Cellular Immunology xxx (2009) xxx–xxx

Contents lists available at ScienceDirect

Cellular Immunology

journal homepage: www.elsevier.com/locate/ycimm

Interleukin-15 expression affects homeostasis and function of B cells through NK cell-derived interferon-γ

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ARTICLE INFO

Article history:
Received 3 February 2009
Accepted 12 March 2009
Available online xxxx

Keywords:
IL-15
NK cells
B cells

ABSTRACT

Interleukin-15 (IL-15) is a cytokine important for the development, maturation, and function of many cells of the immune system including NK, NKT, γδT, and $CD8^+$ T cells. The relationship between IL-15 and B lymphocytes however, is not well characterized and is the focus of our study. Previous *in vitro* reports have shown that IL-15 increases proliferation of B lymphocytes and increases antibody secretion however, this relationship remains inadequately defined *in vivo*. The focus of this study was to examine the role of IL-15 in B cell homeostasis and function *in vivo* using mice that either over express IL-15 (IL-15tg mice) or are deficient in IL-15 (IL-$15^{-/-}$ mice) production. Here we report significant differences between the B cell populations of IL-$15^{-/-}$, C57BL/6, and IL-15tg mice. In fact, increased expression of IL-15 resulted in a significant decrease in the percentage and absolute number of $CD19^+$ cells. *In vitro* B cell co-cultures implicate interferon-gamma (IFN-γ) as the factor responsible for inhibiting B cell proliferation. We also show that IL-15 expression affects B cell function, as B cells from IL-15 transgenic mice produce greater amounts of IgG and IgA than IL-15 knockout mice *in vitro*. Interestingly, despite significant differences in B cell numbers in these strains, there were no significant differences in total antibody titers in serum and vaginal washes of these mice. Results from our *in vivo* and *in vitro* experiments suggest that altered expression of IL-15 affects B cell homeostasis through the induction of NK cell-derived IFN-γ.

1. Introduction

B lymphocytes have important antigen specific effector functions, which make them central to the adaptive immune response. Mice that lack B cells or have non-functional B cell populations are more susceptible to infection and colonization, and cannot mount a significant antibody memory response [1–5]. It is well established that B cell homeostasis *in vivo* is continuously influenced by a variety of cytokines, such as transforming growth factor beta (TGF-β) and IFN-γ, which are known to modulate the strength of B cell responses [6]. This knowledge increases the feasibility of creating a practical and effective form of immunotherapy, in which cytokines important to B cell homeostasis are manipulated. The ability to effectively alter the proliferation and/or function of B cells *in vivo* would have applications in vaccine development, cancer therapy, and the treatment of autoimmune diseases [7,8].

Interleukin-15, a cytokine discovered in 1994, has been shown to have potent effects on numerous cells of the immune system. IL-15 closely resembles interleukin-2 (IL-2) in its tertiary structure and these two cytokines share the β and γ chains of the IL-2 receptor for signal transduction [9–11]. However, IL-15 binds with high affinity to its α chain receptor (IL-15Rα) which enables it to elicit functions distinct from IL-2 *in vivo* [12–14]. IL-15 is produced by many cell types, including monocytes and macrophages, dendritic cells, and bone marrow stromal cells. Its receptor components, including the α chain, are present on the surface of lymphoid, myeloid, and non-hematopoietic cells [9,12]. It is well documented that IL-15 plays a crucial role in the development, maturation, and activation of NK cells [9,15–17]. IL-15 deficient (IL-$15^{-/-}$) and IL-15 α chain receptor deficient (IL-15R$\alpha^{-/-}$) mice are almost completely devoid of all NK and NKT cell populations and their associated functions [18]. IL-15 is also able to augment proliferation of naïve and memory $CD8^+$ T cells [18–21]. Furthermore it has been shown to play a role in upregulation and activation of antigen presenting cells such as dendritic cells [22,23].

Although the role of IL-15 on many hematopoietic cells has been well documented, its effects on B cells have received little attention in comparison. B cells express the receptor components necessary for binding of IL-15 and subsequent signal transduction [24]. Multiple *in vitro* studies have shown that the presence of recombinant IL-15 (rIL-15) is able to increase proliferation of B cells, up to 20-fold [24–26]. In addition, it was reported that rIL-15 is also able to increase IgM, IgG1, and IgA secretion when added to B cell cultures [25]. In contrast to these reports, a very recent study

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doi:10.1016/j.cellimm.2009.03.010

showed that, *in vitro*, IL-15 caused a decrease in B cell numbers through the production of IFN-γ which has been shown to negatively regulate B cells [27]. *In vivo* studies remain limited, although it has been suggested that IL-15 can increase IgA responses in B-1 cells [26].

