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Illustration 1

Illustration 2

Legend for Illustration 1

Legend for Illustration 2

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Abstract

The immune response in IL-4-knockout mice to murine interleukin-4 (IL-4) at immunization doses in range of 1-10 μg was evaluated. Different adjuvants, anti-IL-4 carrier antibodies, and cross-activation monoclonal antibodies were used in immunization protocols. Antibody titers were measured by enzyme-linked immunosorbent assay. At the immunization with 1 μg and 10 μg of IL-4 at the total doses of 18-20 μg antibody titers were very low or indefinable. The data presented here demonstrate that the use of low dose immunization in range of 1-10 μg of autologous IL-4 still is not enough to stimulate the sufficient level of immune response even in the presence of cross-activating monoclonal antibodies and combined immunization protocols.

Introduction

Cytokines are unequal protein antigens, because in vivo they are rapidly utilized by specific target cells or captured by natural antagonist and removed with urine [1, 2]. For the majority of cytokines lifespan ranges from several minutes to several hours. Thus, only small part of injected foreign cytokines may be captured and processed by antigen-presenting cells and presented as an antigen to induce the humoral immune response. On the other hand, the cascade mechanism of interaction the cytokines with target cells may unexpectedly to change character and direction of immune response. In particular, the suppressor mechanisms may be induced by abnormal stimulation of macrophages with high doses of interleukin-4 (IL-4) [3] as well as the increased secretion of interferon-γ that antagonizes the biological effects of IL-4, which is necessary for B-cell development, survival, and immunoglobulin (Ig) synthesis [4-6].

It was shown that mice lacking a specific gene product due to targeted gene inactivation can be used to raise mouse polyclonal antiserum to prion protein [7]. With help this technology mouse-anti-mouse monoclonal antibodies (mAbs) specific to mouse IgD were generated [8]. This new technology opened a potential window for generation of murine mAbs to different autologous antigens.

As known, IL-4-deficient mice develop a normal or higher IgG2a and IgG2b but not IgG1 antigen (Ag)-specific immune response [9, 10]. Recently, we have developed protocols for high level polyclonal responses in IL-4- and IL-13-deficient mice to autologous IL-4 and IL-13 at immunization with high doses of the cytokines (20 μg and 30 μg per a dose) [11] and generated mouse-anti-mouse mAbs that were specific to these cytokines [12].

In this study, we explored the immune response in IL-4-knockout (IL-4KO) mice at low immunization doses of murine IL-4 (1 μg and 10 μg) with use of different adjuvants, anti-IL-4 carrier mAbs, and anti-IgD cross-activating mAbs to evaluate the possibility of reducing the total immunization dose.

Materials and Methods

Experimental Animals

IL-4KO mice (BALB/c strain), female 18-20-week old, were kindly given by Dr. S. Morris. The mice were maintained in the pathogen-free Animal Care Facility at the Cincinnati Veterans Affairs Medical Center. All experiments involving the use of mice were performed in accordance with protocols approved by the Animal Care and Use Committee of the University of Cincinnati College of Medicine.

Antibodies and Cytokines

Hybridomas BVD4-1D11.2 that produce rat IgG2b anti-mouse IL-4-neutralizing mAbs and BVD6-24G2.3 that produce rat IgG1 anti-mouse IL-4-non-neutralizing mAbs [13] and hybridoma HBδ7 that produce rat IgG2a anti-mouse IgD mAbs that effectively activate B cells [14] were used as sources of mAbs prepared from ascites by ammonium sulfate precipitation and ion-exchange chromatography. Biotin-conjugated anti-IgG-specific IgG fraction of goat anti-mouse antiserum cross absorbed against rat Ig that reacts with the heavy and light chains of mouse IgG1, IgG2a, IgG2b and IgG3 and also with the light chains of mouse IgM and IgA was purchased from Southern Biotechnology Associates, Inc. (Birmingham, AL; Cat. No.1034-08). These goat antibodies (Abs) were used as the second reagent for enzyme-linked immunosorbent assay (ELISA). Murine recombinant IL-4 that was used for immunization and ELISA was

donated by PeproTech, Inc. (Rocky Hill, NJ).

