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A Humanized Monoclonal Antibody against Heat Shock Protein 60 Suppresses Murine Arthritis and Colitis and Skews the Cytokine Balance toward an Anti-Inflammatory Response

Rina Ulmansky,* Dorit Landstein,† Eli Moallem,* Virginie Loeb, † Avi Levin,* Ronit Meyuhas, † Galia Katzavian, † Shira Yair, † and Yaakov Naparstek*†

We have previously shown that naturally occurring as well as acquired Abs against the Mycobacterium tuberculosis heat shock protein (HSP)65 protect against the induction of murine autoimmune inflammatory arthritis. In the present work, we have studied the anti-inflammatory effect of prozumab, a humanized anti-HSP mAb in murine inflammatory arthritis and colitis, and its effects on cytokine secretion. Prozumab was shown to bind to HSP60, the highly conserved mammalian homolog of the bacterial protein, and it was found to be effective in protecting and suppressing autoimmune arthritis in the models of adjuvant arthritis and collagen-induced arthritis in rats and mice, respectively, as well as in acute hapten-mediated colitis and chronic, spontaneous colitis models. Mechanistically, prozumab induces IL-10 secretion from naïve human PBMCs and suppresses the secretion of IFN-γ and IL-6 from anti-CD3-activated human PBMCs. These findings make prozumab a promising potential drug for treating human rheumatoid arthritis and inflammatory bowel disease, as well as a wide range of autoimmune inflammatory diseases. The Journal of Immunology, 2015, 194: 5103–5109.

M murine models of autoimmune diseases serve as important tools for understanding the mechanisms of the human diseases as well as the development of novel therapeutic agents. One classical example for such a model is the murine adjuvant arthritis (AA), which contributed to the elucidation of the mechanisms of human rheumatoid arthritis (RA) as well as the development of treatments for this disease.

AA can be induced in certain susceptible strains of rats such as the Lewis rat upon immunization with CFA, whereas other strains such as the Brown Norway rat are resistant to the induction of this disease. Moreover, differently from the human disease, the susceptible Lewis rats recover from arthritis spontaneously and develop resistance to reinduction of the disease.

We have previously shown that the resistance to arthritis is associated with the presence of natural polyclonal rat Abs directed to peptide-6, a surface epitope of the Mycobacterium tuberculosis heat shock protein (HSP)65, and that these Abs can suppress AA in rats (1, 2). Recognition of the exposed peptide-6 by these Abs correlated with binding to the whole M. tuberculosis HSP65 molecule as well as mammalian HSP60, a highly conserved stress protein with immunomodulatory activity. These Abs were found to stimulate IL-10 secretion by rat and human PBMCs (2), suggesting that resistance may be due to a local skewing of the cytokine profile toward an anti-inflammatory response.

Based on those findings, we have now humanized a murine anti-peptide-6 mAb, designated as prozumab, and studied its potential role as a therapeutic immunomodulatory drug.

In the present work, we have tested the effects of prozumab on murine models of arthritis and colitis and cytokine secretion from human PBMCs. We show that this Ab binds to mammalian HSP60 and is effective in protecting and suppressing autoimmune arthritis in the murine models AA and collagen-induced arthritis (CIA) in rats and mice, respectively, as well as in acute 2,4,6-trinitrobenzene sulfonic acid (TNBS)–induced colitis and chronic, spontaneous colitis models. Treatment was associated with upregulation of IL-10 secretion from naïve human PBMCs and suppression of the secretion of IFN-γ and IL-6 from anti-CD3–activated human PBMCs.

These findings make prozumab a promising drug for treating human RA and inflammatory bowel disease (IBD), as well as a wide range of autoimmune inflammatory diseases.

Materials and Methods

Animals

Six-week-old female Lewis rats, 8- to 10-wk-old male DBA/1 mice, and 6- to 8-wk-old male BALB/c mice were purchased from Harlan Laboratories (Jerusalem, Israel). Male 7- to 8-wk-old B6.129P2-H10I-1ter/1ter (IL-10 deficient) were purchased from The Jackson Laboratory (Bar Harbor, ME). The animals were handled in accordance with the guidelines of the institutional authority for biological and biomedical models.

