

行政院國家科學委員會專題研究計畫 期中進度報告

STATs 在維持顆粒球及淋巴球數目恆定機轉之研究(1/3)

計畫類別：個別型計畫

計畫編號：NSC92-2320-B-002-189-

執行期間：92年08月01日至93年07月31日

執行單位：國立臺灣大學醫學院免疫學研究所

計畫主持人：李建國

計畫參與人員：博士班研究生 周威君，碩士班研究生 劉大鳴

報告類型：精簡報告

處理方式：本計畫可公開查詢

中 華 民 國 93 年 5 月 31 日

行政院國家科學委員會補助專題研究計畫

成果報告

期中進度報告

(計畫名稱) STATs在維持顆粒球及淋巴球數目恆定機轉之研究

計畫類別： 個別型計畫 整合型計畫

計畫編號：NSC 91-2320-B-002-189

執行期間：92年8月01日至93年07月31日

計畫主持人：李建國

共同主持人：

計畫參與人員：博士班研究生 周威君，碩士班研究生 劉大鳴

成果報告類型(依經費核定清單規定繳交)： 精簡報告 完整報告

本成果報告包括以下應繳交之附件：

赴國外出差或研習心得報告一份

赴大陸地區出差或研習心得報告一份

出席國際學術會議心得報告及發表之論文各一份

國際合作研究計畫國外研究報告書一份

處理方式：除產學合作研究計畫、提升產業技術及人才培育研究計畫、

列管計畫及下列情形者外，得立即公開查詢

涉及專利或其他智慧財產權， 一年 二年後可公開查詢

執行單位：國立臺灣大學醫學院免疫學研究所

中華民國 93 年5月31日

中文摘要

關鍵字: STAT1, STAT3, SDF-1, B 淋巴球的發生, 趨化作用

STAT 蛋白質能夠被造血細胞素如 G-CSF 及 IL-7 及趨化素如 SDF-1 所活化, 可是 STATs 在造血過程的角色則不是很清楚。在此我們利用骨髓細胞中缺乏 STAT1 或 STAT3 的基因轉殖鼠去探討 STAT1 及 STAT3 在造血過程的角色, 我們觀察到 STAT1 或 STAT3 缺失的老鼠的週邊血液中的 B 細胞有降低的現象, 而骨髓中的 pre-B 和 immature B 的數目亦減半, 對淋巴球發生極為重要的細胞素如 IL-7 在較高劑量下對這些缺乏 STAT1 或 STAT3 的骨髓細胞所引發的細胞增生現象則是受到抑制。相反地, 趨化素對骨髓細胞的趨化作用則不受 STAT1 或 STAT3 的調控, 即使 G-CSF 對 CXCR4 的負調節作用在缺乏 STAT1 或 STAT3 的骨髓細胞中已經消失。這些結果顯示 STAT1 或 STAT3 對 IL-7 所引發的 B 淋巴球的發生的確有重要的功能。然而, 其真正的機轉則有待進一步的生化分析才能瞭解。

Abstract

Keywords: STAT1, STAT3, SDF-1, B lymphopoiesis, Chemotaxis

STAT proteins are activated by hematopoietic cytokines like G-CSF and IL-7 and chemokines like SDF-1. However, the role of STATs in hematopoiesis is not clear. Here the function of STAT1 and STAT3 in hematopoiesis is addressed using mice lacking STAT1 and STAT3 in the bone marrow. We have demonstrated that STAT1KO and STAT3KO mice displayed B lymphopenia with reduced mature B lymphocytes in the periphery and reduced pre-B and immature B cells in the bone marrow. A reduced proliferative activity was also observed in mice lacking STAT1 or STAT3 in response to IL-7, a critical cytokine for development of B lymphocytes. In contrast to the defect in IL-7-mediated proliferation, SDF-1-driven chemotactic activity was not significantly affected in the mutant mice despite that the down-regulation of CXCR4 by G-CSF is impaired in the mutant cells.. In sum these results suggest that STAT1 and STAT3 play important roles for IL-7-mediated B lymphopoiesis. The detailed mechanisms, however, require further investigation using biochemical analysis.