Immunization

IL-4 was dissolved in saline and suspended in equivalent volume of Complete and Incomplete Freund's Adjuvants (CFA and IFA; Difco) or absorbed overnight at 4oC in equivalent volume (100 μg/dose) of 50% aluminum hydroxide (Alum) [15]. In some cases, IL-4 was conjugated with 5-fold concentrations of rat anti-mouse IL-4-neutralizing mAbs BVD4-1D11.2 or rat anti-mouse IL-4-non-neutralizing mAbs BVD6-24G2.3 for 3-5 minutes at room temperature. Rat anti-mouse IgD mAbs HBδ7 were used in some protocols at dose of 200 μg in 0.2 ml saline for intraperitoneal (i.p.) or intravenous (i.v.) injections. Regular immunizations of IL-4KO mice were fulfilled at the cytokine doses of 1 μg or 10 μg per injection (the total dose of 18 μg or 20 μg) with 3-day or 1-2-week intervals i.p. or i.v. in base of the tail. Blood samples were taken from a tail vein on day 6-7 after each immunization series. Serum samples were prepared with standard procedure and kept at –20oC until use. Specific details for each immunization protocol are shown where it is applicable.

ELISA and Evaluation of Ab Titers

Luminescent based ELISA for evaluation of Ab titers in immune serums was performed by standard procedure according to the manufacture's instructions with our modifications as described [12]. In brief, 96-well flat-bottom plates (Greiner Labortechnik, Germany) were coated with mouse IL-4 (5 μg/ml, 50 μl/well) overnight at 4oC in 0.1 M Tris buffer saline (pH 8.3). Wells were treated with a blocking buffer (Super-block, Pierce), and plates were washed with deionized water. Following three incubations were fulfilled step by step for 30 min at room temperature firstly with testing samples, then with biotin-conjugated goat anti-mouse IgG and finally with streptavidin-horseradish peroxidase conjugate (25 μl/well at the each step). Finally, the wells were washed with Tris buffer saline (pH 7.2) for 3 min (150 μl/well), and fresh prepared Femto "SuperSignal" substrate (Pierce) was added in the wells (150 μl/well). Signal intensity was measured immediately with a Fluoroskan Ascent FL (Labsystems, Helsinki, Finland) at 425 nm use Ascent Software for Fluoroskan Ascent FL. Titers of Abs were defined as the highest dilutions of serum samples that still showed at least 2-fold positive reaction at values of $p < 0.05$ versus a normal BALB/c IL-4KO serum (nSer) as the negative control at the same dilutions. Results were expressed as the mean ± SD of three replications for each serum dilution.

Statistical Assay

A paired t-test was used to compare values where it was appropriate. The values of $p < 0.05$ were considered statistically significant.

Results

Immune Response of IL-4KO Mice to Immunization with 1 μg of IL-4

Two groups of IL-4KO mice (three mice in each group) were primed i.v. with HBδ7 anti-IgD mAbs (200 μg per injection in 0.2 ml saline) on day 0 and i.p. on day 14. The next six injections were fulfilled i.v. on days 2, 5, 8 and i.p. on days 16, 19, 22 with 1 μg of murine IL-4 conjugated with 5 μg of anti-IL-4-neutralizing mAbs BVD4-1D11.2 (1st group) or anti-IL-4-non-neutralizing mAbs BVD6-24G2.3 (2nd group) in 0.2 ml saline. On day 31 all mice were immunized i.p. with complex of IL-4 (1 μg) + BVD4-1D11.2 or BVD6-24G2.3 (5 μg) in 0.1 ml saline + CFA (0.1 ml). The immunization procedure was repeated on day 43 with 1 μg of IL-4 and on day 50 with 10 μg of IL-4 conjugated with 5-fold concentration of corresponding anti-IL-4 mAbs + IFA. The total immunization dose of IL-4 was 18 μg per mouse, carrier anti-IL-4 mAbs – 90 μg and cross activation HBδ7 anti-IgD mAbs – 400 μg. Serum samples were collected on day 28 (6-fold immunization), day 38 (7-fold immunization) and day 57 (9-fold immunization), diluted in ten times and analyzed for anti-IL-4, anti-HBδ7, anti-BVD4-1D11.2 and anti-BVD6-24G2.3 Ab activity.