The purpose of the present study was to determine the effects of IL-15 on B cell populations *in vivo* by examining mouse strains with altered IL-15 expression. Our results show that increased IL-15 expression (in IL-15tg mice) decreases both the percentage and absolute number of B cells in the spleen and the bone marrow of these mice. Furthermore, stimulated B cell cultures suggest that the results observed *in vivo* are due to the upregulation of NK cell-derived IFN-γ. In terms of function, IgG and IgA are produced more efficiently by B cells derived from IL-15tg mice compared to IL-15$^{-/-}$ mice. However, these isotypes are found in similar concentrations in the serum and vaginal washes of both strains of mice. These results suggest that IL-15 is indeed a negative regulator of B cell proliferation through the induction of NK cell-derived IFN-γ.

2. Materials and methods

2.1. Mice

Female C57BL/6 (B6) mice 8–12 weeks of age were purchased from Charles River Laboratory (Saint-Constant, Que.). IL-15$^{-/-}$ mice on a C57BL/6 background were obtained from Taconic Farms Inc. (Germantown, NY). IL-15tg mice breeding pairs on a C57BL/6 background were kindly provided by Dr. Caligiuri (Ohio State University). All mice were kept and bred in the barrier facilities at McMaster University, Hamilton, Ont., Canada. All mice followed a 12-h day and 12-h night schedule and were maintained under standard temperature-controlled conditions.

2.2. Flow cytometry

Bone marrow from the femur and tibia bones and whole spleens were harvested, and single cell suspensions were prepared. Cells were stained with CD19, CD3 and NK1.1 (BD Biosciences, Mississauga, ON), for flow cytometry analysis.

2.3. NK cell depletion

C57BL/6 mice were depleted of NK cell populations using anti-NK1.1 specific antibody (Centre for Gene Therapeutics, McMaster University). Mice were given 200 μL intraperitoneal injections containing antibody at a concentration of 1 mg/mL. The injection regimen occurred on days 1, 2, and 6, 9, and 12 with sacrifice on day 14.

2.4. Purification and culture of B cells and NK cells

Splenocytes were obtained as outlined above. Enriched cells were prepared using a negative B cell selection kit or a positive NK cell selection kit (Stem Cell Technologies Inc., Vancouver, BC). B cells/well (4×10^5) were added in triplicate to 96-well plates in 100 μL of RPMI 1640 containing 10% fetal bovine serum. Wells were stimulated with lipopolysaccharide (LPS) (Sigma, Oakville, ON), rIL-15 (Cedarlane Laboratories, Burlington, ON), and rIFN-γ (R&D Systems, Minneapolis, MN) at a concentration of 100 ng/mL, 500 ng/mL, and 10 ng/mL, respectively. Some wells were co-cultured with NK cells (1×10^5 NK cells/well). Plates were then cultured at 37 °C/5% CO_2 for a period of 72 h. Hundred microliters of supernatant was collected at 48 h post stimulation for antibody quantification. Proliferation was quantified by the uptake of 1 μCi of [^{3}H]thymidine/well, added in 100 μL of 10% RPMI for the final 24 h of culture.

2.5. ELISA for total IgG and IgA

Maxisorp 96-well ELISA plates (Invitrogen, Burlington, ON) were coated with 100 μL/well of rat anti-mouse IgG or IgA (Southern Biotech, Birmingham, AL) at a concentration of 0.5 μg/mL and incubated overnight at 4 °C. The plates were then blocked with 2% bovine serum albumin at room temperature (RT) for 2 h. Samples were diluted in 0.1% BSA, added to ELISA plates, and incubated overnight at 4 °C. Hundred microliters per well of biotin-labeled goat anti-mouse IgG or IgA detection antibody (Southern Biotech, Birmingham, AL) was added at a concentration of 1 μg/mL for 1 h in the dark at RT. Extravidin peroxidase conjugate (Sigma, Oakville, ON) was added to each well and incubated for an additional 45 min at RT. TMB microwell peroxidase (KPC, Gaithersburg, MD) was added to each well and allowed to incubate for 30 min at RT. Finally, 100 μL of 1 M H_2SO_4 stop solution was added, and the plates were read with a Sapphire absorbance reader (Tecan, Durham, NC) at an optical density of 450 nm.