Introduction

Cytokines required for hematopoiesis, such as IL-3, IL-5, IL-7, GM-CSF, and G-CSF, activate STATs. While *in vitro* studies have demonstrated a critical role for STAT proteins in cytokine-mediated survival, proliferation and differentiation of hematopoietic cells, *in vivo* evidence for the requirement of STAT1 and STAT3 is less clear. Previously, we have shown that mice lacking STAT3 in the bone marrow displayed neutrophilia and lymphopenia, suggesting the involvement of STAT3 in hematopoiesis. A 2-3-fold increase of neutrophils was observed in the periphery of STAT3-deficient mice. Similarly, 2-fold reduction of B but not T cells was seen in the periphery of the mutant mice. Bone marrow cells of STAT3-null mice were hyper-responsive to G-CSF stimulation with enhanced proliferation but comparable apoptosis. Interestingly, prolonged activation of STAT1 and not STAT5 was observed in STAT3-null bone marrow in response to G-CSF, suggesting that STAT3 is a negative regulator and STAT1 is a positive regulator of cytokine-mediated hematopoiesis (Lee et al., 2002).

During development, IL-7 plays an important role for supporting the proliferation and differentiation of B lineage. Mice lacking IL-7 or IL-7 receptors are defective in the development of T and B cell, suggesting that IL-7 signal transduction is critical for lymphopoiesis (Stoddart et al., 2000; von Freeden-Jeffry et al., 1995). While IL-7 activates mainly STAT1 and STAT5, STAT3 is also activated (Lin et al., 1995). The *in vivo* evidence from STAT1- or STAT5-deficient mice suggest that STAT1 or STAT5 alone is not critical for IL-7-mediated B-lymphopoiesis. It is of interest to study the role of STAT3 in the IL-7-driven hematopoiesis.

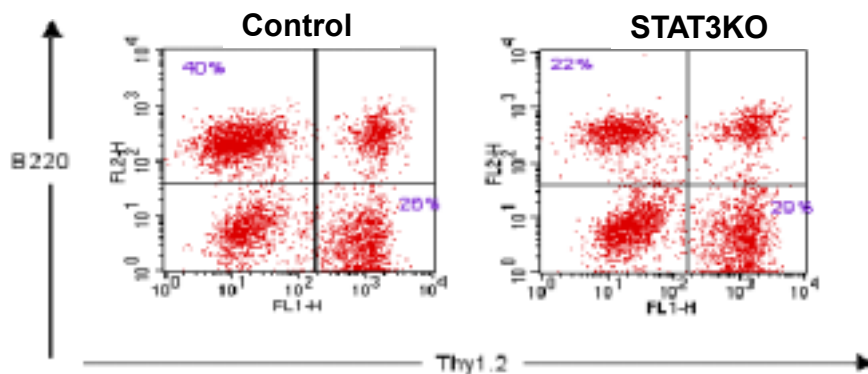
Homeostasis of leukocytes are mediated by two main processes, including hematopoiesis i.e. synthesis of new leukocytes and mobilization i.e. transportation of mature leukocytes from bone marrow to periphery. Several chemokines and their corresponding receptors are required for controlling the process of mobilization. For example, stromal cell-derived factor-1 (SDF-1) and its receptor CXCR4 are important for regulating the mobilization of neutrophils and maybe B lymphocytes. Mice lacking SDF-1 or CXCR4 exhibit impaired development of both lymphoid and myeloid lineage in the bone marrow. However, increased number of neutrophils is observed in the periphery due to the inability of retention of neutrophils in the bone marrow (Ara et al., 2003; Ma et al., 1999b).

Here we used mice lacking STAT1 or STAT3 in the bone marrow to study the role of STAT1 and STAT3 in hematopoiesis and mobilization of neutrophils and B-lymphocytes.