Reagents

PBS, RPMI 1640, L-glutamine, sodium pyruvate, streptomycin, penicillin, n-glucose, HEPES, NaHCO3, sodium pyruvate medium, and FBS were purchased from Biological Industries (Beit Haemek, Israel). Bovine collagen type II was purchased from BioLegend (San Diego, CA). Prozumab (covered by patent application WO2011/027349 and granted U.S. patent 8,680,241) was purified from a Chinese hamster ovary cell line using standard techniques by Xcellerex (Marlborough, MA). Enbrel (etanercept; Wyeth Pharmaceuticals) was used as a positive control biologic

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agent. Avastin (bevacizumab; Roche) and rituximab (MabThera; Roche) were used as negative control Abs. Recombinant human HSP60 was purchased from ProSpec (Rehovot, Israel). Cryopreserved human PBMCs and the appropriate serum-free medium were purchased from Cellular Technologies (Shaker Heights, OH).

Development of a monoclonal humanized anti–peptide-6 Ab

Murine monoclonal anti–peptide-6 Abs were prepared by the hybridoma technique, and one clone was selected for humanization by Antitope (Cambridge, U.K.) utilizing the composite human Abs technology (3). A panel of humanized anti–peptide-6 mAb variants were produced (as described in patent application WO2011027349 Al), and one humanized variant, termed prozumab, was successfully cloned into a stable, high Ab expression Chinese hamster ovary mammalian cell line grown in serum-free media.

Binding of prozumab to recombinant HSP60 by ELISA

Flat-bottom MaxiSorp 96-well microtiter plates (Nunc, Roskilde, Denmark) were coated with 5 µg/ml recombinant human HSP60 in PBS (100 µl/well) overnight at 4°C. Plates were washed with wash buffer (0.1% Tween 20 in citrate buffer [pH 5.5] containing 0.15 M NaCl) and blocked for 1 h with 1% BSA in wash buffer. Plates were washed and goat anti-human Fc-specific Ab conjugated to peroxidase, prepared in blocking buffer, was added. Plates were incubated for 1 h at room temperature, washed, and the presence of Abs was detected by addition of 100 µl/well TMB substrate for 10 min at room temperature followed by addition of 1% hydrochloric acid (50 µl/well). Absorbance was detected at 450 nm by an absorbance microplate reader (BioTek, Winoski, VT).

Induction and clinical assessment of AA

AA was induced in 6-wk-old female Lewis rats by an intradermal injection of 1 mg M. tuberculosis H37Ra (Difco, Detroit, MI) in CFA at the base of the tail. Severity of arthritis (arthritis score) was assessed by a blinded observer as follows: 0, no arthritis; 1, redness of the joint; 2, redness and swelling of the joint. The tarsal–metatarsal and metatarsal–tibial joints of the hindpaws and carpal–metacarpal and metacarpal–radial joints of the forepaws were scored. The sum of scores for each rat was calculated so a maximum score of 16 can be obtained.

Induction and clinical assessment of collagen-induced arthritis

Collagen-induced arthritis (CIA) was induced in 8- to 10-wk-old male DBA/1 mice by an s.c. injection of 0.1 ml bovine collagen type II emulsified in CFA and Mycobacterium tuberculosis H37Ra at the base of the tail (200 µg/mouse; 50 µl emulsion at two sites). Three weeks later the mice were boosted with the same amount of collagen in incomplete Freund’s adjuvant. Severity of CIA was assessed daily by measuring the diameter of the tarsal–metatarsal joint of the hindpaws and the carpal–metacarpal joint of the fore paws with 0- to 10-mm calipers (Mitutoyo, Kawasaki, Japan) by a blinded observer. Starting from day 3 after the boost injection, every mouse with first signs of arthritis (i.e., redness of one paw) was assigned to one of the treatment groups to begin the treatment before the diameter of the paw increased.

Histopathology assessment in AA and CIA

Rats or mice were euthanized and hindpaws were removed and fixed in PBS containing 4% formalin. A longitudinal section was prepared from the tarsal–metatarsal bone and cross-section was prepared from the tibia–fibula–tarsal joint. Joints were stained with H&E and evaluated by a specialized veterinary pathologist. Histopathological changes were scored using semiquantitative grading (0–4) according to lesion/change severity: 0, no lesion; 1, minimal change; 2, mild change; 3, moderate change; 4, marked change. The arthritis changes evaluated were inflammation, presence of fibrin in the joint, cartilage loss, fibrosis, synovial cell loss, bone resorption, and periosteal apposition.

Induction and clinical assessment of hapten-mediated (TNBS) colitis

BALB/c mice were sensitized with 160 µl haptenating agent TNBS (Sigma-Aldrich, St. Louis, MO) at a concentration of 2.5% in 50% ethanol by skin painting. A week later, 120 µl 1% TNBS in 50% ethanol was administered intracutaneously via a 2.5-French catheter. Mice were sacrificed 3 d after intracutaneous TNBS administration. Animals were weighted at the time of sacrifice, intracutaneous administration, and every day following such until sacrifice. Clinical assessment was carried out by evaluating weight loss and histopathology of the colon tissue postmortem.