2.6. ELISA for IFN-γ

IFN-γ ELISAs were conducted using Quantikine Murine Kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

2.7. Statistical analysis

Statistical differences in cell populations, proliferation, and antibody production were determined using an unpaired *t*-test. A p value of <0.05 was considered statistically significant.

3. Results

3.1. Increased IL-15 expression results in reduced B cell populations in vivo

We first examined whether differences in IL-15 expression would have an effect on B cell populations *in vivo*. For these experiments we compared B cell populations in normal congenic control B6 mice to IL-15tg mice, which express elevated levels of IL-15, and IL-15$^{-/-}$ mice, which are deficient in IL-15. Fig. 1a demonstrates higher percentages of CD19$^+$ cells in the bone marrow of IL-15$^{-/-}$ mice compared to B6 mice and decreased proportions of CD19$^+$ cells in IL-15tg mice. A similar trend is observed in the splenocytes of these mouse strains (Fig. 1b) with IL-15$^{-/-}$ mice displaying the largest population of CD19$^+$ cells and IL-15tg mice the lowest percentage of these cells. Furthermore, the absolute number of CD19$^+$ cells is significantly increased in the bone marrow of IL-15$^{-/-}$ mice compared to IL-15tg mice and this population is also significantly greater in B6 mice compared to IL-15tg mice (Fig. 1c).

3.2. B cell populations increase in response to NK cell depletion in vivo

The above results suggest that IL-15 negatively regulates B cell proliferation, however it is not clear whether this is a direct effect of IL-15 or an indirect effect mediated via NK cells. Since it is well established that IL-15 drives the proliferation of NK cells and that IL-15$^{-/-}$ mice are deficient in NK cells, we hypothesized that NK cells were in fact responsible for inhibiting B cell populations. In order to assess this hypothesis we selectively depleted NK cells *in vivo* by injecting anti-NK1.1 specific antibody, and subsequently quantified the change in CD19$^+$ cells. Anti-NK1.1 specific antibody successfully depleted NK cells in the bone marrow and spleen of wild type B6 after 2 weeks, significantly reducing NK cell populations to the background staining levels observed in IL-15$^{-/-}$ mice

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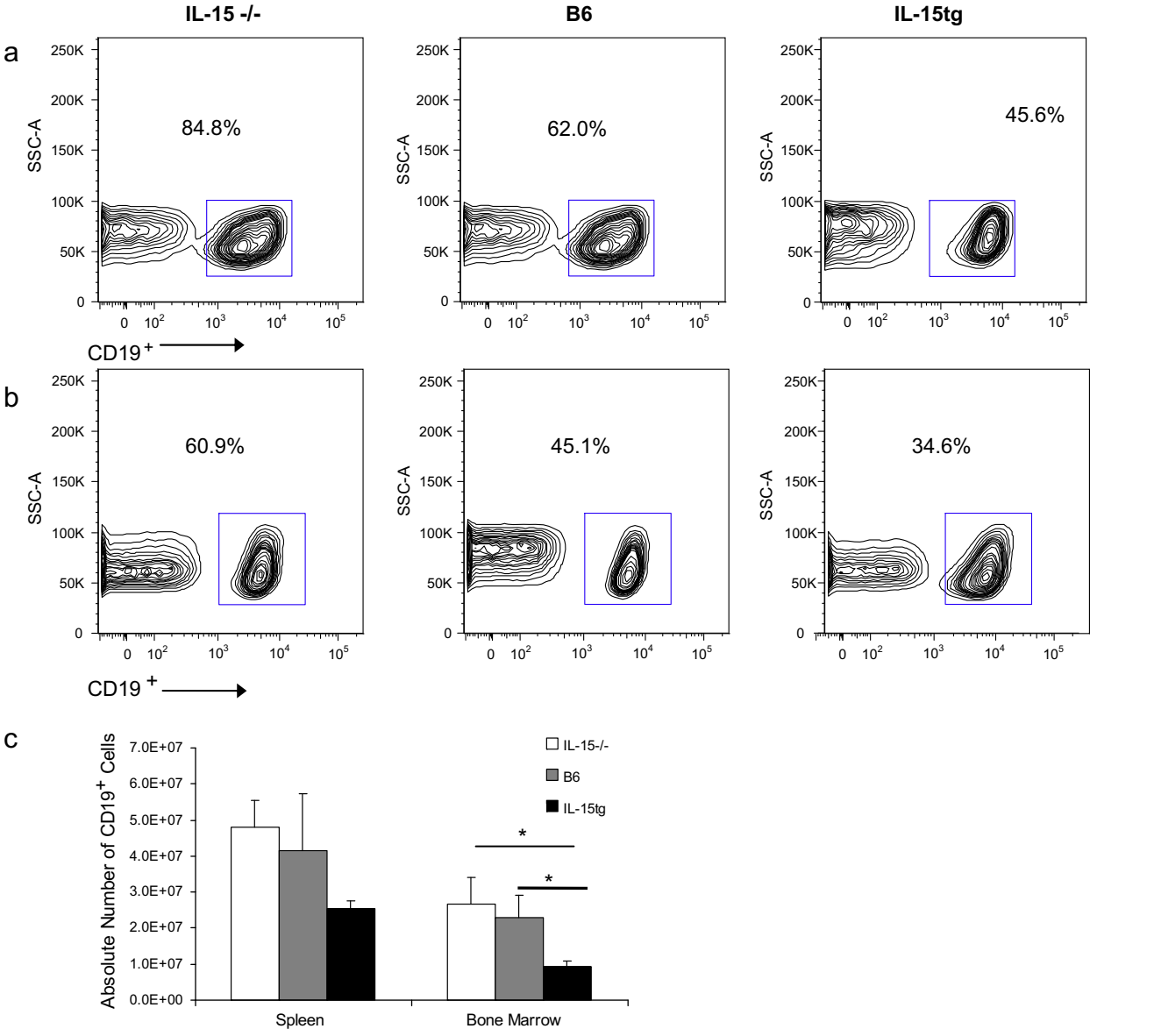