Results

To study the role of STAT3 in hematopoiesis, STAT3 gene was targeted deletion using loxP-Cre system. STAT3 was induced deletion after treatment of MxCre-STAT3^{f/f} mice with poly (I:C). Previously we have shown that mice lacking STAT3 in the bone marrow developed neutrophilia and lymphopenia (Lee et al., 2002). To further dissect the defect in the development of lymphocytes in the absence of STAT3, peripheral blood of control or mutant mice was prepared for FACS analysis using antibody to Thy1.2 and B220. As shown in Fig. 1A, percentage of B-lymphocytes, but not T lymphocytes, was significantly reduced. Since B220 is also expressed on some activated T lymphocytes, antibodies to IgD and IgM were used to further confirm the reduction of B population in STAT3- deficient mice. As shown in Fig. 1B, surface IgD and IgM double positive mature B cells of STAT3KO mice were decreased in peripheral blood as much as ~2 folds compared to that of control animal.

A.



B.

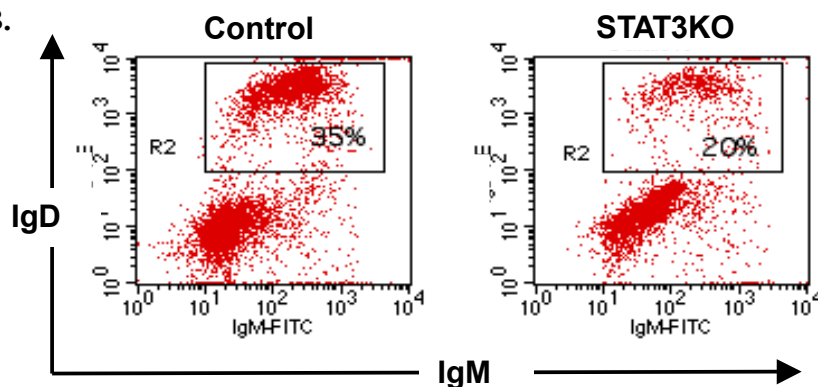


Fig. 1 Reduced B, but not T, cells in the mice lacking STAT3. Peripheral blood cells of control or STAT3KO mice were stained with antibodies to B220-PE and Thy1.2-FITC (A) or IgD-PE and IgM-FITC (B) followed by FACS analysis.

To investigate the effect of STAT3 on the development of B lineage, bone marrow cells from control, STAT1KO, or STAT3KO mice were triple stained with antibodies to CD43, IgM, and B220. As shown in Fig. 2A, while percentage of CD43⁺B220⁺ pro-B cells was similar in both mice, CD43⁻B220⁺ pre-B or immature B population was dramatically reduced in the bone marrow of STAT3KO mice when compared to control mice. To investigate if the role of STAT3 in B development was specific, mice lacking STAT1 were also included. As shown in Fig. 2B, while the percentage of pre-B was reduced significantly in STAT1KO and STAT3KO mice, immature B cells were only decreased in STAT3KO mice. These results suggested that STAT1 and STAT3 were both required for the development of pre B cells, while STAT3 was needed for both pre-B and immature B stage during B cell development.

