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Brief communication

Monoclonal anti-interleukin-6 receptor antibody attenuates donor-specific antibody responses in a mouse model of allosensitization

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ABSTRACT

Interleukin 6 is an immune regulatory cytokine that impacts the development and maturation of T-cell, B-cell, and antibody producing plasma cells. A monoclonal antibody to the IL-6R (Tocilizumab®) was recently approved by the FDA for treatment of rheumatoid arthritis. Although anti-IL-6R anitbodies can reduce autoantibody levels in human disease, the use of anti-IL-6R for alloantibody suppression has not been examined. Here, we report on our experience with a mousenized rat-anti-mouse IL-6R (mMR16-1) for attenuating donor-specific antibody (DSA) responses. C57BL/6 mice were sensitized with skin allografts from a HLA.A2 transgenic mouse, and treated with intraperitoneal injections of mMR16-1 or control antibody. DSA responses were monitored weekly for 5 weeks by measurement of serum anti-HLA.A2 antibodies in a flow cytometric antibody binding assay. Results show that mMR16-1 significantly reduced DSA IgM, IgG2a and IgG1 responses, respectively, while normalizing serum amyloid A (SAA), an acute phase reactant induced by IL-6 ($p < 0.01$ vs. control). mMR16-1 injections increased mononuclear cell apoptosis in the spleens, as detected by annexin V staining and TUNEL. In conclusion, anti-IL6R attenuates de novo DSA responses and suppresses inflammatory markers (SAA). The data indicate that antibody therapy targeting the IL-6/IL-6R pathway may serve as a strategy to suppress DSA generation.

1. Introduction

Interleukin 6 is a pleiotropic cytokine that has potent effects on T-cells, B-cells and plasma cells [1]. Dysregulation of IL-6 production can occur in a number of disease states that ultimately are characterized by lymphadenopathy, excessive and unregulated antibody production and autoimmunity [2]. Animals transgenic for IL-6 have a phenotype similar to human Castleman's disease (fever, lymphadenopathy, autoimmune nephritis and hyperglobulinemia). When these animals are treated with an antibody to the IL-6 receptor (anti-IL-6R), all features of dysregulated immunity abate [2]. These observations led to clinical trials of anti-IL-6R therapy in Castleman's disease, rheumatoid arthritis, inflammatory bowel disease and systemic lupus erythematosus using a humanized version of the anti-IL-6R antibody, Tocilizumab® [2–6]. Tocilizumab® is a first in class humanized anti-IL-6R antibody that is FDA approved for treatment of rheumatoid arthritis and juvenile rheumatoid arthritis [7,8]. Tocilizumab® blocks activation of IL-6 receptors expressed on neutrophils, T-cells, mitogen-activated B-cells, peripheral monocytes and macrophages, therefore, preventing the inflammatory and immune stimulatory effects of IL-6 on target cells [7]. In addition, Tocilizumab® binds to soluble IL-6 receptor (sIL-6R), blocking circulating IL-6 interaction with sIL-6R and inhibiting subsequent trans signaling of gp130 membrane protein, which is present on most mammalian cells [1,2]. By blocking immune cell activation through the IL-6 receptors (classic signaling) and blocking IL-6 trans signaling through gp130, anti–IL-6R antibody improves the symptoms of these diseases and normalizes acute-phase proteins, such as C-reactive protein and serum amyloid A (SAA), and reduces B-cell activation and antibody production [8–11].

The role IL-6 plays in transplant rejection has been increasingly recognized [12,13]. Studies have shown that production of IL-6 is up-regulated in allografts undergoing acute rejection [14]. IL-6 promotes CD4 T-cell activation and expansion toward Th17 responses, while inhibiting regulatory T cells [15–17]. In animal models, IL-6 neutralization reduces intragraft $CD4^+$ cell infiltration, attenuates Th1 responses and suppresses serum alloantibodies [13]. In addition, antibodies to IL-6R are powerful inhibitors of CD4+ T-cell mediated lethal graft versus host disease (GVHD). This was associated with a reduction of Th17 cell activation and increased CD4+/CD25+/FoxP3+ cell populations in the spleen of treated animals [18]. Thus, targeting the IL-6/IL-6R pathway may have applications in prevention and treatment of allograft rejection and GVHD [19,20].

mMR16-1 is a mouse version of a rat-anti-mouse IL6R monoclonal (MR16-1) developed by Chugai Pharmaceutical in Japan [21]. The original MR16-1 was studied for treatment efficiency in various mouse models of autoimmune/inflammatory diseases [7]. Use of MR16-1 in mice has limited efficacy since the rat IgG1 monoclonal is highly

Abbreviations: DSA, donor-specific antibody; FITC, fluorescein isothiocyanate; IL-6R, Interleukin-6 receptor; IP, intraperitoneal injection; MFI, mean fluorescence intensity; mIL-6R, membrane-bound IL-6R; SAA, serum amyloid A; sIL-6R, soluble IL-6R; TUNEL, terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling.

