Brief Report

Analysis Tissue Expression of IFN-γ in IL-12 and/or IL-18 Gene Ablated Naïve Mice

Jianhong Zhang[1, 2], Haiming Wei[1] and Zhigang Tian[1, 3]

Interleukin 12 (IL-12) and/or interleukin 18 (IL-18) gene ablated mice were applied for the investigation of the tissue expression of interferon γ (IFN-γ). For IL-12$^{-/-}$, IL-18$^{-/-}$, IL-12$^{-/-}$/18$^{-/-}$ and wt mice, reproductive performance were recorded and IFN-γ concentrations in heart, lung, liver, spleen, kidney and serum were quantified by ELISA. There were no significant differences of IFN-γ in heart, lung and kidney between 4 strains although control group was higher. It was observed that for IL-12$^{-/-}$ mice, compared with other 3 groups, IFN-γ in liver and spleen were decreased ($p < 0.05$) and reproductive performance appeared to be impaired. Serum IFN-γ level of IL-12$^{-/-}$/18$^{-/-}$ mice was significantly higher ($p < 0.05$). It was showed that IFN-γ productions under the normal condition were independent upon IL-12 and IL-18, its expressions in various tissues were different, and optimal IFN-γ is necessary for the normal growth and development of mammals. This study is helpful for clinical cytokines therapy. *Cellular & Molecular Immunology*. 2005;2(1):68-72.

Key Words: IFN-γ, IL-12, IL-18, gene ablated mice

Introduction

Interferon γ (IFN-γ), as a type 1 cytokine that plays a key role in the regulation of both innate and acquired immunity, regulate many hundreds of genes and exert a multitude of cellular biological effects (1, 2). IFN-γ is produced mainly by activated lymphocytes and has receptors on virtually all cell types of the body, so that many organs and systems undergo the action of IFN-γ. The expression of IFN-γ is regulated by a set of complex interactions between accessory cells, such as macrophages and dendritic cells, and T lymphocytes and natural killer (NK) cells (3).

IL-12, a heterodimeric cytokine composed of p40 and p35 chains, is the most important stimulus for upregulation of IFN-γ mRNA expression and IFN-γ production in different T, NK cell subsets (4). IL-12 was initially recognized as an inducer of IFN-γ synthesis in resting human peripheral blood mononuclear cells *in vitro* (5). It is now recognized that IL-12 induces IFN-γ production in both resting and activated human T and NK cells *in vivo* (6-8). IL-18, originally called interferon-γ inducing factor, is a cytokine of 18 kD synthesised by Kupffer cells and activated macrophages (9, 10). Striking synergy has been observed between IL-12 and IL-18 in induction and release of IFN-γ (11). However, Mice doubly deficient for IL-12p40 and IL-18 are viable and fertile (12) and the relative importance of IL-12 and IL-18 has not been fully examined.

To address the roles of IL-12 and IL-18 in the IFN-γ production of naïve mice, and back up clinical choice for cytokine therapy against related diseases, IFN-γ concentrations of various tissues extraction of IL-12$^{-/-}$, IL-18$^{-/-}$, IL-12$^{-/-}$/18$^{-/-}$ mice and their congenic strain (wt) were studied.

Materials and Methods

Animals

Mice genetically ablated for IL-12/18 on a C57BL/6J background were kept under barrier husbandry. Intercrosses were used to establish 4 sublines: IL-12$^{+/+}$/18$^{+/+}$ (used as normal congenic controls, wild type); IL-12$^{-/-}$/18$^{+/+}$ (IL-12$^{-/-}$); IL-12$^{+/+}$/18$^{-/-}$ (IL-18$^{-/-}$) and IL-12$^{-/-}$/18$^{-/-}$. Genotypes of those mice were obtained by PCR using tail DNA. For IL-12$^{-/-}$ mice, primers a and b were used to detect the wild-type allele, and primers b and c to mutated allele. For IL-18$^{-/-}$ mice, primers d and e were used to detect the wild-type allele, and

[1]School of Life Sciences, Institute of Immunology, University of Science and Technology of China, Hefei, Anhui 230027, China.

[2]Laboratory Animal Center, Shanxi Medical University, Taiyuan, Shanxi 030001, China.

