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(54) Title: ATTENUATION OF THE ADAPTIVE IMMUNE RESPONSE

(57) Abstract: The present invention encompasses compositions and methods for attenuating a host's immune system response to a graft, by administering an IL-1 receptor antagonist to a host, thereby improving clinical outcomes for host recipients of grafts.

TITLE OF THE INVENTION

Attenuation of the Adaptive Immune Response

CROSS REFERENCE TO RELATED APPLICATIONS

Application claims priority under 35 U.S.C.§119(e) to U.S. Provisional Application No. 60/880,885, filed on January 17, 2007 which is incorporated by reference herein in its entirety.

BACKGROUND OF THE INVENTION

For dysfunctional and/or diseased organs of the body, after therapeutic intervention with drugs, organ transplantation is an alternative, and sometimes a treatment of last resort for a patient. For patients with leukemia, end-stage renal, cardiac, pulmonary or hepatic failure in particular, organ transplantation is becoming increasingly common. Allografts (organ grafts harvested from human donors other than the patient him/herself or host/recipient of the graft) of various types, e.g. kidney, heart, lung, liver, bone marrow, pancreas, cornea, small intestine and skin (e.g. epidermal sheets) are currently routinely performed. Xenografts (organ grafts harvested from non-human animals), such as porcine heart valves, are also being used clinically to replace their dysfunctional human counterparts.

Graft arteriosclerosis is the major cause of clinical graft failure after the first year posttransplantation and is characterized by a diffuse narrowing of the conduit arteries of the graft (Libby and Pober, 2001, Immunity 14:387-397). Animal models clearly demonstrate a role for the host antigraft adaptive immune response in the development of graft arteriosclerosis, as such lesions either do not form, or form much more slowly, in syngeneic grafts or in grafts transplanted into immunocompromised recipients (Tullius and Tilney, 1995, Transplantation 59:313-318). Affected arteries contain infiltrates of T cells subjacent to the luminal endothelium. Graft endothelial cells (EC) are a likely target of the host alloimmune response, as human EC express MHC class I and class II in vivo and can activate allogeneic resting memory T cells to proliferate and produce effector cytokines in vitro (Choi et al., 2004, Annu. Rev. Immunol. 22:683-709).

T cell production of IFN-γ in particular plays a key role in the development of graft arteriosclerosis. Genetic ablation of IFN- γ or its receptors

ameliorates graft arteriosclerosis in mouse heterotopic heart transplant models (Wiseman, et al., 2001, J. Immunol. 167:5457-5463' Nagano et al., 1997, J. Clin. Invest. 100:550-557). In humanized mouse models, IFN- γ alone is sufficient to induce arteriosclerosis in human artery grafts (Tellides et al., 2000, Nature 403:207-211), whereas neutralization of IFN- γ protects human artery grafts from allogeneic T cell-mediated remodeling (Wang et al., 2004, FASEB J. 18:606-608). Although the role of IL-17 in graft arteriosclerosis is less clear, T cell derived IL-17 has been implicated in acute allograft rejection, as blockade of the IL-17R prolongs survival of murine heterotopic heart allografts (Antonysamy et al., 1999, J. Immunol. 162:5577-584; Tang et al., 2001, Transplantation 72:348-350).

Nonimmune factors also contribute to the development of graft arteriosclerosis and dictate in part the lifespan of allografts (Choi et al., 2004, Annu. Rev. Immunol. 2:683-709). In human kidney transplantation, cadaver allografts fail at a faster rate than living donor grafts, often despite better MHC matching (Terasaki et al., 1995, N. Eng. J. Med. 333:333-336). This difference in graft survival has been attributed to the greater degree of injury incurred by cadaver grafts, which includes incubation in a hemodynamically unstable donor, longer cold ischemic times, and in the case of non-heart beating donors, additional warm ischemic time (Salahudeen et al., 2004, Kidney Int. 65:713-718). In addition, cadaver renal allografts with delayed graft function, a condition that correlates strongly with prolonged ischemic time, have a significantly shorter half-life than those without delayed function (Perico,e t al., 2004, Lancet 364:1814-1827). One possible explanation of these observations is the "burden of injury" hypothesis, which proposes that early graft injury is one of several types of injury experienced by a graft, including acute rejection episodes, chronic rejection, and preexisting comorbidities, such that when all of the forms of injury are summed, grafts exposed to increased perioperative injury simply reach the threshold of failure earlier (Weis and von Scheidt, 1997, Circulation 96:2069-2077). Alternatively, the "immune modulation" hypothesis posits that early graft injury changes a graft such that it interacts with the host adaptive immune system differently, eliciting an altered rejection response with increased production of pathogenic cytokines (Libby and Pober, Immunity 14:387-397; Halloran, et al., 1997, Transplant Proc. 29:79-81). Experiments using murine cardiac allograft models have provided some support for this latter hypothesis, as grafts recently exposed to ischemia-reperfusion injury are more rapidly rejected by an adoptively transferred

effector T cell populations than are healed-in grafts (Chalasani et al., 2004, J. Immunol. 172:7813-7820).

Currently, allograft rejection is controlled using a combination of potent immunosuppressive agents including calcineurin inhibitors such as cyclosporine and tacrolimus; inhibitors of the mammalian target of rapamycin (mTOR) pathway including rapamycin also known as sirolimus and the related everolimus; anti-proliferatives including Azathioprine and mycophenolic acid or mycophenolate mofetil; coricosteroids including prednisolone and hydrocortisone; and antibodies including monoclonal anti-IL-2Rα receptor antibodies, such as Basiliximab and Daclizumab, as well as polyclonal anti-T-cell antibodies, such as anti-thymocyte globulin (ATG) and anti-lymphocyte globulin (ALG).

Although the development of new immunosuppressive drugs has led to substantial improvement in the survival of patients, these drugs are associated with a high incidence of side effects such as nephrotoxicity, hepatotoxicity, as well as significant cardiovascular complications including drug-induced hypertension, hyperlipidemia, and diabetes. Thus, there still exists the need for treatment and prophylaxis for managing a host's immune response to allografts with improved toxicity profiles. The present invention fills this need.

SUMMARY OF THE INVENTION

One embodiment of the present invention comprises a method of attenuating a host immune system's T cell response to an antigen present on a graft, the method comprising administering to the host a therapeutically effective amount of IL-1 receptor antagonist prior to establishing host circulation to the graft during transplantation of the graft into the host. In one aspect, the host is human. In another aspect, the graft comprises a cell, a tissue, an organ, or any combination thereof, excluding a cell derived from bone marrow. In another aspect, the IL-1 receptor antagonist is administered systemically to the host up to at least 1 day but not more than 3 days after the host receives the graft. In yet another aspect, the IL-1 receptor antagonist is administered systemically to the host up to at least 1 day but not more than 7 days after the host receives the graft. In still another aspect, the IL-1 receptor antagonist is administered systemically to the host up to at least 1 day but not more

than 14 days after the host receives the graft. In still another aspect, the IL-1 receptor antagonist is administered systemically to the host up to at least 1 day but not more than 21 days after the host receives the graft.

Another embodiment of the present invention comprises a method of enhancing the long-term survival of an allograft in a host, the method comprising administering to the host a therapeutically effective amount of IL-1 receptor antagonist prior to establishing host circulation to the allograft during transplantation of the allograft into the host. In one aspect, the host is human. In another aspect, the allograft comprises a cell, a tissue, an organ, or a combination thereof, excluding a cell derived from bone marrow. In another aspect, the IL-1 receptor antagonist is administered systemically to the host up to at least 1 day but not more than 3 days after the host receives the allograft. In yet another aspect, the IL-1 receptor antagonist is administered systemically to the host up to at least 1 day but not more than 7 days after the host receives the allograft. In still another aspect, the IL-1 receptor antagonist is administered systemically to the host up to at least 1 day but not more than 14 days after the host receives the allograft. In still another aspect, the IL-1 receptor antagonist is administered systemically to the host up to at least 1 day but not more than 21 days after the host receives the allograft.

Another embodiment of the present invention comprises a method of treating a patient experiencing a stroke, the method comprising administering to the patient a therapeutically effective amount of IL-1Ra within 24 hours of the onset of the stroke. In one aspect, the IL-1 receptor antagonist is administered systemically to the patient up to at least 1 day but not more than 3 days post-onset of said stroke. In another aspect, the IL-1 receptor antagonist is administered systemically to the patient up to at least 1 day but not more than 7 days post-onset of the stroke. In still another aspect, the IL-1 receptor antagonist is administered systemically to the patient up to at least 1 day post-stroke, but not more than 14 days post-onset of the stroke. In another aspect, the IL-1 receptor antagonist is administered systemically to the patient up to at least 1 day but not more than 21 days post-onset of the stroke.

BRIEF DESCRIPTION OF THE DRAWINGS

For the purpose of illustrating the invention, there are depicted in the drawings certain embodiments of the invention. However, the invention is not limited

to the precise arrangements and instrumentalities of the embodiments depicted in the drawings.

Figure 1, comprising Figure 1A through Figure 1C, is a series of graphs depicting mediators contained within human endothelial cell (EC) lysates, including high mobility group protein B1 (HMGB1), that enhance IFN-γ production in human EC-T cell cocultures. Figure 1A is a graph depicting the concentration of IFN- γ in the culture medium as assayed by ELISA at 24 hours. EC lysates were added to cocultures of $CD4^+$ T cells with allogeneic $HLA\text{-}DR^+$ EC. Mean±SD of triplicate samples is shown. Figure 2B is a photograph of an immunoblot depicting both the presence of HMGB1 in EC lysates and the effectiveness of depleting HMGB1 by immunoadsorption of EC lysates by incubation with isotype control- or anti-HMGB1-coated beads. Figure 1C depicting the concentration of IFN- γ in the culture medium as assayed by ELISA at 24 hours. The effect of adding control- or HMGB1-depleted EC lysates to cocultures of $CD4^+$ T cells with allogeneic $HLA\text{-}DR^+$ EC on IFN- γ concentration in the culture medium is shown. Mean±SD of triplicate samples is shown. *, $p < 0.001$; **, $p < 0.02$. One of six (A) or one of three (C) independent experiments using two different donors is shown with similar results.

Figure 2, comprising Figure 2A through Figure 2C, is a series of graphs that depict the effect of recombinant HMGB1 on $CD4^+$ T cell IFN- γ production. Figure 2A is a graph depicting the concentration of IFN- γ in the culture medium as assayed by ELISA at 24 hours. The effect of recombinant HMGB1 (5 μg/ml) added to cultures of $CD4^+$ T cells alone or to cocultures of $CD4^+$ T cells with allogeneic $HLA\text{-}DR^+$ or $HLA\text{-}DR^+$ EC is shown. Mean±SD of quadruplicate samples is shown. Figure 2B is a series of graphs depicting the effect of coculturing $CD4^+$ T cells with allogeneic EC and stimulating with 1 μg/ml PHA in the presence or absence of recombinant HMGB1 (5 μg/ml). Brefeldin A was added for the final 4 hours of the 24-hour culture, at which time cells were permeabilized and stained for intracellular IFN- γ and cell surface CD4. Figure 2C is a graph depicting percent IFN-γ, CD4+ T cells. The mean±SD of triplicate samples was analyzed as in Figure 2B. *, $p < 0.0005$; ** $p < 0.002$. One of eight (A) or one of three (B) independent experiments is shown using three different donors with similar results.

Figure 3, comprising Figure 3A through Figure 3C, is a series of graphs that depict the effect of HMGB1 on alloreactive T cell IFN-γ production in the

absence of monocytes. Figure 3A is a pair of graphs depicting the results of flow cytometric analysis for CD4 and CD14. Positively isolated $CD4^+$ T cells were depleted of monocytes using an anti-CD14 Ab and compared with depletion with a control Ab. Figure 3B is a graph depicting the concentration of IFN-γ present in the culture medium as assayed by ELISA at 24 hours for $CD4^+$ T cell populations either depleted of $CD14^+$ monocytes or not depleted of monocytes and cocultured with allogeneic HLA-DR^+ EC and treated with HMGB1 (5 μg/ml) or control buffer. Mean± SD of triplicate samples is shown. Figure 3C is a graph depicting the effect of depleting monocytes from $CD4^+$ T cell isolates as in Figure 3A, and negatively isolated autologous monocytes added back to $CD4^+$ T cell isolates or not before coculture with HLA-DR^+ allogeneic EC. HMGB1 (5 μg/ml) or control buffer was added. IFN-γ was measured in culture medium by ELISA at 24 h. Mean±SD of triplicate samples is shown. *, $p < 0.001$; **, $p < 0.0001$. One of three independent experiments using three different donors with similar results.

Figure 4, comprising Figure 4A through Figure 4C, is a series of graphs that depict the effect of treating monocytes with HMGB1 to enhance alloreactive T cell IFN-γ production through secretion of IL-1β. Figure 4A is a graph depicting the concentration of IFN-γ in the culture medium as assayed by ELISA at 24 hours. Negatively isolated monocytes were treated with HMGB1 (5 μg/ml) or control buffer for 24 h. Conditioned medium (CM) from HMGB1- or control-treated monocytes was added to cocultures of $CD4^+$ T cells, thoroughly depleted of monocytes, with allogeneic HLA-DR^+ EC at the indicated dilutions, and Mean±SD of triplicate samples is shown. Figure 4B is a graph depicting the concentration of IFN-γ in the culture medium as assayed by ELISA at 24 hours. Monocyte conditioned medium was added to EC-T cell cocultures at a 1/30 dilution with a neutralizing Ab to IL-1β or an isotype control Ab (10 μg/ml). Mean±SD of triplicate samples is shown. Figure 4C is a graph depicting depicting the concentration of IFN-γ in the culture medium as assayed by ELISA at 24 hours. HMGB1 (5 μg/ml) was added to cocultures of HLA-DR^+ EC with allogeneic $CD4^+$ T cells not depleted of monocytes. A neutralizing Ab to IL-1β or an isotype control was added (10 μg/ml) at the start of the culture. Mean±SD of triplicate samples is shown. *, $p < 0.05$; **, $p < 0.005$. One of three independent experiments is shown using two different donors with similar results.