In this experiment, the first immunization series (6-fold immunization with 1 μg of IL-4) did not induce any significant Ab response to IL-4 in both groups of mice (Illustration 1) (1st bleed; titer $p > 0.05$), except one mouse (1-3) which showed the Ab titer ≥ 10 ($p < 0.05$). After the next immunization with 1 μg of IL-4 (7-fold immunization), when complex of IL-4 + anti-IL-4 mAbs was suspended in CFA, only one well-responding mouse (1-3) showed the sufficient increase of Ab titer (> 10). More significant tendency to acceleration of Ab titers (titer >10, $p < 0.05$) was observed only after two additional immunization, first with 1 μg and then with 10 μg of IL-4 (9-fold immunization) conjugated with BVD4-1D11.2 IL-4-neutralizing mAbs (mouse 1-1, 1-2 and 1-3; 3rd bleed). This tendency was insufficient in group of mice immunized with IL-4 conjugated with anti-IL-4-non-neutralizing mAbs (mouse 2-1, 2-2 and 2-3; titer < 10, $p > 0.05$). At the same time, immune response to rat BVD4-1D11.2, BVD6-24G2.3, and HB δ7 mAbs was expressed 10-30-fold higher than to IL-4 (data not shown).

Immune Response of IL-4KO Mice to Immunization with 10 μg of IL-4

For next experiment, Alum instead of CFA and IFA and BVD4-1D11.2 IL-4-neutralizing mAbs were used

as adjuvants and a carrier protein, correspondingly. The total dose of injected IL-4 was 20 μg per mouse. Specifically, two groups of mice (three mouse in each group) were primed i.p. on day 0 and day 14 with 10 μg of IL-4 + Alum (1st group) or 10 μg of IL-4 + 50 μg of BVD4-1D11.2 + Alum (2nd group) in 0.5 ml saline. Mice of the 3rd group (three mice) were primed i.p. on day 0 with 10 μg of IL-4 + Alum, and i.v. on day 14 with mAbs HBδ7 and two days later with 10 μg of IL-4 + BVD4-1D11.2 in 0.2 ml saline. Serum samples were collected on day 20 after 2-fold immunization with IL-4, diluted in ten times and analyzed for anti-IL-4 Ab activity.

As Illustration 2 shows, there is no sufficient differences of immune responses between these three groups of mice treated at the three different immunization protocols with 10 μg of IL-4 at the total dose of 20 μg (Ab titers > 10 in mice 1-2, 2-2 and 3-1, and Ab titers < 10 in all other mice) as well as between two first groups of mice in the previous experiment, which were immunized 8-fold with 1 μg and additionally once with 10 μg of IL-4 (Illustration 1).

Discussion

Results of early studies demonstrated that antigen-specific humoral immune response can be increased by cross activation of cooperating CD4+ T cells following the cross-linking of membrane IgD on surface of naïve B-cells with foreign anti-IgD Abs [14, 16]. We believed that low quantity of IL-4 (1-10 μg per injection) might be used in this case to induce anti-IL-4 immune response in IL-4KO mice.

Rat IgG2a anti-mouse mAbs HBδ7 specific to mouse surface IgD was used to achieve this aim. Furthermore, rat anti-mouse neutralizing BVD4-1D11.2 (IgG2b) and non-neutralizing BVD6-24G2.3 (IgG1) anti-IL-4 mAbs were used to prolong IL-4 persistence in the mice and induce immune response to two different antigen determinants of IL-4 conjugated with these mAbs. However, use of neutralizing and non-neutralizing mAbs as carrier proteins in combination with classic adjuvants such as CFA, IFA and Alum could not induce the significant anti-IL-4 immune response to the low doses of the antigen at the different immunization protocols. Only noticeable tendency to increase of immune response was observed when IL-4-neutralizing mAbs were used as a carrier protein at the increased final dose of IL-4 up to 10 μg. At the same time, immune response to the foreign carrier anti-IL-4 mAbs and activation anti-IgD mAbs, concentrations of which were much higher, was sufficiently higher. Twofold immunization with 10 μg of IL-4 in presence of the carrier protein and adjuvants also could not change the immune response cardinally. Thus, the data presented here clearly demonstrate that the use of low dose immunization in range of 1 – 10 μg of autologous IL-4 still is not enough to stimulate the sufficient level immune response even in presence of cross-activating mAbs, and for induction of a sufficient immune response much more doses of IL-4 should be used.

