recipients to induce basophil production without Nb infection correlates with the T cell IL-3 production needs to be clarified. Lymph node cells were thus harvested 10 days after transfer, *ex vivo* stimulated with PMA plus ionomycin and measured for IL-3 production by intracellular staining. As shown in Fig. 2(C), T cells from Nb-infected Rag2−/− recipients produced IL-3 (11.9 ± 4.3%), while T cells from non-infected Rag2−/− recipients produced little IL-3 (1.8 ± 1.1%). No IL-3 production from IL-3-deficient T cells was found. Taken together, these results strongly suggest that T cell IL-3 production closely correlates with basophil production *in vivo*.

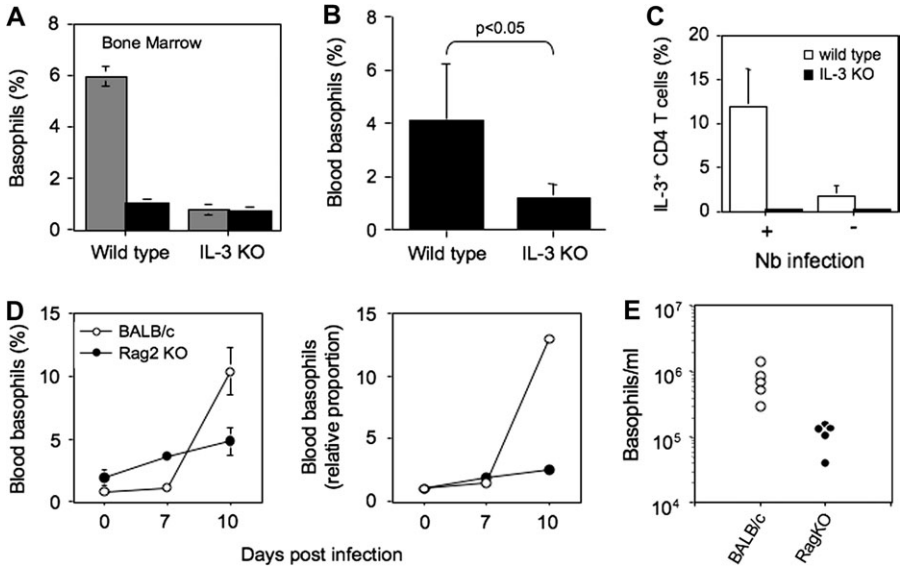

Fig. 2. T cell-derived IL-3 is critical for Nb infection-induced basophil production *in vivo*. (A) Rag2−/− mice ($n = 8$) were adoptively transferred with 4×10^6 T cells isolated from either WT or IL-3-deficient mice. The recipient mice were subsequently infected with Nb. At 10 days post-infection, basophils (FcεRI$^+$ CD49b$^+$) in the bone marrow were determined by FACS analysis (gray bars). Basophils in Rag2−/− recipients of T cells without Nb infection were compared with control (filled bars). Shown are representatives of three independent experiments. (B) Blood basophils were determined as described in Fig. 1. Shown are mean ± SD of five individually tested mice. (C) Lymph node T cells from groups described above were stimulated with PMA plus ionomycin. IL-3 production was measured by intracellular staining. Shown are mean ± SD of individually tested mouse ($n = 3$). (D) Groups ($n = 5$) of BALB/c and Rag2−/− mice were infected with Nb. Blood samples were drawn on 0, 7 and 10 days. Basophils were measured by FACS analysis as described in Fig. 1. Relative proportion of basophils compared with uninfected mice is also shown. (E) Absolute numbers of blood basophils were calculated from groups of five mice. Each circle represents individual mouse.

It was previously reported that basophils in the blood of Nb-infected 4get/Rag−/− mice were present at levels similar to infected WT 4get mice (19), strongly suggesting that basophil production itself does not require T cells. Instead, basophil tissue migration was shown to be severely impaired in the absence of T cells (19). To examine this possibility, groups of BALB/c and Rag2−/− mice were infected with Nb. Blood basophils were examined 7 and 10 days post-infection. Blood basophils determined by CD45^{intermediate} FcγR^{high} expression dramatically increased (>10-fold) 10 days post-infection in WT mice. In contrast, circulating basophils in Nb-infected Rag2−/− mice only marginally increased (~2-fold) (Fig. 2D). When absolute numbers of blood basophils were compared, ~7- to 8-fold difference was found between WT and Rag2−/− mice (Fig. 2E). These results strongly suggest that both basophil production and tissue recruitment require the presence of adaptive immune system, particularly IL-3-producing T cells.