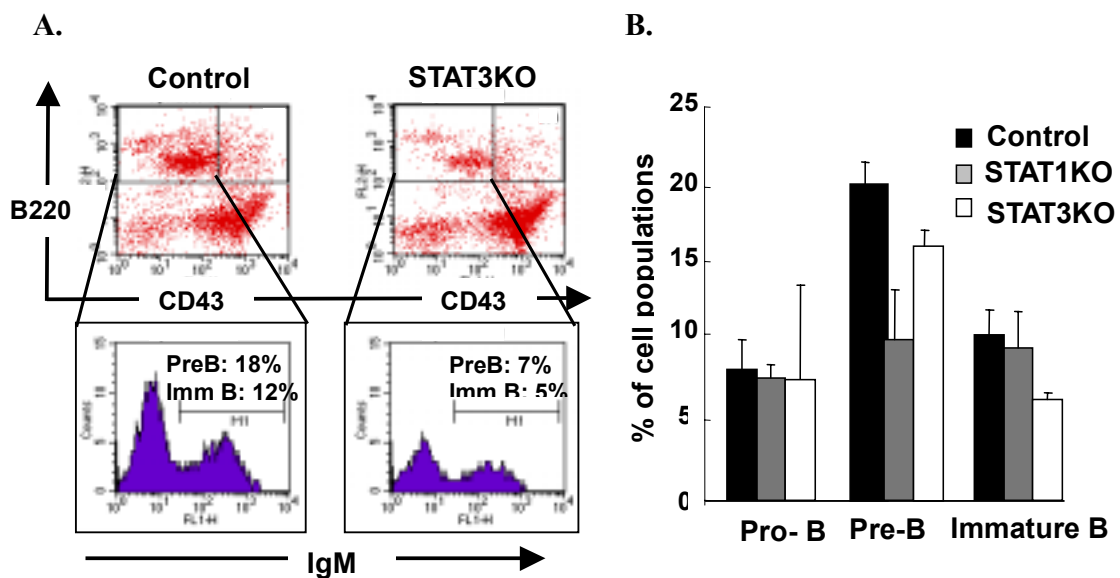


Fig. 2 Reduced pre-B and immature B cells in mice lacking STAT3. Bone marrow of control, STAT3KO (A) was prepared for FACS analysis using antibodies to CD43, IgM, and B220. CD43⁺B220⁻ cells were gated for further analysis for IgM expression (lower panels). CD43⁺B220⁺IgM⁻ cells are pro-B population. CD43⁻B220⁺IgM⁻ cells are pre-B population. CD43⁻B220⁺IgM⁺ cells are immature B population. (B) Bone marrow of control (solid), STAT1KO (shade), and STAT3KO (empty) mice was prepared and analyzed using the same method as described in (A). n=4

Mice lacking IL-7 or IL-7 receptors are defective in the development of T and B cell, suggesting that IL-7 signal transduction is critical for lymphopoiesis (Stoddart et al., 2000; von Freuden-Jeffry et al., 1995). The development of B cells is arrested at pro-B stage in the bone marrow in both mutant mice. Since IL-7 activates STAT1, STAT3, and STAT5, it

is of interest to investigate if the response to IL-7 is affected in the absence of STAT1 or STAT3. Bone marrow of control or STAT3KO mice was subject o proliferation assay using different concentration of IL-7. As shown in Fig. 3A, while the proliferative response in control and mutant mice are comparable at low dose of IL-7 (1 ng/ml), a reduced activity was observed in mice lacking STAT1 or STAT3 in response to higher concentration of IL-7. In fact, the proliferation of bone marrow from STAT3KO mice at was not further enhanced by high IL-7 treatment. The impaired proliferative activity of bone marrow from mutant mice was specific to IL-7 because IL-3-mediated proliferation was comparable between these three mice (Fig. 3B). These results suggested that the impaired development of B lymphocyte maybe due to altered signals in the absence of STAT1 or STAT3.