Histopathology assessment in TNBS colitis

Tissues were fixed in PBS containing 4% formalin and embedded in paraffin. Sections (5 µm) were stained with H&E. Histological grading of colitis included four parameters: extent of inflammation, degree of inflammation, degree of damage/necrosis, and Regeneration. Each parameter was scored by a blinded, experienced histopathologist using the following scoring system: from 0 to 3. Extent of inflammation: 0, none; 1, focal; 2, limited to one segment (proximal, middle, and distal); 3, involving more than one segment. Inflammation: 0, none; 1, mild; 2, moderate; 3, severe. Degree of damage/necrosis: 0, none; 1, mild (superficial); 2, moderate (involving muscularis mucosa); 3, severe (transmural, involving muscularis propria). Regeneration: 0, complete; 1, re-epithelialization; 2, focal migration and mitotic features; 3, none.

Spontaneous colitis in IL-10–deficient mice

Male mice (B6.129P2-Ilt2+/−;129Seri) (IL-10 deficient) 7 to 8 wk of age were selected and monitored for signs of disease, such as loss of weight, diarrhea, and prolapsed colon. When external clinical signs of disease appeared in two mice, at 19 wk of age, the mice were allocated to treatment groups of seven mice per group. Severity of disease was scored as follows: 0, none; 0.5–1.5, mild redness without diarrhea; 1.5–2.5, redness and diarrhea; 3.0, diarrhea and prolapse of colon. Mice exhibiting severe disease were sacrificed prior to study termination, at which time all remaining mice were sacrificed.

Cytokine levels in supernatants of human (PBMCs) and animal sera

All human samples were collected after informed consents were signed and the study was approved by the Helsinki Committee at Hadassah University Hospital (Jerusalem, Israel). Human venous blood was collected from healthy donors and layered on a Ficoll–Hypaque gradient to separate and enrich the WBC fraction. After centrifugation (1800 rpm, 30 min), the mononuclear cell band was collected, transferred into a new tube, washed with 20 ml PBS, and centrifuged (11000 rpm, 10 min). Cells were resuspended in 1 ml RPMI 1640 supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 100 µg/ml streptomycin, 100 U/ml penicillin, and 2% heat-inactivated human serum (Sigma-Aldrich) and incubated (1.5 × 10⁷ cells/ml) in the presence of prozumab or control treatments in flat-bottom 96-well cell culture plates for 24 or 48 h at 37°C. Supernatants were collected and stored at −80°C until tested.

For PBMC activation experiments, flat-bottom 96-well cell culture plates were coated with 5 µg/ml anti-CD3 Ab for 4 h at room temperature. Plates were washed with PBS and the cells (150 µl/well) were incubated in the presence of prozumab or control treatments in the coated wells for 24 h at 37°C with 5% CO₂.

For activation of cryopreserved human PBMCs, cells were thawed according to manufacturer’s instructions in serum-free medium. Anti-CD3 Ab was added at 2 µg/ml and the cells were dispensed in flat-bottom 96-well cell culture plates and incubated in the presence of prozumab or control treatments as described above for fresh donor cells.

Evaluation of cytokine levels in human cell culture or in the serum of animals was carried out utilizing specific ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions.

Jurkat cell culture

Jurkat E6.1 human T cell leukemia cells (American Type Culture Collection, Manassas, VA) were cultured in RPMI 1640 plus L-glucose (4.5 g/l), 10 mM HEPES, 1.5 g/l NaHCO₃, and 1 mM sodium pyruvate medium, supplemented with 10% FBS, 2 mM L-glutamine and 1% penicillin-streptomycin solution). Cells were grown at 37°C, 5% CO₂. Experiments were performed on cells that had achieved a density of 8 × 10⁵ cells/ml and contained <5% of dead cells as determined by trypan blue staining.