IL-3 prevents basophil apoptosis in vitro

IL-3 has been demonstrated to promote basophil survival (14). Thus, it is possible that the diminution in basophil numbers within IL-3-deficient environment is due to decreased basophil survivability in the periphery. In order to test this possibility, we first performed *in vitro* co-culture experiment recently reported (5). Basophils were isolated from mice treated with IL-3 (5). We used FcεRI antibody to positively isolate basophils. It should be noted that we did not find activation of basophils during FcεRI-based selection process (data not shown). Basophils labeled with CFSE were co-cultured with OVA 323-339 specific TCR transgenic OT-II CD4 T cells and splenic CD11c$^+$ DCs. When OVA was included in the culture, basophil recovery was strikingly enhanced (data not shown). Basophil recovery seemed to correlate with the magnitude of T cell activation as increasing antigen dose further enhanced the recovery. Since basophils are considered terminally differentiated cells, it is unlikely that increased basophil recovery is the result of proliferation. Indeed, co-cultured basophils did not show any signs of cell division as determined by CFSE profile (Fig. 3A). Instead, we measured basophil apoptosis in the absence of T cell activation to test whether activated T cell-derived factors prevented basophil apoptosis. As shown in Fig. 3(A), a substantial proportion (~70%) of basophils became Annexin-V$^+$ when they were co-cultured with OT-II CD4 T cells without antigen; the proportion of Annexin-V$^+$ basophils dramatically diminished when T cells were activated. Data shown in the Fig. 3(A) represent Annexin-V staining at 48-h culture. Annexin-V staining was also measured 24 and 72 h post-stimulation, and similar patterns of Annexin-V staining were observed (data not shown). Increased survival was also found even when the basophils were separated from the stimulated OT-II CD4 T cells using a Transwell culture system, supporting the notion that soluble factors are involved (Fig. 3B).

Activation-induced basophil survival was less pronounced when OVA-activated OT-I CD8 T cells were used (Fig. 3C). Interestingly, separating basophils from the activated OT-I CD8 T cells in a Transwell culture significantly enhanced basophil survival (Fig. 3C). The discrepancy is likely due to

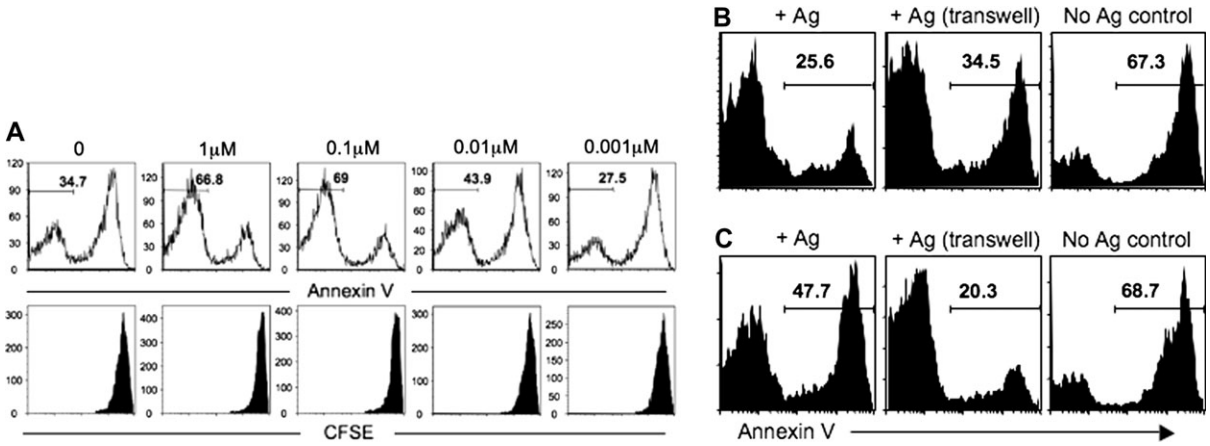

Fig. 3. T cell activation enhances basophil survival *in vitro*. (A) CFSE-labeled basophils were co-cultured with OT-II TCR Tg CD4 T cells and splenic DCs in the presence or absence of OVA. Cells were harvested 48 h after stimulation, and basophil survival was examined by Annexin-V staining. CFSE profile of basophils was also examined. Shown are representatives of three independent experiments. (B and C) CFSE-labeled basophils were co-cultured with OT-II TCR Tg CD4 T cells (B) and OT-I TCR Tg CD8 T cells (C) in the presence of peptide antigen and splenic DCs. In a Transwell culture experiment, basophils were separated from T cells and DCs. Basophil survival was determined by Annexin-V staining 48 h after stimulation. Similar results were observed in two independently performed experiments.