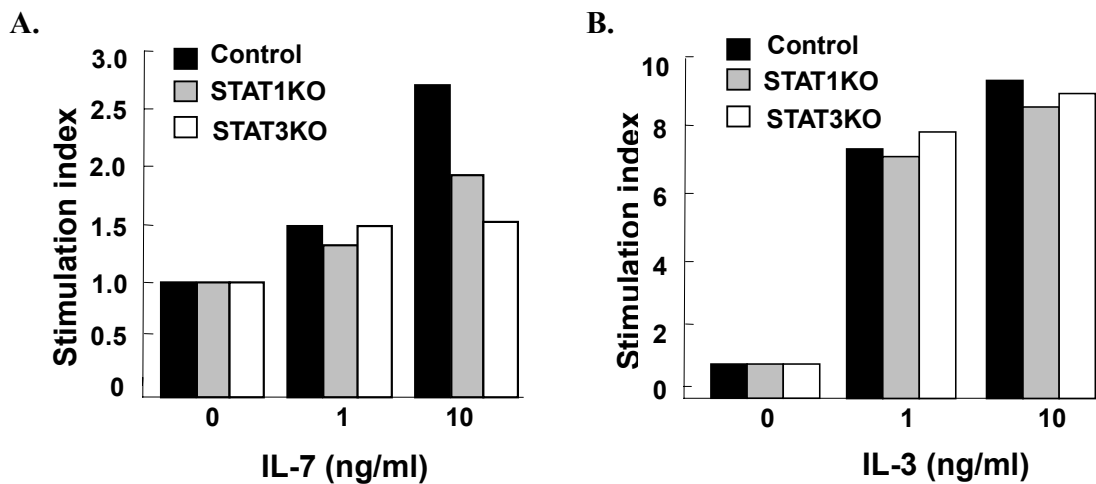


Fig. 3 Reduced proliferative activity of STAT1KO or STAT3KO bone marrow cells in response to IL-7. 2×10^5 bone marrow cells of control (solid), STAT1 (shade), or STAT3KO (empty) mice were stimulated with IL-7 (A) or IL-3 (B) for 24 h at indicated doses followed by proliferative assay using ^3H -thymidine incorporation assay. Stimulation index was calculated by the following formula. $\text{cpm}^{(\text{with cytokine})} / \text{cpm}^{(\text{medium alone})}$.

In fact, the phenotypes of neutrophilia and B lymphopenia seen in STAT3 conditional knockout mice were very similar to mice lacking SDF-1 (CXCL12) or its receptor, CXCR4 (Ma et al., 1999a). The chemokine SDF-1 signals through CXCR4 receptors expressed on a variety of lymphohematopoietic cells. It is a potent chemotactic factor for mobilization of leukocytes from bone marrow to periphery and for recruiting leukocytes to inflammatory sites (Nagasawa et al., 1999). G-CSF has been shown to down-regulate the expression of SDF-1 on the hematopoietic progenitor cells resulting in mobilization of granulocytes from bone marrow to periphery (Petit et al., 2002). Indeed, the mRNA

levels of SDF-1 and CXCR4 on bone marrow cells are significantly decreased in control cells after G-CSF treatment for 24h (Fig. 4, left panels). To investigate the regulation of expression of CXCR4 and SDF-1 in mice lacking STAT1 or STAT3, bone marrow cells from these two mutant mice were also subject to G-CSF treatment. As shown in the Fig. 4 middle and right panels, the levels of both CXCR4 and SDF-1 were decreased in the absence of STAT1 or STAT3. While the expression of CXCR4 remained throughout the course of treatment, SDF-1 expression was reduced in STAT3KO but not STAT1KO cells after 24 h treatment of G-CSF. These results suggested that the STAT1 or STAT3 was required for down-regulation of CXCR4 by G-CSF.

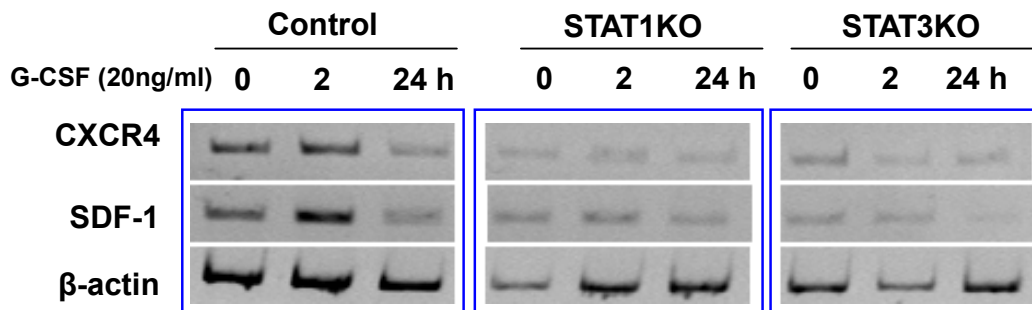


Fig. 4. Impaired down-regulation of CXCR4 in bone marrow of STAT1KO or STAT3KO mice in response to G-CSF. Bone marrow of control, STAT1KO, or STAT3KO mice was treated with or without G-CSF 10 ng/ml for 2 h and 24h. Total RNA was prepared for RT-PCR using primers specific for SDF-1, CXCR4 and β -actin.

