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# Targeting the IL-15 Receptor with an Antagonist IL-15 Mutant/Fc $\gamma$ 2a Protein Blocks Delayed-Type Hypersensitivity<sup>1</sup>

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Owing to shared receptor components, the biologic activities of IL-15 are similar to those of IL-2. However, the patterns of tissue expression of IL-2/IL-2R $\alpha$  and IL-15/IL-15R $\alpha$  differ. The development of agents targeting the receptor and signaling elements of IL-15 may provide a new perspective for treatment of diseases associated with expression of IL-15/IL-15R. We designed, genetically constructed, and expressed a receptor site-specific IL-15 antagonist by mutating glutamine residues within the C terminus of IL-15 to aspartic acid and genetically linked this mutant IL-15 to murine Fc $\gamma$ 2a. These mutant IL-15 proteins specifically bind to the IL-15R, competitively inhibit IL-15-triggered cell proliferation, and do not activate the STAT-signaling pathway. Because the receptor site-specific antagonist IL-15 mutant/Fc $\gamma$ 2a fusion proteins had a prolonged  $t_{1/2}$  in vivo and the potential for destruction of IL-15R<sup>+</sup> leukocytes, we examined the immunosuppressive activity of this agent. An IL-15 mutant/Fc $\gamma$ 2a fusion protein markedly attenuated Ag-specific delayed-type hypersensitivity responses and decreased leukocyte infiltration within the delayed-type hypersensitivity sites. These findings suggest that 1) IL-15/IL-15R<sup>+</sup> cells are crucial to these T cell-dependent immune responses, and 2) treatment with IL-15 mutant/Fc $\gamma$ 2a protein may ameliorate T cell-dependent immune/inflammatory diseases. *The Journal of Immunology*, 1998, 160: 5742–5748.

**B**ecause cytokines influence immunity and inflammation, interventions that modify cytokine pathways or destroy cytokine receptor-bearing cells can be effective for modulating harmful inflammatory responses (1). Owing to shared receptor components, IL-15 is a cytokine with similar activity to that of IL-2 as 1) an activator of T lymphocyte growth, 2) an inducer of cytotoxic effector cells, 3) a chemoattractant for T cells, and 4) a stimulator of NK cell proliferation and activation, and facilitator of IFN- $\gamma$  and TNF- $\alpha$  synthesis (2–7). The production of IL-15 is regulated at transcriptional and posttranscriptional levels (4). Unlike IL-2, IL-15 transcripts are not detected in resting or activated T cells (4, 5). Nonetheless, IL-15 expression is associated with exacerbations of autoimmune diseases such as rheumatoid arthritis (8–10), inflammatory bowel disease (11), and allograft rejection (12, 13).

The IL-15R comprises three elements; IL-15R $\alpha$ , IL-2R $\beta$ , and IL-2R $\gamma$  (also called common  $\gamma$  or  $\gamma_c$ ) (14–16). Thus, the IL-2R and IL-15R share two components. IL-15R $\alpha$  mRNA is expressed in a wide range of cell types, including activated T cells, activated NK cells, activated B cells, activated macrophages, activated vascular endothelial cells, as well as thymic and bone marrow stromal

cells. IL-15R $\alpha$  mRNA also has wide range of tissue expression such as liver, heart, spleen, lung, and skeletal muscle (4, 16). The development of agents targeting IL-15R may provide a new perspective for the treatment of immune and inflammatory diseases.

Cytokines possess a very high affinity for their receptor, but their short lives and agonist activity, triggering activation of receptor-bearing target cells, limit or preclude their utility as receptor site antagonists or a vehicle for targeting cytotoxic agents to cytokine receptor-bearing cells without transiently stimulating the target cells. The relatively short in vivo  $t_{1/2}$  of many cytokines hamper their therapeutic efficacy and require frequent injection or constant administration (1). To overcome the problem associated with the short  $t_{1/2}$ , we and others have generated long-lived cytokine/IgG-related fusion proteins (17–19). Depending upon the desired application, the Fc region can be chosen to express or preclude cytotoxic activity against the target cells (17, 19).

Petit et al. (20) demonstrated that mutation of the glutamine at residue 108 in human IL-15 to serine creates an IL-15R site-specific antagonist. In our laboratory, an IL-15 antagonist was also constructed by replacing the codons for the C-terminal glutamine amino acid residues with codons for aspartic acid (i.e., Q101 and Q108) and we developed a strategy for selective targeting high affinity IL-15R $\alpha$ -bearing cells by use of IL-15 mutant/Fc $\gamma$ 2a fusion proteins. IL-15 mutant/Fc $\gamma$ 2a proteins have a high affinity, receptor site-specific IL-15R binding, and antagonist properties; fail to activate the STAT system; and possess a prolonged  $t_{1/2}$  in vivo. Treatment with the IL-15 mutant/Fc $\gamma$ 2a fusion protein markedly attenuates Ag-specific DTH<sup>4</sup> responses and cellular infiltration within the DTH sites. These findings suggest that IL-15 and/or IL-15R<sup>+</sup> cells are crucial for, at least, some Ag-specific T cell-mediated immune response in vivo. Hence, IL-15 mutant/Fc $\gamma$ 2a

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<sup>4</sup> Abbreviations used in this paper: DTH, delayed-type hypersensitivity; CsA, cyclosporine; FLAG, International Biotechnologies-Kodak trade name for marker octapeptide N-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys-C; HMK, heart muscle kinase recognition site; MBSA, methylated bovine serum albumin; rh, recombinant human.

proteins may provide therapeutic benefit for certain T cell-dependent immune diseases and other IL-15-rich inflammatory states.