basophil expression of MHC I molecules and their capacity to act as target cells, presenting peptide–MHC I complexes to CD8 T cells (T. Shen and B. Min, unpublished observation) and thus being killed by T cells. Collectively, these results suggest that soluble products made by activated CD4 and CD8 T cells can prevent basophil apoptosis *in vitro*.

T cell-derived IL-3 prevents basophil apoptosis in vitro

IL-3 is a hemopoietic cytokine mainly produced by activated T cells and anti-apoptotic effect of IL-3 has previously been demonstrated on human basophils *in vitro* (14). Since T cell IL-3 production induces basophil production *in vivo* and T cell activation prolongs basophil survival *in vitro*, we next tested if activated T cell mediates anti-apoptotic effects via IL-3 production *in vitro*. Panels of cytokines were tested for anti-apoptotic effect (Fig. 4A). Anti-apoptotic effect was found in response to IL-3 and IL-18, both of which are also known to stimulate basophils to produce IL-4 (10). Addition of both IL-3 and IL-18 did not significantly improve basophil survival (Annexin-V$^+$ cells from IL-3 alone: 46 ± 5%; IL-18 alone: 51 ± 7% and IL-3/IL-18: 41 ± 5%). To test if activated T cells prolong basophil survival through IL-3 production, a co-culture experiment was performed in the presence or absence of neutralizing anti-IL-3 antibody (8F8). Anti-IL-3 antibody abrogated the anti-apoptotic effect exerted by activated T cells (Fig. 4B). Anti-IL-3 antibody-mediated basophil apoptosis was observed when CD4 and CD8 T cells were used in Transwell experiments (data not shown), further suggesting that IL-3 is the common factor produced by activated T cells that prevents basophils from undergoing apoptosis. Furthermore, CD4 T cells isolated from IL-3-deficient mice were stimulated with soluble anti-CD3 plus anti-CD28 antibodies and T-depleted splenocytes in the presence of CFSE-labeled basophils. Activation of IL-3-deficient CD4 T cells failed to reverse basophil apoptosis while activated WT CD4 T cells were fully capable of preventing basophil apoptosis (Fig. 4C). Again, basophil proliferation was not found, suggesting that basophil proliferation is not involved. Taken together, these data strongly suggest that IL-3 produced by activated T cells is primarily involved in preventing basophil apoptosis *in vitro*.

IL-3 is dispensable for basophil survival in vivo

T cell-derived IL-3 prevents basophil apoptosis *in vitro* and induces basophil production *in vivo*. Therefore, it is possible that T cell-derived IL-3 not only promotes basophil production in the bone marrow and accumulation in the peripheral tissues but also prevents basophil apoptosis *in vivo*. To directly test the contribution of IL-3 to basophil survival *in vivo*, we repeated Nb infection experiments and examined basophil survival by Annexin-V staining *ex vivo*. Nb-infected Rag2−/− mice that had received WT or IL-3-deficient T cells were sacrificed 10 days after infection. Basophils from both bone marrow and liver were examined for Annexin-V staining. To our surprise, Annexin-V staining of basophils was indistinguishable between the two groups (Fig. 5A). Comparable Annexin-V staining between Rag2−/− recipients of WT and IL-3-deficient T cells was also found 4 days post-infection and without infection (data not shown). Moreover, such IL-3 independency of *in vivo* basophil survival was also observed in Nb-infected IL-3-deficient mice (Fig. 5B). Little or no differences in basophil survival were found in IL-3-competent and IL-3-deficient mice throughout the infection as well as without Nb infection shown as day 0 (Fig. 5B). Of note, Annexin-V staining in the liver increased 10 days post-infection, while that in the bone marrow was significantly lower. Higher Annexin-V$^+$ liver basophils are likely due to increased apoptosis in the liver compared with bone marrow, considering short half-life of basophils in the periphery.