SDF-1-CXCR4 axis is critical for mobilization and migration of mature granulocyte and B-lymphocytes from bone marrow to periphery. The mobilization and migration is mainly mediated by chemotaxis between the chemokine SDF-1 and its receptor, CXCR4. To investigate the effect of STAT3 on the chemotactic activity, transwell migration assay was performed. Bone marrow cells of control mice were loaded on the upper chamber separated with a 5 A pore-sized membrane coated with or without collagen IV. The transmigrated cells were stained with antibodies to Gr-1 (for granulocytes) or CD19 (for B cells). As shown in Fig. 5B, the transmigrated cells increased as the concentration of SDF-1 in the lower chamber increased. The highest number of migrated cells was using 500 ng/ml SDF-1. However, no obvious effect on migration was observed with or without coating of collagen on the membrane. A 50% reduction of CD19⁺ B cells but slightly increased percentage of Gr-1⁺ granulocytes in the input bone marrow cells of STAT3KO mice (Fig. 5A upper panels). After transmigration, there was a decreased

CD19⁺ B-cells of control and STAT3KO mice. However, the ratio of transmigrated cells between control and STAT3KO mice was very unchanged. These results suggested that the SDF-1-mediated chemotaxis was not affected in the absence of STAT3.

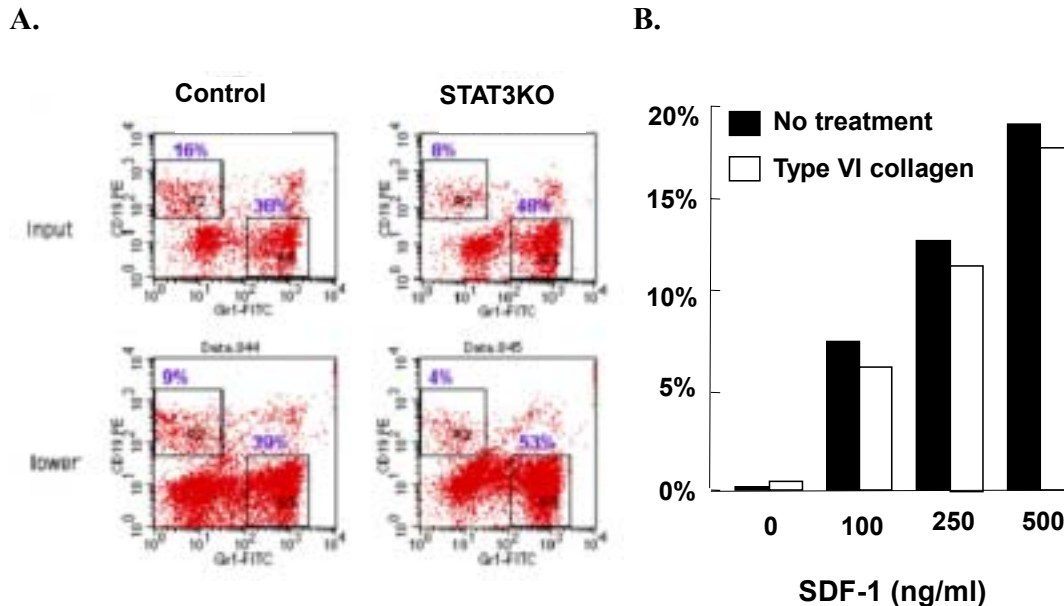


Fig. 5 SDF-1-CXCR4-mediated chemotaxis was not affected by the absence of STAT3. (A) Transwell migration assay was performed by loading 1×10^6 bone marrow cells of control (left panels) or STAT3KO mice (right panels) on the upper wells of transwell plate. The membrane in the upper well was coated with collagen type VI followed by adding 100 ng/ml SDF-1 in the lower chamber. The transmigrated cells in the lower wells were collected and stained with CD19-PE and Gr-1-FITC followed by FACS analysis. (B) The transwell migrated was performed as described in (A). The membrane in the upper well was either coated with or without collagen type IV (1 μ g/ml) followed by adding indicated concentration of SDF-1 in the lower well. The percentage of migrated cells was calculated by dividing the number of transmigrated cells with the input number (1×10^6).