## Materials and Methods

### Genetic construction of IL-15 mutant/Fc $\gamma$ 2a

Human IL-15 and murine Fc $\gamma$ 2a cDNAs were generated from mRNA extracted from PHA-stimulated human PBMCs and IgG2a-secreting hybridoma (American Type Cell Culture (ATCC) HB129, Rockville, MD), respectively, using reverse-transcriptase MMLV-RT (Life Technologies, Gaithersburg, MD) and synthetic oligo(dT) oligonucleotides (Life Technologies). The IL-15 mutant cDNAs were designed to target select glutamine codons of human IL-15 (Q101 and Q108) for mutation to aspartic acid sequences by PCR-assisted site-directed mutagenesis. For the construction of mutant plasmids, a 322-bp cDNA fragment encoding mature human IL-15 with relevant mutations at positions 101 and 108 was amplified by PCR utilizing synthetic sense: 5'-GAAGCTTGAACCTGGGTGAATGTAATAAGT-3' (*Hind*III site plus bases) and antisense oligonucleotides corresponding to the C-terminal fragment of human IL-15, followed by a *Bam*HI site (the mutated codons are underlined and in bold): 5'-TCTGGGATCCGAAGTGTGATGAACATGTCGACAATATGTACAAAAGTGTCCAAAAT-3'. Synthetic oligonucleotides used for the amplification of the Fc $\gamma$ 2a domain cDNA change the first codon of the hinge region from Glu to Asp to create a unique *Bam*HI site spanning in the first codon of the hinge and introduce a unique *Xba*I site 3' to the termination codon. Ligation of cytokine and Fc $\gamma$ 2a components in the correct translational reading frame yields a 1059-bp-long open frame encoding a single 353-amino-acid polypeptide. The mature secreted homodimeric IL-15 mutant/Fc $\gamma$ 2a is predicted to have a m.w. of 80 kDa, exclusive of glycosylation. Proper genetic construction of IL-15 mutant/Fc $\gamma$ 2a was confirmed by DNA sequence analysis following cloning of the fusion genes into the eukaryotic expression plasmid pSecTag (Invitrogen, San Diego, CA). This plasmid carries a CMV promoter, IgG  $\kappa$  leader sequence, and a gene for selection against Zeocin (Invitrogen).

### Expression and purification of IL-15 mutant/Fc $\gamma$ 2a

Plasmids carrying fusion genes were transfected into NS.1 cells (ATCC) by electroporation (1.5 kV/3  $\mu$ F/0.4 cm/PBS) and selected in serum-free U1-traculture media (BioWhittaker, Walkersville, MD) containing 100  $\mu$ g/ml Zeocin. After subcloning, high producing clones were selected by screening supernatants for IgG2a by ELISA. IL-15 mutant/Fc $\gamma$ 2a fusion proteins were purified from culture supernatant by protein A-Sepharose affinity chromatography (Pharmacia, Piscataway, NJ), followed by dialysis against PBS and 0.22- $\mu$ m filter sterilization. Purified proteins were stored at -20°C until use. The size and specificity of purified IL-15 mutant/Fc $\gamma$ 2a were confirmed by SDS-PAGE under reducing (+DTT) and nonreducing (-DTT) conditions, followed by Western blot analysis using polyclonal anti-human IL-15 (PeproTech, Rocky Hill, NJ) and anti-murine IgG2a Abs (PharMingen, San Diego, CA).

### Determination of IL-15 mutant/Fc $\gamma$ 2a circulating t<sub>1/2</sub>

The serum concentration of IL-15 mutant/Fc $\gamma$ 2a was determined at various time points following a single bolus i.v. injection of the fusion protein that was administered to 8- to 10-wk-old BALB/c mice (The Jackson Laboratory). Serial 100- $\mu$ l blood samples were obtained by retroorbital bleeding at intervals of 0.1, 6, 24, 48, 72, and 96 h after administration. Measurements of IL-15 mutant/Fc $\gamma$ 2a were made by ELISA using rabbit anti-human IL-15 Ab as the capture Ab and horseradish peroxidase-conjugated anti-mouse IgG2a mAb as the detection Ab (PharMingen). This assured that the ELISA was specific for the IL-15 mutant/Fc $\gamma$ 2a protein, and not IL-15 or mouse IgG2a.

### Proliferation assays

IL-3-dependent BAF-BO3 cells expressing IL-2R $\beta$  chains were washed twice to remove the growth factor and starved for 6 h in RPMI 1640 medium supplemented with 1% FCS, penicillin, and streptomycin. Cells were then plated ( $2 \times 10^4$  cells/well) and cultured for 48 h at 37°C with medium alone or medium supplemented with IL-3-rich supernatants from WEHI cells, rhIL-2, or rhIL-15 in an atmosphere containing 5% CO<sub>2</sub>. Following this incubation, cells were pulsed for 6 h with 1  $\mu$ Ci [<sup>3</sup>H]TdR and harvested onto Whatman 934-AH glass microfiber filters using a PHD cell harvester (Cambridge Technology, Cambridge, MA). Cell-associated [<sup>3</sup>H]TdR was measured using a Beckman LS 2800 scintillation counter (Beckman, Fullerton, CA). To probe for receptor site-specific antagonist activity, growth factors (IL-3-rich media, rhIL-2, or rhIL-15) were added simultaneously with the indicated concentrations of IL-15 mutant/Fc $\gamma$ 2a

proteins. BAF-BO3 cells were then harvested, and cell-associated radioactivity was measured by scintillation counting, described as above. BAF-BO3 cells cultured with IL-15 mutant/Fc $\gamma$ 2a proteins for 3 days were stained with trypan blue to determine cell viability. No evidence of cell toxicity was observed in IL-15 mutant/Fc $\gamma$ 2a-treated cells in comparison with controls.