[3]Corresponding to: Dr. Zhigang Tian, Institute of Immunology, School of Life Sciences, University of Science & Technology of China, Hefei 230027, China. Tel: +86-551-360-7379, E-mail: tzg@ustc.edu.cn.

Received Sep 30, 2004. Accepted Dec 24, 2004.

Abbreviations: IFN-γ, interferon γ; IL-12, interleukin 12; IL-18, interleukin 18; NK, natural killer.

primers e and c to the mutated allele. The primer sequences were: a, 5'-CAC TTG CCA AAC TCC TGT GAG CTA TGA-3'; b, 5'-TTC TTG TGG AGC AGC AGA TGT GAG TGG-3'; c, 5'-ATC GCC TTC TAT CGC CTT CTT GAC GAG-3'; d, 5'-TAA TGG GTG GTC TTC TCA TCT CTG TGT-3'; e, 5'-TTG CTG CAC CTA GAG GTA TGT ACT GAC-3'. The PCR conditions were used that hot start at 94°C for 4 min, and 35 cycles of 94°C for 30 sec, 67°C for 30 sec, 74°C for 1 min, then 74°C for 7 min prior to cooling to 4°C.

Tissue acquisition for IFN-γ quantification

To quantify IFN-γ concentration, blood was collected before cervicall dislocation, followed by centrifuge at 4°C to get sera. Mice heart, lung, liver, kidney and spleen were also collected and weighted, then minced in 1.5 ml microcentrifuge tubes containing 800 μl RPMI 1640 medium with 10% fetal calf serum (FCS) and immediately homogenized using a micropestle (Fisher Scientific, Nepean, ON) on ice. Samples were centrifuged (800 g, 4°C, 15 min). All supernatants were collected and stored at -20°C until analyzed for IFN-γ.

Quantification of IFN-γ by ELISA

Dilute capture antibody (purified rat anti-mouse IFN-γ antibody, Pharmingen, Mississauga, ON) at a concentration of 1 μg/ml in coating buffer (0.1 M Na_2HPO_4), was added in a volume of 50 μl to each well of the ELISA plate (Dynex Technologies Inc., Chantilly, VA). Plates were sealed with plastic film and incubated overnight at 4°C. Plates were washed, blocked (10% FCS in PBS) and emptied. Samples and recombinant mouse IFN-γ standards (Sigma, Oakville, Canada) were added to triplicate wells (100 μl/well) and plates were incubated (4°C, overnight). After washing, 50 μl 0.5 μg/ml of biotinylated detection antibody (XMG1.2 rat anti-mouse IFN-γ antibody, Pharmingen) were added for 60 min at RT, followed by 100 μl horseadish peroxidase avidin D (1:3,000; Vector Laboratories, Burlingame, CA) for 30 min at RT. Substrate (100 μl; 2, 2'-azino 3-ethylbenzthiazoline-6-sulfonic acid, 1 mg/ml; 0.003% H_2O_2; 0.1 M citric acid) was added to each well. After 60 min at RT, absorbance was measured at a wavelength of 405 nm. IFN-γ concentrations were determined using a SAS program against serially diluted recombinant mouse IFN-γ. Final results for IFN-γ of heart, lung, liver, kidney and spleen were correlated to the net tissue weight.

Statistical analysis

Results were analyzed by Student's *t*-test, $p < 0.05$ was accepted as the level of significance. All values were shown as mean ± SD.

Results

Reproductive performance of IL-12 and/or IL-18 gene knockout mice

Litter size at birth was recorded over 18 months of breeding for IL-12$^{-/-}$, IL-18$^{-/-}$, IL-12$^{-/-}$/18$^{-/-}$ mice and their congenic, genetically normal subline (Figure 1A). Only IL-12$^{-/-}$ mice showed significant difference from the wt in numbers born (litter size is fewer) ($p < 0.05$). For females killed during gestation, fetal resorption was occasionally observed. Resorptions were significantly higher in the IL-12$^{-/-}$ strain compared to the other strains (Figure 1B) ($p < 0.05$). No significant difference was observed between IL-18$^{-/-}$, IL-12$^{-/-}$/18$^{-/-}$ and wt mice.