Fig. 1. $CD19^+$ populations are affected by levels of IL-15 expression. Six- to eight-week-old female B6, IL-$15^{-/-}$ and IL-15tg mice were sacrificed. Single cell suspensions were prepared for the bone marrow and spleen cells and stained for CD19 as described in materials and methods. (a) Bone marrow cells from IL-15tg mice showed a significant reduction in $CD19^+$ cells and (b) spleen cells from IL-15tg mice also showed a decrease in $CD19^+$ cells. (c) Significant differences are also observed when comparing the absolute number of $CD19^+$ cells in the spleen and bone marrow. $^*p < 0.05$. Experiments represent five mice per group.

(Fig. 2a). This NK cell depletion resulted in a significant increase in B cell populations in the bone marrow of these mice by approximately 20% (Fig. 2b). These results suggest that IL-15 derived NK cells may directly or indirectly inhibit B cell homeostasis.

3.3. B cell proliferation decreases in response to IFN-γ and increases in response to IL-15 in vitro

Based on these findings, we next set out to determine how NK cells mediate their effect on B cells. To achieve this, B cell cultures were stimulated for 72 h in the presence of IFN-γ, NK cells, or rIL-15 following stimulation with LPS, a known B cell mitogen. Fig. 3 shows that rIFN-γ was the only tested factor that was able to significantly reduce the proliferation of B cells. Furthermore, our results confirmed the findings of others that rIL-15 increases B cell proliferation, while NK cells did not significantly alter B cell proliferation. B cells isolated from IL-15tg, B6, and IL-$15^{-/-}$ mice all show similar results (data not shown).

3.4. B cells from IL-15tg mice produce greater amounts of total IgG and IgA in vitro

Since we found that B cells from the three strains of mice responded similarly to *in vitro* stimulation with IFN-γ, rIL-15 and NK cells, we were interested in assessing whether the functional capabilities of these B cells was also similar. Supernatants from B cell cultures were analyzed by ELISA for both IgG and IgA and compared based on endpoint dilutions. B cells derived from IL-15tg mice show an increase in IgG production (Fig. 4a) and significantly greater IgA secretion than those derived from B6 and IL-$15^{-/-}$ mice (Fig. 4b).