Figure 5, comprising Figure 5A through Figure 5D, is a series of graphs that depict the efficacy of HMGB1 to induce monocyte secretion of IL-1β via TLR4 and CD14. Figure 5A is a graph depicting the concentration of IL-1β in the culture medium as assayed by ELISA at 24 hours. Monocytes isolated by negative selection were treated with increasing concentrations of HMGB1. Figure 5B is a graph depicting the concentration of IL-1β in the culture medium as assayed by ELISA at 24 hours. Monocytes were pretreated with blocking Abs to TLR2, TLR4, CD14, or AGER or an isotype control Ab (20 μg/ml) for 30 min before addition of HMGB1 (10 μg/ml). Figure 5C is a graph depicting the concentration of IL-1β in the culture medium as assayed by ELISA at 24 hours. Monocytes were treated with HMGB1 or LPS. Mean±SD of triplicate samples is shown. *, $p < 0.00005$. Figure 5D comprises an image of a gel depicting the results of incubating the HMGB1 stock solution with beads coated with a control Ab or an anti-HMGB1 Ab, and then analyzing an aliquot of each by immunoblotting for HMGB1. Both the HMGB1 stock solution and the control buffer were subjected to immunoadsorption and then added to monocytes as in Figure 5A.. Figure 5D further comprises a graph depicting the concentration of IL-1β in the culture medium as assayed by ELISA at 24 hours. One of three independent experiments is shown using two different donors with similar results.

Figure 6, comprising Figure 6A through Figure 6C, is a series of graphs that depict the ability of neutralization of IL-1α and IL-1β to block the effect of EC lysates on T cell IFN-γ and IL-17 production. Figure 6A is a graph depicting the concentration of IFN-γ (top) and IL-17 (bottom) in the culture medium as assayed by ELISA at 24 hours. EC lysates generated by freeze-thaw were added to cocultures of HLA-DR^+ EC with allogeneic $CD4^+$ T cells thoroughly depleted of monocytes. A neutralizing Ab to IL-1α or an isotype control was added at 10 μg/ml. Mean±SD of triplicate samples is shown. Figure 6B is a graph depicting the concentration of IFN-γ in the culture medium as assayed by ELISA at 24 hours. EC lysates were added to cocultures of HLA-DR^+ EC and allogeneic $CD4^+$ T cells that contained a small number of monocytes. Neutralizing Abs to IL-1α and/or IL-1β were added as indicated at 10 μg/ml. Mean±SD of triplicate samples is shown. Figure 6C is a graph depicting the concentration of IFN-γ in the culture medium as assayed by ELISA at 24 hours. Increasing concentrations of IL-1R antagonist were added as indicated to

cocultures treated with EC lysates (f) or control buffer (u). IFN-γ in the culture medium were assayed by ELISA at 24 h. Mean±SD of triplicate samples is shown. *, $p < 0.05$; #, $p < 0.005$; **, $p < 0.0005$; ***, $p < 0.00005$. One of three independent experiments is shown using three different donors with similar results.

Figure 7, comprising Figure 7A through Figure 7C, is a series of graphs that depict IL-1 increases IFN-γ and IL-17 production from memory $CD4^+$ T cells. Figure 7A comprises a pair of graphs depicting the concentration of IFN- γ (top) and IL-17 (bottom) in the culture medium as assayed by ELISA at 24 hours. Increasing concentrations of IL-1α were added to cocultures of $CD4^+$ T cells depleted of monocytes with allogeneic $HLA\text{-}DR^+$ or $HLA\text{-}DR^+$ EC. A neutralizing Ab to IL-1α (10 μg/ml) was also added where indicated. Mean±SD of triplicate samples is shown. Figure 7B comprises a pair of graphs depicting the concentration of IFN- γ (top) and IL-17 (bottom) in the culture medium as assayed by ELISA at 24 hours. $CD4^+$ T cells were depleted of monocytes and separated into $CD45RA^+$ or $CD45RO^+$ subsets by negative selection. T cell populations were activated by anti-CD3 (5 μg/ml) and anti-CD28 (2.5 μg/ml) mAbs with increasing doses of IL-1. Mean±SD of triplicate samples is shown. #, $p < 0.01$; *, $p < 0.005$; **, $p < 0.001$; ***, $p < .0005$. One of three independent experiments using three different donors with similar results is shown. Figure 7C is a graph depicting the results of quantitative RT-PCR for IL-1R1 expression in $CD4^+$ T cells activated by anti-CD3 and anti-CD28 mAbs. Each point represents a separate T cell donor. IL-1R1 expression is normalized to GAPDH expression and is shown as a relative fold change compared with resting $CD4^+$ T cells for each donor.

Figure 8, comprising Figure 8A through Figure 8C, is a series of graphs that depict that IL-1 promotes preferential expansion of alloreactive human memory $CD4^+$ T cells that produce IL-17. Figure 8A is a series of three graph depicting the fold change of IFN-γ (left), IL-17 (middle), and IL-5 (right) in the culture medium as assayed by ELISA after 24 hours of restimulation. Purified memory $CD4^+$ T cells were activated by coculture with allogeneic EC with the indicated concentrations of IL-1α and then restimulated by coculture with EC from the same donor as the primary coculture without further addition of IL-1. Pooled data from three T cell donors are shown. Figure 8B is a pair of representative dot plots of intracellular cytokine staining (ICS) for IFN-γ and IL-17 obtained from one donor.

Purified memory $CD4^+$ T cells were activated by coculture with CD32-transduced EC coated with anti-CD3 mAb with or without 100 pg/ml IL-1α and then restimulated with PMA plus ionomycin and analyzed by ICS. Figure 8C is a series of graphs depicting the fold change in the amount of cytokine produced (Figure 8A) or the number of cytokine-positive T cells (Figure 8C) upon restimulation between IL-1-treated cocultures and control cocultures was calculated for each donor. Pooled data from four T cell donors treated as in Figure 8B are shown. Data were analyzed by a one-sample *t* test and represent the mean±SE in ($n = 3$ experiments) *A* or ($n = 4$ experiments) *C*. *, $p < 0.05$. The range of values for amount of cytokine measured in Figure 8A were IFN-γ (47–737 pg/ml), IL-17 (15–1012 pg/ml), and IL-5 (10–437 pg/ml). The range of values for the percentage of cells positive for each cytokine in *C* were IFN-γ (14–34%), IL-17 (0.7–5%), and IL-4 (2.4–4.8%).

Figure 9, comprising Figure 9A and Figure 9B, is a series of graphs that depict that under inflammatory conditions, such as those produced by tumor necrosis factor (TNF) EC enhance alloreactive memory CD4+ T cell cytokine production via IL-1α. Figure 9A is a graph that depicts the concentration of IFN-γ in the culture supernatant were measured by ELISA at 24hours. Figure 9B is a graph that depicts the concentration of IL-17 in the culture supernatant were measured by ELISA at 24hours. TNF-treated EC are more potent inducers of allogeneic T cell cytokine production, and a significant portion of this activity is due to EC production of IL-1α. * $p < 0.05$ compared to untreated EC. ** $p < 0.05$ compared to TNF-treated EC with isotype control.

Figure 10, comprising Figure 10A through Figure 10C, is a series of graphs that depict that under inflammatory conditions, such as those produced by tumor necrosis factor (TNF), living EC skew the alloreactive memory CD4+ population towards IL-17 production via IL-1α. Figure 10A is a graph depicting the concentration of IFN-γ in the culture supernatant as measured by ELISA. Figure 10B is a graph depicting the concentration of IL-17 in the culture supernatant as measured by ELISA. Figure 10C is a graph depicting the concentration of IL-5 in the culture supernatant as measured by ELISA. Co-culture of memory CD4+ T cells with TNF-treated EC skews the expanded alloreactive CD4+ T cell population towards an IL-17 producing phenotype. * $p < 0.05$ compared to untreated EC. ** $p < 0.05$ compared to TNF-treated EC with isotype control.

Figure 11, comprising Figure 11A and Figure 11B, is a pair pf photomicrographs depicting the effect of ischemia perfusion injury on IL-1α expression in human coronary artery interposition grafts transplanted into the femoral aorta of CB-17 SCID/beige immunodeficient mouse recipients. Figure 11A is a photomicrograph depicting staining for human IL-1α in sham treated arteries. Figure 11B is a photomicrograph depicting focally increased staining for human IL-1α within the endothelium of ischemia-reperfused grafts.

Figure 12, comprising Figure 12A through Figure 12C, is a series of graphs depicting the effect of IL-1 blockade by IL-1 receptor antagonist on T cell-derived IL-17 production and intimal lesion size in human coronary artery interposition grafts transplanted into an immunodeficient mouse host as in Figure 11, and then exposed to adoptively transferred human T cells from a donor allogeneic to the human artery donor. Figure 12A is a graph depicting the relative expression of IL-17 and GAPDH in PBS treated (controls) and IL-1Ra treated arteries. Figure 12B is a graph depicting relative expression of IL-17 and CD3e in PBS treated (controls) and IL-1Ra treated arteries. Figure 12C is a graph depicting the intimal area in control and IL-1Ra treated arteries. There appears to be a trend towards reduced total amount of IL-17 expression, reduced IL-17 expression per CD3+ T cell, and reduced intimal expansion in rejecting arteries with IL-1 blockade (n=7).

DETAILED DESCRIPTION OF THE INVENTION

The invention is based on the discovery that long term prognosis for the acceptance of a transplanted graft in a recipient (host) is dependent on whether or not the donated graft expresses and/or induces high levels of IL-1 at the time of transplantation into the host. Damaged donor grafts express large amounts of IL-1. They also release mediators, such as HMGB1, that cause the host to make large amounts of IL-1. Undamaged donor grafts neither substnatially produce nor induce IL-1 expression in the host. When a damaged graft is introduced into a recipient, the recognition of the graft-expressed or host-induced IL-1 by the T cells of the host's immune system induces a host immune response that has a deleterious effect on the long term acceptance of the graft. This reaction is different from IL-1 induced inflammation that can also occur during graft rejection. Thus, the present invention is very specifically focused on attenuating the host adaptive immune response that

occurs in response to host T cells "sighting" the graft-expressed IL-1 or host-induced IL-1 at the instant when such IL-1 comes in contact with the host, by administering to the host an IL-1 receptor antagonist (IL-1Ra) at the time host circulation is established to the organ (such as, for example, release of the surgical clamps that prevent host blood flow to the organ) during the transplantation surgery.

According to the present invention, it is therefore contemplated that administration of IL-1Ra to the host would occur during transplant surgery, and would extend for only a limited time thereafter. This is in contrast to conventional anti-IL-1 treatments contemplated for graft recipients, where IL-1 is administered generally following transplant surgery, and treatment is continued for a prolonged period of time. This latter treatment is referred to herein as "treatment of inflammation" whereas the treatment contemplated in the present invention is referred to as "attenuating the host adaptive immune response that occurs in response to graft-expressed IL-1 or host-induced IL-1" during the final stages of the transplant procedure.

Accordingly, an essential distinctive feature of the method of the present invention is the narrow therapeutic window during which a practitioner of the method is required to administer an IL-1Ra to a host. Specifically, IL-1Ra is administered systemically to a host during the surgical graft of an allogeneic tissue or organ into the host, such that at the moment of initial perfusion of a graft tissue or organ by the host circulatory system, sufficient IL-1Ra is present to attenuate the host adaptive immune response that occurs in response to graft-expressed IL-1 or host-induced IL-1. Systemic IL-1Ra administration to the graft recipient may be continued throughout the remaining surgical procedure or throughout the post-operative recovery period for at least one day after the graft or transplant has been surgically completed and not more than 21 days after the graft or transplant has been surgically completed.

The methods of the present invention include compositions and methods to attenuate the host adaptive immune response that occurs in response to host T cells "sighting" the graft-expressed IL-1 or host-induced IL-1 when the host has received an allogeneic, vascularized graft comprising a cell, a tissue, an organ, or a combination thereof. Still another embodiment of the present invention comprises compositions and methods of preventing allograft rejection in a host.

The invention should not be construed to be limited to treatment of a host recipient of an allogeneic graft. Rather, the invention may also be useful in situations where host damaged tissue expresses sudden large amounts of IL-1 due to tissue damage for example, at the onset of stroke. Accordingly, another embodiment of the present invention comprises compositions and methods of minimizing neurological tissue loss during stroke.

Definitions:

As used herein, each of the following terms has the meaning associated with it in this section.

The articles "a" and "an" are used herein to refer to one or to more than one (i.e. to at least one) of the grammatical object of the article. By way of example, "an element" means one element or more than one element.

The term "about" will be understood by persons of ordinary skill in the art and will vary to some extent on the context in which it is used.

"Acute rejection or AR" is the rejection by the immune system of a tissue transplant recipient when the transplanted tissue is immunologically foreign. Acute rejection is characterized by infiltration of the transplanted tissue by immune cells of the recipient, which carry out their effector function and destroy the transplanted tissue. The onset of acute rejection is rapid and generally occurs in humans within a few weeks after transplant surgery. Generally, acute rejection can be inhibited or suppressed with immunosuppressive drugs such as calcineurin inhibitors, mTOR inhibitors, anti-proliferatives, coricosteroids, antibodies, and the like.

"Chronic transplant rejection or CR" generally occurs in humans within several months to years after engraftment, even in the presence of successful immunosuppression of acute rejection. Replacement fibrosis of organ parenchyma and luminal loss of conduit arteries, generally called graft arteriosclerosis, are common factors in chronic rejection of all types of organ transplants. Chronic rejection can typically be described by a range of specific disorders that are characteristic of the particular organ. For example, in lung transplants, such disorders include fibroproliferative destruction of the airway (bronchiolitis obliterans); in heart transplants, such disorders include fibrotic arteriosclerosis leading to cardiac allograft vasculopathy; in kidney transplants, such disorders include, obstructive nephropathy,

nephrosclerorsis, tubulointerstitial nephropathy; and in liver transplants, such disorders include disappearing bile duct syndrome.

The term "autogeneic," "autologous," or, "self," as used herein, indicates the origin of a cell with respect to a recipient of the cell. Thus, a cell is autogeneic if the cell was derived from an individual (the "donor") or a genetically identical individual and is to be readministered to the same individual. An autogeneic cell can also be a progeny of an autogeneic cell. The term also indicates that cells of different cell types are derived from the same donor or genetically identical donors. Thus, an effector cell and an antigen presenting cell are said to be autogeneic if they were derived from the same donor or from an individual genetically identical to the donor, or if they are progeny of cells derived from the same donor or from an individual genetically identical to the donor.