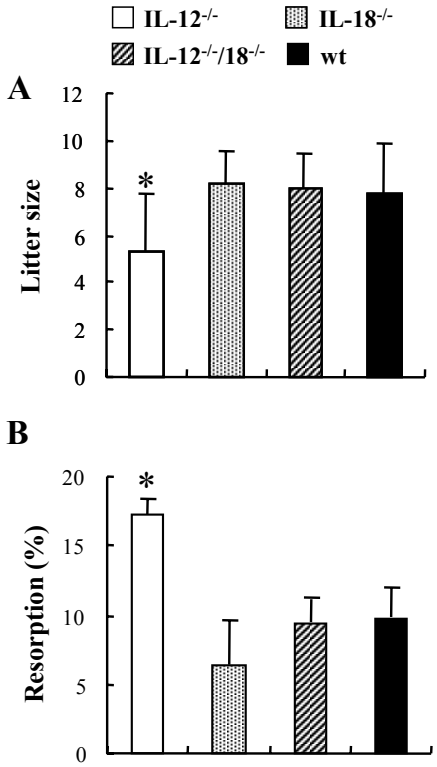

Figure 1. Reproductive performance of IL-12 and/or IL-18 gene ablated mice. Litter size at birth and resorption ratios of implantation sites were carefully recorded over 18 months of breeding for IL-12$^{-/-}$, IL-18$^{-/-}$, IL-12$^{-/-}$/18$^{-/-}$ and genetically normal subline (wt) mice (*$p < 0.05$).

IFN-γ concentrations in various tissues' extractions

There is no significant difference of IFN-γ concentrations in heart and kidney between 4 strains, although the value of wt mice was highest (Figures 2A, 2B). It was observed that IFN-γ concentrations in liver and spleen of IL-12$^{-/-}$ were significantly lower compared with IL-18$^{-/-}$, IL-12$^{-/-}$/18$^{-/-}$and wt mice ($p < 0.05$) (Figures 2D, 2E). For IL-12$^{-/-}$/18$^{-/-}$, IFN-γ concentration of lung was decreased but no significant difference compared with IL-12$^{-/-}$, IL-18$^{-/-}$ and wt mice (Figure 2C), meanwhile we got unexpected results that those mice didn't display impaired production of IFN-γ (Figures 2A, 2B, 2D, 2E) in the absence of two important IFN-γ inducers, IL-12 and IL-18. Also data showed significantly higher IFN-γ concentration in serum of IL-12$^{-/-}$/18$^{-/-}$ mice compared with others ($p < 0.05$) (Figure 2F).