3.5. Levels of IgG and IgA are similar in serum and mucosa of IL-15tg, B6, and IL-$15^{-/-}$ mice

Finally, since we observed that over expression of IL-15 leads to a decreased population of B cells *in vivo*, but results in increased

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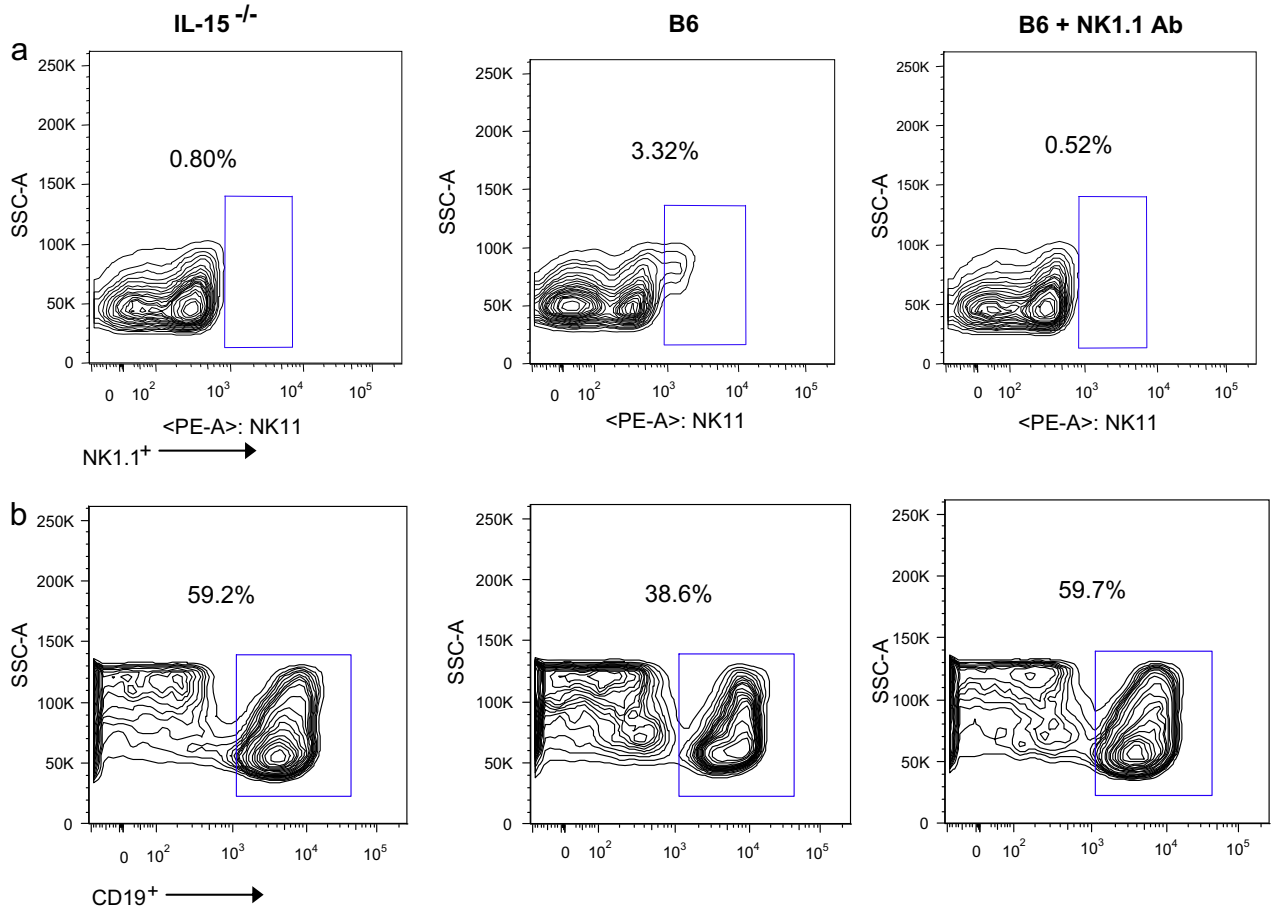

Fig. 2. Depletion of NK cell populations using anti-NK1.1 antibody increases the percentage of B cells in the bone marrow. Six- to eight-week-old female B6 mice were injected IP with anti-NK1.1 antibody to deplete NK cells. Seven days post injection, NK cell depleted mice, wild type B6 and IL-15$^{-/-}$ mice were sacrificed and bone marrow cells were harvested and stained for flow cytometry analysis. (a) Analysis of NK1.1^{+} cells using flow cytometry revealed that the antibody was effective at depleting this population. (b) A subsequent increase in CD19^{+} cells was observed in anti-NK1.1 injected mice comparable to levels observed in IL-15$^{-/-}$ mice. These results are representatives of 5 mice per group.

production of antibody *in vitro*, we tested whether *in vivo* B cell functions differed in these mice. We measured total IgG and IgA levels systemically in the serum and in vaginal secretions to assess mucosal antibody levels. Fig. 5 demonstrates that the antibody concentrations are similar in all three strains of mice in both the serum and the vaginal washes. Furthermore, antigen specific antibody levels were also similar in immunized B6, IL-15$^{-/-}$ and IL-15tg mice (data not shown).