### Immunoblotting for STAT proteins

IL-3-dependent BAF-BO3 cells expressing IL-2R $\beta$  chains were washed twice to remove the growth factor and starved for 6 h in RPMI 1640 medium supplemented with 1% FCS, penicillin, and streptomycin. Cells were washed again, resuspended in RPMI 1640 (10<sup>7</sup> cells/ml), and stimulated with medium alone, or medium supplemented with either 50 U/ml of rhIL-2, 10 ng of rhIL-15, or IL-15 mutant/Fc $\gamma$ 2a proteins. Following interaction with these proteins for 2 min at 37°C, the cells were washed with ice-cold PBS and lysed for 15 min on ice in 50  $\mu$ l of buffer containing NaCl (150 mM), Nonidet P-40 (1%), Tris-HCl, pH 7.5 (25 mM), Na<sub>3</sub>VO<sub>4</sub> (1 mM), PMSF (1  $\mu$ M), leupeptin (10  $\mu$ g/ml), and aprotinin (10  $\mu$ g/ml). Cellular debris was removed by centrifugation, and proteins present in the supernatants of these cell lysates were separated on 7.5% SDS-PAGE under reducing conditions and transferred onto polyvinylidene difluoride membranes. The membranes were blocked for 1 h at room temperature in buffer containing Tris-HCl (20 mM, pH 7.5), NaCl (150 mM), Tween-20 (0.1%), and BSA (3%), and then incubated for 1 h with phosphospecific STAT3 Ab (New England Biolabs, Beverly, MA). The membrane was washed and developed using the SuperSignal Western blotting kit (Pierce, Rockford, IL), according to the manufacturer's protocol. For subsequent staining, the membranes were incubated at 50°C for 20 min in stripping buffer containing 100 mM 2-ME, 2% SDS, and 62.5 mM Tris-HCl, pH 6.7, followed by a 1-h incubation in blocking buffer, and immunodetection was performed using anti-STAT3 Ab (Santa Cruz Biotechnology, Santa Cruz, CA). For the detection of phosphorylation of STAT5 proteins, cell lysates from BAF-BO3 cells expressing IL-2R $\beta$ , stimulated described as above, were separated on SDS-PAGE. After transfer onto polyvinylidene difluoride membranes, immunodetection of phosphorylated proteins was performed using horseradish peroxidase-conjugated anti-phosphotyrosine Ab (Zymed, South San Francisco, CA). This membrane was blotted again using anti-STAT5 Ab (Transduction Laboratories, Lexington, KY) after stripping described as above.

### Cell staining for flow cytometry

BAF-BO3 cells expressing IL-2R $\beta$  (10<sup>6</sup> cells/tube) were washed twice with ice-cold PBS/0.02% sodium azide. After blocking with control mouse IgG (PharMingen), cells were incubated in medium alone (control) or medium containing IL-15 mutant/Fc on ice for 30 min, washed with PBS, and incubated for 30 min with FITC-conjugated goat anti-mouse Fc Ab (Pierce). To define the receptor-ligand specificity, molar excess rhIL-2, rhIL-15, or rat anti-mouse IL-2R $\gamma$  Ab (4G3/3E12; kind gift of Dr. Thomas R. Malek, Miami, FL) was added to medium containing IL-15 mutant/Fc $\gamma$ 2a. Cells were stained with FITC-conjugated goat anti-mouse Fc Ab. Cell staining was analyzed using FACSscan (Becton Dickinson, Mountain View, CA) and CellQuest software.

### DTH responses

BALB/c (The Jackson Laboratory) mice were sensitized to methylated BSA (MBSA; Sigma, St. Louis, MO) by an intradermal injection of 50  $\mu$ l of 5 mg/ml MBSA in CFA (Sigma) at two sites on the abdomen. Eight days after immunization, the mice were rechallenged by injection of 20  $\mu$ l of 5 mg/ml MBSA into one rear footpad, while the other rear footpad received a comparable volume of PBS. Measurements of footpad swelling were taken at 24, 48, and 72 h after challenge by use of two different micrometers (Mitutoyo, Tokyo, Japan, and LS Starrett, Athol, MA). The magnitude of the DTH responses was determined as the differences in footpad thickness between the Ag- and PBS-injected footpads. DTH responses were measured in a blinded fashion, in which measurements were obtained by an individual who did not know the treatment protocol for each subject. Treatment protocols were: 1) i.p. injection of 1.5  $\mu$ g of either IL-15 mutant/Fc $\gamma$ 2a or mouse IgG2a daily, starting 30 min before the rechallenge with MBSA and continued for 3 days with or without concomitant cyclosporine (CsA; Novartis, Hanover, NJ) (2 mg/kg of CsA i.p. as loading dose, and 0.5 mg/kg on the following 2 days), or 2) i.p. injection of 1.5  $\mu$ g of either IL-15 mutant/Fc $\gamma$ 2a or mouse IgG2a daily, starting 30 min before the initial challenge of MBSA and continued daily for 8 days. For histologic examination of the DTH reaction sites in the first treatment protocol, soft tissue samples from the foot were collected at 12 and 24 h after the second MBSA challenge.