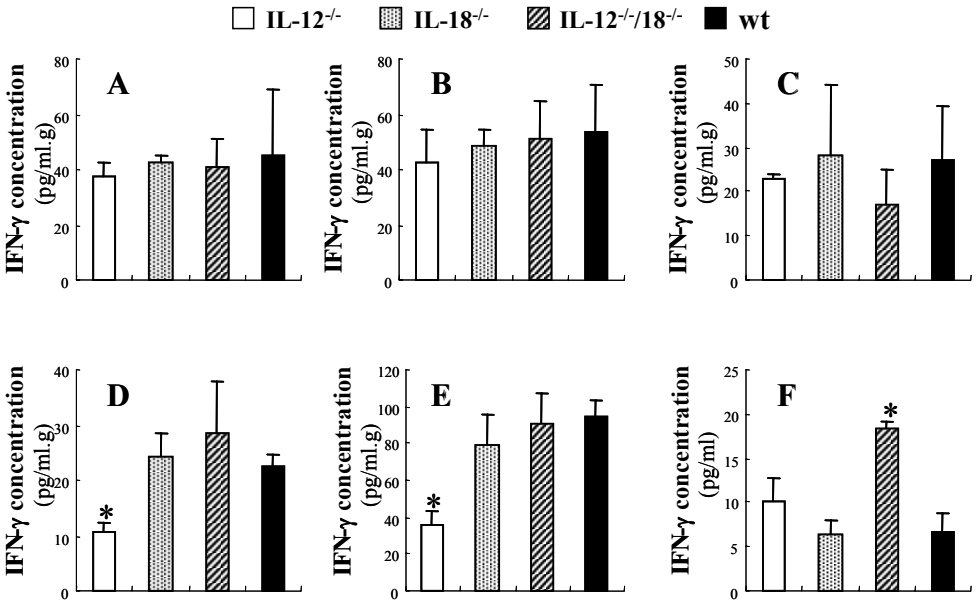

Figure 2. **IFN-γ concentrations in various tissues' extractions.** For IL-12$^{-/-}$, IL-18$^{-/-}$, IL-12$^{-/-}$/18$^{-/-}$ and genetically normal subline (wt) mice, different tissue including A-heart, B-kidney, C-lung, D-liver, E-spleen, F-serum were obtained and the levels of IFN-γ were determined by ELISA study. Final results were correlated to the net tissue weight (n = 4, *$p < 0.05$).

Discussion

The goal of this study was to investigate tissue expression of IFN-γ of mice with or without IL-12 and IL-18. Our previously study has shown that optimal IFN-γ level is needed for uterine blood vessel remodeling of normal mice pregnancy (13). At present, litter size and fetal resorptions of IL-12$^{-/-}$ mice were significantly lower than IL-18$^{-/-}$, IL-12$^{-/-}$/18$^{-/-}$ and wt strain. This was consistent with the lower IFN-γ concentrations of mice tissues' extractions, especially liver and spleen ($p < 0.05$). Combined with Nakamura and Zhang's study (14, 15) that IL-12/IL-18 treatment, through IFN-γ-dependent or IFN-γ-independent process, could induce severe adverse effects, we suggest that optimal IFN-γ level is necessary for normal growth and development of mammals, but the range still need further investigation.

As a typical lymphokine, IFN-γ was produced almost exclusively by properly activated NK cells and certain subpopulations of T lymphocytes. In fact, most lymphocytes produce soluble factors-cytokines, chemokines, which can serve as endogenous inducers or enhancers of IFN-γ production, also some resting NK cells can produce certain amount of IFN-γ (16). Varma et al. (17) proved that approximately 60% of IFN-γ-producing cells are natural killer (NK) cells (CD3$^-$DX5$^+$) and 25% are NKT cells (CD3$^+$DX5$^+$), most of the remaining IFN-γ-producing cells are T cells (CD3$^+$DX5$^-$), macrophages, and dendritic cells. Our present study demonstrated that absolute IFN-γ concentration in spleen (> 35.5 pg/ml.g for all 4 strains) is higher than that in liver (< 28.6 pg/ml.g for all 4 strains). It was noted that, in liver, around 12.18% hepatic mononuclear cells (NK1.1$^+$, 63.4%; CD3$^+$, 19.6%) produced IFN-γ, while the spleen only 0.68% (CD3$^+$, 44.6%; CD19$^+$, 53.6%) (18). In fact, the absolute number of IFN-γ-producing cells in each organ per mouse were 8.9×10^4 in the liver, 8.2×10^5 in the spleen. Therefore, the contribution of spleen cell systemic IFN-γ production as an absolute number was greater than that in liver.

Whenever IFN-γ is produced somewhere in the body, it also enters the bloodstream circulation but is quickly cleared, so that it becomes detectable in serum in usually small concentrations and for relatively short durations. It was observed in our study that serum IFN-γ concentrations of IL-12$^{-/-}$/18$^{-/-}$ mice were significantly higher than that of IL-12$^{-/-}$, IL-18$^{-/-}$ and wt mice, indicating certain kind of compensating for the loss of both IL-12 and IL-18, which were important co-stimulator to induce IFN-γ production. The roles of IL-12 and IL-18 in the developing Th1-type immunity are widely known and were already discussed (19), and our data here, IFN-γ level in serum of wt (IL-12$^{+/+}$/18$^{+/+}$) mice was lower than that of IL-12$^{-/-}$ and IL-12$^{-/-}$/18$^{-/-}$ mice, was contradictory to classical one. But it can be partly explained by the study of Zenclussen that *ex vivo* stimulation of the peripheral blood cells with increasing doses of IL-12 resulted in a significant decrease of IFN-γ, whereas TNF-α level unaffected (20).