Apoptotic cells could rapidly be taken up by phagocytes *in vivo*, thus *ex vivo* Annexin-V staining might not necessarily reflect the *in vivo* apoptosis, although *ex vivo* Annexin-V staining is still being used to assess *in vivo* apoptosis (20). *In vivo* basophil proliferation was then examined by *in vivo*

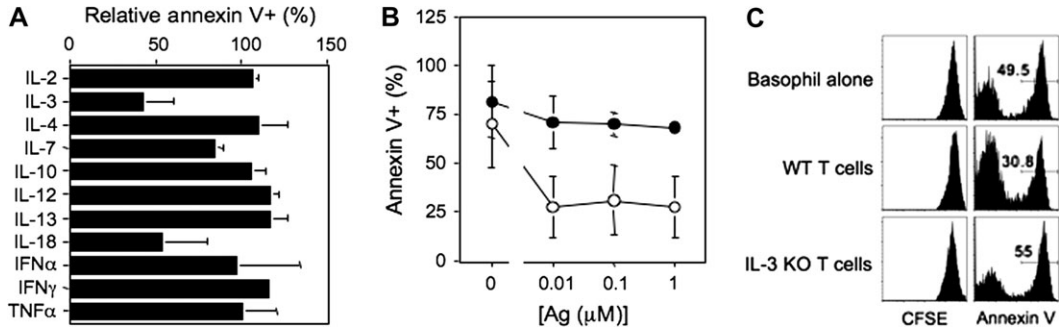

Fig. 4. T cell-derived IL-3 is responsible for basophil survival. (A) Basophils were incubated with the indicated cytokines for 48 h. Annexin-V staining without cytokine was measured, and relative Annexin-V staining compared with no cytokine control was accordingly determined. Concentrations of cytokines were as follows: 10 U ml^{-1} IL-2, 10 ng ml^{-1} IL-3, 25 ng ml^{-1} IL-4, 25 ng ml^{-1} IL-7, 5 ng ml^{-1} IL-10, 50 ng ml^{-1} IL-12, 25 ng ml^{-1} IL-13, 25 ng ml^{-1} IL-18, 500 U ml^{-1} IFNα and 25 ng ml^{-1} IFNγ. Shown are mean ± SD of two independently performed experiments. (B) CFSE-labeled basophils were cultured with OT-II CD4 T cells plus DCs in the presence of the indicated concentrations of OVA. In all, 10 μg ml^{-1} of anti-IL-3 antibody (closed circles) or control antibody (open circles) was included in the culture. Annexin-V expression was subsequently determined 48 h post-stimulation. Shown are mean ± SD of actual percentage of Annexin-V staining of two independently performed experiments. (C) CD4 T cells were isolated from either WT or IL-3-deficient mice and subsequently stimulated with anti-CD3/anti-CD28 antibodies together with irradiated T-depleted splenocytes. CFSE-labeled basophils were included in the culture. Annexin-V expression of basophils was determined. CFSE profiles of cultured basophils are also shown. The experiments were repeated three times with similar results.

5-bromo-2-deoxyuridine (BrdU) labeling experiments. As shown in Fig. 5(C), basophils in WT and IL-3-deficient mice infected with Nb equally incorporated BrdU *in vivo*. No statistically significant difference of BrdU incorporation was found in the bone marrow and the liver. It is important to note that proliferation rate of bone marrow basophils was >10-fold higher than that of liver basophils, suggesting that basophils found in the bone marrow might represent newly generated 'immature' basophils that are highly proliferating, while basophils found in the periphery might represent terminally differentiated cells that are rarely dividing. BrdU-expressing basophils (~3%) found in the liver may reflect cells proliferating in the periphery. Alternatively, these basophils might be labeled in the bone marrow and migrate to the periphery during the labeling period. Further study will be needed to clarify the possibilities. In addition, IL-3 did not influence the expression of anti-apoptotic factor, bcl-2 (Fig. 5D). Lastly, basophils were CFSE labeled and adoptively transferred into either WT or IL-3 KO recipients. Proportions of circulating $CFSE^+$ basophils were examined by FACS 24 h post-transfer. As shown in Fig. 5(E), the absence of IL-3 in recipients did not appear to affect *in vivo* survival of basophils. Therefore, these results suggest that IL-3 appears to play an important role in basophil production during parasite infection, yet its contribution to *in vivo* basophil survival and proliferation might be less critical.