Really, as we showed previously [11] increase of the cytokine dose to 20 μg per injection was able to induce the significant Ab response in IL-4-deficient mice even in absence of the carrier mAbs with serum titer within the scope of 640-2560. However, the increase of the total dose of IL-4 beyond of 100 μg failed to induce further increase in Ab response and suppression of the immune response was observed [11].

Early, the suppressor effect of IL-4 on IgE and IgG1 Ab responses was demonstrated in mice injected with foreign anti-IgD stimulating Abs, and this suppression was associated with decreased CD4+ T-cell activation [17]. Other authors demonstrated that in vitro, early and brief exposure of B cells to IL-4 enhances Ig secretory response to subsequent stimulation with dextran-conjugated anti-IgD mAbs in the presence of Th1-dependent cytokines, while continuous exposure results in the inhibition of the response [18]. In accord with these data, our previously published results [11, 12] suggest a possibility for induction of high-zone T-B-cell tolerance that may explain the absence of increase in Ab response at the total dose of IL-4 beyond of 100 μg. In particular, blocking effect of high doses of IL-4 on differentiation and antigen-presenting function of dendritic cells [19] may be responsible for induction of such T-B-cell tolerance. On the other hand, this phenomenon may be explained by abnormal accumulation of macrophages/monocytes with increased 12-lipoxygenase suppressor activity in spleens of mice immunized with IL-4 [3] that might be increased by multifold use of adjuvants [20].

Thus, taken together our data demonstrate that murine IL-4 at the total dose of 20 μg still does not induce the notable immune respond in IL-4KO mice with production of anti-IL-4 specific Abs, and 100 μg is the maximal total dose of IL-4 that may be used for immunization of IL-4KO mice without development of suppression the immune response, and the highest level of Ab response may be reached in range of 80 – 100 μg of IL-4.

Abbreviations

Abs, antibodies; Alum, aluminum hydroxide; CFA, complete Freund's adjuvant; ELISA, enzyme-linked immunosorbent assay; IFA, incomplete Freund's adjuvant; Ig, immunoglobulin; IL-4, interleukin-4; IL-4KO, IL-4-knockout; i.p., intraperitoneal; i.v., intravenous; mAbs, monoclonal antibodies; nSer, normal serum.

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Illustration legends

Illustration 1. Immune response of IL-4KO mice to immunization with 1 μg of murine IL-4. Mice were primed 2-fold with rat anti-mouse IgD mAbs HBδ7 and immunized 9-fold with IL-4 (18 μg total dose) conjugated with anti-IL-4-neutralizing mAbs BVD4-1D11.2 (1st group) or anti-IL-4-non-neutralizing mAbs BVD6-24G2.3 (2nd group) in CFA and IFA. Blood was collected on day 28 (1st bleed, 6-fold immunization, 6 μg), day 38 (2nd bleed, 7-fold immunization, 7 μg) and day 57 (3rd bleed, 9-fold immunization, 18 μg). Details of the immunization protocols are shown in the Results. Serum samples were diluted 10-fold and analyzed for anti-IL-4 Ab activity by ELISA versus to nSer. Data are represented as the mean ± SD of three replications. Each bar represents one individual mouse (*$p < 0.05$, Ab titer ≥ 10; $p > 0.05$, Ab titer < 10).