4. Discussion

The relationship between IL-15 and B lymphocytes has received little attention in the last several years. A few studies have suggested that in an *in vitro* culture system IL-15 can induce B cell proliferation, antibody production, or both [24–26]. On the other hand, a recent report found that IL-15 inhibits pre B cell proliferation through NK cell-derived IFN-γ production *in vitro* [27]. The focus of our paper was to clarify the effect of IL-15 on B cell development, proliferation and function *in vivo*. This was accomplished by utilizing mice that were either deficient in IL-15 production or had excessive amounts of IL-15. Here, we have shown that *in vivo* IL-15 alters the homeostasis of B cells through NK cell-derived IFN-γ. We also show that the inherent ability of B cells to produce antibody may be affected by the presence of IL-15.

In this study, we first examined whether there were differences in the B cell population of IL-15$^{-/-}$ and IL-15tg mice compared to wild type B6 mice. In mice lacking IL-15 we found very high percentages and absolute number of B cells compared to wild type B6 mice. In contrast, IL-15tg mice showed the lowest percentage and absolute number of CD19^{+} cells. These results suggest that indeed IL-15 does inhibit B cell homeostasis and proliferation *in vivo*. However, this introduced further questions as to the mechanism by which IL-15 was regulating B cell homeostasis. It is possible that IL-15 may have a direct effect on B lymphocytes, or that the decrease in B cells is the result of an indirect effect of IL-15 perhaps via NK cell activation.

IL-15 has been widely characterized to be essential for NK cell development, maturation and activation [9,15–21]. In the mouse model, IL-15$^{-/-}$ mice do not have NK cells, whereas IL-15tg mice have more NK cells that B6 mice. As such, we were interested in examining whether IL-15 may be indirectly inhibiting B cell proliferation through NK cells. To achieve this, we depleted wild type B6 mice of NK cells and then examined the effect of this depletion on the B cell population in these mice. We found that by depleting the 3.5% of NK cells in B6 mice, there was a 20% increase in B cells, which resembled the phenotype observed in IL-15$^{-/-}$ mice. These results suggest that NK cells do play a role in inhibiting B cell proliferation *in vivo*. This is strengthened by the observation that IL-15tg mice which have a greater percentage of NK cells have a very small B cell population.

In order to address the question of whether NK cells inhibited B cell proliferation via a direct cell-to-cell contact or indirect mecha-

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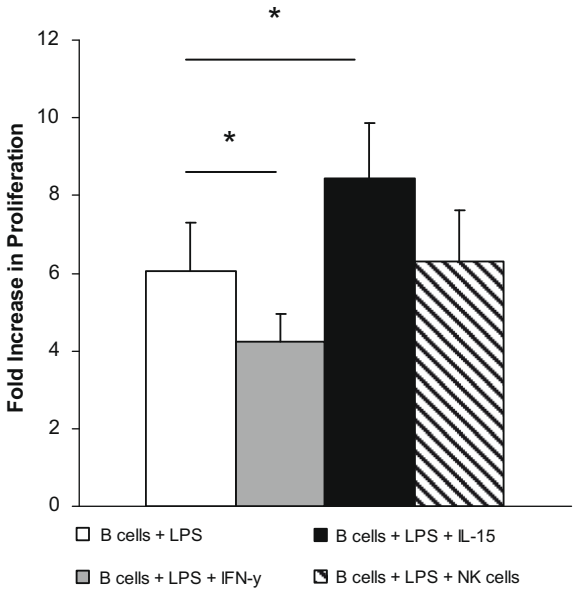

Fig. 3. IFN-γ is able to decrease LPS-induced B cell proliferation in culture. B cells were isolated from the spleens of 6- to 8-week-old female B6, mice using a negative selection B cell isolation kit. B cells (4×10^5) were plated in a 96-well plate and cultured in the presence of LPS (100 ng/mL). Cells were also treated with either rIFN-γ (10 ng/mL), rIL-15 (500 ng/mL) or purified NK cells (1×10^5) and a proliferation assay was carried out. Treatment with rIFN-γ significantly decreased B cell proliferation, while treatment with rIL-15 significantly increased proliferation. Results depict the means from four B6 mice. This experiment was repeated with B cells from IL-15$^{-/-}$ and IL-15tg mice with similar results (data not shown). $^{*}p < 0.05$.