Flow cytometry analysis

Jurkat cells were harvested using ice-cold PBS. Cells were then washed with FACS medium (0.5% BSA plus 1% goat serum in PBS). Staining and washing were performed in FACS medium using a standard indirect immunofluorescence procedure. Primary mAbs and isotype controls were used at the concentration of 80–100 µg/ml. Secondary staining was performed using aliphophycocyanin-conjugated affinity pure, Fab’₂ fragment goat anti-human IgG (H+L) Ab (Jackson ImmunoResearch Laboratories) diluted 1:200 in FACS medium. Washing stages were performed on ice. Cells were then fixed in cold 3.7% buffered formalin phosphate and fluorescence data were acquired on an LSR II flow cytometer (BD Biosciences). Gating for viable cell population was applied for all analyses.
Materials and Methods

We have previously shown that polyclonal rat Abs to peptide-6 of the *M. tuberculosis* HSP65 bind to mammalian HSP60, suppress AA, and induce IL-10 secretion from murine and human PBMCs (1, 2). To further explore the therapeutic potential of such Abs, prozumab, a humanized anti–peptide-6 mAb, was prepared as described in Materials and Methods. As shown for the polyclonal Abs, prozumab binds to recombinant mammalian HSP60 in ELISA. Fig. 1 demonstrates that this binding is dose-dependent.

**Prozumab suppresses autoimmune arthritis in murine animal models**

The effect of prozumab in reducing AA severity was tested in established AA. PBS was used as a negative control and methylprednisolone succinate (MPS) steroids were used as a positive control. Lewis rats were immunized with CFA to induce arthritis and monitored for signs of arthritis via clinical scoring. On day 15, before the peak of disease, rats were treated i.p. with prozumab (2.5 mg/kg) or MPS. MPS (5 mg/kg) was injected s.c. on day 15. As shown in Fig. 2A, treatment with prozumab significantly ameliorated the severity of arthritis (*p* < 0.05 compared with PBS, on days 20–24).

In a similar experiment, levels of the pro- and anti-inflammatory cytokines IL-6, IFN-γ, IL-4, IL-10, IL-17, IL-1, and TNF-α were measured in the sera of representative AA-induced Lewis rats, treated with either PBS or prozumab. Levels of the anti-inflammatory cytokine IL-10 in the sera of prozumab-treated rats were higher compared with PBS-treated rats (mean ± SE of 71 ± 23 compared with 0 pg/ml). Reduced serum levels were detected for the proinflammatory cytokines IL-6 (mean ± SE of 127 ± 30 compared with 184 ± 105 pg/ml) and IFN-γ (mean ± SE of 33 ± 15 compared with 62 ± 36 pg/ml) in prozumab-treated rats compared with PBS-treated rats, respectively. These differences, however, did not reach significance. The levels of TNF-α and IL-1 were not detected, and the levels of IL-17 (ranging from 3.9 to 6.2 pg/ml) and IL-4 (ranging from 9.6 to 10.5 pg/ml) were similar in the sera of the representative rats (data not shown). Histopathology analysis revealed reduction in parameters of leukocyte infiltration in and around joint, fibrosis surrounding the joint or replacing bone and cartilage, synovial surface integrity, and cartilage erosions following treatment with prozumab compared with PBS, as represented in Fig. 2B. Normal articular surfaces with no evidence of inflammation or fibrosis can be seen in the prozumab-treated rats compared with intensive inflammatory infiltrate and marked fibrosis in the PBS-treated rats.

We also tested the effect of prozumab on arthritis in the CIA mouse model. Enbrel, a fusion protein composed of human TNF-type II receptor fused to human IgG1 Fc component, served as a biologic agent positive control, whereas rituximab, a chimeric Ab comprised of human IgG1 and directed at human CD20 specifically, was used as an isotype control. DBA/1 mice were injected s.c. with bovine collagen type II as described in Materials and Methods and randomly assigned to treatment groups as soon as redness appeared in one paw. Mice were treated i.p. on the day of arthritis onset and 4 d later according to the following treatment groups: PBS, rituximab (20 mg/kg), Enbrel (10 mg/kg), and prozumab (20 mg/kg). CIA severity was monitored by caliper measurement during an 8-d period and was calculated as the delta of paw diameter on each day compared with day 1 (onset of disease). Mean paw diameter on day 1 did not differ significantly between the groups and was between 1.59 and 1.61 mm.

Treatment with prozumab suppressed the disease, which reached significance (*p* < 0.05) on days 4–8 compared with rituximab treatment (Fig. 2C), similar to disease suppression achieved by Enbrel. Pathology assessment from three prozumab-treated mice and three rituximab-treated mice showed significant improvement (*p* < 0.02) in the following parameters: inflammation (score of 1.2 compared with 3.7, respectively) and fibrosis (score of 0.5 compared with 1.7, respectively). The levels of IL-10 were evaluated in the serum of the mice upon sacrifice, and IL-10 levels were significantly higher (*p* < 0.05) in the sera of prozumab-treated mice compared with all other treatment groups (Fig. 2D).