Since IL-7 and SDF-1 were reported to activate STATs in cells (Hernanz-Falcon et al., 2004), it is likely that the signaling pathways transduced by these two cytokines were affected in the absence of STAT1 and/or STAT3. Bone marrow or spleen cells were treated with SDF-1 or IL-7 for 30 min and the activation of STAT1 or STAT3 was monitored. As shown in Fig. 6, IL-7 readily activated STAT1 and STAT3 in the spleen, however, the response in the bone marrow was hardly detectable. In contrast, STAT1 but not STAT3 was slightly activated by SDF-1 in the spleen, although no signal was detected in the bone marrow. Interestingly, the protein levels of STAT3 were greatly increased in spleen as opposed to in the bone marrow. It is not clear if the activation of STATs will be

defected in the absence of STAT1 or STAT3 in response to IL-7 or SDF-1.

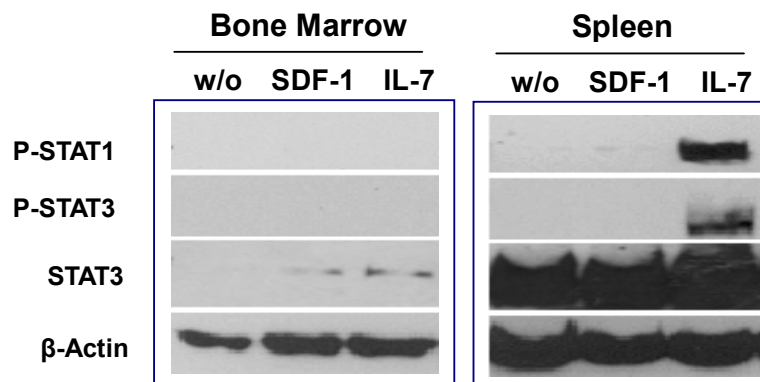


Fig. 6 Activation of STAT1 and STAT3 in response to IL-7. Total cell extracts prepared from bone marrow (left panels) or spleen (right panels) were subject to Western blotting using antibodies to phosphotyrosyl-STAT1 (P-STAT1), phosphotyrosyl-STAT3 (P-STAT3), STAT3, or β -actin.

Discussion

Here we have demonstrated a role of STAT1 and STAT3 in the development of B lymphocytes. A reduced number of peripheral B, but not T, cells are observed in the mice deficient in STAT3. Reduced numbers of pre-B and immature B cells are also observed in the bone marrow of STAT3-deficient mice. Since STAT1 and STAT3 is activated by IL-7, a critical cytokine for T and B development, it is likely that the response of IL-7 is impaired in the absence of STAT3. Indeed, a reduced proliferative response was observed in the bone marrow of STAT1- or STAT3-deficient mice when stimulated with higher doses of IL-7. It is still not clear why B but not T cells are affected, although IL-7 apparently exerts its effect on both populations. One possibility is that other than IL-7, a B cell-specific factor that is equally important for B cell development is impaired in the absence of STAT1 or STAT3.

In contrast to the defect in the development of B lymphocytes, mobilization of B cells from bone marrow is not affected in STAT1- or STAT3-deficient mice. In the trans-migration assay, a comparable chemotactic activity of bone marrow B lineage of STAT3-deficient mice was observed when stimulated with SDF-1.

Obviously, there are several directions that needed to be addressed to unveil the function of STAT3 on B lymphopoiesis and mobilization. First of all, although STAT1 and STAT3 is activated by IL-7, but the main effect of IL-7 is thought mediated mainly by STAT5. It is therefore somewhat surprising to see the dramatic effect of STAT3. One immediate question is the status of STAT1 and STAT5 activation by IL-7 in the absence

of STAT3. Of course, we also need to examine if IL-7 receptor levels are comparable in the STAT3-deficient cells. Recently, STAT3 has been demonstrated to be required for Flt3L-mediated DC development. Mice lacking STAT3 in the DC resulted in impaired development and function of DC (Laouar et al., 2003). Since Flt3L is also required for B cell development, it is of great interest to investigate if Flt3L signaling is affected in the absence of STAT1 or STAT3. In sum, we have demonstrated the unexpected role of STAT1 and STAT3 in the B lymphopoiesis. We will further study the underlying mechanisms using biochemical and genetic approaches in the near future.