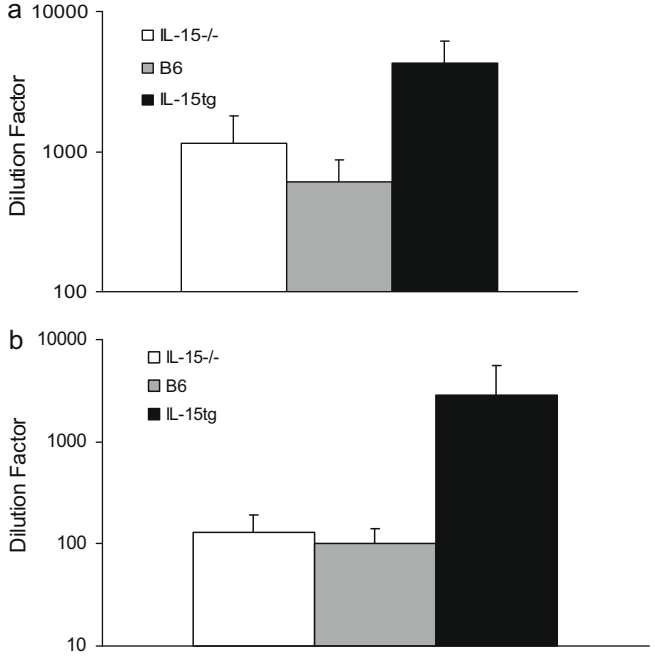

Fig. 4. B cells from IL-15tg mice produce more antibody in response to stimulation *in vitro*. B cells were isolated from the spleens of 6- to 8-week-old female B6, IL-15$^{-/-}$ and IL-15tg mice using a negative selection B cell isolation kit. B cells (4×10^5) were plated in a 96-well plate and cultured in the presence of LPS (100 ng/mL). Supernatants were collected 72 h post stimulation for examination of total IgG and IgA antibodies using an ELISA. (a) IgG levels were found to be the highest in B cells isolated from IL-15tg mice. (b) IL-15tg mice also produced significantly more IgA in response to stimulation compared to both IL-15$^{-/-}$ and C57Bl/6 mice. Results represent the means of four mice from each strain.

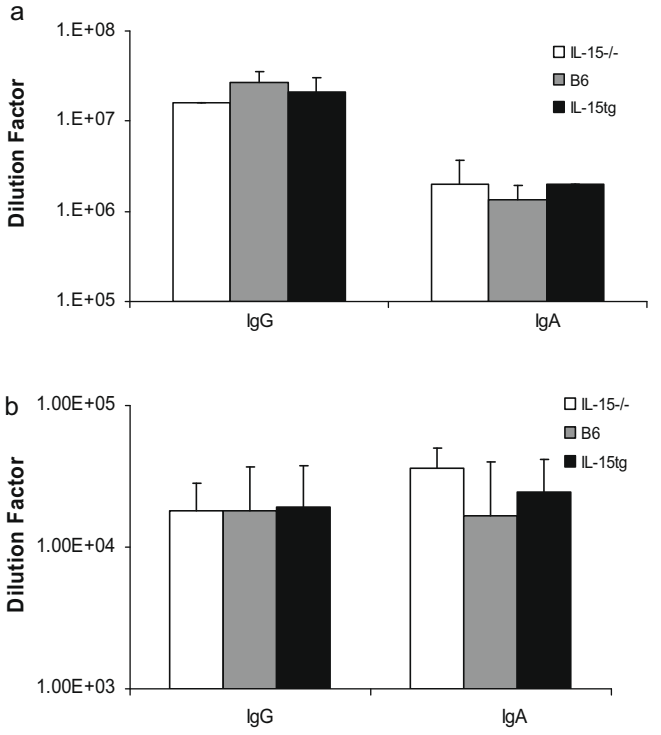

Fig. 5. IL-15$^{-/-}$, B6, and IL-15tg mice produce similar levels of antibody *in vivo*. Six- to eight-week-old female B6, IL-15$^{-/-}$ and IL15tg mice were examined for systemic and mucosal antibody levels. (a) Serum was collected from mice and levels of IgG and IgA were measured using an ELISA. There were no significant differences found in the levels of IgG and IgA between the three strains of mice. (b) Vaginal washes were collected for 5 days and pooled for measurement of IgG and IgA using an ELISA. Again, no significant differences were found in the antibody levels between the three strains of mice. All graphs depict the means of five mice from each strain.