**Prozumab ameliorates colitis in mouse models of IBD**

To evaluate whether the anti-inflammatory effect of prozumab is relevant for autoimmune diseases other than arthritis, we tested prozumab in animal models of IBD. Furthermore, because the humanized Ab was developed against a peptide of HSP65, which is part of the adjuvant, one might argue that the effect of prozumab is through suppression of induction of the disease rather than the disease itself. Importantly, note that adjuvant (CFA) is not involved in the induction of disease in the IBD models that were tested.

Cytokines may be crucially involved in the pathogenesis of IBD, and, specifically, there have been references to the involvement of IL-10, IFN-γ, and IL-6 in the immunopathogenesis of IBD (4–6). Based on our preliminary results showing induction of IL-10 and reduction of IFN-γ and IL-6 secretion by treatment with prozu-mab, we have expanded the efficacy analysis with prozumab to the animal model of TNBS-induced colitis, which presents many features of IBD in humans (7, 8).

BALB/c mice were sensitized via TNBS skin painting, followed by intrarectal administration of TNBS a week later. The animals were sacrificed 3 d later and histopathological analysis of the colon tissue was carried out. Prozumab (0.8 and 8 mg/kg) was administered i.p. twice, that is, 2 d prior to and 1 d following intrarectal induction with TNBS. PBS-treated mice served as a negative control and rituximab (8 mg/kg) served as an isotype control Ab, both administered similar to prozumab.

Prozumab (8 mg/kg) was significantly effective in preventing weight loss (*p* < 0.05 compared with PBS- and rituximab-treated mice) (Fig. 3A). In mice treated with PBS, rituximab (8 mg/kg), or low-dose prozumab (0.8 mg/kg), weight loss (sacrifice versus intrarectal administration) was significantly higher (14.1, 13.1, and 14.0%, respectively) compared with prozumab (8 mg/kg)–treated mice (2.7%).

Pathology analysis of the colon revealed that treatment with prozumab (8 mg/kg) resulted in a significant reduction in the extent of tissue inflammation, necrosis, and regeneration compared with PBS- or rituximab-treated groups (Fig. 3B, 3C).

**Statistical analysis**

Analysis of statistical significance was performed using a Student *t* test for paired data. A *p* value <0.05 was considered significant.
All of the former experiments were carried out in acute autoimmune animal models induced by various substances. To evaluate the efficacy of prozumab in a spontaneous and chronic model, which better resembles the human disease, we tested its effect in IL-10–deficient mice, which develop spontaneous chronic colitis starting generally from 16 wk of age. The disease is characterized by loss of weight, diarrhea, and prolapse of the colon.

Male 7- to 8-wk-old mice (B6.129P2-IL10tm1Cgn/J) were assigned to four groups for treatment as follows: PBS (negative control; i.p. every other week), MPS (positive control; 50 mg/kg; s.c. every other week), prozumab (2.5 mg/kg; i.p. every other week), or rituximab (8 mg/kg; i.p. every other week). Two injections were given i.p. on the day of arthritis onset and 4 d later. Paw diameter change was calculated based on the difference between paw diameter on the specified day and the initial paw diameter on the day of arthritis onset, as assessed by caliper. The mean ± SE of eight to nine mice is shown. *p < 0.05 for prozumab compared with rituximab.

FIGURE 2. Prozumab suppresses AA and CIA. (A) AA was induced in Lewis rats as described in Materials and Methods. Rats were treated i.p. with prozumab (2.5 mg/kg) or PBS on day 15. MPS (5 mg/kg) was administered s.c. on day 15. Disease severity was evaluated every few days. Arthritis score is the mean ± SE of seven animals. *p < 0.05 for prozumab compared with PBS-treated rats. (B) Representative joint images of formalin-fixed paraffin-embedded sections prepared from rats treated with PBS or prozumab, as described in (A). Arrows indicate inflammation and fibrosis. Scale bars, 1 mm. (C) CIA was induced in DBA/1 mice as described in Materials and Methods. Mice were treated with PBS, prozumab (20 mg/kg), rituximab (20 mg/kg), or Enbrel (10 mg/kg). Two injections were given i.p. on the day of arthritis onset and 4 d later. Paw diameter change was calculated based on the difference between paw diameter on the specified day and the initial paw diameter on the day of arthritis onset, as assessed by caliper. The mean ± SE of eight to nine mice is shown. *p < 0.05 for prozumab compared with rituximab. (D) IL-10 levels (mean ± SE) in the sera of eight to nine CIA-induced mice, treated as described in (C) and sacrificed at the end of the study. *p < 0.05 for prozumab compared with all other treatment groups.