Discussion

In this report, we demonstrate that IL-3 produced by activated T cells is directly involved in enhancing basophil production in the bone marrow as well as accumulation in the peripheral tissues following Nb infection. Although IL-3 produced by activated T cells prolongs basophil survival *in vitro*, we did not find evidence supporting that IL-3 is critical for basophil survival *in vivo*.

IL-3 has been considered a major hemopoietic cytokine that stimulates bone marrow progenitors to differentiate into various blood cell types, including granulocytes, macrophages, megakaryocytes, erythroid progenitors and mast cells (21). Paradoxically, IL-3 deficiency does not result in any noticeable defect in uninfected mice, suggesting that there might be other redundant factors *in vivo* that contribute to the lineage differentiation (15). Substantial defects in basophil and mast cell production were found in IL-3-deficient mice in response to parasite infection (12), suggesting that, IL-3, although dispensable for homeostatic maintenance of these cells, appears to play key roles in infection-induced production of basophils. Our results reported in this study further provide evidence that activated T cells are important source of IL-3 necessary for this process. Therefore, IL-3 may become a determining factor that influences basophil production from the progenitors only when there are ongoing infections or inflammations and IL-3 level increases.

IL-3 production of T cells rapidly increases upon activation. Like IL-2, IL-3 mRNA expression peaks around 4–6 h after activation and then slowly disappears thereafter (L. Wang and B. Min, unpublished observation). It is interesting to note that T cells transferred into Rag2−/− recipients do not produce IL-3 during proliferation process within lymphopenic conditions. Since these T cells undergoing 'homeostatic proliferation' were shown to produce effector cytokines including IL-2, tumor necrosis factor α and IFNγ (17, 18), it is possible that IL-3 production may require rather different signals in activated T cells. The fact that T cells produce IL-3 and induce basophil production only after Nb infection further supports this possibility. However, it should be emphasized that IL-3-producing activated T cells do not always induce basophil production without parasite infection. This strongly implies that T cell IL-3 production is not likely the sole factor that promotes basophil production *in vivo*. Parasite antigens may directly induce production of additional factors. Soluble Nb extracts or Nb-associated antigens co-immunized with peptide antigens were shown to induce T_h2 differentiation of antigen-specific T cells *in vivo* (22, 23). Proteases or other structural proteins such as