Similarly, the term "allogeneic," or "non-self," as used herein, indicates the origin of a cell, tissue, or organ with respect to a recipient of the cell, tissue, or organ. Thus, a cell and progeny thereof is allogeneic if the cell was derived from an individual not genetically identical to the recipient to whom it is to be administered; in particular, the term relates to non-identity in expressed MHC molecules. The term also indicates that cells of different cell types are derived from genetically non-identical donors, or if they are progeny of cells derived from genetically non-identical donors. For example, an APC is said to be allogeneic to an effector cell if they are derived from genetically non-identical donors.

As used herein, the term “allograft” refers to an allogeneic, vascularized graft comprising a cell, a tissue, an organ, or a combination thereof, derived from a non-self human donor and administered, implanted, or transplanted into a host such that the allograft is contacted by the host’s immune system.

As used herein, “syngeneic” refers to biological material derived from a genetically-identical individual (e.g. identical twin) as the individual into whom the material will be introduced.

The term “graft,” as used herein, refers to a cell, tissue, organ, or combination thereof, excluding cells derived from bone marrow, that can be transplanted into a host.

The term "host," as used herein, generally refers to any mammal, such as humans, non-human primates, rodents, and the like, which is to be the recipient of the particular treatment. In the present invention, this treatment is specifically a graft

or transplant of biological material obtained from a donor comprising a cell, a tissue, or an organ for the purpose of supplementing or replacing biological material of the host that is diseased, disordered, damaged, or otherwise no longer optimally functioning in the host. Typically, the terms "host," "recipient," and "patient" are used interchangeably herein to refer to a human subject.

The term "Interleukin -1 Receptor antagonist (IL-1Ra)," as used herein includes any therapeutically effective molecule that inhibits, antagonizes, or interferes with the interaction of IL-1 with its receptor. Accordingly, an IL-1Ra of the present invention should be construed to include therapeutically effective forms of a native IL-1 receptor antagonist (nIL-1Ra) protein that occurs naturally in the body, or therapeutically effective variants, analogs, or fragments thereof; a therapeutically effective form of an antibody directed against an IL-1 receptor, or therapeutically effective variants, analogs, or fragments thereof; a soluble IL-1R, and any other IL-1 receptor antagonist disclosed herein.

By "attenuate the host immune response," as used herein, it is intended that administering an IL-1 receptor antagonist, as defined herein, to a host attenuates the host T cell response directed against a transplanted graft, leading to better graft function, graft survival, and improved clinical outcomes for the host. This term includes any accompanying up- or down-regulation of cytokines released by cells of the host immune system.

As used herein, "sighting" refers to a host's adaptive immune system's ability to detect and respond to a foreign antigen, such as the non-self MHC molecules present on an graft. Antigen recognition by T cells (i.e. "sighting") triggers T cell proliferation, clonal expansion, and acquisition of effector functions such as cytokine secretion by cells of the host's immune system. In the present invention, therefore, elevated IL-1 levels produced, secreted, or released by an graft, or made by the host's innate immune system in response to a transplanted graft, modulate the host T cell response that follows antigen recognition ("sighting"), thereby changing the effector functions that mediate the immune response directed against antigens, for example by increasing expression of certain cytokines while decreasing others. Further, elevated levels of IL-1 produced, secreted, or released by a patient's own damaged tissue, such as might be the case in stroke, can induce a self-directed immune response. When such IL-1 is produced and T cell function is thereby modulated by elevated IL-1 expression, a poor outcome is predicted.

As used herein, to "treat" means reducing the frequency with which symptoms of a disease, defect, disorder, or adverse condition, and the like, are experienced by a patient.

As used herein, a "therapeutically effective amount" is the amount of a composition of the invention sufficient to provide a beneficial effect to the individual to whom the composition is administered. For instance, with respect to the administration of an IL-1Ra, a therapeutically effective amount is that amount of IL-1Ra that attenuates the host T cell response directed against an graft, leading to improved graft function, graft survival, and improved clinical outcomes for the host, .

A "therapeutic" treatment is a treatment administered to a subject who exhibits at least one symptom of pathology for the purpose of treating or alleviating the at least one symptom.

"Encoding" refers to the inherent property of specific sequences of nucleotides in a polynucleotide, such as a gene, a cDNA, or an mRNA, to serve as templates for synthesis of other polymers and macromolecules in biological processes having either a defined sequence of nucleotides (i.e., rRNA, tRNA and mRNA) or a defined sequence of amino acids and the biological properties resulting therefrom. Thus, a gene encodes a protein if transcription and translation of mRNA corresponding to that gene produces the protein in a cell or other biological system. Both the coding strand, the nucleotide sequence of which is identical to the mRNA sequence and is usually provided in sequence listings, and the non-coding strand, used as the template for transcription of a gene or cDNA, can be referred to as encoding the protein or other product of that gene or cDNA.

Unless otherwise specified, a "nucleotide sequence encoding an amino acid sequence" includes all nucleotide sequences that are degenerate versions of each other and that encode the same amino acid sequence. Nucleotide sequences that encode proteins and RNA may include introns.

An "isolated nucleic acid" refers to a nucleic acid segment or fragment which has been separated from sequences which flank it in a naturally occurring state, i.e., a DNA fragment which has been removed from the sequences which are normally adjacent to the fragment, i.e., the sequences adjacent to the fragment in a genome in which it naturally occurs. The term also applies to nucleic acids which have been substantially purified from other components which naturally accompany the nucleic acid, i.e., RNA or DNA or proteins, which naturally accompany it in the cell. The

term therefore includes, for example, a recombinant DNA which is incorporated into a vector, into an autonomously replicating plasmid or virus, or into the genomic DNA of a prokaryote or eukaryote, or which exists as a separate molecule (i.e., as a cDNA or a genomic or cDNA fragment produced by PCR or restriction enzyme digestion) independent of other sequences. It also includes a recombinant DNA which is part of a hybrid gene encoding additional polypeptide sequence.

In the context of the present invention, the following abbreviations for the commonly occurring nucleic acid bases are used. "A" refers to adenosine, "C" refers to cytosine, "G" refers to guanosine, "T" refers to thymidine, and "U" refers to uridine.

"Recombinant polynucleotide" refers to a polynucleotide having sequences that are not naturally joined together. An amplified or assembled recombinant polynucleotide may be included in a suitable vector, and the vector can be used to transform a suitable host cell. A recombinant polynucleotide may serve a non-coding function (e.g., promoter, origin of replication, ribosome-binding site, etc.) as well.

"Polypeptide" refers to a polymer composed of amino acid residues, related naturally occurring structural variants, and synthetic non-naturally occurring analogs thereof linked via peptide bonds, related naturally occurring structural variants, and synthetic non-naturally occurring analogs thereof. Synthetic polypeptides can be synthesized, for example, using an automated polypeptide synthesizer.

The term "protein" typically refers to large polypeptides.

The term "peptide" typically refers to short polypeptides.

The term "antibody," as used herein, refers to an immunoglobulin molecule which is able to specifically bind to a specific epitope on an antigen. Antibodies can be intact immunoglobulins derived from natural sources or from recombinant sources and can be immunoreactive portions of intact immunoglobulins. Antibodies are typically tetramers of immunoglobulin molecules. The antibodies in the present invention may exist in a variety of forms including, for example, polyclonal antibodies, monoclonal antibodies, Fv, Fab and $F(ab)_2$, as well as single chain antibodies and humanized antibodies (Harlow et al., 1999, Using Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, NY; Harlow et al., 1989,

Antibodies: A Laboratory Manual, Cold Spring Harbor, N.Y.; Houston et al., 1988, Proc. Natl. Acad. Sci. USA 85:5879-5883; Bird et al., 1988, Science 242:423-426).

By the term "synthetic antibody" as used herein, is meant an antibody which is generated using recombinant DNA technology, such as, for example, an antibody expressed by a bacteriophage as described herein. The term should also be construed to mean an antibody which has been generated by the synthesis of a DNA molecule encoding the antibody and which DNA molecule expresses an antibody protein, or an amino acid sequence specifying the antibody, wherein the DNA or amino acid sequence has been obtained using synthetic DNA or amino acid sequence technology which is available and well known in the art.

A "portion" of a polynucleotide means at least at least about fifteen to about fifty sequential nucleotide residues of the polynucleotide. It is understood that a portion of a polynucleotide may include every nucleotide residue of the polynucleotide.

By the term "specifically binds," as used herein, is meant an antibody which recognizes and binds an antigen of interest, but does not substantially recognize or bind other molecules in a sample.

A "prophylactic" treatment is a treatment administered to a subject who does not exhibit signs of a disease or exhibits only early signs of the disease for the purpose of decreasing the risk of developing pathology associated with the disease.

"Preventing" a disease, as the term is used herein, means that the onset of the disease is delayed, and/or that the symptoms of the disease will be decreased in intensity and/or frequency, when an IL-1 receptor antagonist is administered compared with the onset and/or symptoms in the absence of an Il-1 receptor antagonist.

By "pharmaceutically acceptable carrier" is meant any carrier, diluent or excipient which is compatible with the biological component of a pharmaceutical composition and not deleterious to the recipient.

Description

I. Compositions

The present invention provides novel therapeutic uses for compounds that inhibit or antagonize IL-1 receptor type I. In one embodiment, a native

interleukin-1 receptor antagonist (nIL-1Ra) comprises a naturally occurring protein that antagonizes the IL-1 interaction with its receptor. Other compounds useful in the practice of the methods of the present invention include any molecule that inhibits or antagonizes IL-1 receptor type I, including an antibody specific for IL-1 receptor type I, a soluble IL-1 receptor, an antisense oligonucleotide, or an siRNA.

A. Native Interleukin-1 Receptor Antagonist

Native IL-1 receptor antagonist (nIL-1Ra) is a naturally occurring protein that inhibits the activity of the proinflammatory cytokine interleukin-1 (IL-1). The IL-1 pathway consists of the two agonists IL-α and IL-1β, IL-1 converting enzyme, a native receptor antagonist (nIL-1Ra) produced in different isoforms and two high affinity receptors. IL-1α and IL-1β bind to two distinct IL-1 receptor types, IL-1 receptor type I (IL-1RI) and IL-1 receptor type II (IL-1RII), both of which are members of the immunoglobulin superfamily of receptors. Both types of receptors are usually coexpressed, although type I is the predominant form in fibroblasts and T cells, while type II is preferentially expressed on B cells, monocytes and neutrophils. IL-1RI and IL-1RII have different affinities for the three ligands of the IL-1 family (IL-α, IL-1β and IL-1Ra). In particular, nIL-1Ra binds to the type I receptor with an affinity similar to that of IL-1α, while nIL-1Ra binds to the type II receptor 100-fold less efficiently than the type I receptor. There is evidence indicating that IL-1 induced activities are mediated exclusively via the type I receptor, whereas the type II receptor has no signaling activity and inhibits IL-1 activities by acting as a decoy for IL-1.

nIL-1Ra binds to the IL-1 receptor with affinity similar to that of IL-1 but has no IL-1-like activity, even at very high concentrations, and thus inhibits (antagonizes) the activity of IL-1. The purified nIL-1Ra molecule has a molecular weight of approximately 25 kD and is believed to be glycosylated. An unglycosylated recombinant form of nIL-1Ra that has a molecular weight of approximately 17 kD is commercially available from a number of sources including Amgen (Thousand Oaks, CA) and R & D Systems (Minneapolis, MN). nIL-1Ra has limited sequence similarity to IL-1α and IL-1β at the amino acid level (19% and 26%, respectively). There appear to be at least two isoforms of nIL-1Ra, including a soluble form and an intracellular form generated by an alternative splicing event. nIL-1Ra appears to be produced by monocytes, macrophages, neutrophils and fibroblasts; keratinocytes and cells of epithelial origin produce almost exclusively the intracellular form. In humans, the

gene for nIL-1Ra has been localized to the long arm of chromosome 2, which is the same region where IL-1α and IL-1β, as well as IL-1RI and IL-1RII, are found.

The term "variant" (or "analog") as used herein refers to any polypeptide differing from naturally occurring polypeptides by amino acid insertions, deletions, and substitutions, created using, e g., recombinant DNA techniques. Variants that comprise amino acid sequence having at least about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, or higher sequence identity to IL-1Ra protein, and that retain the desired biological activity of IL-1Ra, are contemplated in the uses according to the present invention. Guidance in determining which amino acid residues may be replaced, added or deleted without abolishing activities of interest, may be found by comparing the sequence of the particular polypeptide with that of homologous peptides and minimizing the number of amino acid sequence changes made in regions of high homology (conserved regions) or by replacing amino acids with consensus sequence. Guidance can also be provided by various three-dimensional protein modeling programs known in the art. In general, conservative substitutions are expected to provide a variant that retains biological activity of wild type polypeptide.

Preferably, amino acid "substitutions" are the result of replacing one amino acid with another amino acid having similar structural and/or chemical properties, i.e., conservative amino acid replacements. "Conservative" amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues involved. For example, nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and methionine; polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine; positively charged (basic) amino acids include arginine, lysine, and histidine; and negatively charged (acidic) amino acids include aspartic acid and glutamic acid. "Insertions" or "deletions" are typically in the range of about 1 to 5 amino acids. The variation allowed may be experimentally determined by systematically making insertions, deletions, or substitutions of amino acids in a polypeptide molecule using recombinant DNA techniques and assaying the resulting recombinant variants for activity.

Alternatively, where alteration of function is desired, insertions, deletions or non-conservative alterations can be engineered to produce altered

polypeptides. Such alterations can, for example, alter one or more of the biological functions or biochemical characteristics of the polypeptides of the invention. For example, such alterations may change polypeptide characteristics such as ligand-binding affinities, interchain affinities, or degradation/turnover rate. Further, such alterations can be selected so as to generate polypeptides that are better suited for expression, scale up and the like in the host cells chosen for expression. For example, cysteine residues can be deleted or substituted with another amino acid residue in order to eliminate disulfide bridges.

Any variant, analog, or fragment of IL-1 receptor antagonist that retains therapeutic efficacy in preventing sighting of allograft expressed IL-1 as described herein should be understood to be included in the invention. Such a variant, analog, or ragment is referred to herein as a therapeutically effective form of IL-1 receptor antagonist.