### Immunohistochemistry

Cryostat tissue sections (6  $\mu\text{m}$ ) were placed on slides coated with poly(L-lysine) (Sigma) and fixed for 10 min in 95% ethanol at 4°C. Endogenous peroxidase activity, present in tissue sections, was blocked by treatment with 0.6%  $\text{H}_2\text{O}_2$  and 0.2%  $\text{NaN}_3$  for 10 min. Tissue sections were then incubated for 15 min each in avidin solution, followed by biotin solution (Vector Laboratories, Burlingame, CA) to block endogenous biotin. Non-specific IgG binding was prevented by pretreatment of tissue sections for 30 min with 10% normal rabbit serum and 10% BSA. Tissue sections were then incubated overnight with rat anti-mouse primary Ab (10  $\mu\text{g}/\text{ml}$ ) in 1% BSA at 4°C. Bound primary Ab was then labeled with rabbit anti-rat IgG conjugated with biotin for 1 h at room temperature. The sections were then incubated with avidin-biotin-horseradish peroxidase complex (Vectastain ABC reagent; Vector Laboratories) for 1 h at room temperature. Diaminobenzidine substrate solution (Vector Laboratories) was then added to tissue sections, resulting in a brown color at sites of immunoenzymatic labeled Ag. Tissue sections were then counterstained with hematoxylin to detect cell nuclei. Positively stained cells were counted in 10 randomly selected fields (each 100  $\mu\text{m}^2$ ). The following primary Abs were used for immunostaining: rat anti-mouse CD4 IgG2a clone RM4-5 (PharMingen) and rat anti-mouse F4/80 IgG2b (prepared from hybridoma supernatant, ATCC HB198). The negative isotype control Abs used were rat IgG2a clone R35-95 and rat IgG2b clone R35-38 (PharMingen). The secondary Ab used for all immunostaining was rabbit anti-rat IgG conjugated with biotin (Vector Laboratories).

### Statistics

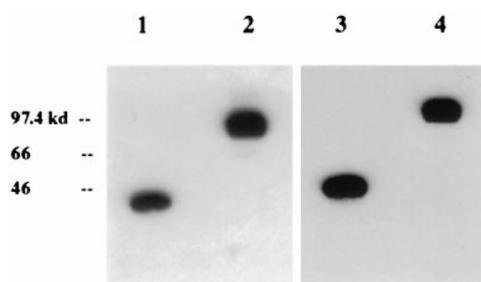
Student's *t* test was used.

## Results

### Characterization of IL-15 mutant/Fc $\gamma$ 2a fusion proteins

In previous studies, we demonstrated that FLAG-HMK-IL-15 specifically binds to IL-15R expressed on PHA-activated PBMCs (21) and T84 colonic cryptlike intestinal epithelial carcinoma cells (22). Mutations targeting glutamine residues localized in the C-terminal  $\alpha$ -helix of human IL-15 do not destroy the ability of these FLAG-HMK-IL-15 mutant proteins to bind to IL-15R (manuscript submitted<sup>5</sup>). In keeping with the observations of Pettit et al. (20), an IL-15-related glutamine to aspartic acid mutant, i.e., FLAG-HMK-IL-15 Q101D,Q108D proteins, specifically and competitively block IL-15-triggered cell proliferation (data not shown). This FLAG-HMK-IL-15 Q101D,Q108D mutant protein is an antagonist for rhIL-15-triggered proliferation. As the FLAG epitope is immunogenic, and the  $t_{1/2}$  of unmodified cytokine is short (1), these features limit therapeutic application. Thus, we developed an IL-15 mutant/Fc $\gamma$ 2a fusion protein to provide a receptor site-specific antagonist with a prolonged circulating  $t_{1/2}$  and cytotoxic potential. To confirm the molecular size and the cytokine/isotype specificity, the affinity-purified fusion protein was characterized by Western blot analysis following 12% SDS-PAGE. As shown in Figure 1, the IL-15 mutant/Fc $\gamma$ 2a fusion proteins migrated under reducing (+DTT) conditions as a single species at a molecular size of 46 kDa (Fig. 1, lanes 1 and 3). Under nonreducing (-DTT) conditions, each IL-15 mutant/Fc $\gamma$ 2a fusion protein runs as a single species at a molecular size of 95 kDa (Fig. 1, lanes 2 and 4), which indicates that the IL-15 mutant/Fc $\gamma$ 2a fusion protein is expressed as a homodimer. Moreover, the IL-15 mutant/Fc $\gamma$ 2a fusion protein is immunoreactive with both anti-human IL-15 Ab (Fig. 1, lanes 1 and 2) and anti-mouse IgG2a Ab (Fig. 1, lanes 3 and 4), confirming the cytokine and isotype specificity of the IL-15 moiety and Fc $\gamma$ 2a domain, respectively.

Flow-cytometric analysis revealed that the IL-15 mutant/Fc $\gamma$ 2a fusion protein binds to IL-15R expressed upon IL-2R $\beta^+$  BAF-BO3 cells (Fig. 2A). The specificity of the IL-15 mutant/Fc $\gamma$ 2a



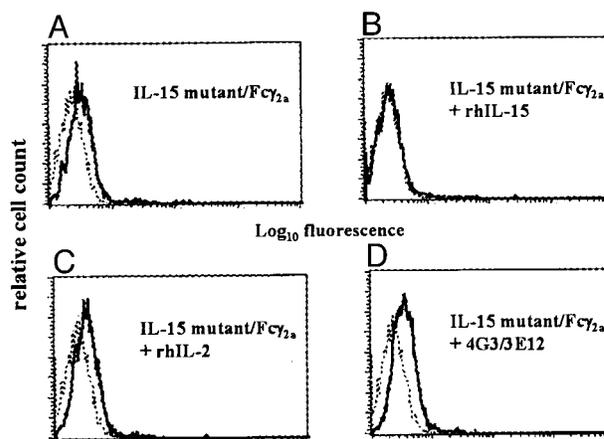
**FIGURE 1.** Western analysis of IL-15 mutant/Fc $\gamma$ 2a fusion protein. SDS-PAGE of IL-15 mutant/Fc $\gamma$ 2a fusion protein under reducing (lanes 1 and 3) and nonreducing (lanes 2 and 4) conditions shows homodimerization of single species of protein. This protein is immunoreactive with both anti-IgG2a Ab (lanes 1 and 2) and anti-IL-15 Ab (lanes 3 and 4).

binding for IL-15 binding sites was established through a study in which the binding of the IL-15 mutant/Fc $\gamma$ 2a to target cells was blocked by provision of a molar excess of rhIL-15 (Fig. 2B) and not inhibited by a molar excess of rhIL-2 (Fig. 2C) or 4G3/3E12 rat anti-mouse IL-2R $\gamma$  Ab (Fig. 2D).