FIGURE 3. Prozumab ameliorates colitis in IBD mouse models. (A) Colitis was induced by TNBS as described in Materials and Methods. Reduction of body weight was measured in TNBS-induced colitis mice treated with prozumab (0.8 or 8 mg/kg), PBS, or rituximab (8 mg/kg). Treatment was administered i.p. twice, 2 d prior to and 1 d following intrarectal induction with TNBS. Body weight loss was calculated comparing mice weight at sacrifice to their weight at the intrarectal TNBS administration. Results are mean ± SE of eight mice per group. *p < 0.05 for prozumab (8 mg/kg) compared with PBS and rituximab-treated mice. (B) Representative images of formalin-fixed paraffin-embedded colon sections of mice treated with PBS and prozumab (8 mg/kg), as described in (A). Scale bars, 200 μm. (C) Histopathological analysis of the colon tissues taken from the TNBS-induced mice. Colon sections were scored for histopathologic changes. Results are the mean ± SE of seven to eight mice per group. *p < 0.05 for prozumab (8 mg/kg) compared with PBS and rituximab. (D) Disease score of prozumab (8 mg/kg) and control-treated mice in spontaneous colitis, developed in IL-10–deficient mice. Results are mean ± SE of six to seven mice per group. *p < 0.05 for prozumab compared with PBS and rituximab. (E) Mice survival in the various treatment groups of the spontaneous colitis model. *p < 0.05 for prozumab compared with PBS.
day), prozumab (8 mg/kg; i.p. every other week), and rituximab (isotype control; 8 mg/kg; every other week). As soon as two mice showed reduction in weight, on week 19, we initiated the treatment, which ended on week 29. Every other week, administration was selected based on initial pharmacokinetic analysis with prozumab, indicating an approximate 10-d t1/2 in rodents. This administration regimen corresponds to a potential application in clinical studies. However, reduction in weight was not a sensitive parameter, as external clinical signs of disease were obvious whereas the change in weight was delayed. Thus, from week 23 onward, we assessed the disease by scoring as detailed in Materials and Methods.

Treatment with prozumab suppressed the clinical signs of colitis significantly and was as effective as treatment with MPS (given every other day), compared with treatment with PBS or rituximab (Fig. 3D). Survival of prozumab-treated mice was significantly longer ($p < 0.05$ on weeks 27 and 28 compared with PBS) (Fig. 3E).

The levels of the proinflammatory cytokines (IL-6 and IFN-γ) were evaluated in the sera of the mice. Although the levels of IFN-γ were below the quantification limit, a trend toward reduction in IL-6 levels was found in the prozumab-treated mice compared with PBS-treated mice; however, this did not reach significance (data not shown).

**Prozumab modulates cytokine secretion from human PBMCs**

The effect of prozumab on the secretion of IL-10 from human PBMCs was evaluated, as elevated IL-10 levels were found following polyclonal anti–peptide-6 treatment (2). Incubation of PBMCs from various naive donors with prozumab resulted in increased IL-10 levels of up to 12-fold higher than the control (data not shown). However, this effect was highly variable and not observed in some of the donors upon incubation with prozumab.

Because prozumab efficacy was not solely dependent on IL-10, as shown in the spontaneous colitis model in IL-10 deficient mice, we investigated further the effect of prozumab on secretion of other pro- and anti-inflammatory cytokines using human PBMCs from naive donors with or without activation by various activators such as LPS, anti-CD3 Ab, or a combination of anti-CD3 and anti-CD28 Abs. These cells were treated with prozumab or control vehicle and their supernatant was tested by multicytokine analysis. Incubation of prozumab with cells following activation with LPS or anti-CD3/anti-CD28 did not result in a specific consistent effect. Considerable reduction of IL-6 and IFN-γ secretion from anti-CD3–activated PBMCs was, however, observed for all donors. Fig. 4A and 4B depict both the individual and mean relative reduction of IFN-γ and IL-6 secretion, respectively, with PBMCs from six donors activated by anti-CD3 and treated with prozumab compared with an isotype control. We then tested the effect of prozumab and its dose dependence on IFN-γ and IL-6 secretion from commercial cryopreserved human PBMCs. Commercial cryopreserved human PBMCs were used given ease of access to supplies of large amounts of same-donor PBMCs and in preparation for development of a cell-based bioassay with prozumab. As shown in Fig. 4C and 4D, reduction of IFN-γ and IL-6 secretion from anti-CD3–activated human PBMCs, obtained from two cryopreserved donors, is highly dependent on the prozumab dose.