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immunogenic [22]. Neutralizing antibodies against rat IgG rapidly develop after MR16-1 injection into the mouse, reducing therapeutic effect. Recently, MR16-1 was modified by Roche-Genentech (South San Francisco, CA). The new anti-IL-6R mAb is a rat (Fab)-mouse (FC) chimeric. The expectation was that by replacing the rat IgG1 FC with a mouse IgG2a FC, the new mousensized mMR16-1 would be less immunogenic and retain its IL-6R blocking potency.

Therefore, the purpose of this study was to examine the efficacy of mMR16-1 in suppressing alloantibody responses. We tested the hypothesis that mMR16-1, when used alone, would be effective in attenuating donor-specific antibody (anti-HLA-A2) responses to skin allografts. We used an established mouse model of sensitization in which skin grafts expressing a transgenic HLA.A2 antigen were transplanted on wild type mice [23,24]. We also examined serum levels of IL-6, IL-6R and serum amyloid A (SAA) in controls and mMR16-1 treated animals.

2. Materials and methods

2.1. Mouse model and anti-IL-6R treatment

A mouse model of allosensitization involving a skin graft transplanted from a C57BL/6-Tg(HLA-A2.1)1Enge/J mouse to a wild-type C57BL/6 (Jackson Laboratory, Bar Harbor, Maine; jaxmice.jax.org) recipient was employed [23,24]. In this model, the donor strain and recipient strain share a common B6 mouse background, but differ for a single transgenic HLA.A2 antigen, which can be recognized as an allopeptide capable of evoking allogenic immune responses by the recipient [25]. The use of animals and all the procedures performed were approved by Institutional Animal Care and Use Committee. All animals received humane care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Research (ILAR), published by the National Academies Press (1996 version; http://www.ncbi.nlm.nih.gov/books/NBK44152/).

Allograft recipients were divided into 3 groups (n = 6 in each group): (1) Isotype control, in which an anti-Ragweed mAb (mouse IgG2a) was given by intraperitoneal injection (IP) at a dosage schedule of 10 mg/kg, 3 times per week for 4 weeks; (2) low dose anti-IL-6R (IP injection of mMR16-1, 10 mg/kg, 3×/week for 4 weeks) and (3) high dose anti-IL-6R (30 mg/kg, 3×/week for 4 weeks).

Anti-IL-6R mAb (mMR16-1, IgG2a) and isotype control mAb (IgG2a, anti-ragweed) were provided by Genentech (South San Francisco, CA; www.gene.com).

2.2. Measurement of donor-specific anti-HLA.A2 antibodies

Serum samples weekly collected through retro-orbital bleeding were analyzed in a flow cytometric antibody binding assay in which a HLA.A2 expressing human T cell lymphoma line (CCL-120.1; ATCC, Manassas, VA; www.atcc.org) served as target cells. The procedure was described in previous publications [23,24].

2.3. ELISA for serum amyloid A2 (SAA2), sIL-6R and IL-6

Mouse SAA2 ELISA kit was purchased from Life Diagnostics (West Chester, PA; www.lifediagnostics.com). Mouse IL-6 ELISA Kit and IL-6R ELISA kit were obtained from RayBiotech (Norcross, Georgia; www.raybiotech.com). The assays were conducted per set of manufactory's manual. Samples were measured in duplicates. Reference standards comprised of known protein concentrations were used in each ELISA experiment to generate a standard curve, which was used for calibration of protein concentration in samples.

2.4. Flow cytometric analysis of splenocyte apoptosis

An FITC Annexin V Apoptosis Detection Kit (BD Biosciences, San Jose, California; www.bdbiosciences.com) was employed to study the early events of splenic cell apoptosis. Freshly isolated splenocytes in single cell suspension were stained with FITC annexin V in conjunction with 7-Amino-Actinomycin. The stained cells were then analyzed in a multiparameter flow cytometer (CyAn™ ADP, Dako USA, Carpinteria, CA; www.dako.com).

A two color TUNEL (Terminal deoxynucleotide transferase dUTP Nick End Labeling) assay (APO-BRDUTM Kit, Phoenix Flow Systems, San Diego, CA; www.phnxflow.com) was conducted according to the kit's manual. Each TUNEL experiment included use of positive and negative cells provided in the kit.

2.5. Statistics

Experimental data generated in the studies were reported as mean ± SEM. Significance levels were determined using a two-tailed two sample Student's *t*-test. A P value of <0.05 was considered statistically significant.