### IL-15 mutant/Fc $\gamma$ 2a fusion proteins fail to support cell proliferation and to trigger tyrosine phosphorylation of STAT3 and STAT5 proteins

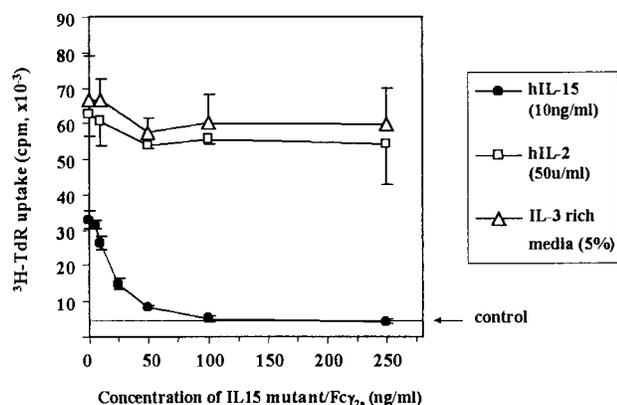
The impact of mutation of the C-terminal glutamine residues and linking of the mutant IL-15 to the Fc domain on the biologic activity of IL-15 was probed. The IL-15 mutant/Fc $\gamma$ 2a fusion protein fails to support the proliferation of IL-15-sensitive IL-2R $\beta^+$  BAF-BO3 cells. Furthermore, simultaneous addition of the mutant IL-15 protein blocks rhIL-15-driven cell proliferation in dose-dependent manner (Fig. 3), while rhIL-2- or IL-3-rich medium-dependent cell proliferation is not inhibited by the addition of IL-15 mutant/Fc $\gamma$ 2a, even in excess amount of fusion proteins (Fig. 3).

The tyrosine phosphorylation of STAT3 and STAT5 proteins is critical to IL-15-triggered cell proliferation (23, 24). Therefore, we



**FIGURE 2.** IL-15 mutant/Fc $\gamma$ 2a protein specifically binds to the IL-15R. IL-3-sensitive BAF-BO3 cells were washed and incubated with medium alone (dotted line) or medium supplemented with IL-15 mutant/Fc $\gamma$ 2a protein (bold line), followed by interaction with FITC-conjugated goat anti-mouse Fc Ab (A). The stained IL-15R $^+$  cells were analyzed by flow cytometry. This binding was blocked by molar excess of rhIL-15 (B). Excess amounts of rhIL-2 or 4G3/3E12 rat anti-mouse IL-2R $\gamma$  Ab did not inhibit the binding of IL-15 mutant/Fc $\gamma$ 2a protein to its receptors (C and D). The data presented are representative of results achieved in three separate experiments.

<sup>5</sup> Y. S. Kim, D.-W. Chae, Y. Nosaka, A. C. Stevens, T. B. Strom, and W. Maslinski. Certain substitutions at the Glu<sup>108</sup> residue of human IL-15 create receptor site specific antagonist proteins. Submitted for publication.

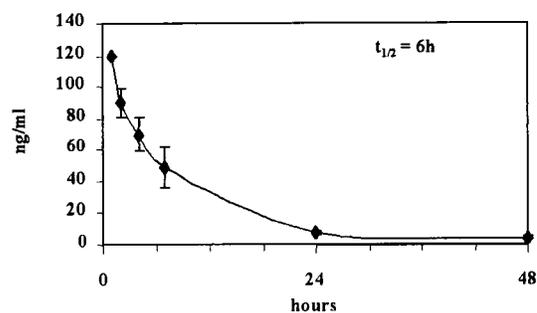


**FIGURE 3.** IL-15 mutant/Fc $\gamma$ 2a protein competitively blocks IL-15, but not IL-2- or IL-3-rich medium-triggered cell proliferation. IL-2R $\beta^+$  BAF-BO3 cells were incubated with rhIL-15 (10 ng/ml) in the presence of different concentrations of IL-15 mutant/Fc $\gamma$ 2a protein (filled circle) for 48 h, followed by 6 h of [ $^3$ H]TdR pulse, and cell-incorporated radioactivity was counted in a scintillation counter. The IL-15 mutant/Fc $\gamma$ 2a protein blocked rhIL-15-driven BAF-BO3 cell proliferation in dose-dependent manner. But rhIL-2 (open square)- or IL-3-rich medium (open triangle)-dependent cell growth was not lessened in the presence of IL-15 mutant/Fc $\gamma$ 2a proteins. The data = mean  $\pm$  SD of triplicate experiments. Similar results were obtained in three separate experiments.

tested the ability of the IL-15 mutant/Fc $\gamma$ 2a fusion protein to trigger tyrosine phosphorylation of STAT3 and STAT5 proteins in IL-2R $\beta^+$  BAF-BO3 cells. Unlike rhIL-15, IL-15 proteins bearing the Q101D and Q108D mutations fail to stimulate tyrosine phosphorylation of STAT3 and STAT5 (Fig. 4), thereby linking the failure of tyrosyl phosphorylation of STAT3 and STAT5 proteins with the failure to trigger target cell proliferation.