nism mediated through cytokines such as IFN-γ, we carried out an *in vitro* proliferation assay, stimulating B cells with rIL-15, NK cells or rIFN-γ. In previous *in vitro* experiments, IL-15 has been shown to increase proliferation of B cells by up to 20-fold [24–26]. Our results are in agreement with these observations, although proliferation did not occur to the same extent as previously reported. However, the observations *in vivo* suggest that the relationship between IL-15 and B lymphocytes is more complex than suggested. Since IL-15tg mice have higher levels of IL-15, they would be expected to have an increase in B cells according to the reports by other authors, yet they have less B cells than control B6 mice. Our results indicate that IFN-γ may be the factor most important for B cell homeostasis in mice expressing varying levels of IL-15, as it was able to reduce LPS-induced proliferation of B cells; this is in agreement with the *in vitro* results reported by Nakajima et al. [27]. Furthermore, IFN-γ has previously been shown to decrease B cell proliferation in other models, possibly through the cyclic AMP-responsive element-binding protein-1 (CREB-1) signaling pathway [28–30]. NK cell/B cell co-cultures in our experiments did not significantly alter B cell proliferation. Our results suggest that IL-15 likely affects B cell populations *in vivo* indirectly, through the activation of NK cells which secrete IFN-γ.

Since we have established that the over expression of IL-15 did in fact suppress B cell numbers, we were interested in exploring what effect IL-15 had on the function of B cells. Previous studies have reported that IL-15 is able to induce antibody secretion *in vitro* [25,26]. In our study, we found that B cells from IL-15tg mice produced the greatest amount of both IgG and IgA antibody in response to LPS stimulation, compared to IL-15$^{-/-}$ and B6 B cells. This would suggest that there would also be differences observed

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in antibody levels *in vivo*. In fact, a report by Hiroi et al. suggested that IL-15 increases IgA secretion at certain mucosal sites [26]. However, when we examined total antibody in serum and vaginal washes of IL-15tg, IL-$15^{-/-}$ and B6 mice, we found no significant differences in the antibody levels. This can be explained in part by the differences in the absolute number of B cells in IL-15tg and IL-$15^{-/-}$ mice. IL-15tg mice have fewer B cells, however their B cells are capable of producing more antibody per B cell, while IL-$15^{-/-}$ mice have far more B cells, but they produce lower levels of antibody per cell, resulting in similar levels of antibody in the two strains of mice. The study by Hiroi et al. examined B cells treated *in vitro* with rIL-15 and also wild type mice that were treated with rIL-15 *in vivo*, which may not have been enough to cause changes in the NK cell population and subsequently the B cell population [26]. The results of these experiments suggest that in fact, IL-15 can augment B cell function by inducing antibody production. However, in an *in vivo* environment excess IL-15 also indirectly alters the total B cell population, dampening its positive effects on B cell function.

In this report we have demonstrated the role of IL-15 on B cell proliferation and function using both *in vivo* and *in vitro* techniques. Our results clearly show that IL-15 acts indirectly through NK cell-derived IFN-γ to inhibit B cell proliferation and hence impact B cell homeostasis. Our study also indicates that IL-15 can influence B cell function and increase antibody production; however the mechanism of this action remains to be clarified. This study also brings to light the need to study relationships between immune factors in the environment in which they act. It has been reported that treatment of B lymphocytes with IL-15 does increase proliferation *in vitro*, and our results agree with these findings as well. However, the same results are not obtained *in vivo*, since IL-15 also affects other immune parameters. To our knowledge, this report is the first to describe the *in vivo* role of IL-15 and B cells. The effects of IL-15 on various other cell types is well established, and understanding the relationship between B cells and IL-15 is essential in designing effective and safe therapies using IL-15.

Acknowledgments

We thank Ying Chuyan for preparation of the anti-NK1.1 antibody. We thank Elizabeth Davies and Jennifer Newton for technical assistance. This work was supported by a grant from the Canadian Institutes of Health Research (CIHR) to Ali A Ashkar. AAA is a recipient of Career award from Rx&D/CIHR. Navkiran Gill is a recipient of the Ontario Graduate Scholarship.

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Please cite this article in press as: N. Gill et al., Interleukin-15 expression affects homeostasis and function of B cells through NK cell-derived interferon-γ, Cell. Immunol. (2009), doi:10.1016/j.cellimm.2009.03.010