3. Results

3.1. mMR16-1 attenuates alloantibody responses to primary skin allografts

To assess the efficacy of mMR16-1 on modulating anti-HLA-A2 responses we analyzed serum samples from recipients of HLA-A2+ skin grafts (n = 6 in each group) weekly for 5 weeks post-transplantation. As shown in Fig. 1, titers of donor-specific IgM, IgG1 and IgG2a obtained from flow cytometric antibody binding assays were displayed as mean fluorescence intensity (MFI). The kinetics of DSA responses in isotype control mice showed early IgM production, which peaked at the second week post-transplant, and a subsequent strong IgG2a and moderate IgG1 response. Both low and high doses of mMR16-1 significantly inhibited anti-HLA-A2 responses, resulting in reduction of IgM (high dose vs. control: 12.7 ± 2.3 MFI vs. 22.9 ± 3.2 MFI at day 14, p = 0.00022), IgG2a (99.41 ± 35.31 MFI vs. 300.6 ± 53.04 MFI at day 28, p = 6.621E-05) and IgG1 (25.6 ± 9.8 MFI vs. 40.01 ± 7.7 MFI at day 28, p = 0.029), respectively.

3.2. mMR16-1 normalizes acute phase protein SAA2 induced by skin allografts

Serum amyloid A (SAA) is an acute phase reactant induced by IL-6. Tocilizumab® treatment significantly reduces SAA blood levels in patients [26]. Thus, we examined the effects of mMR16-1 vs. control antibody on serum SAA levels obtained after skin grafting using a mouse SAA2 ELISA. As shown in Fig. 2, normal mice showed low titers of serum amyloid A2 in sera (0.54 ± 0.04 μg/ml). This was also seen in sera from mMR16-1 treated mice without allografts (0.77 ± 0.17 μg/ml). SAA2 titers in sera of isotype control mice increased by >100-fold at Day 1 post-transplant (66.3 ± 2.14 μg/ml, P = 6.79E-09 vs. normal sera), and then gradually decreased to normal levels at Day 7 (39.02 ± 5.9, 42.75 ± 15.31 and 3.7 ± 0.08 μg/ml at Days 2, 3, and 7, respectively). In contrast, SAA2 titers in sera from mMR16-1 treated mice remained at baseline levels (2.14 ± 1.7, 0.8 ± 0.1, 2.35 ± 1.6 and 0.67 ± 0.08 μg/ml at Days 1, 2, 3 and 7, respectively), indicating SAA suppression by mMR16-1.

3.3. mMR16-1 treatment significantly increases sIL-6R and IL-6 concentrations in the blood

A prominent immunobiologic feature of the IL-6 receptor system is the existence of a naturally occurring form of soluble IL-6R in the blood [1,2]. To understand the impact of anti-IL-6R treatment on the homeostasis of sIL-6R we used ELISA to determine the serum concentrations of sIL-6R and IL-6, respectively. A striking finding (Fig. 3a) was that sIL-6R concentrations in mMR16-1 treated sera increased by 14-fold (77.8 ± 11.9 ng/ml) over that of isotype control sera (5.2 ± 0.56 ng/ml, p = 0.00016) within 24 h. The titers of sIL-6R in the treated sera reached 152.6 ± 6.1 ng/ml at day 7 and 156.5 ± 21.6 ng/ml at day 14 while the titers in the isotype control sera remained at low levels (Day 7, 4.9 ± 0.5 ng/ml, p = 4.533E-14 vs. treated; Day 14, 8.97 ± 0.4 ng/ml, p = 1.4955E-07). Titers of IL-6R in normal sera (Day 0) were 11.87 ± 4.4 ng/ml.

We also measured serum IL-6 using ELISA. As shown in Fig. 3b, normal (Day 0) mouse sera contained trace amount of IL-6 (23.4 ± 3.6 pg/ml). Serum IL-6 titers of isotype control mice increased dramatically (235.8 ± 15.5 pg/ml at Day 1 (p = 6.08079E-05 vs. normal), and gradually returned to normal levels at Day 7 post-transplant (19.1 ± 8.3 pg/ml). In contrast, serum IL-6 in mMR16-1 treated recipients were higher at Day 1 (700.8 ± 46.4 pg/ml, p = 4.10066E-06 vs. control: 235.8 ± 15.5 pg/ml), Day 2 (752.9 ± 126.8 pg/ml, p = 0.000673 vs. control: 160.8 ± 78.4 pg/ml), Day 3 (439.4 ± 219.3 pg/ml, p = 0.013 vs. control: 36.2 ± 26.9 pg/ml) and Day 7 (295.6 ± 187 pg/ml, p = 0.011 vs. control: 19.1 ± 8.3 pg/ml). These findings are consistent with data reported for use of Tocilizumab® in humans, suggesting a significant perturbation of IL-6/sIL-6R homeostasis [8].