#### The properties of IL-15 mutant/Fc $\gamma$ 2a fusion protein studied in vivo: circulating $t_{1/2}$

We determined the circulating  $t_{1/2}$  of the IL-15 mutant/Fc $\gamma$ 2a fusion protein using a unique dual-probe ELISA that detects the IL-15 mutant/Fc $\gamma$ 2a fusion protein, but not IL-15 nor mouse IgG2a. The circulating  $t_{1/2}$  of the IL-15 mutant/Fc $\gamma$ 2a fusion protein was 6 h (Fig. 5). Thus, the  $t_{1/2}$  of the IL-15 mutant/Fc $\gamma$ 2a



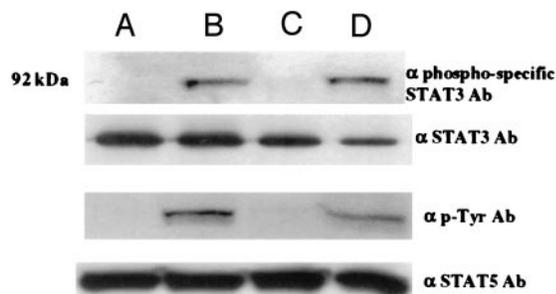
**FIGURE 5.** IL-15 mutant/Fc $\gamma$ 2a protein has prolonged circulating  $t_{1/2}$  (6 h). The time-related serum concentration was determined following a single bolus i.v. dose (6  $\mu$ g) of the fusion protein. Blood samples were obtained by retroorbital bleeding at the indicated intervals. The levels of mutant fusion protein were detected by ELISA with rat anti-human IL-15 Ab as capture Ab and horseradish peroxidase-conjugated rat anti-mouse IgG2a Ab as the detection Ab. Four mice were used for determining the  $t_{1/2}$ . Data = mean  $\pm$  SD.

fusion protein is prolonged in comparison with the  $t_{1/2}$  of unmodified IL-15, which is 2 to 3 min (20).

#### IL-15 mutant/Fc $\gamma$ 2a fusion proteins block DTH in normal mice

To determine whether IL-15 mutant/Fc $\gamma$ 2a treatment blocks T cell-dependent in vivo responses to an Ag, DTH responses were evaluated. After the initial immunization with MBSA, mice were treated with either the IL-15 mutant/Fc $\gamma$ 2a fusion protein or mouse IgG in control group starting just before rechallenge of MBSA with or without concomitant CsA. As shown in Table I, control mouse IgG-treated mice mounted a brisk DTH response to a rechallenge of MBSA. Treatment with CsA and control IgG did not markedly attenuate the DTH response, while treatment with IL-15 mutant/Fc $\gamma$ 2a protein blocked the DTH response. This reduction in DTH was reflected by a decreased influx of macrophages (Fig. 6) and CD4 $^+$  T cells (Fig. 7) within the footpad dermis in IL-15 mutant/Fc $\gamma$ 2a-treated mice vs control mice. Combined treatment with IL-15 mutant/Fc $\gamma$ 2a plus CsA reduced the DTH response synergistically and further suppressed the cellular infiltration (Table I; Figs. 6 and 7).

Because inflammatory reactions to Ag, albeit suppressed, were evident with the short-term treatment of IL-15 mutant/Fc $\gamma$ 2a, we tested the efficacy of prolonged treatment with IL-15 mutant/Fc $\gamma$ 2a proteins. IL-15 mutant/Fc $\gamma$ 2a was administered just before the initial challenge of MBSA and continued daily until the day of Ag rechallenge (*Materials and Methods*). Control mouse IgG-treated



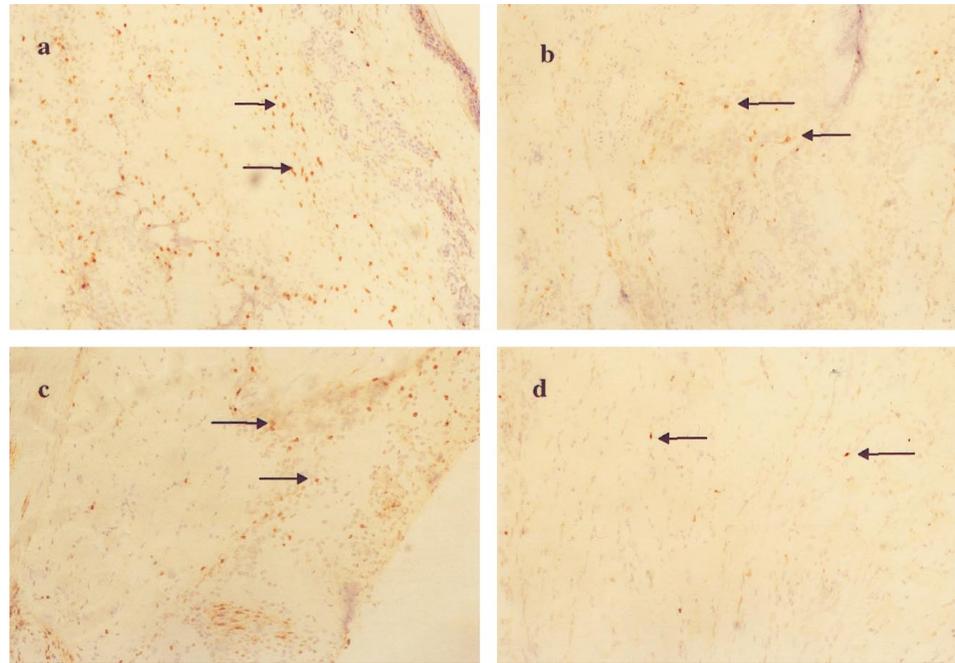
**FIGURE 4.** IL-15 mutant/Fc $\gamma$ 2a protein fails to trigger tyrosyl phosphorylation of STAT3/STAT5. IL-2R $\beta^+$  BAF-BO3 cells were washed, and restimulated with buffer alone, rhIL-2 (50 U/ml), or 10 ng/ml of either human rIL-15 or IL-15 mutant/Fc $\gamma$ 2a protein for 2 min at 37°C. Followed by SDS-PAGE and transfer onto membrane, immunostaining with phosphospecific STAT3 Ab (first row), anti-STAT3 Ab (second row), anti-phosphotyrosine Ab (third row), and anti-STAT5 Ab (fourth row) showed lack of phosphorylation of STAT3 and STAT5 in case of stimulation by IL-15 mutant/Fc $\gamma$ 2a protein. Cell lysates were obtained after stimulation with media (A), rhIL-2 (B), IL-15 mutant/Fc $\gamma$ 2a (C), or rhIL-15 (D).