Our results confirmed that endogenous IL-12 play a central role in Th1 cell response, as IFN-γ concentration shown in heart, lung, kidney, liver and spleen (Figure 2), compared IL-12$^{-/-}$ with IL-18$^{-/-}$ and wt group. It was also known IFN-γ production by highly purified T cells is IL-12 independent. Recently two new cytokines, IL-23 and IL-27

were reported contributing to the proliferation and activation of IFN-γ production in NK cells and T cells (13, 21, 22). IL-23 is a member of IL-6 family composed of the IL-12p40 soluble subunit and an IL-12p35 related chain named p19. The p19 chain is inactive biologically without its partner (23). IL-23 signals through IL-12Rβ1 and a novel IL-23R chain (24). IL-27 is composed of 2 chains, p28 and EBI3, analogous to IL-12p40 and IL-12p35 respectively. Compared with IL-12, IL-27 is proved to be a much more potent proliferative stimulus for introducing proliferation of naïve $CD4^+$ T cells *via* the receptor WSX-1/TCCR (25). IL-27 appears to act at an early stage in Th1 response in a manner distinct from IL-12, and IL-18, which act later in Th1 development (26). Thus, IL-12 or IL-18 deficient mice were impaired but not completely lacking in the ability to produce IFN-γ. In the case of deficient both IL-12 and IL-18, there is tendency that IFN-γ production appears to be lower, induced by those cytokines. Because IFN-γ signaling serve as a pivotal role in the immune surveillance system, for example, a decreased level of IFN-γ production play a functional role in the immunosuppression that occurs after major injury or sepsis, SLE patients (27, 28). Lack of endogenous IFN-γ synthesis results in significantly higher type 2 cytokines (IL-4, IL-5, IL-10 and IL-13) and chemokine (TARC) production following exogenous antigen exposure (29). Accordingly, to keep the balance of Th1/Th2 paradigm, IFN-γ production is evoked and enhanced through a novel pathway by complex interactions among cytokines, such as IL-23 and IL-27, in the case of absence of IL-12 and IL-18.

In conclusion, we demonstrated that IFN-γ expressions in various tissues were different, some organs, such as liver and spleen, were more sensitive to Th1 response, and IFN-γ productions were independent upon IL-12 and IL-18, indicating that other factors involved in the Th1 development in naïve mice under normal condition. Further studies, including determination of IFN-γ mRNA expression in different tissues, are needed to give stronger supports for the clinical cytokines therapy.

Acknowledgements

We appreciate the excellent help by Dr. B. Anne Croy (Queenss University, Kingston, Ontario, Canada). We also thank the staff of the OMAFRA Isolation Unit for the outstanding level of husbandry provided to our immunodeficient mice.