**Prozumab binds to the surface of the Jurkat human T cell line**

Induction of cytokine secretion by anti-CD3 involves activation of T lymphocytes. We postulated that one of the mechanisms by which prozumab may exert its effect on cytokine secretion is by binding to T cells. Whereas human HSP60 is a mitochondrial chaperonin, its presence outside the mitochondria and outside the cell, for example, in circulating blood and on the surface of cells, has been extensively reported (9–11). We therefore examined prozumab binding to the human T cell line, Jurkat. As shown in Fig. 5A, FACS analysis demonstrates that prozumab binds to Jurkat cells specifically, compared with two isotype control Abs. Furthermore, recombinant HSP60 inhibits prozumab binding to Jurkat cells as shown in Fig. 5B. Presence of HSP60 protein on the cell surface of Jurkat cells was confirmed by FACS analysis using commercial anti-HSP60 Abs (data not shown).

**Discussion**

In the present study, we have shown that prozumab, a humanized mAb originally developed against the peptide-6 epitope of the *M. tuberculosis* HSP65, cross-reacts with the mammalian HSP60 and ameliorates experimental autoimmune inflammatory disease in various murine models by skewing the overall cytokine balance toward an anti-inflammatory response.

HSPs are a family of highly conserved stress proteins that function as intracellular molecular chaperones of newly synthesized polypeptide chains, preventing their aggregation during folding. In addition to stress-induced members, most HSP families also contain members that are constitutively expressed. Self and microbial HSPs also play an important role in the control of the immune response, and several HSP-derived immunotherapeutic agents have been developed and tested in clinical trials of autoimmune diseases (12).

Expression of the mammalian (or self) HSP60 is upregulated in stressed or damaged tissues, such as inflamed synovia of rats with AA (13) and RA patients (14). HSP60 expression also increases in colon mucosa from Crohn’s disease and ulcerative colitis patients (15). Although the exact mechanisms by which HSP60 is secreted into the extracellular medium are not understood, it is clear that extracellular HSP60 is a link between body tissues and the immune system (16). Soluble HSP60 has been shown to activate macrophages via TLR4 (17), B cells via a TLR4/MyD88 pathway (18), and T cells via TLR2 (19). One of the multiple effects of extracellular HSP60 on these components of the immune system is a change in cytokine secretion (20, 21). HSP60 is both a proinflammatory signal and an anti-inflammatory signal. The integrated effects of HSP60 on the immune response depend on the concentration, the particular HSP60 epitope (bacterial or self), and the cohort of cells present at the site where HSP60 acts (16).

Involvement of HSP60 in inflammation makes it a potential target for immune-mediated therapy and indeed several HSP60-based therapeutic agents have been developed and tested in preclinical and clinical settings of autoimmune diseases. An immunogenic peptide of the human HSP60 (aa 437–460), termed DiaPep277, arrested β cell destruction in NOD mice and was successful in preserving β cell function in phase II trials. It is now being tested in advanced phase III trials in new-onset type 1 diabetes (22, 23). Altered peptide of the aa 180–188 T cell epitope that targets and modulates HSP60-specific T cells induced highly effective protection against AA in rats (24). Recently, two altered peptides designed based on novel T cell epitopes of human HSP60 were shown to efficiently inhibit the course of AA and CIA (25). The immunomodulatory effect of these peptides is based on modulating the T cell function.

In the present work, we demonstrate that prozumab, an mAb targeting HSP60, suppresses autoimmune arthritis and colitis in murine models. This approach is based on our previous findings demonstrating that a polyclonal humoral rather than cellular response to this epitope characterizes natural resistance to AA and that monoclonal murine Abs against it can prevent and suppress autoimmune arthritis by upregulation of the anti-inflammatory cytokines. Our results showing that the humanized mAb prozumab ameliorates arthritis in AA and CIA models as well as IBD in...
acute TNBS-induced colitis and spontaneous colitis, and changes
the cytokine balance, support those findings. Interestingly, one of
the T cell–modulating therapeutic HSP60 peptides described by
Domínguez et al. (25), APL-2, resides in the same region and shares
significant sequence homology with the original Ag recognized by
the protecting Abs (peptide-6).

Prozumab, being an mAb that reacts with the mammalian HSP60,
represents the natural protective repertoire of polyclonal anti-HSP
Abs that are found in AA-resistant strains of rats and can transfer
resistance to the susceptible strains. Binding both the bacterial HSP
as well as the mammalian HSP raises an interesting question of
whether these Abs are part of the organism’s primary anti–self-
protective repertoire of Abs or rather the reaction of the organism
to environmental pathogens either external or its own microbiota.
Given that prozumab has been generated following immunization
with a peptide originated from the bacterial HSP may support the
latter hypothesis.