B. Antibodies specific for IL-1 receptor type I

In another embodiment or the present invention, an IL-1 receptor antagonist is an antibody targeted against the IL-1 receptor in the compositions and methods of the invention is a polyclonal antibody (IgG), the antibody is generated by inoculating a suitable animal with the targeted cell surface molecule. Antibodies produced in the inoculated animal which specifically bind to the cell surface molecule are then isolated from fluid obtained from the animal. Antibodies may be generated in this manner in several non-human mammals such as, but not limited to goat, sheep, horse, camel, rabbit, and donkey. Methods for generating polyclonal antibodies are well known in the art and are described, for example in Harlow, et al. (1988, In: Antibodies, A Laboratory Manual, Cold Spring Harbor, NY).

Monoclonal antibodies directed against a full length targeted cell surface molecule or fragments thereof may be prepared using any well known monoclonal antibody preparation procedures, such as those described, for example, in Harlow et al. (1988, In: Antibodies, A Laboratory Manual, Cold Spring Harbor, NY) and in Tuszynski et al. (1988, Blood, 72:109-115). Human monoclonal antibodies may be prepared by the method described in U.S. patent publication 2003/0224490. Monoclonal antibodies directed against an antigen are generated from mice immunized with the antigen using standard procedures as referenced herein. Nucleic acid encoding the monoclonal antibody obtained using the procedures described

herein may be cloned and sequenced using technology which is available in the art, and is described, for example, in Wright et al. (1992, Critical Rev. in Immunol. 12(3,4):125-168) and the references cited therein.

When the antibody used in the methods of the invention is a biologically active antibody fragment or a synthetic antibody corresponding to antibody to a targeted cell surface molecule, the antibody is prepared as follows: a nucleic acid encoding the desired antibody or fragment thereof is cloned into a suitable vector. The vector is transfected into cells suitable for the generation of large quantities of the antibody or fragment thereof. DNA encoding the desired antibody is then expressed in the cell thereby producing the antibody. The nucleic acid encoding the desired peptide may be cloned and sequenced using technology which is available in the art, and described, for example, in Wright et al. (1992, Critical Rev. in Immunol. 12(3,4):125-168) and the references cited therein. Alternatively, quantities of the desired antibody or fragment thereof may also be synthesized using chemical synthesis technology. If the amino acid sequence of the antibody is known, the desired antibody can be chemically synthesized using methods known in the art as described elsewhere herein.

The present invention also includes the use of humanized antibodies specifically reactive with targeted cell surface molecule epitopes. These antibodies are capable of binding to the targeted cell surface molecule. The humanized antibodies useful in the invention have a human framework and have one or more complementarity determining regions (CDRs) from an antibody, typically a mouse antibody, specifically reactive with a targeted cell surface molecule.

When the antibody used in the invention is humanized, the antibody can be generated as described in Queen, et al. (U.S. Patent No. 6, 180,370), Wright et al., (supra) and in the references cited therein, or in Gu et al. (1997, Thrombosis and Hematocyst 77(4):755-759), or using other methods of generating a humanized antibody known in the art. The method disclosed in Queen et al. is directed in part toward designing humanized immunoglobulins that are produced by expressing recombinant DNA segments encoding the heavy and light chain complementarity determining regions (CDRs) from a donor immunoglobulin capable of binding to a desired antigen, attached to DNA segments encoding acceptor human framework regions. Generally speaking, the invention in the Queen patent has applicability toward the design of substantially any humanized immunoglobulin. Queen explains

that the DNA segments will typically include an expression control DNA sequence operably linked to the humanized immunoglobulin coding sequences, including naturally-associated or heterologous promoter regions. The expression control sequences can be eukaryotic promoter systems in vectors capable of transforming or transfecting eukaryotic host cells or the expression control sequences can be prokaryotic promoter systems in vectors capable of transforming or transfecting prokaryotic host cells. Once the vector has been incorporated into the appropriate host, the host is maintained under conditions suitable for high level expression of the introduced nucleotide sequences and as desired the collection and purification of the humanized light chains, heavy chains, light/heavy chain dimers or intact antibodies, binding fragments or other immunoglobulin forms may follow (Beychok, Cells of Immunoglobulin Synthesis, Academic Press, New York, (1979), which is incorporated herein by reference).

Human constant region (CDR) DNA sequences from a variety of human cells can be isolated in accordance with well known procedures. Preferably, the human constant region DNA sequences are isolated from immortalized B-cells as described in WO 87/02671. CDRs useful in producing the antibodies of the present invention may be similarly derived from DNA encoding monoclonal antibodies capable of binding to the targeted cell surface molecule. Such humanized antibodies may be generated using well known methods in any convenient mammalian source capable of producing antibodies, including, but not limited to, mice, rats, camels, llamas, rabbits, or other vertebrates. Suitable cells for constant region and framework DNA sequences and host cells in which the antibodies are expressed and secreted, can be obtained from a number of sources, such as the American Type Culture Collection, Manassas, VA.

One of skill in the art will further appreciate that the present invention encompasses the use of antibodies derived from camelid species. That is, the present invention includes, but is not limited to, the use of antibodies derived from species of the camelid family. As is well known in the art, camelid antibodies differ from those of most other mammals in that they lack a light chain, and thus comprise only heavy chains with complete and diverse antigen binding capabilities (Hamers-Casterman et al., 1993, Nature, 363:446-448). Such heavy-chain antibodies are useful in that they are smaller than conventional mammalian antibodies, they are more soluble than conventional antibodies, and further demonstrate an increased stability compared to

some other antibodies. Camelid species include, but are not limited to Old World camelids, such as two-humped camels (C. bactrianus) and one humped camels (C. dromedarius). The camelid family further comprises New World camelids including, but not limited to llamas, alpacas, vicuna and guanaco. The production of polyclonal sera from camelid species is substantively similar to the production of polyclonal sera from other animals such as sheep, donkeys, goats, horses, mice, chickens, rats, and the like. The skilled artisan, when equipped with the present disclosure and the methods detailed herein, can prepare high-titers of antibodies from a camelid species. As an example, the production of antibodies in mammals is detailed in such references as Harlow et al., (1988, Antibodies: A Laboratory Manual, Cold Spring Harbor, New York).

V_H proteins isolated from other sources, such as animals with heavy chain disease (Seligmann et al., 1979, Immunological Rev. 48:145-167, incorporated herein by reference in its entirety), are also useful in the compositions and methods of the invention. The present invention further comprises variable heavy chain immunoglobulins produced from mice and other mammals, as detailed in Ward et al. (1989, Nature 341:544-546, incorporated herein by reference in its entirety). Briefly, V_H genes are isolated from mouse splenic preparations and expressed in E. coli. The present invention encompasses the use of such heavy chain immunoglobulins in the compositions and methods detailed herein.

Antibodies useful as targeting moieties in the invention may also be obtained from phage antibody libraries. To generate a phage antibody library, a cDNA library is first obtained from mRNA which is isolated from cells, e.g., the hybridoma, which express the desired protein to be expressed on the phage surface, e.g., the desired antibody. cDNA copies of the mRNA are produced using reverse transcriptase. cDNA which specifies immunoglobulin fragments are obtained by PCR and the resulting DNA is cloned into a suitable bacteriophage vector to generate a bacteriophage DNA library comprising DNA specifying immunoglobulin genes. The procedures for making a bacteriophage library comprising heterologous DNA are well known in the art and are described, for example, in Sambrook et al. (2001, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).

C. Soluble Receptors

In another embodiment of the invention, an IL-1 receptor antagonist comprises a soluble IL-1 receptor protein (sIL-1R) that binds both IL-1α and IL-1β (referred to collectively herein as IL-1) is useful in the practice of the present invention. sIL-1R competes with IL-1α and IL-1β binding to cell-bound IL-1R. Accordingly, sIL-1R can be used to reduce or prevent the binding of IL-1 to cell bound IL-1R and thereby act as an antagonist of IL-1. Soluble receptors have been used to bind cytokines or other ligands to regulate their function (Thomson, (1998) Cytokine Handbook, Academic Press). A commercially available soluble IL-1R is available from Regeneron (Tarrytown, NY) known as Rilonacept or an IL-1 Trap.

The invention should be construed to include all additional soluble IL-1R that are either previously known or yet to be discovered. A soluble receptor is one which is generally present in solution, or not bound to a membrane. Soluble receptors may arise because the segment of the molecule which spans or associates with the membrane is absent. This segment is commonly referred to in the art as the transmembrane domain of the gene, or membrane binding segment of the protein. Preferably, the fragment contains at least six, e.g., ten, fifteen, twenty, twenty-five, thirty, forty, fifty, sixty, or seventy amino acids, provided it retains its desired activity, in this case, it's ability to bind IL-1.

In certain embodiments of the invention, the structure of the segment that associates with the membrane is modified (e.g., DNA sequence polymorphism or mutation in the gene) so the receptor is not tethered to the membrane, or the receptor is inserted, but is not retained within the membrane. Thus, a soluble receptor, in contrast to the corresponding membrane bound form, differs in one or more segments of the gene or receptor protein that are important to its association with the membrane.

The present invention encompasses cDNA encoding a soluble IL-1 receptor protein which is isolated from IL-1R producing cells or is recombinantly engineered from IL-1R-encoding DNA. Soluble IL-1R, as used herein, refers to a protein which can specifically bind to IL-1 without eliciting undesired downstream effects including, but not limited to, attenuation of a host's T cell response following alloantigen recognition.

IL-1 receptors known in the art include two high affinity receptors, IL-1 receptor type I (IL-1RI) and IL-1 receptor type II (IL-1RII), both of which are members of the immunoglobulin superfamily of receptors, and are included in the

term IL-1 receptor, as used herein. However, the invention should not be considered to be limited to the use of these receptors. Any IL-1 receptor identified may serve as the basis for the generation of a soluble IL-1 receptor.

Any of a variety of procedures may be used to molecularly clone soluble IL-1R cDNA. These methods include, but are not limited to, direct functional expression of the sIL-1R gene following the construction of an sIL-1R-containing cDNA library in an appropriate expression vector system.

It is readily apparent to those skilled in the art that suitable cDNA libraries may be prepared from cells or cell lines which have IL-1R activity. The selection of cells or cell lines for use in preparing a cDNA library to isolate IL-1R cDNA may be done by first measuring IL-1R activity using a IL-1 binding assay.

Preparation of cDNA libraries can be performed by standard techniques well known in the art. Well known cDNA library construction techniques can be found for example, in Maniatis, T., Fritsch, E. F., Sambrook, J., Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 2001).

It is also readily apparent to those skilled in the art that DNA encoding IL-1R may also be isolated from a suitable genomic DNA library. Construction of genomic DNA libraries can be performed by standard techniques well known in the art. Well known genomic DNA library construction techniques can be found in Maniatis, T., Fritsch, E. F., Sambrook, J. in Molecular Cloning: A Laboratory Manuel (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 2001).

sIL-1R molecules may also be obtained by recombinantly engineering them from DNA encoding the partial or complete amino acid sequence of an IL-1R. Using recombinant DNA techniques, DNA molecules are constructed which encode at least a portion of an IL-1 receptor capable of binding IL-1. Standard recombinant DNA techniques are used such as those found in Maniatis, et al., supra.

DNA encoding sIL-1R is constructed from a DNA sequence encoding an IL-1 receptor. Restriction endonuclease cleavage sites are identified within the receptor DNA and can be utilized directly to excise the extracellular-encoding portion. In addition, PCR techniques well known in the art may be utilized to produce the desired portion of DNA. It is readily apparent to those skilled in the art that other techniques, which are standard in the art, may be utilized to produce sIL-1R

molecules in a manner analogous to those described above. Such techniques are found, for example, in Maniatis et al., supra.

The cloned sIL-1R cDNA obtained through the methods described above may be recombinantly expressed by molecular cloning into an expression vector containing a suitable promoter and other appropriate transcription regulatory elements, and transferred into prokaryotic or eukaryotic host cells to produce recombinant sIL-1R. Techniques for such manipulations are fully described in Maniatis, T, et al., supra, and are well known in the art.

Expression vectors are defined herein as DNA sequences that are required for the transcription of cloned copies of genes and the translation of their mRNAs in an appropriate host. Such vectors can be used to express eukaryotic genes in a variety of hosts such as bacteria, bluegreen algae, fungal cells, yeast cells, plant cells, insect cells and animal cells.

Specifically designed vectors allow the shuttling of DNA between hosts such as bacteria-yeast or bacteria-animal or bacteria-insect cells. An appropriately constructed expression vector should contain: an origin of replication for autonomous replication in host cells, selectable markers, a limited number of useful restriction enzyme sites, a potential for high copy number, and active promoters. A promoter is defined as a DNA sequence that directs RNA polymerase to bind to DNA and initiate RNA synthesis. A strong promoter is one which causes mRNAs to be initiated at high frequency. Expression vectors may include, but are not limited to, cloning vectors, modified cloning vectors, specifically designed plasmids or viruses.

A variety of mammalian expression vectors may be used to express recombinant sIL-1R in mammalian cells. Commercially available mammalian expression vectors which may be suitable for recombinant sIL-1R expression, include but are not limited to, pMC1neo (Stratagene), pXT1 (Stratagene), pSG5 (Stratagene), EBO-pSV2-neo (ATCC 37593) pBPV-1(8-2) (ATCC 37110), pdBPV-MMTneo(342-12) (ATCC 37224), pRSVgpt (ATCC 37199), pRSVneo (ATCC 37198), pSV2-dhfr (ATCC 37146), pUCTag (ATCC 37460), and gZD35 (ATCC 37565).

DNA encoding sIL-1R may also be cloned into an expression vector for expression in a recombinant host cell. Recombinant host cells may be prokaryotic or eukaryotic, including but not limited to bacteria, yeast, mammalian cells including but not limited to cell lines of human, bovine, porcine, monkey and rodent origin, and

insect cells including but not limited to drosophila, moth, mosquito and armyworm derived cell lines. Cell lines derived from mammalian species which may be suitable and which are commercially available, include but are not limited to, CV-1 (ATCC CCL 70), COS-1 (ATCC CRL 1650), COS-7 (ATCC CRL 1651), CHO-K1 (ATCC CCL 61), 3T3 (ATCC CCL 92), NIH/3T3 (ATCC CRL 1658), HeLa (ATCC CCL 2), C127I (ATCC CRL 1616), BS-C-1 (ATCC CCL 26) and MRC-5 (ATCC CCL 171). Insect cell lines which may be suitable and are commercially available include but are not limited to 3M-S (ATCC CRL 8851) moth (ATCC CCL 80) mosquito (ATCC CCL 194 and 195; ATCC CRL 1660 and 1591) and armyworm (Sf9, ATCC CRL 1711).