References

1. Boehm U, Klamp T, Groot M, Howard JC. Cellular responses to interferon-γ. Annu Rev Immunol. 1997;15:749-795.
2. Boehm U, Guethlein L, Klamp T, et al. Two families of GTPases dominate the complex cellular response to IFN-γ. J Immunol. 1998;161:6715-6723.
3. Shtrichman R, Samuel C. The role of γ interferon in antimicrobial immunity. Curr Opin Microbiol. 2001;4:251-259.
4. Fehniger TA, Shah MH, Turner MJ, et al. Differential cytokine and chemokine gene expression by human NK cells following activation with IL-18 or IL-15 in combination with IL-12: implications for the innate immune response. J Immunol. 1999;162:4511-4520.
5. Kobayashi M, Fitz L, Ryan M, et al. Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. J Exp Med. 1989;170:827-845.
6. Chan SH, Perussia B, Gupta JW, et al. Induction of interferon γ production by natural killer cell stimulatory factor: characterization of the responder cells and synergy with other inducers. J Exp Med. 1991;173:869-879.
7. Gately MK, Desai BB, Wolitzky AG, et al. Regulation of human lymphocyte proliferation by a heterodimeric cytokine, IL-12 (cytotoxic lymphocyte maturation factor). J Immunol. 1991;147: 874-882.
8. Gately MK, Renzetti LM, Magram J, et al. Interleukin-12/ interleukin-12-receptor system: role in normal and pathologic immune responses. Annu Rev Immunol. 1998;16:495-521.
9. Okamura H, Tsutsui H, Komatsu T, et al. Cloning of a new cytokine that induces IFN-γ production by T cells. Nature. 1995; 378:88-91.
10. Okamura H, Tsutsui H, Kashiwamura S, Yoshimoto T, Nakanishi K. Interleukin-18, a novel cytokine that augments both innate and acquired immunity. Adv Immunol. 1998;70: 281-312.
11. Lauwerys BR, Garot N, Renauld JC, Houssiau FA. Cytokine production and killer activity of NK/T-NK cells derived with IL-2, IL-15, or the combination of IL-12 and IL-18. J Immunol. 2000;65:1847-1853.
12. Takeda K, Tsutsui H, Yoshimoto T, et al. Defective NK cell activity and Th1 response in IL-18-deficient mice. Immunity. 1998;8:383-390.
13. Zhang JH, He H, Borzychowski AM, et al. Analysis of cytokine regulators inducing interferon production by mouse uterine natural killer cells. Biol Reprod. 2003;69:404-411.
14. Nakamura S, Otani T, Ijiri Y, Motoda R, Kurimoto M, Orita K.. IFN-γ-dependent and -independent mechanisms in adverse effects caused by concomitant administration of IL-18 and IL-12. J Immunol. 2000;164:3330-3336.
15. Zhang TT, Kazuyoshi K, Mahboob H, Tang Y, Atsushi S. Combined therapy of interleukin-12 and interleukin-18 against cryptococcus neoformans infection in a murine model. Chinese Med J. (Engl) 2003;116:669-672.
16. Rajagopalan S, Fu J, Long EO. Cutting edge: induction of IFN-γ production but not cytotoxicity by the killer cell Ig-like receptor KIR2DL4 (CD158d) in resting NK cells. J Immunol. 2001;167: 1877-1881.
17. Varma TK, Lin CY, Toliver-Kinsky TE, Sherwood ER. Endotoxin-induced γ interferon production: contributing cell types and key regulatory factors. Clin Diagn Lab Immunol. 2002;9:530-543.
18. Otani T, Nakamura S, Toki M, Motoda R, Kurimoto M, Orita K. Identification of IFN-γ-producing cells in IL-12/IL-18-treated mice. Cell Immunol. 1999;198:111-119.
19. Munder M, Mallo M, Eichmann K, Modolell M. Murine macrophages secrete interferon γ upon combined stimulation with interleukin (IL)-12 and IL-18: a novel pathway of autocrine macrophage activation. J Exp Med. 1998;187:2103-2108.
20. Zenclussen AC, Fest S, Busse P, Joachim R, Klapp BF, Arck PC. Questioning the Th1/Th2 paradigm in reproduction: peripheral levels of IL-12 are down-regulated in miscarriage patients. Am J Reprod Immunol. 2002;48:245-251.

21. Oppmann B, Lesley R, Blom B, et al. Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. Immunity. 2000; 13:715-725.
22. Van Seventer JM, Nagai T, van Seventer GA. Interferon-β differentially regulates expression of the IL-12 family members p35, p40, p19 and EBI3 in activated human dendritic cells. J Neuroimmunol. 2002;133:60-71.
23. Parham C, Chirica M, Timans J, et al. A receptor for the heterodimeric cytokine IL-23 is composed of IL-12Rβ1 and a novel cytokine receptor subunit, IL-23R. J Immunol. 2002;168: 5699-5708.
24. Chen Q, Ghilardi N, Wang H, et al. Development of Th1-type immune responses requires the type I cytokine receptor TCCR. Nature. 2000;407:916-920.
25. Pflanz S, Timans JC, Cheung J, et al. IL-27, a heterodimeric cytokine composed of EBI3 and p28 protein, induces proliferation of naïve $CD4^+$ T cells. Immunity. 2002;16:779-790.
26. Robinson DS, O'Garra A. Further checkpoints in Th1 development. Immunity. 2002;16:755-758.
27. Enzler T, Gillessen S, Manis JP, et al. Deficiencies of GM-CSF and interferon γ link inflammation and cancer. J Exp Med 2003; 197:1213-1219.
28. Docke WD, Randow F, Syrbe U, et al. Monocyte deactivation in septic patients: restoration by IFN-γ treatment. Nat Med. 1997; 3:678-681.
29. Lewkowich IP, HayGlass KT. Endogenous IFN-γ and IL-18 production directly limit induction of type 2 immunity in vivo. Eur J Immunol. 2002;32:3536-3545.