In the present work, we have shown that prozumab is capable of
reducing the anti–CD3 induction of IFN-γ and IL-6 secretion from
human PBMCs ex vivo and to induce the production of the anti-
inflammatory cytokine IL-10 from human PBMCs ex vivo and
in vivo in the experimental model of AA. Although enhanced IL-10
secretion from human PBMCs was observed following prozumab
addition ex vivo, the Ab was able to ameliorate spontaneous colitis
developing in IL-10–deficient mice. Hence, elevated IL-10 is not
essential for the effect of prozumab in reducing colitis. Given that we
did not observe significant reduction of IL-6 in the serum of the mice
and the levels of IFN-γ were undetectable, it is possible that pro-
zumab may exert its effect via local suppression of proinflammatory
cytokines from activated mononuclear cells in the inflamed colon.
Further investigation would be warranted to evaluate this hypothesis.

The mechanism by which prozumab induces this cytokine shift
has not been completely deciphered. Because HSP60 can be found
on the cell surface or in extracellular fluids, prozumab may either
bind to and sequester soluble HSP60 or bind to cells via its HSP60
protein or the specific peptide recognition. It is postulated that such
binding eventually leads to modulation of cytokine secretion from
immune cells, which results in an overall anti-inflammatory balance.
Indeed, in a preliminary work, we have found that the Ab binds
to cell lines of mononuclear origin, such as Jurkat cells. Moreover,
HSP60 inhibits the binding, suggesting that prozumab interacts with
the cell surface–expressed HSP60 protein on Jurkat cells. Further
investigations of prozumab binding to primary cells and its mode of
action are ongoing.

Cytokines are important modulators of autoimmune diseases, and
the balance between proinflammatory cytokines, such as TNF-α, IFN-γ,
and IL-6, and anti-inflammatory cytokines, such as IL-10, plays a
major role in the development of autoimmune diseases both in animals
and humans. Since the launch of anti–TNF-α drugs, development of
therapies for autoimmune diseases based on cytokine modulation is

FIGURE 4. Prozumab modulates cytokine secretion from human PBMCs. IFN-γ (A) and
IL-6 (B) levels in supernatants from anti–CD3-activated human PBMCs, incubated without
(no Ab) or with 200 μg/ml prozumab or an isotype control Ab for 24 h, were determined
by ELISA. Results obtained from each of the six naive donors tested are shown. The inset
depicts the calculated mean ± SE of relative reduction in cytokine secretion from Ab-treated
cells compared with nontreated cells (no Ab) for these six donors. For the calculation, cyto-
kine secretion of nontreated cells (no Ab) for each donor was defined as 1. (C and D) Effect
of prozumab dose on cytokine secretion (IFN-γ and IL-6, respectively) from anti–CD3-acti-
vated cryopreserved human PBMCs from two naive donors. Mean ± SE of results obtained
from triplicate wells is presented.

FIGURE 5. Prozumab binding to Jurkat cells by FACS analysis. (A) Jurkat cells (0.7–1 × 10⁶/ml) stained using prozumab and the isotype control mAbs, rituximab or Avastin, at a concentration of 100 μg/ml followed by allophycocyanin-conjugated anti-human Ab or the secondary Ab only. (B) Percentage binding of prozumab (80 μg/ml) or prozumab in the presence of 120 μg/ml soluble human HSP60 to Jurkat cells. Rituximab served as the isotype control. Detection was done using allophycocyanin-conjugated anti-human Ab. Mean ± SE of results obtained from duplicates is shown.
increasing and new such drugs are being approved. These drugs are mostly aimed at neutralization of a single cytokine.

A single Ab such as prozumab that skews the combined cytokine balance of IFN-γ, IL-6, and IL-10 toward an anti-inflammatory response via modulation of the immune cells rather than depletion or addition of individual cytokines represents a novel therapeutic approach and makes prozumab a promising drug for human RA, IBD, and potentially additional autoimmune inflammatory diseases. HSP60 appears to be an ideal Ag for immunotherapy given that expression of HSP60 is upregulated at the site of inflammation. Thus, immunotherapy directed against HSP60 via an mAb such as prozumab would be site specific and there is potential for targeting various autoimmune inflammatory indications.

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Disclosures
Y.N. is the Chief Scientific Officer of ProtAb Ltd. D.L. and S.Y. are current employees of ProtAb Ltd. V.L., R.M., and G.K. are former employees of ProtAb Ltd. The remaining authors have no financial conflicts of interest.

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