The expression vector may be introduced into host cells via any one of a number of techniques including but not limited to transformation, transfection, liposome or protoplast fusion, and electroporation. The expression vector-containing cells are clonally propagated and individually analyzed to determine whether they produce sIL-1R protein. Identification of sIL-1R expressing host cell clones may be done by several means, including but not limited to immunological reactivity with anti-sIL-1R antibodies, binding to radiolabelled IL-1, and the presence of host cell-secreted sIL-1R activity.

Expression of sIL-1R DNA may also be performed using in vitro produced synthetic mRNA. Synthetic mRNA can be efficiently translated in various cell-free systems, including but not limited to wheat germ extracts and reticulocyte extracts, as well as efficiently translated in cell based systems, including but not limited to microinjection into frog oocytes.

Levels of sIL-1R protein produced by host cells may be quantitated by immunoaffinity and/or ligand affinity techniques. sIL-1R-specific affinity beads or sIL-1R-specific antibodies are used to isolate ^{35}S-methionine labeled or unlabeled sIL-1R protein. Labeled sIL-1R protein is analyzed by SDS-PAGE. Unlabeled sIL-1R protein is detected by Western blotting, ELISA or RIA assays employing sIL-1R specific antibodies, or by ligand blotting with labeled IL-1.

Following expression of sIL-1R in a recombinant host cell, sIL-1R protein may be recovered to provide sIL-1R in active form, capable of binding IL-1 without stimulating lung epithelial cell DNA damage, apoptosis, pathological collagen deposition, and alveolar remodeling. Several sIL-1R purification procedures are available and suitable for use. sIL-1R may be purified from cell lysates and

extracts, or from conditioned culture medium, by various combinations of, or individual application of salt fractionation, ion exchange chromatography, size exclusion chromatography, hydroxylapatite adsorption chromatography, reversed phase chromatography, heparin sepharose chromatography, sIL-1R ligand affinity chromatography, and hydrophobic interaction chromatography.

In addition, recombinant sIL-1R can be separated from other cellular proteins by use of an immuno-affinity column made with monoclonal or polyclonal antibodies specific for full length sIL-1R, or polypeptide fragments of sIL-1R. The sIL-1R protein can be expressed using a baculovirus expression system. The recombinantly produced sIL-1R is purified from the recombinant host cell extracts or cell culture fluid using heparin-sepharose column chromatography which specifically binds the sIL-1R protein. The heparin-sepharose bound sIL-1R column is washed using a suitable buffer containing between b 0.1M and 0.6M NaCl which removes contaminating proteins without significant loss of sIL-1R. The sIL-1R is eluted from the heparin-sepharose column using a suitable buffer containing about 1M NaCl, yielding substantially purified sIL-1R.

D. siRNA

In one embodiment, siRNA is used to decrease the level of IL-1 or IL-1R protein. RNA interference (RNAi) is a phenomenon in which the introduction of double-stranded RNA (dsRNA) into a diverse range of organisms and cell types causes degradation of the complementary mRNA. In the cell, long dsRNAs are cleaved into short 21-25 nucleotide small interfering RNAs, or siRNAs, by a ribonuclease known as Dicer. The siRNAs subsequently assemble with protein components into an RNA-induced silencing complex (RISC), unwinding in the process. Activated RISC then binds to complementary transcript by base pairing interactions between the siRNA antisense strand and the mRNA. The bound mRNA is cleaved and sequence specific degradation of mRNA results in gene silencing. See, for example, U.S. Patent No. 6,506,559; Fire et al., 1998, Nature 391(19):306-311; Timmons et al., 1998, Nature 395:854; Montgomery et al., 1998, TIG 14 (7):255-258; David R. Engelke, Ed., RNA Interference (RNAi) Nuts & Bolts of RNAi Technology, DNA Press, Eagleville, PA (2003); and Gregory J. Hannon, Ed., RNAi A Guide to Gene Silencing, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (2003). Soutschek et al. (2004, Nature 432:173-178) describe a chemical

modification to siRNAs that aids in intravenous systemic delivery. Optimizing siRNAs involves consideration of overall G/C content, C/T content at the termini, Tm and the nucleotide content of the 3' overhang. See, for instance, Schwartz et al., 2003, Cell, 115:199-208 and Khvorova et al., 2003, Cell 115:209-216. Therefore, the present invention also includes methods of decreasing levels of IL-1 or IL-1R protein using RNAi technology.

Modification of siRNA

Following the generation of the siRNA polynucleotide of the present invention, a skilled artisan will understand that the siRNA polynucleotide will have certain characteristics that can be modified to improve the siRNA as a therapeutic compound. Therefore, the siRNA polynucleotide may be further designed to resist degradation by modifying it to include phosphorothioate, or other linkages, methylphosphonate, sulfone, sulfate, ketyl, phosphorodithioate, phosphoramidate, phosphate esters, and the like (see, e.g., Agrwal et al., 1987 Tetrahedron Lett. 28:3539-3542; Stec et al., 1985 Tetrahedron Lett. 26:2191-2194; Moody et al., 1989 Nucleic Acids Res. 12:4769-4782; Eckstein, 1989 Trends Biol. Sci. 14:97-100; Stein, In: Oligodeoxynucleotides. Antisense Inhibitors of Gene Expression, Cohen, ed., Macmillan Press, London, pp. 97-117 (1989)).

Any polynucleotide of the invention may be further modified to increase its stability *in vivo*. Possible modifications include, but are not limited to, the addition of flanking sequences at the 5' and/or 3' ends; the use of phosphorothioate or 2' O-methyl rather than phosphodiester linkages in the backbone; and/or the inclusion of nontraditional bases such as inosine, queosine, and wybutosine and the like, as well as acetyl- methyl-, thio- and other modified forms of adenine, cytidine, guanine, thymine, and uridine.

Vectors

In other related aspects, the invention includes an isolated nucleic acid encoding anIL-1 receptor inhibitor, wherein the inhibitor such as an siRNA, inhibits IL-1, an IL-1 receptor operably linked to a nucleic acid comprising a promoter/regulatory sequence such that the nucleic acid is preferably capable of directing expression of the protein encoded by the nucleic acid. Thus, the invention encompasses expression vectors and methods for the introduction of exogenous DNA

into cells with concomitant expression of the exogenous DNA in the cells such as those described, for example, in Sambrook et al. (2001, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York), and in Ausubel et al. (1997, Current Protocols in Molecular Biology, John Wiley & Sons, New York).

In another aspect of the invention, IL-1 or an IL-1 receptor can be inhibited by way of inactivating and/or sequestering IL-1 or an IL-1 receptor. As such, inhibiting the effects of IL-1 can be accomplished by using a transdominant negative mutant.

In another aspect, the invention includes a vector comprising an siRNA polynucleotide. Preferably, the siRNA polynucleotide is capable of inhibiting the expression of a target polypeptide, wherein the target polypeptide is selected from the group consisting of IL-1 or an IL-1R. The incorporation of a desired polynucleotide into a vector and the choice of vectors is well-known in the art as described in, for example, Sambrook et al., *supra*, and Ausubel et al., *supra*.

The siRNA polynucleotide can be cloned into a number of types of vectors. However, the present invention should not be construed to be limited to any particular vector. Instead, the present invention should be construed to encompass a wide plethora of vectors which are readily available and/or well-known in the art. For example, an siRNA polynucleotide of the invention can be cloned into a vector including, but not limited to a plasmid, a phagemid, a phage derivative, an animal viruse, and a cosmid. Vectors of particular interest include expression vectors, replication vectors, probe generation vectors, and sequencing vectors.

In specific embodiments, the expression vector is selected from the group consisting of a viral vector, a bacterial vector and a mammalian cell vector. Numerous expression vector systems exist that comprise at least a part or all of the compositions discussed above. Prokaryote- and/or eukaryote-vector based systems can be employed for use with the present invention to produce polynucleotides, or their cognate polypeptides. Many such systems are commercially and widely available.

Further, the expression vector may be provided to a cell in the form of a viral vector. Viral vector technology is well known in the art and is described, for example, in Sambrook et al. (2001), and in Ausubel et al. (1997), and in other virology and molecular biology manuals. Viruses, which are useful as vectors include, but are not limited to, retroviruses, adenoviruses, adeno-associated viruses, herpes viruses, and lentiviruses. In general, a suitable vector contains an origin of

replication functional in at least one organism, a promoter sequence, convenient restriction endonuclease sites, and one or more selectable markers. (See, e.g., WO 01/96584; WO 01/29058; and U.S. Pat. No. 6,326,193.

For expression of the siRNA, at least one module in each promoter functions to position the start site for RNA synthesis. The best known example of this is the TATA box, but in some promoters lacking a TATA box, such as the promoter for the mammalian terminal deoxynucleotidyl transferase gene and the promoter for the SV40 genes, a discrete element overlying the start site itself helps to fix the place of initiation.

Additional promoter elements, i.e., enhancers, regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 bp upstream of the start site, although a number of promoters have recently been shown to contain functional elements downstream of the start site as well. The spacing between promoter elements frequently is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another. In the thymidine kinase (tk) promoter, the spacing between promoter elements can be increased to 50 bp apart before activity begins to decline. Depending on the promoter, it appears that individual elements can function either co-operatively or independently to activate transcription.

A promoter may be one naturally associated with a gene or polynucleotide sequence, as may be obtained by isolating the 5′ non-coding sequences located upstream of the coding segment and/or exon. Such a promoter can be referred to as "endogenous." Similarly, an enhancer may be one naturally associated with a polynucleotide sequence, located either downstream or upstream of that sequence. Alternatively, certain advantages will be gained by positioning the coding polynucleotide segment under the control of a recombinant or heterologous promoter, which refers to a promoter that is not normally associated with a polynucleotide sequence in its natural environment. A recombinant or heterologous enhancer refers also to an enhancer not normally associated with a polynucleotide sequence in its natural environment. Such promoters or enhancers may include promoters or enhancers of other genes, and promoters or enhancers isolated from any other prokaryotic, viral, or eukaryotic cell, and promoters or enhancers not "naturally occurring," *i.e.,* containing different elements of different transcriptional regulatory

regions, and/or mutations that alter expression. In addition to producing nucleic acid sequences of promoters and enhancers synthetically, sequences may be produced using recombinant cloning and/or nucleic acid amplification technology, including PCR™, in connection with the compositions disclosed herein (U.S. Patent 4,683,202, U.S. Patent 5,928,906). Furthermore, it is contemplated the control sequences that direct transcription and/or expression of sequences within non-nuclear organelles such as mitochondria, chloroplasts, and the like, can be employed as well.

Naturally, it will be important to employ a promoter and/or enhancer that effectively directs the expression of the DNA segment in the cell type, organelle, and organism chosen for expression. Those of skill in the art of molecular biology generally know how to use promoters, enhancers, and cell type combinations for protein expression, for example, see Sambrook et al. (2001). The promoters employed may be constitutive, tissue-specific, inducible, and/or useful under the appropriate conditions to direct high level expression of the introduced DNA segment, such as is advantageous in the large-scale production of recombinant proteins and/or peptides. The promoter may be heterologous or endogenous.

A promoter sequence exemplified in the experimental examples presented herein is the immediate early cytomegalovirus (CMV) promoter sequence. This promoter sequence is a strong constitutive promoter sequence capable of driving high levels of expression of any polynucleotide sequence operatively linked thereto. However, other constitutive promoter sequences may also be used, including, but not limited to the simian virus 40 (SV40) early promoter, mouse mammary tumor virus (MMTV), human immunodeficiency virus (HIV) long terminal repeat (LTR) promoter, Moloney virus promoter, the avian leukemia virus promoter, Epstein-Barr virus immediate early promoter, Rous sarcoma virus promoter, as well as human gene promoters such as, but not limited to, the actin promoter, the myosin promoter, the hemoglobin promoter, and the muscle creatine promoter. Further, the invention should not be limited to the use of constitutive promoters. Inducible promoters are also contemplated as part of the invention. The use of an inducible promoter in the invention provides a molecular switch capable of turning on expression of the polynucleotide sequence which it is operatively linked when such expression is desired, or turning off the expression when expression is not desired. Examples of inducible promoters include, but are not limited to a metallothionine promoter, a glucocorticoid promoter, a progesterone promoter, and a tetracycline promoter.

Further, the invention includes the use of a tissue specific promoter, which promoter is active only in a desired tissue. Tissue specific promoters are well known in the art and include, but are not limited to, the HER-2 promoter and the PSA associated promoter sequences.

In order to assess the expression of the siRNA, the expression vector to be introduced into a cell can also contain either a selectable marker gene or a reporter gene or both to facilitate identification and selection of expressing cells from the population of cells sought to be transfected or infected through viral vectors. In other embodiments, the selectable marker may be carried on a separate piece of DNA and used in a co-transfection procedure. Both selectable markers and reporter genes may be flanked with appropriate regulatory sequences to enable expression in the host cells. Useful selectable markers are known in the art and include, for example, antibiotic-resistance genes, such as neo and the like.

Reporter genes are used for identifying potentially transfected cells and for evaluating the functionality of regulatory sequences. Reporter genes that encode for easily assayable proteins are well known in the art. In general, a reporter gene is a gene that is not present in or expressed by the recipient organism or tissue and that encodes a protein whose expression is manifested by some easily detectable property, e.g., enzymatic activity. Expression of the reporter gene is assayed at a suitable time after the DNA has been introduced into the recipient cells.

Suitable reporter genes may include genes encoding luciferase, beta-galactosidase, chloramphenicol acetyl transferase, secreted alkaline phosphatase, or the green fluorescent protein gene (see, e.g., Ui-Tei et al., 2000 FEBS Lett. 479:79-82). Suitable expression systems are well known and may be prepared using well known techniques or obtained commercially. Internal deletion constructs may be generated using unique internal restriction sites or by partial digestion of non-unique restriction sites. Constructs may then be transfected into cells that display high levels of siRNA polynucleotide and/or polypeptide expression. In general, the construct with the minimal 5' flanking region showing the highest level of expression of reporter gene is identified as the promoter. Such promoter regions may be linked to a reporter gene and used to evaluate agents for the ability to modulate promoter-driven transcription.