Illustration 2. Immune response of IL-4KO mice to immunization with 10 μg of murine IL-4. Mice were primed 2-fold with 10 μg of IL-4 + Alum (1st group) or IL-4 + BVD4-1D11.2 + Alum (2nd group). Mice of the 3rd group (3-1, 3-2, 3-3) were primed with 10 μg of IL-4 + Alum, then on day 14 with mAbs HBδ7 and two days later with 10 μg of IL-4 + BVD4-1D11.2. Blood was collected on day 20. Details of the immunization protocols are shown in the Results. Serum samples were diluted 10-fold and analyzed for anti-IL-4 Ab activity by ELISA versus to nSer. Data are represented as the mean ± SD of three replications. Each bar represents one individual mouse (*$p < 0.05$, Ab titer ≥ 10; $p > 0.05$, Ab titer < 10).

Illustrations

Illustration 1

Illustration 1. Immune response of IL-4KO mice to immunization with 1 ÃŽÂ¼g of murine IL-4. Mice were primed 2-fold with rat anti-mouse IgD mAbs HBÃŽÂ´7 and immunized 9-fold with IL-4 (18 ÃŽÂ¼g total dose) conjugated with anti-IL-4-neutralizing mAbs BVD4-1D11.2 (1st group) or anti-IL-4-non-neutralizing mAbs BVD6-24G2.3 (2nd group) in CFA and IFA. Blood was collected on day 28 (1st bleed, 6-fold immunization, 6 ÃŽÂ¼g), day 38 (2nd bleed, 7-fold immunization, 7 ÃŽÂ¼g) and day 57 (3rd bleed, 9-fold immunization, 18 ÃŽÂ¼g). Details of the immunization protocols are shown in the Results. Serum samples were diluted 10-fold and analyzed for anti-IL-4 Ab activity by ELISA versus to nSer. Data are represented as the mean Ã‚Â± SD of three replications. Each bar represents one individual mouse (*p < 0.05, Ab titer Ã¢?Â¥ 10; p > 0.05, Ab titer < 10).