Self Evaluation

We have documented a novel role of STAT3 in B lymphopoiesis. This probably is due to the defect of IL-7 responsiveness in the absence of STAT3. Further studies need to be done to confirm this possibility. STAT1 and STAT3 are both activated by IL-7, although the main signal transducer is STAT5. It is of interest to investigate if STAT5 activated is modulated or compensated in the absence of STAT1 or STAT3. We have observed the effect of STAT1 and STAT3 in B-lymphopoiesis. However, we have not assessed the biological significance of this defect. It is of great interest to understand the immune response after challenging the mutant mice with defined T-dependent or T-independent antigens, or well-characterized pathogens like viruses or bacteria.

Although the effect of STAT3 in the development of neutrophils is more or less well characterized, it is yet to clarify the role of STAT3 in mobilization of neutrophils. Obviously, more experiments are required to unveil the mechanisms.

For the moment, the causes of immunodeficiency or autoimmunity are still unclear. The abnormal development or homeostasis of B cells or neutrophils resulting from mutations in STAT1 or STAT3 may account for the defect or imbalance of immune responses. Therefore, the study in STAT3-deficient mice may provide us with a clue to understand and maybe to treat the abnormality of the immune system.

References

- Ara, T., Tokoyoda, K., Sugiyama, T., Egawa, T., Kawabata, K., and Nagasawa, T. (2003). Long-term hematopoietic stem cells require stromal cell-derived factor-1 for colonizing bone marrow during ontogeny. *Immunity* 19, 257-267.
- Hernanz-Falcon, P., Rodriguez-Frade, J. M., Serrano, A., Juan, D., del Sol, A., Soriano, S. F., Roncal, F., Gomez, L., Valencia, A., Martinez, A. C., and Mellado, M. (2004). Identification of amino acid residues crucial for chemokine receptor dimerization. *Nat*

Immunol 5, 216-223.

Laouar, Y., Welte, T., Fu, X. Y., and Flavell, R. A. (2003). STAT3 is required for Flt3L-dependent dendritic cell differentiation. *Immunity* 19, 903-912.

Lee, C. K., Raz, R., Gimeno, R., Gertner, R., Wistinghausen, B., Takeshita, K., DePinho, R. A., and Levy, D. E. (2002). STAT3 is a negative regulator of granulopoiesis but is not required for G-CSF-dependent differentiation. *Immunity* 17, 63-72.

Lin, J. X., Migone, T. S., Tsang, M., Friedmann, M., Weatherbee, J. A., Zhou, L., Yamauchi, A., Bloom, E. T., Mietz, J., John, S., and et al. (1995). The role of shared receptor motifs and common Stat proteins in the generation of cytokine pleiotropy and redundancy by IL-2, IL-4, IL-7, IL-13, and IL-15. *Immunity* 2, 331-339.

Ma, Q., Jones, D., and Springer, T. A. (1999a). The chemokine receptor CXCR4 is required for the retention of B lineage and granulocytic precursors within the bone marrow microenvironment. *Immunity* 10, 463-471.

Ma, Q., Jones, D., and Springer, T. A. (1999b). The chemokine receptor CXCR4 is required for the retention of B lineage and granulocytic precursors within the bone marrow microenvironment. *Immunity* 10, 463-471.

Nagasawa, T., Tachibana, K., and Kawabata, K. (1999). A CXC chemokine SDF-1/PBSF: a ligand for a HIV coreceptor, CXCR4. *Adv Immunol* 71, 211-228.

Petit, I., Szyper-Kravitz, M., Nagler, A., Lahav, M., Peled, A., Habler, L., Ponomaryov, T., Taichman, R. S., Arenzana-Seisdedos, F., Fujii, N., *et al.* (2002). G-CSF induces stem cell mobilization by decreasing bone marrow SDF-1 and up-regulating CXCR4. *Nat Immunol* 3, 687-694.

Stoddart, A., Fleming, H. E., and Paige, C. J. (2000). The role of the preBCR, the interleukin-7 receptor, and homotypic interactions during B-cell development. *Immunol Rev* 175, 47-58.

von Freeden-Jeffry, U., Vieira, P., Lucian, L., McNeil, T., Burdach, S., and Murray, R. (1995). Lymphopenia in interleukin (IL)-7 gene-deleted mice identifies IL-7 as a nonredundant cytokine. *J Exp Med* 181, 1519-1526.