E. Antisense nucleic acids

In one embodiment of the invention, an antisense nucleic acid sequence which is expressed by a plasmid vector is used to inhibit IL-1 or IL-1R expression. The antisense expressing vector is used to transfect a mammalian cell or the mammal itself, thereby causing reduced endogenous expression of IL-1 or IL-1R.

Antisense molecules and their use for inhibiting gene expression are well known in the art (*see, e.g.,* Cohen, 1989, In: Oligodeoxyribonucleotides, Antisense Inhibitors of Gene Expression, CRC Press). Antisense nucleic acids are DNA or RNA molecules that are complementary, as that term is defined elsewhere herein, to at least a portion of a specific mRNA molecule (Weintraub, 1990, Scientific American 262:40). In the cell, antisense nucleic acids hybridize to the corresponding mRNA, forming a double-stranded molecule thereby inhibiting the translation of genes.

The use of antisense methods to inhibit the translation of genes is known in the art, and is described, for example, in Marcus-Sakura (1988, Anal. Biochem. 172:289). Such antisense molecules may be provided to the cell via genetic expression using DNA encoding the antisense molecule as taught by Inoue, 1993, U.S. Patent No. 5,190,931.

Alternatively, antisense molecules of the invention may be made synthetically and then provided to the cell. Antisense oligomers of between about 10 to about 30, and more preferably about 15 nucleotides, are preferred, since they are easily synthesized and introduced into a target cell. Synthetic antisense molecules contemplated by the invention include oligonucleotide derivatives known in the art which have improved biological activity compared to unmodified oligonucleotides (*see* U.S. Patent No. 5,023,243).

Cloning and expression of antisense molecules is well known in the art and can be performed generally as described for the cloning and expression of siRNA.

II. Methods

The present invention comprises novel methods of using an IL-1 receptor antagonist to enhance the long-term survival of an allograft in a host. An allogeneic graft, or allograft, comprises a cell, a tissue, an organ, or a combination thereof, obtained from one human, and administered to, or transplanted into, a second, genetically non-identical human host to supplement or replace the host's cell, tissue, or organ function. The ability of IL-1Ra therapy to enhance the long-term

survival of an allograft in a host may be assessed by one skilled in the art using any of a number of clinically relevant parameters to determine the clinical status or health of a host recipient of an allograft or the physiological or functional status of the graft. By way of a non-limiting example, a skilled artisan may assess the number, frequency, or severity of incidences of acute graft rejection, chronic rejection, clinical outcome, quality of life, host survival, graft survival, host's need for or strength of immunosuppressive therapy required, or any other relevant clinical parameter that can be measured in a host receiving or that has received IL-1Ra therapy. These data can be compared to data obtained from a host recipient, or a population of host recipients, that never received IL-1Ra as a means of determining the ability of IL-1Ra to enhance the long-term survival of an allograft.

A host immune system detects and responds to a foreign antigen or alloantigen, such as the non-self MHC molecules, present on graft tissue. Antigen recognition by T cells (i.e. "sighting") triggers T cell proliferation and clonal expansion specific for those alloantigens, as well as acquisition of effector functions, such as cytokine secretion. In the present invention, elevated IL-1 levels produced, secreted, or released by an allograft, or made by the host's innate immune system in response to an allograft, modulate the host T cell response that follows alloantigen recognition ("sighting"), changing the effector functions that mediate the immune response directed against alloantigens, for example increasing certain cytokines while decreasing others, and exacerbating the long term T cell response directed against the allograft. Further, elevated levels of IL-1 produced, secreted, or released by a patient's own damaged tissue, such as might be the case in stroke, can induce similar changes in a self-directed adaptive immune response.

Accordingly, the present invention encompasses the use of an IL-1Ra or a variant, analog, or fragment thereof, to attenuate the host immune response to graft alloantigens, as defined herein, wherein the subsequent T cell response directed against the alloantigens of the graft is attentuated.

In one embodiment, the present invention comprises a novel method of ameliorating the deleterious effects of allograft injury on the viability of an allograft by administering IL-1Ra or a therapeutic form thereof to the host to counter the effects of molecules released by a damaged allograft that direct the host adaptive immune response toward a more pathogenic response characterized herein by increased IFN- γ and IL-17 production.

Another embodiment of the present invention comprises a method of attenuating a host's adaptive immune response to a vascularized allograft by administering to the host a therapeutically effective amount of an IL-1Ra or a therapeutic form thereof during a narrow therapeutic window that begins the moment the graft comes in contact with the host's circulatory system, and continues for an acute period of time not more than 1 day, 3 days, 7 days, 14, days, or 21 days after the host has received the graft.

Another embodiment of the present invention comprises a method of preventing acute or chronic allograft rejection in a host who has received an allogeneic vascularized graft by administering to the host a therapeutically effective amount of an IL-1Ra, or a therapeutically effective variant, analog, or fragment thereof, during a narrow therapeutic window that begins the moment the graft comes in contact with the host's circulatory system, and consequently the host's immune system, and continues for an acute period of time not more than 1 day, 3 days, 7 days, 14, days, or 21 days after the host has received the graft.

The present invention also contemplates situations where the methods of the present invention are useful in treating a patient whose own tissues are expressing large amount of IL-1 due to physical damage or trauma. Accordingly, the present invention is useful in treating stroke, defined herein as a central nervous system vascular disorder, a venous thrombosis, a transient ischemic attack, an ischemic neurological deficit, or a hemorrhage within the central nervous system. The present invention is also useful in treating a patient that has experienced physical trauma to the brain or spinal cord resulting in bleeding in the central nervous system. The present invention is also useful in treating a patient who has experienced indirect trauma to the brain or spinal cord such as might occur from trauma or bleeding originating from the meninges covering the brain and spinal cord, or trauma to the bony coverings of the brain and spinal cord. The patient may have a peripheral vascular disorder, a venous thrombosis, a pulmonary embolus, a myocardial infarction, a transient ischemic attack, unstable angina, cardiac arythmia a reversible ischemic neurological deficit, sickle cell anemia or a stroke disorder which causes damage or trauma to the brain or spinal cord. The patient may be undergoing heart surgery, lung surgery, spinal surgery, brain surgery, vascular surgery, abdominal surgery, or organ transplantation surgery, which creates a condition that produces damage or trauma to the brain or spinal cord.

In one embodiment of the present invention a patient experiencing stroke or central nervous system trauma is administered a therapeutically effective amount of IL-1Ra, or a therapeutically effective variant, analog, or fragment thereof, as soon as possible after the commencement of the stroke or trauma. IL-1Ra administration continues throughout a therapeutic window not to exceed 1 day, 3 days, 7 days, 14, days, or 21 days after the onset of the stroke or trauma.

In another embodiment of the present invention, a patient at risk of experiencing stroke is administered a therapeutically effective amount of IL-1Ra prophylatically, until such time that it is determined that the patient is no longer at risk of experiencing stroke.

The methods of the present invention can be used in combination with other treatment regimens, including immune suppressive therapy, virostatic and virotoxic agents, antibiotic agents, antifungal agents, anti-inflammatory agents (steroidal and non-steroidal), antidepressants, anxiolytics, pain management agents, (acetaminophen, aspirin, ibuprofen, opiates (including morphine, hydrocodone, codeine, fentanyl, methadone), steroids (including prednisone and dexamethasone), and antidepressants (including gabapentin, amitriptyline, imipramine, doxepin) antihistamines, antitussives, muscle relaxants, brondhodilaters, beta-agonists, anticholinergics, corticosteroids, mast cell stabilizers, leukotriene modifiers, methylxanthines, nucleic acid based therapeutic agents, as well as combination therapies, and the like. The compounds of the present invention may be administered before, during, after, or throughout administration of any therapeutic agents used in the treatment of a subject's disease or disorder.

III. Pharmaceutical Composition and Formulation

Pharmaceutical compositions for use in accordance with the present invention thus may be formulated in a conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. These pharmaceutical compositions may be manufactured in a manner that is itself known, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes. Proper formulation is dependent upon the route of administration chosen. Pharmaceutical compositions that are useful in the methods of

the invention may be prepared, packaged, or sold in formulations suitable for oral, rectal, vaginal, parenteral, topical, pulmonary, intranasal, buccal, ophthalmic, intrathecal or another route of administration. Other contemplated formulations include projected nanoparticles, liposomal preparations, resealed erythrocytes containing the active ingredient, and immunologically-based formulations.

When a therapeutically effective amount of IL-1Ra or other active ingredient of the present invention is administered orally, IL-1Ra or other active ingredient of the present invention will be in the form of a tablet, capsule, powder, solution or elixir. When administered in tablet form, the pharmaceutical composition of the invention may additionally contain a solid carrier such as a gelatin or an adjuvant. The tablet, capsule, and powder contain from about 5 to 95% IL-1Ra or other active ingredient of the present invention, and preferably from about 25 to 90% IL-1Ra or other active ingredient of the present invention. When administered in liquid form, a liquid carrier such as water, petroleum, oils of animal or plant origin such as peanut oil, mineral oil, soybean oil, or sesame oil, or synthetic oils may be added. The liquid form of the pharmaceutical composition may further contain physiological saline solution, dextrose or other saccharide solution, or glycols such as ethylene glycol, propylene glycol or polyethylene glycol. When administered in liquid form, the pharmaceutical composition contains from about 0.5 to 90% by weight of I1-1Ra or other active ingredient of the present invention, and preferably from about 1 to 50% IL-1Ra or other active ingredient of the present invention.

When a therapeutically effective amount of IL-1Ra or other active ingredient of the present invention is administered by intravenous, cutaneous or subcutaneous injection, IL-1Ra or other active ingredient of the present invention will be in the form of a pyrogen-free, parenterally acceptable aqueous solution. The preparation of such parenterally acceptable protein or other active ingredient solutions, having due regard to pH, isotonicity, stability, and the like, is within the skill in the art. A preferred pharmaceutical composition for intravenous, cutaneous, or subcutaneous injection should contain, in addition to IL-1Ra or other active ingredient of the present invention, an isotonic vehicle such as Sodium Chloride Injection, Ringer's Injection, Dextrose Injection, Dextrose and Sodium Chloride Injection, Lactated Ringer's Injection, or other vehicle as known in the art. The pharmaceutical composition of the present invention may also contain stabilizers, preservatives, buffers, antioxidants, or other additives known to those of skill in the art. For

injection, the agents of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks's solution, Ringer's solution, or physiological saline buffer. Pharmaceutical formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., sterile pyrogen-free water, before use. For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

For oral administration, the compounds can be formulated readily by combining the active compounds with pharmaceutically acceptable carriers well known in the art. Such carriers enable the compounds of the invention to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a patient to be treated. Pharmaceutical preparations for oral use can be obtained solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate. Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments

may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules can contain the active ingredients in admixture with filler such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added. All formulations for oral administration should be in dosages suitable for such administration. For buccal administration, the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by inhalation, the compounds for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebuliser, with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, e.g., gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch. The compounds may be formulated for parenteral administration by injection, e.g., by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents.

In addition to the formulations described previously, the compounds may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the compounds may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

A pharmaceutical carrier for the hydrophobic compounds of the invention is a cosolvent system comprising benzyl alcohol, a nonpolar surfactant, a water-miscible organic polymer, and an aqueous phase. The cosolvent system may be the VPD co-solvent system. VPD is a solution of 3% w/v benzyl alcohol, 8% w/v of the nonpolar surfactant polysorbate 80, and 65% w/v polyethylene glycol 300, made up to volume in absolute ethanol. The VPD co-solvent system (VPD:5W) consists of VPD diluted 1:1 with a 5% dextrose in water solution. This co-solvent system dissolves hydrophobic compounds well, and itself produces low toxicity upon systemic administration. Naturally, the proportions of a co-solvent system may be varied considerably without destroying its solubility and toxicity characteristics. Furthermore, the identity of the co-solvent components may be varied: for example, other low-toxicity nonpolar surfactants may be used instead of polysorbate 80; the fraction size of polyethylene glycol may be varied; other biocompatible polymers may replace polyethylene glycol, e.g. polyvinyl pyrrolidone; and other sugars or polysaccharides may substitute for dextrose.

Alternatively, other delivery systems for hydrophobic pharmaceutical compounds may be employed. Liposomes and emulsions are well known examples of delivery vehicles or carriers for hydrophobic drugs. Certain organic solvents such as dimethylsulfoxide also may be employed, although usually at the cost of greater toxicity. Additionally, the compounds may be delivered using a sustained-release system, such as semipermeable matrices of solid hydrophobic polymers containing the therapeutic agent. Various types of sustained-release materials have been established and are well known by those skilled in the art. Sustained-release capsules may, depending on their chemical nature, release the compounds for a few weeks up to over 100 days. Depending on the chemical nature and the biological stability of the therapeutic reagent, additional strategies for protein or other active ingredient stabilization may be employed.

The pharmaceutical compositions also may comprise suitable solid or gel phase carriers or excipients. Examples of such carriers or excipients include but are not limited to calcium carbonate, calcium phosphate, various sugars, starches, cellulose derivatives, gelatin, and polymers such as polyethylene glycols. Many of the active ingredients of the invention may be provided as salts with pharmaceutically compatible counterions. Such pharmaceutically acceptable base addition salts are those salts which retain the biological effectiveness and properties of the free acids

and which are obtained by reaction with inorganic or organic bases such as sodium hydroxide, magnesium hydroxide, ammonia, trialkylamine, dialkylamine, monoalkylamine, dibasic amino acids, sodium acetate, potassium benzoate, triethanol amine and the like.

The pharmaceutical composition of the invention may be in the form of a liposome in which protein of the present invention is combined, in addition to other pharmaceutically acceptable carriers, with amphipathic agents such as lipids which exist in aggregated form as micelles, insoluble monolayers, liquid crystals, or lamellar layers in aqueous solution. Suitable lipids for liposomal formulation include, without limitation, monoglycerides, diglycerides, sulfatides, lysolecithins, phospholipids, saponin, bile acids, and the like. Preparation of such liposomal formulations is within the level of skill in the art, as disclosed, for example, in U.S. Pat. Nos. 4,235,871; 4,501,728; 4,837,028; and 4,737,323, all of which are incorporated herein by reference.