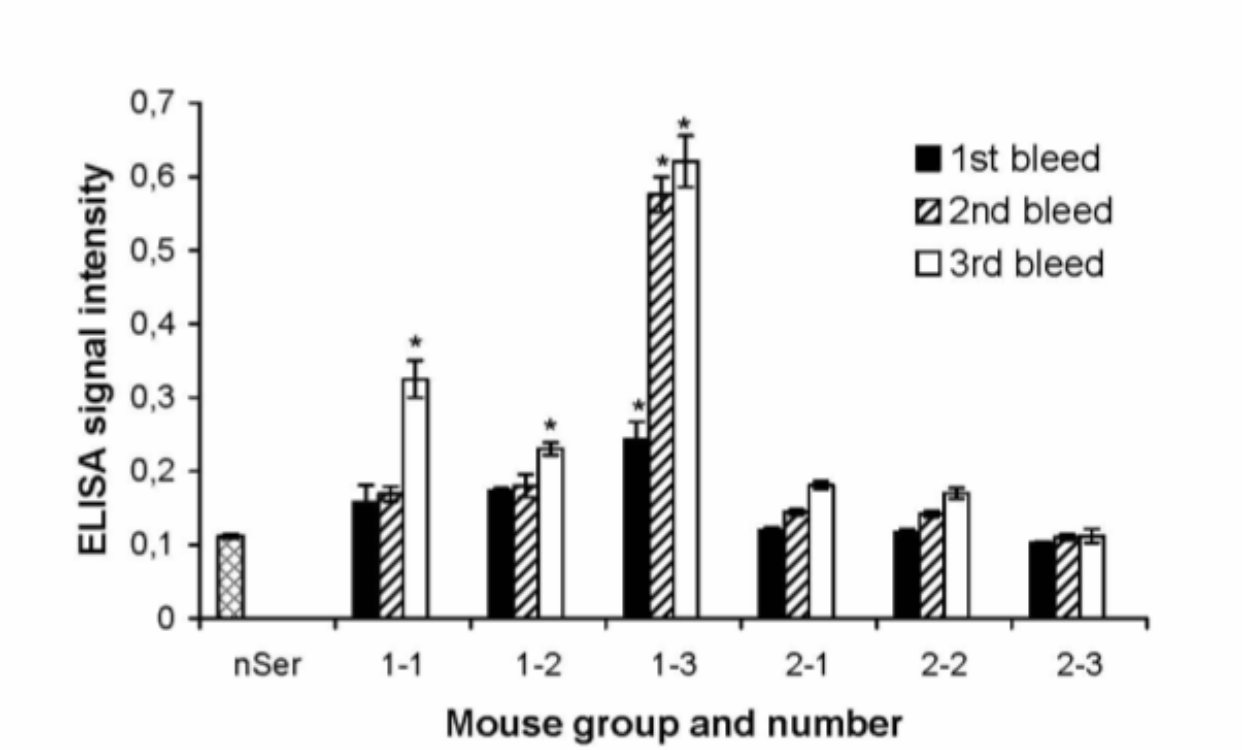
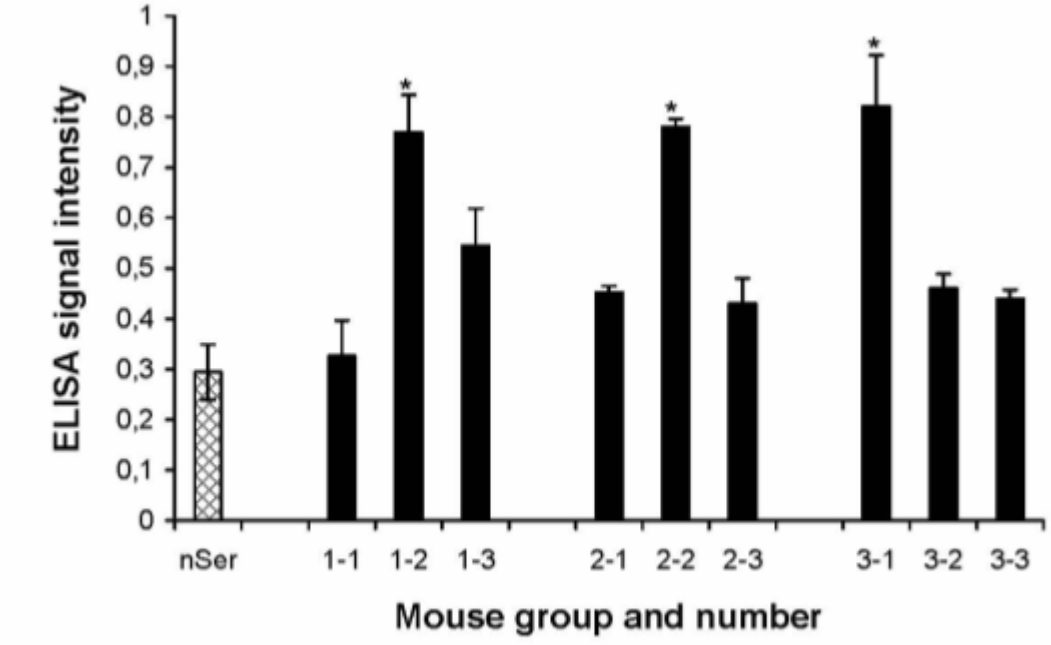


Illustration 2

Illustration 2. Immune response of IL-4KO mice to immunization with 10 ÃŽÂ¼g of murine IL-4. Mice were primed 2-fold with 10 ÃŽÂ¼g of IL-4 Alum (1st group) or IL-4 BVD4-1D11.2 Alum (2nd group). Mice of the 3rd group (3-1, 3-2, 3-3) were primed with 10 ÃŽÂ¼g of IL-4 Alum, then on day 14 with mAbs HBÃŽÂ´7 and two days later with 10 ÃŽÂ¼g of IL-4 BVD4-1D11.2. Blood was collected on day 20. Details of the immunization protocols are shown in the Results. Serum samples were diluted 10-fold and analyzed for anti-IL-4 Ab activity by ELISA versus to nSer. Data are represented as the mean Ã‚Â± SD of three replications. Each bar represents one individual mouse (*p < 0.05, Ab titer Ã¢?Â¥ 10; p > 0.05, Ab titer < 10).



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