Table I. Change of footpad thickness according to treatment

| Day | Treatment       | Thickness Change (mm; mean $\pm$ SD) | $p^a$  |
|-----|-----------------|--------------------------------------|--------|
| 1   | mIgG            | 0.51 $\pm$ 0.120                     |        |
|     | mutant/Fc       | 0.27 $\pm$ 0.105                     | < 0.01 |
|     | CsA + mIgG      | 0.59 $\pm$ 0.106                     | NS     |
| 2   | mutant/Fc       | 0.18 $\pm$ 0.173                     | < 0.01 |
|     | mIgG            | 0.56 $\pm$ 0.154                     |        |
|     | CsA + mIgG      | 0.40 $\pm$ 0.098                     | < 0.05 |
| 3   | mutant/Fc       | 0.09 $\pm$ 0.036                     | < 0.01 |
|     | mIgG            | 0.42 $\pm$ 0.171                     |        |
|     | CsA + mIgG      | 0.15 $\pm$ 0.042                     | < 0.01 |
|     | CsA + mutant/Fc | 0.39 $\pm$ 0.125                     | NS     |
|     | CsA + mutant/Fc | 0.03 $\pm$ 0.039                     | < 0.01 |

<sup>a</sup> Comparing with mIgG-treated mice. NS, not significant; mIgG, mouse IgG; eight mice were included in each group.

**FIGURE 6.** Immunohistochemical staining for the presence of macrophages/monocytes (12 h after Ag rechallenge) with anti-mouse F4/80 IgG2b. The arrows denote for F4/80<sup>+</sup> cells in specimens obtained from mice undergoing: mouse IgG treatment (a), IL-15 mutant/Fc $\gamma$ 2a protein treatment (b), mouse IgG and CsA treatment (c), and IL-15 mutant/Fc $\gamma$ 2a and CsA treatment (d). The decrease in footpad swelling and cell infiltration in IL-15 mutant/Fc $\gamma$ 2a  $\pm$  CsA-treated groups is parallel.



mice showed a brisk DTH response to rechallenge of MBSA, while the DTH responses in mice given IL-15 mutant/Fc $\gamma$ 2a were markedly attenuated (control mouse IgG-treated vs IL-15 mutant/Fc $\gamma$ 2a-treated mice:  $0.86 \pm 0.084$  mm vs  $0.23 \pm 0.143$  at 24 h after rechallenge,  $0.53 \pm 0.174$  vs  $0.13 \pm 0.052$  at 48 h, and  $0.33 \pm 0.041$  vs  $0.09 \pm 0.053$  at 72 h, respectively; mean  $\pm$  SD,  $p < 0.01$ ; 7 mice/each group).

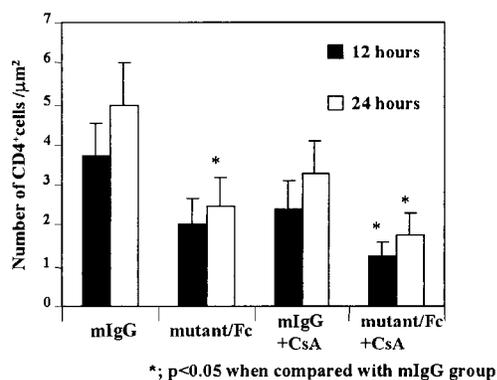
## Discussion

IL-15 is a 14- to 15-kDa member of the 4 $\alpha$ -helix bundle family of cytokines that possess T cell growth-factor activity (2, 6). In contrast to IL-2, a T cell product, IL-15 mRNA is expressed by a wide variety of cells, including macrophages, B cells, thymic, activated vascular endothelial cells, and bone marrow stromal cells, as well as tissues such as liver, heart, spleen, lung, and skeletal muscle (4, 6). Despite their differing cellular origins, IL-15 and IL-2 exert overlapping activities due to their shared

$\beta$ - and  $\gamma$ -chain receptor components (3, 25). While the expression of IL-2R $\alpha$  and IL-15R $\alpha$  upon mononuclear leukocytes is limited to recently activated cells, the tissue distribution of the unique IL-15R $\alpha$  component on nonimmune cells suggests that IL-15 has activity outside the immune system, such as anabolic activities on myocytes (26) and increasing transepithelial resistance on colonic epithelial cells (22).

IL-15 expression is associated with exacerbations of rheumatoid arthritis (8–10), sarcoidosis (27), and inflammatory bowel disease (11), as well as allograft rejection (12, 13). Because the importance of IL-15/IL-15R<sup>+</sup> cells to these immune/inflammatory disease states is not certain, we sought to target IL-15R<sup>+</sup> cells with a very high affinity receptor site-specific antagonist possessing a prolonged circulating  $t_{1/2}$  and the potential for cytotoxic targeting of IL-15R<sup>+</sup> cells. In this study, we report the design and properties of an IL-15 mutant/Fc $\gamma$ 2a (Q101D,Q108D) immunoligand protein (Fig. 1) that 1) specifically binds with high affinity to IL-15R (Fig. 2), 2) specifically inhibits IL-15-stimulated proliferative responses (Fig. 3), 3) fails to activate STAT-signaling pathway (Fig. 4), and 4) has a prolonged in vivo serum  $t_{1/2}$  of 6 h (Fig. 5). Importantly, the potential therapeutic value of the IL-15 mutant/Fc $\gamma$ 2a is hinted by the attenuation of T cell-dependent Ag responses (DTH) (Table I; Figs. 6 and 7).