The amount of IL-1Ra or other active ingredient of the present invention in the pharmaceutical composition of the present invention will depend upon the nature and severity of the condition being treated, and on the nature of prior treatments which the patient has undergone. Ultimately, the attending physician will decide the amount of IL-1Ra or other active ingredient of the present invention with which to treat each individual patient. Initially, the attending physician will administer low doses of IL-1Ra or other active ingredient of the present invention and observe the patient's response. Larger doses of protein or other active ingredient of the present invention may be administered until the optimal therapeutic effect is obtained for the patient, and at that point the dosage is not increased further. It is contemplated that the various pharmaceutical compositions used to practice the method of the present invention should contain about 0.01 μg to about 100 mg (preferably about 0.1 μg to about 10 mg, more preferably about 0.1 μg to about 1 mg) of IL-1Ra or other active ingredient of the present invention per kg body weight.

Compositions of the present invention include therapeutic method administering the composition topically, systematically, or locally as an implant or device. When administered, the therapeutic composition for use in this invention is, of course, in a pyrogen-free, physiologically acceptable form. Further, the composition may desirably be encapsulated or injected in a viscous form for delivery to the

affected tissue. Therapeutically useful agents other than IL-1Ra or other active ingredient of the invention which may also optionally be included in the composition as described above, may alternatively or additionally, be administered simultaneously or sequentially with the composition in the methods of the invention. Pharmaceutical compositions suitable for use in the present invention include compositions wherein the active ingredients are contained in an effective amount to achieve its intended purpose. More specifically, a therapeutically effective amount means an amount effective to prevent development of or to alleviate the existing symptoms of the subject being treated. Determination of the effective amount is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein. For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from appropriate in vitro assays. For example, a dose can be formulated in animal models to achieve a circulating concentration range that can be used to more accurately determine useful doses in humans. For example, a dose can be formulated in animal models to achieve a circulating concentration range that includes the IC_{50} as determined in cell culture (i.e., the concentration of the test compound which achieves a half-maximal inhibition of IL-1 induced IFN-γ production). Such information can be used to more accurately determine useful doses in humans.

A therapeutically effective dose refers to that amount of the compound that results in amelioration of symptoms, prevention of graft rejection, suppression of an adaptive immune response to a graft, or a prolongation of survival in a patient. Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio between LD_{50} and ED_{50}. Compounds which exhibit high therapeutic indices are preferred. The data obtained from these cell culture assays and animal studies can be used in formulating a range of dosage for use in human. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED_{50} with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. The exact formulation, route of administration and dosage can be chosen by the individual

physician in view of the patient's condition. See, e.g., Fingl et al., 1975, in "The Pharmacological Basis of Therapeutics", Ch. 1 p. 1. Dosage amount and interval may be adjusted individually to provide plasma levels of the active moiety which are sufficient to maintain the desired effects, or minimal effective concentration (MEC). The MEC will vary for each compound but can be estimated from in vitro data. Dosages necessary to achieve the MEC will depend on individual characteristics and route of administration. However, HPLC assays or bioassays can be used to determine plasma concentrations.

Dosage intervals can also be determined using MEC value. Compounds should be administered using a regimen which maintains plasma levels above the MEC for 10-90% of the time, preferably between 30-90% and most preferably between 50-90%. In cases of local administration or selective uptake, the effective local concentration of the drug may not be related to plasma concentration.

An exemplary dosage regimen for IL-1Ra will be in the range of about 0.01 to 100 mg/kg of body weight daily, with the preferred dose being about 0.1 to 25 mg/kg of patient body weight daily, varying in adults and children. Dosing may be once daily, or equivalent doses may be delivered at longer or shorter intervals.

The amount of composition administered will, of course, be dependent on the subject being treated, on the subject's age and weight, the severity of the affliction, the manner of administration and the judgment of the prescribing physician.

Previous clinical trials aimed largely at the use of IL-1Ra as an anti-inflammatory compound have determined that administration of IL-1Ra is well tolerated and does not cause serious side effects. For example, in patients suffering from rheumatoid arthritis, 30, 75, or 150 mg/day of recombinant IL-1Ra was self-administered as a single subcutaneous injection at the site of arthritis. This treatment caused a dose dependent reduction in the number of swollen joints and overall patient scores; decrease in C-reactive and sedimentation rates; and 50% reduction in new bone erosions. (See Brenihan, Ann. Rheum. Dis. 58:196-198, 1999).

In patients suffering from septic shock, IL-1Ra was administered as a loading bolus of 100 mg followed by 3 day infusion of 17, 67, or 133 mg/hr of IL-1Ra. In Phase II clinical trials, a dose dependent decrease in mortality was observed where 44% mortality in patients receiving the lowest dose and 16% mortality in group receiving the highest dose. (Fisher et al., Crit. Care Med. 22: 12-21, 1994). In further

Phase III clinical trials, however, no statistically significant reduction in mortality was observed with IL-1Ra treatment.

Patients exhibiting graft-versus-host disease following allogeneic bone marrow transplants received 400-3400 mg/day of IL-1Ra continuously every 24 hours for 7 days as intervenous infusions. This treatment resulted in an improvement in 16 out of 17 patient as measured by an organ specific acute disease scale. (Antin et al., Blood 84: 1342-48, 1994).

Packaging

The compositions may, if desired, be presented in a pack or dispenser device which may contain one or more unit dosage forms containing the active ingredient. The pack may, for example, comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration. Compositions comprising a compound of the invention formulated in a compatible pharmaceutical carrier may also be prepared, placed in an appropriate container, and labeled for treatment of an indicated condition.

EXPERIMENTAL EXAMPLES

The invention is further described in detail by reference to the following experimental examples. These examples are provided for purposes of illustration only, and are not intended to be limiting unless otherwise specified. Thus, the invention should in no way be construed as being limited to the following examples, but rather, should be construed to encompass any and all variations which become evident as a result of the teaching provided herein.

The materials and methods employed in the experiments disclosed herein are now described.

Reagents and Antibodies (Abs)

PHA, PMA, and ionomycin were purchased from Sigma-Aldrich (St. Louis, MO). Mouse anti-human IL-1α, mouse anti-human IL-1β, mouse anti-human AGER, mouse anti-human CD14, mouse anti-human TNF, mouse anti-human IL-12p40, mouse anti-human IL-18Rα, and mouse anti-human HMGB1 were purchased from R&D Systems (Minneapolis, MN). Mouse anti-human CD3 (OKT3), mouse antihuman CD28 (clone 28.2), mouse anti-human TLR4, mouse anti-human TLR2,

mouse anti-human CD45RA, mouse anti-human CD45RO, and isotype control Abs were purchased from eBioscience (San Diego, CA). Rabbit anti-human phospho-p38 and anti-human phospho-JNK were purchased from Cell Signaling Technology. PE-conjugated anti-IFN-γ and the isotype control were purchased from BD Pharmingen (San Diego, CA). FITC-conjugated anti-human ICAM-1 and an isotype control were purchased from Beckman Coulter (Fullerton, CA). Anti-human HLA-DR (LB3.1) was a gift from J. Strominger (Harvard University, Cambridge, MA). Ultra-pure LPS was purchased from InvivoGen (San Diego, CA). Recombinant human IL-1α and IL-1β were purchased from PeproTech (Rocky Hill, NJ). IL-1Ra was purchased from Amgen (Thousand Oaks, CA). Recombinant human TNF was purchased from R&D Systems (Minneapolis, MN). Recombinant human IFN-γ was purchased from Invitrogen Life Technologies (Carlsbad, CA0. Recombinant HMGB1 was prepared as previously described (Li et al., 2004, J. Immunol. Methods 289: 211–223). Endotoxin content in the HMGB1 preparations was found to be 0.6 pg/μg of HMGB1 by the Chromogenic *Limulus* amebocyte lysate assay (BioWhittaker) (Li et al., 2004, J. Immunol. Methods 289: 211–223).

Isolation and culture of human cells

All human cells were obtained under protocols approved by the Yale Human Investigations Committee. PBMCs were isolated by density centrifugation of leukapheresis products from anonymized adult volunteer donors. To isolate $CD4^+$ T cells, PBMCs were incubated in RPMI 1640 supplemented with 10% FBS on tissue culture plates for 30 min at 37°C to deplete adherent PBMCs. $CD4^+$ T cells were isolated from nonadherent PBMCs by positive selection using magnetic bead separation (Invitrogen Life Technologies; Carlsbad, CA). Monocytes were depleted from CD4-selected populations by further negative selection using anti-CD14 and anti-HLA-DR Abs at 5 μg/ml for 20 min. Naive and memory $CD4^+$ T cell subsets were isolated from CD4-selected populations by negative selection using anti-CD45RA or anti-CD45RO Abs at 5 μg/ml. Cells were then washed twice and incubated with beads coated with goat anti-mouse Abs (Invitrogen Life Technologies; Carlsbad, CA). Monocytes were isolated from PBMCs by negative selection using magnetic bead separation (Invitrogen Life Technologies). To generate monocyte conditioned medium, monocytes cultured at 10^6 cells/ml were treated with HMGB1 at

5 μg/ml or the control buffer for 24 h. Conditioned medium was passed through a 0.45-micron filter before addition to EC-T cell co-cultures.

Human umbilical vein EC (HUVEC) were isolated from umbilical cords by collagenase (Worthington Biochemical; Lakewood, NJ) digestion and cultured on gelatin-coated (Fisher Scientific; Pittsburgh, PA) tissue culture plates in M199 supplemented with 20% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μμg/ml streptomycin (all from Invitrogen Life Technologies), 0.1% endothelial cell growth supplement (Collaborative Biomedical Products; Bedford, MA), and 100 μg/ml porcine heparin (Sigma-Aldrich; St. Louis, MO) as previously described (Pober et al., 1986, J. Immunol. 136:1680-1687). Serially passaged cells were used at the third or fourth subculture. Such cultures are uniformly positive for von Willebrand factor and CD31 and lack detectable contamination by CD45-expressing leukocytes. For EC activation assays, confluent EC monolayers were washed once with HBSS and then treated with stimuli as indicated.

To generate lysates, 3–4 million EC were washed in HBSS (Invitrogen Life Technologies) and incubated in a thin layer of trypsin-EDTA (Invitrogen Life Technologies; Carlsbad, CA) for 1 minute. Detached cells were collected in EC culture medium, washed twice in HBSS, then resuspended in RPMI 1640 and subjected to four rounds of rapid freezing in liquid nitrogen and thawing in a 37°C water bath. Incubation of an aliquot in 0.2% trypan blue (Sigma-Aldrich; St. Louis, MO) confirmed >90% cell lysis. Lysates were centrifuged at over 10,000 × *g* for 10 min at 4°C, and the soluble fraction was collected. Lysates were kept cold throughout the preparation until immediately before addition to EC-T cell co-cultures.

T cell activation in vitro

Where indicated, EC were treated with 50 ng/ml recombinant human IFN-γ for 72 hours to restore HLA-DR expression lost during cell culture. EC were washed three times with HBSS before co-culture with allogeneic $CD4^+$ T cells. No residual IFN-γ is detected in IFN-γ-treated EC culture wells after washing the cells with HBSS. $CD4^+$ T cells were added at a density of 3×10^6 cells/ml in RPMI 1640 (Invitrogen Life Technologies; Carlsbad, CA) supplemented with 10% FBS, 2 mM L-glutamine, and 100 U/ml penicillin, and 100 μg/ml streptomycin.

For activation assays using anti-CD3 and anti-CD28 mAbs, tissue culture

plates were coated with anti-human CD3 Ab in PBS at the indicated concentrations for 2 hours at 37°C. Plates were washed three times in PBS, and then $CD4^+$ T cells were added at a density of 10^6 cells/ml. Anti-human CD28 Ab was added directly to T cell cultures.

For restimulation assays, memory $CD4^+$ T cells were co-cultured with allogeneic EC for 3 days, washed twice, and rested for 3 days with 10 U/ml recombinant IL-2. T cells were then washed twice and cocultured with fresh EC from the same donor as those used in the primary coculture. Alternatively, to increase the number of responding T cells, CD32-transduced EC, generated as previously described (Manes et al., 2007, J. Immunol. 178:3237-3243), were coated with 1 μg/ml anti-CD3 mAb for 30 minutes, washed three times with HBSS, and then cocultured with T cells. T cells were activated and rested as above and then restimulated for 6 hours with 10 ng/ml PMA plus 1 μM ionomycin and analyzed by intracellular cytokine staining (ICS).

Immunoadsorption of HMGB1

Protein G beads (Invitrogen Life Technologies; Carlsbad, CA) were incubated with an mAb against HMGB1 or an isotype control Ab at a concentration of 1mg/ml for 1 hour at room temperature with continuous shaking. Beads were washed four times with PBS, blocked by incubation with PBS containing 1% BSA for 30 minutes, then washed another four times. EC lysates or recombinant HMGB1 stock solution was incubated with the coated beads for 1 hour on ice with continuous shaking. Solutions recovered following the depletion procedure were analyzed by immunoblotting to determine the extent of depletion and added to EC-T cell co-cultures.

Flow cytometric analysis

For cell surface staining of EC, cells were washed in HBSS and incubated

with trypsin-EDTA (Invitrogen Life Technologies; Carlsbad, CA) for 1 minute. Detached cells were collected in M199 containing 20% FBS and centrifuged at 300 × *g* for 10 min. Cells were resuspended in PBS containing 1% BSA and 0.1% sodium azide and incubated with conjugated Abs for 30 minutes on ice. For ICS, 10 μg/ml brefeldin A (Molecular Probes; Carlsbad, CA) was added for the last 4 of 24 hours EC-T cell co-cultures or at the start of 6 hour restimulation periods with PMA plus

ionomycin. Cells were collected by vigorous pipetting, washed once in PBS, and then fixed in Cytofix/Cytoperm (BD Biosciences) for 30 minutes at room temperature. Fixed cells were washed twice in PBS containing 1% BSA (American Bioanalytical) and 0.1% saponin (Sigma-Aldrich) and stained with a conjugated anti-IFN-γ Ab or an isotype control diluted at 1/100 for 1 hour on ice in PBS/BSA/saponin. Cells were washed once in PBS/BSA/saponin and once in PBS. T cells or EC were analyzed on a FACSort using CellQuest software (BD Biosciences; San Jose, CA).