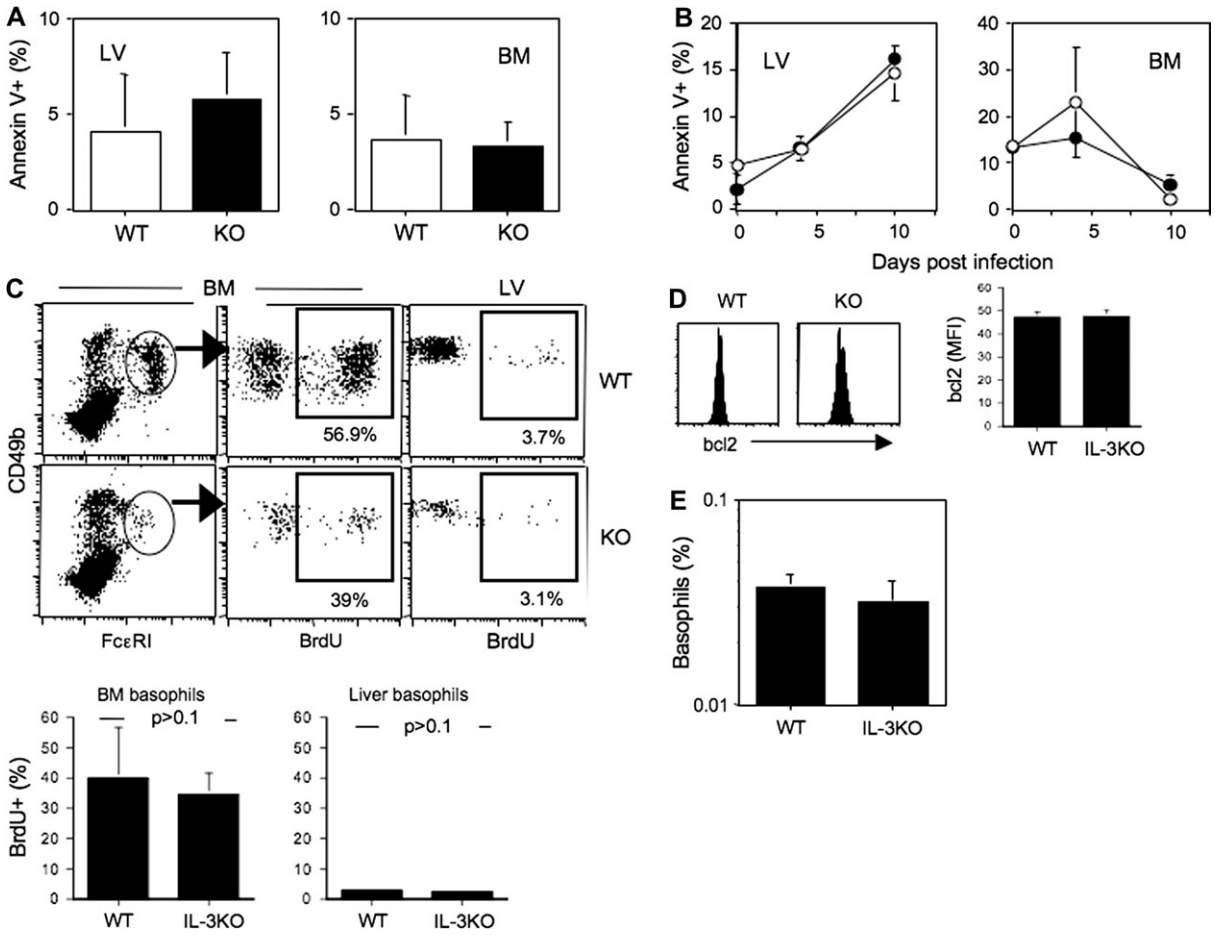

Fig. 5. *In vivo* basophil survival upon Nb infection is IL-3 independent. (A) Nb-infected Rag2−/− recipients of WT (open bars) and IL-3-deficient (filled bars) T cells were examined for basophil survival 10 days after infection by *ex vivo* Annexin-V staining. (B) WT (open circles) and IL-3-deficient mice (closed circles) infected with Nb were examined for basophil survival 4 and 10 days later after infection. Uninfected control groups are indicated as day 0. Annexin-V staining of basophils was examined by FACS analysis. Shown are mean ± SD of individually tested mouse ($n = 3$). Experiments were repeated twice with similar results. (C) Groups of BALB/c and IL-3 knockout (KO) mice were infected with Nb. Mice were injected with 1 mg BrdU 10 days post-infection. Mice were sacrificed 6 h after the BrdU injection. Shown are BrdU incorporation of FcεRI/CD49b-gated basophils in the liver and the bone marrow. (D) bcl-2 expression of bone marrow basophils was also determined by FACS analysis. Shown are mean ± SD of four to five individually tested mice. (E) CFSE-labeled basophils were adoptively transferred into either WT or IL-3-deficient recipients (3×10^6 cells per recipient). Circulating blood basophils were measured by FACS analysis 24 h post-transfer. Shown are mean ± SD of individually tested mouse ($n = 2$).

chitins derived from parasites could induce T_h2 immunity (24, 25). More recently, protease allergen was shown to activate basophils to produce factors that subsequently induce *in vivo* T_h2 differentiation (8). Whether these antigens directly induce basophil production *in vivo* needs to be investigated.

Voehringer *et al.* (19) have recently reported that T cells are not required for basophil production. This conclusion was made based on the observation that blood basophils equally increased in Nb-infected 4get/Rag−/− and 4get mice. However, we found no evidence to support the findings since blood basophils dramatically increased in WT but not in Rag−/− mice following Nb infection (Fig. 2D and E). While Voehringer *et al.* have defined GFP^+ IgE^+ cells as basophils, we defined basophils as $CD45^{intermediate}$ $Fc\gamma R^{high}$ cells, which turns out to be the most reliable markers for blood basophils (Fig. 1B) especially when surface FcεRI is likely occupied by IgE produced in the course of Nb infection (16). It is unclear whether the methods to identify blood basophils are responsible for the discrepancy between the two studies. It is also interesting that the increase was obvious only 10 days post-infection. Absolute numbers of blood basophils also dramatically increased compared with Rag−/− mice. Therefore, our results strongly suggest that adaptive immune system, particularly activated T cells producing IL-3, plays an irreplaceable role in promoting basophil production in the bone marrow as well as migration of basophils into peripheral tissues. Our results further suggest that parasite antigens alone are not sufficient to promote basophil production since Nb infection of Rag2−/− mice fails to do so.