The in vitro binding and proliferative results for IL-15 mutant/Fc $\gamma$ 2a parallel those reported for bacterially expressed IL-15 mutant proteins (manuscript submitted<sup>5</sup>) (20). The IL-15 mutant/Fc $\gamma$ 2a blocked cell proliferation triggered by rhIL-15, but not rhIL-2 (Fig. 3). Even excess amounts of IL-15 mutant/Fc $\gamma$ 2a fusion protein failed to inhibit IL-2-driven cell proliferation, while both rhIL-2- and rhIL-15-dependent IL-2R $\beta$ <sup>+</sup> BAF-BO3 cell proliferation was blocked by 4G3/3E12 rat anti-mouse IL-2R $\gamma$  (data not shown). In addition, binding of this mutant protein was not blocked by different growth factors, even though they share occupation of certain receptor subunits (Fig. 2). Combining the flow-cytometric analysis with cell proliferation results, human IL-15 and the IL-15-related mutant protein bind to mouse IL-15R. Therefore, the IL-15 mutant/Fc $\gamma$ 2a protein can be used to distinguish



**FIGURE 7.** IL-15 mutant/Fc $\gamma$ 2a treatment decreased the infiltration of CD4<sup>+</sup> T cells in the footpads. Immunohistochemical staining was done against CD4<sup>+</sup> T cells (12 and 24 h after Ag rechallenge). Ten randomly selected fields (each  $100 \mu\text{m}^2$ ) were scored on ethanol-fixed tissue sections. Numbers of cells are expressed in mean  $\pm$  SE.

IL-15 from IL-2-mediated responses. Using IL-15-sensitive cells, we now demonstrate that IL-15 mutant/Fc $\gamma$ 2a fails to stimulate phosphorylation of STAT3 and STAT5 proteins that are critical to IL-15 intracellular signaling (23, 24). Clearly, glutamine residues localized in the C-terminal  $\alpha$ -helix of the IL-15 molecule are crucial for STAT protein activation, which is a critical component of the intracellular signaling cascade leading to IL-15-mediated proliferation. Given the similar three-dimensional structures of IL-15 and IL-2 and the fact that a C-terminal glutamine in IL-2 is responsible for IL-2R $\gamma$  chain binding (28), it is reasonable to speculate that Q101D,Q108D IL-15 mutant/Fc $\gamma$ 2a proteins cannot transduce signals through the IL-2R $\gamma$  chain.

Genetic linkage of IL-15 to Fc enhanced the  $t_{1/2}$  of the IL-15 moiety (Fig. 5), as previously reported for fusion proteins involving IL-2 (18), IL-10 (19), and IL-4 (17). The  $t_{1/2}$  of 6 h for IL-15 mutant/Fc $\gamma$ 2a is not as long as the 33-h  $t_{1/2}$  for IL-10/Fc $\gamma$ 2a molecule (19), perhaps due to the larger tissue distribution of IL-15R than IL-10R. A second advantage of immunoligand construction is the opportunity to manipulate the Fc backbone to produce, as previously described, lytic and nonlytic forms of molecules (17, 19, 29). The known complement fixation and Ab-dependent cell cytotoxicity binding sites of the Fc moiety can be mutated to generate immunoligands that are nonlytic (19). In these studies, we used the native Fc $\gamma$ 2a backbone to create the IL-15 mutant/Fc $\gamma$ 2a fusion protein. This sequence provides longevity (1) and the ability to activate complement on receptor-bearing leukocytes.

This laboratory has reported previously that in vivo administration of an IL-2 diphtheria toxin-related fusion protein blocks DTH (30). As IL-15/IL-15R $\alpha$  mRNAs are expressed upon activated lymphocytes as well as tissues targeted by T cell-mediated immune reactions, we postulated that IL-15R-targeted treatment, as previously documented for IL-2R-targeted treatment (30), would also inhibit Th1-dependent in vivo DTH responses (31–33).

Commensurate with an attenuation in inflammation (e.g., footpad swelling) (Table I), IL-15 mutant/Fc $\gamma$ 2a treatment reduced the intralesional infiltration of macrophages and CD4<sup>+</sup> T cells (Figs. 6 and 7). Indeed, treatment with the IL-15 mutant/Fc $\gamma$ 2a proved more potent than a standard dose of CsA. Combined treatment with CsA plus IL-15 mutant/Fc $\gamma$ 2a synergistically inhibited the DTH reaction (Table I; Figs. 6 and 7). Although the mechanism by which the IL-15 mutant protein blocks T cell-dependent DTH responses was not directly addressed in this work, we speculate that IL-15R site antagonism and/or elimination of IL-15R<sup>+</sup> cells account for the effectiveness of IL-15 mutant protein treatment. Since the number of IL-15R<sup>+</sup> (or IL-2R-positive) cells within the inflammatory lesion is very small, we will determine whether cell lysis is responsible, at least in part, for diminishing the inflammatory response by comparing the effects of IL-15 mutant lytic and nonlytic Fc fusion proteins.

This report characterizes the binding and function of an antagonist-type IL-15 mutant/Fc $\gamma$ 2a and demonstrates that targeting of IL-15R can abrogate an in vivo Th1 response (DTH). Hence, based on the inhibition of DTH, we suggest that IL-15 mutant/Fc $\gamma$ 2a protein offers therapeutic promise as an agent for the treatment of Th1-type autoimmune diseases, organ transplantation, and other T cell-dependent disease processes. In short, we have constructed a novel long-lived IL-15 mutant/Fc $\gamma$ 2a molecule whose use may aid in determining the roles of IL-15 and IL-15R<sup>+</sup> cells in certain immune and inflammatory disease states.

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