Processes involved in basophil lineage differentiation are poorly understood. Our data support the possibility that T cell-derived IL-3 enhances basophil production in the bone marrow and subsequent peripheral migration. Uniform

increases of basophils in multiple tissues including blood strongly suggest increased production of basophils in the bone marrow and subsequent migration to the peripheral tissues. IL-3 produced by activated T cells in the draining lymph nodes may circulate and stimulate basophil differentiation in the bone marrow. Alternatively, activated T cells may migrate into the bone marrow, where they stimulate basophilopoiesis (26). However, it is rather unlikely that T cells are reactivated to produce IL-3 by antigens within the bone marrow. Mechanisms by which IL-3 induces basophil production in the bone marrow will require further investigation. Basophil life span in the periphery is believed to be short. However, our understanding related to basophil homeostasis is very limited. Future study should be directed to understand processes controlling generation, peripheral migration, survival and death of basophils.

IL-3 has been known to enhance cell survival and proliferation (14). Indeed, *in vitro* co-culture experiments where basophils are cultured with activated T cells strongly indicate that IL-3 plays a key role in preventing basophil apoptosis *in vitro*. Molecular mechanisms to prevent apoptosis in cell lines may include serine phosphorylation of the IL-3R as well as bcl-2 phosphorylation (27, 28). Nevertheless, *in vivo* results highlight that basophil survival is not significantly altered in the absence of T cell-derived IL-3 or even in the complete absence of IL-3. This conclusion was made based on *ex vivo* Annexin-V staining experiments. We are aware that apoptotic cells may rapidly be eliminated by phagocytes *in vivo*, thus Annexin-V staining *ex vivo* might not necessarily reflect *in vivo* apoptosis, although *ex vivo* Annexin-V staining is still being used to assess apoptotic cell death (20). Instead, it is interesting to find that *in vivo* proliferation of basophils determined by *in vivo* BrdU injection is not influenced by the absence of IL-3 (Fig. 5C), considering that enhanced production of basophils via elevated IL-3 levels would require higher proliferation activity of basophil progenitors. Whether lineage differentiation of basophil progenitors into mature basophils increases by IL-3 will need further investigation. Equal expression of anti-apoptotic factor, bcl-2, of basophils in WT and IL-3-deficient mice further supports IL-3-independent regulation of basophil survival *in vivo*. Nonetheless, basophils cultured *in vitro* are heavily dependent on IL-3, suggesting that IL-3 still plays important role in basophil survival. Instead, there might be a redundant factor involved in maintaining basophil survival. Indeed, stem cell factor was shown to enhance human basophil survival (29).

The finding that blood basophils have lower FcεRI expression during peak response after Nb infection is an unexpected yet interesting observation. It is not clear whether the lower expression is due to elevated serum IgE level that might somehow interfere staining with anti-FcεRI antibody or to actual down-regulation of the receptor. It should be noted that the FcεRI down-regulation was not noticed in basophils from other tissues (data not shown). However, such interference of the anti-FcεRI antibody (MAR-1, specific for FcεRI α chain) has not previously been noticed. It was previously reported that treatment of anti-IgE (Omalizumab) reduces FcεRI expression on human basophils (30). Consistent with this, IgE was shown to enhance FcεRI expression in both mouse basophils (31) and human mast cells (32). Further study will be needed to examine whether FcεRI expression between circulating basophils and tissue basophils is differentially regulated.

In conclusion, there is an increasing evidence suggesting that basophils play critical roles in the development of *in vivo* type 2 immune responses (8). Understanding mechanisms underlying basophil production, tissue accumulation and survival may have important clinical implication to develop therapeutic strategies to prevent pathogenesis mediated by basophils often found in allergic inflammation such as asthma.

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Abbreviations

BrdU	5-bromo-2-deoxyuridine
CFSE	carboxyfluorescein succinimidyl ester
DCs	dendritic cells
KO	knockout
Nb	*Nippostrongylus brasiliensis*
OVA	ovalbumin peptide
PMA	phorbol myristate acetate
WT	wild type

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