Immunoblotting

To assess p38 and JNK phosphorylation, EC samples were prepared as previously described (50). These lysates and freeze-thaw lysates were supplemented with an equal volume of 2 × SDS-PAGE sample buffer (100 mM Tris-Cl (pH 6.8), 200 mM DTT, 4% SDS, 0.2% bromphenol blue, 20% glycerol) and heated at 95°C for 5 minutes. Samples were separated by electrophoresis in a 10% SDS-PAGE gel, and proteins were transferred to a nitrocellulose membrane (Bio-Rad) at 100 V for 1 hour at 4°C. After blocking with TBST containing 5% nonfat milk for 1 hour at room temperature, membranes were probed overnight at 4°C with an anti-HMGB1 Ab at 2 μg/ml in the blocking buffer or with anti-phospho-p38 or anti-phospho-JNK Abs in TBST containing 5% BSA. Bound Abs were detected with a HRP-conjugated goat anti-mouse or goat anti-rabbit secondary Abs (1/10,000; Jackson ImmunoResearch, Laboratories; West Grove, PA) and ECL substrate (Pierce; Rockford, IL).

Measurement of cytokine production

Supernatants from EC cultures, monocyte cultures, and EC-T cell cocultures were collected after 24 hours. ELISA analysis for IL-1β, IL-17 (both R&D Systems; Minneapolis, MN), IFN-γ, IL-8 (both Invitrogen Life Technologies; Carlsbad, CA), and IL-5 (eBioscience; San Diego, CA) were performed according to the manufacturer's instructions.

Quantitative RT-PCR

Total RNA from naive or memory $CD4^+$ T cells was isolated using a Qiagen RNeasy Mini kit. cDNA was synthesized using Taqman R reagents (Applied Biosystems; Foster City, CA) according to the manufacturer's instructions. Quantitative RT-PCR was performed exactly as described (51) using the primers (5' to 3') shown in Table I.

Table 1. RT-PCR Primers

Target	Forward	Reverse
IL-1R1	GTATCTACAGAACAAG CCTCC	GTTTGCAATCCTTATACCACTG
GAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC
AGER	AGATTCTGCCTCTGAA CTCAC	CCTTCACAGATACTCCCTTCTC
TLR2	CTTTCACTGCTTTCAACTGGTAGTT	TGAGGGAATGGAGTTTAAAGATCCT
TLR4	AGAACTGCAGGTGCTGGATTTAT	GTTCTCTAGAGATGCTAGATTTGT

Statistical analysis

Unless otherwise indicated, comparisons were made using the Student's *t* test with the Bonferroni posthoc test as appropriate. Where indicated, a one-sample *t* test was used to analyze the significance of calculated fold changes. Differences were considered significant at $p < 0.05$.

The results of the experiments presented in this Example are now described.

In this study, in vitro human EC-T cell cocultures were used as a model of the allogeneic memory T cell response to investigate the effects of mediators released by damaged EC on graft rejection. Injured EC release mediators that enhance alloreactive human T cell production of IFN-γ and IL-17. Using a candidate approach, HMGB1 was first identified as one of these mediators. HMGB1 enhances T cell IFN-γ production principally through an indirect pathway by inducing monocyte secretion of IL-1β.

IL-1α is identified herein for the first time as the primary mediator released from damaged EC that acts directly on T cells to enhance early cytokine production. IL-1 released by either mechanism further promotes the expansion of human alloreactive memory $CD4^+$ T cells that secrete IL-17 in secondary cultures. Thus, a molecular mechanism is identified for the first time herein by which cell injury alters the adaptive alloimmune response through release of IL-1 providing a mechanism to account for an alloimmune response to damaged graft by a host's immune system.

Example 1: EC lysates contain soluble mediators that enhance alloreactive T cell IFN-γ production

EC lining the vasculature of solid organ allografts are exposed to multiple insults during the transplantation process. Damage to EC can release components that alter the response of human $CD4^+$ T cells to healthy, allogeneic EC. Lysates of EC were generated by repeated freeze-thaw, cleared by centrifugation, and added to cocultures of $CD4^+$ T cells with IFN- γ-pretreated ($HLA\text{-}DR^+$) allogeneic EC. EC that express HLA-DR can induce allogeneic human $CD4^+$ T cells to secrete IFN-γ, and addition of EC lysates significantly increases the amount of IFN-γ produced by alloreactive $CD4^+$ T cells (Figure 1A), consistent with the hypothesis that damaged EC can release mediators that modulate alloreactive T cell cytokine production. One such mediator might be HMGB1, a NF released from necrotic cells. HMGB1 was readily detected in the lysates by immunoblotting (Figure 1B). Lysates were then depleted of HMGB1 by immunoadsorption on magnetic beads coated with a specific anti-HMGB1 Ab; in parallel, lysates were mock depleted using irrelevant Ig-coated beads. Depletion of HMGB1 from the lysates significantly decreases, but does not totally eliminate, the activity of the lysates, indicating that HMGB1 released from damaged cells can contribute to enhanced IFN-γ production in EC-T cell cocultures (Figure 1C).

Example 2: Recombinant HMGB1 effects on IFN-γ production in EC-T co-cultures depend on monocytes

To further assess how HMGB1 affects T cell cytokine production, recombinant HMGB1 rigorously depleted of endotoxin was used as previously described (Li et al., 2004, J. Immunol. Methods 289:211-223). Addition of recombinant HMGB1 to cocultures of $CD4^+$ T cells with allogeneic $HLA\text{-}DR^+$ EC, like EC lysates, increases T cell IFN-γ production (Figure 2A). The effect of HMGB1 on cytokine production requires antigenic stimulation, as HMGB1 does not induce IFN-γ production from cultures of $CD4^+$ T cells alone or from cocultures of $CD4^+$ T cells with EC lacking HLA-DR expression. In allogeneic EC-T cell cocultures, the frequency of $CD4^+$ T cells activated by allogeneic EC is quite low and difficult to analyze without further expansion; therefore, in some experiments, a suboptimal concentration of the polyclonal activator PHA was added to boost the number of

activated T cells. In such cultures, addition of HMGB1 increases both the amount of IFN-γ detected by ELISA (data not shown) and the number of IFN-γ^+ $CD4^+$ T cells detected by ICS at 24 h (Figure 2B).

HMGB1 has been reported to induce activation of EC, which constitutively express both AGER and TLR4 (Fuiza et al., 2003, Blood 101:2652-2660; Treutiger et al., 2003, J. Int. Med. 254:375-385; Wautier et al., 2004, Circ. Res. 95:233-238). It was therefore examined whether HMGB1 might affect IFN-γ production by enhancing the efficiency with which EC can activate allogeneic T cells. However, no evidence of EC activation by HMGB1 as measured by several parameters was observed. Specifically, treatment of EC with up to 20 μg/ml recombinant HMGB1 did not up-regulate surface ICAM-1 expression, did not induce phosphorylation of p38 or JNK, and did not increase IL-8 secretion (data not shown). Thus, it is unlikely that EC are the target of HMGB1 action in these cocultures.

Because responses from EC could not be detected to explain the effect of HMGB1 on IFN-γ production in EC-T cell cocultures, the question of whether the target of this effect is provided by the T cell population was explored. Recently, it has been asserted that T cell responses to allogeneic EC depend upon the presence of monocytes (Xu et al., 2006, J. Immunol. 176:750-761). It's been found that serially passaged EC cultures do not contain $CD45^+$ leukocytes. However, T cell preparations isolated solely by positive selection do contain a small (<1%) number of $CD14^+$ HLA-DR^+ monocytes (Figure 3A). These cells were depleted by an additional step using anti-CD14 or anti-HLA-DR Abs and then compared highly purified T cell responses with those of populations containing monocytes. Depletion of monocytes from the T cell populations before coculture with allogeneic EC does not substantially reduce the amount of IFN-γ produced by alloreactive T cells in the absence of an additional stimulus. In other words, contaminating monocytes are not required for allogeneic T cell responses to cultured EC. However, the ability of HMGB1 to enhance IFN-γ production is markedly reduced in the absence of monocytes (Figure 3B). To confirm a role for monocytes in mediating the effect of HMGB1, $CD4^+$ T cells were isolated and depleted of monocytes, and negatively isolated autologous monocytes were added back at a ratio of 20:1 ($CD4^+$ T cells to monocytes) before coculture with allogeneic EC (Figure 3C). HMGB1 induced much more IFN-γ production in cocultures to which monocytes were added back.

Whether HMGB1 acts directly on highly purified T cells to modulate cytokine production was also investigated. HMGB1 had no discernible effect on resting T cells. In cultures of monocyte-depleted $CD4^+$ T cells activated with anti-CD3 and anti-CD28 mAbs, HMGB1 could increase IFN-γ production in a dose-dependent manner. However, when the same T cell isolates are activated by allogeneic HLA-DR^+ EC, similar concentrations of HMGB1 have little or no effect on IFN-γ production. mRNA for AGER (2.2±1.2% of GAPDH) and TLR2 (0.035± 0.018% of GAPDH) are detected, but not TLR4, in both resting and activated $CD4^+$ T cells. However, the effect of HMGB1 on anti- CD3- and anti-CD28-activated T cells could not be blocked by mAbs against AGER, TLR2, TLR4, or CD14. HMGB1 thus appears to act directly on $CD4^+$ T cells to enhance IFN-γ production following activation with anti-CD3 and anti-CD28, although it remains unclear through which receptor(s) HMGB1 acts on T cells. Importantly, an immunomodulatory effect is not observed when T cells are activated by allogeneic EC, and the effect of HMGB1 on alloreactivity is highly dependent on the presence of monocytes.

Example 3: HMGB1-treated monocytes augment alloreactive T cell IFN-γ production through secretion of IL-1β

Given that a small number of monocytes are sufficient to mediate an HMGB1-induced increase in IFN-γ production in EC-T cell cocultures, it was hypothesized that monocytes respond to HMGB1 by producing a soluble factor that enhances alloreactive T cell cytokine production. Consistent with this hypothesis, monocytes separated from the EC-T cell coculture by Transwell enhance IFN-γ production when treated with HMGB1 (data not shown). Conditioned medium was collected from monocytes treated either with HMGB1 or the control buffer and added to EC-T cell cocultures depleted of monocytes. Addition of conditioned medium from HMGB1-treated monocytes increases IFN-γ production in EC-T cell cocultures in a dose-dependent manner, whereas addition of conditioned medium from control-treated monocytes has no effect (Figure 4A) Neutralizing Abs to TNF or IL-12 did not inhibit

the ability of HMGB1-treated monocyte conditioned medium to enhance IFN-γ production, nor did a blocking Ab to the IL-18R (data not shown). In contrast, a neutralizing Ab to IL-1β completely blocks the ability of the HMGB1-treated

monocyte conditioned medium to increase IFN-γ production (Figure 4B). Consistent with this result, addition of an anti-IL-1β neutralizing Ab to cocultures of monocytes and $CD4^+$ T cells with allogeneic EC strongly inhibits the ability of HMGB1 to increase IFN-γ production (Figure 4C). Thus, in EC-T cell cocultures, HMGB1 enhances alloreactive $CD4^+$ T cell IFN-γ production indirectly by inducing IL-1β secretion from contaminating monocytes.

Example 4: HMGB1 induces monocyte production of IL-1β in a TLR4- and CD14-dependent manner

To further examine the ability of HMGB1 to induce monocyte IL-1β secretion, human monocytes were purified by negative selection and treated with increasing concentrations of HMGB1. HMGB1 increases IL-1β secretion in a dose-dependent manner (Figure 5A). HMGB1-induced IL-1β secretion could be blocked by blocking Abs against TLR4 and CD14, but not against TLR2 or AGER (Figure 5B). Because TLR4 and CD14 are the well-characterized receptors for endotoxin, a common bacterial contaminant, several controls were performed to demonstrate that the IL-1β-inducing activity is conferred by HMGB1. The endotoxin content of the HMGB1 preparations was found to be 0.6 pg/μg HMGB1 by the *Limulus* assay. Addition of ultra-pure LPS at 10 pg/ml, a concentration that exceeds that contained in HMGB1 preparations added to monocytes, induces far less IL-1β secretion than does 10 μg/ml HMGB1 (Figure 5C). In addition, depletion of HMGB1 from the stock solution by immunoadsorption on anti-HMGB1, but not control beads, fully removes the activity of the stock solution (Figure 5C). Further, the ability of HMGB1 to induce IL-1β secretion from monocytes can be fully destroyed by heating at 80°C for 15 min; however, this standard control for LPS may not be specific, as heating low concentrations of ultra-pure LPS before addition to monocyte cultures also diminished the ability of LPS to induce IL-1β secretion (data not shown). Cumulatively, this evidence strongly suggests that the recombinant protein HMGB1 induces IL-1β from monocytes by directly engaging CD14 and TLR4 and that this effect is not attributable to contamination by endotoxin.

Example 5: IL-1α released directly from damaged EC enhances alloreactive T-cell IFN-γ production

Consistent with a previous study, human EC can express IL-1α, which is stored in an unprocessed form that is fully bioactive as a cytokine . Because IL-1α strongly enhances IFN-γ production in EC-T cell cocultures (Figure 4, *B* and *C*), it was hypothesized that IL-1α may contribute to the portion of the activity of the lysates that is not removed by HMGB1 depletion. EC lysates enhance IFN-γ production in cocultures fully depleted of monocytes, and in these cocultures, neutralization of IL-1α blocks almost all of the IFN-γ-stimulating activity of the lysates (Figure 6A). IL-1 has recently been reported to influence murine $CD4^+$ T cell production of IL-17, a cytokine implicated in the development of autoimmune inflammation (Sutton, et al., 2006, J. Exp. Med. 203:1685-1691; Dong et al., 2006, Nat. Rev. Immunol. 6:329-333). Therefore, the effect of IL-1α in EC lysates on alloreactive T cell IL-17 production was also investigated. As with IFN-γ, EC lysates increase the amount of IL-17 produced, and the majority of this activity is also blocked by neutralizing IL-1α. In cocultures that include monocytes, neutralization of both IL-1α and IL-1β blocks almost all of the activity of the lysates (Figure 6B). The fraction of the activity contributed by IL-1α vs IL-1β varies among experiments and is likely due to variation in the lysate preparations and/or differences in the number or responsiveness of monocytes included in the cultures. Nonetheless, dual neutralization of both IL-1α and IL-1β consistently blocks almost all of the ability of mediators released from damaged EC to enhance alloreactive T cell cytokine production. Addition of IL-1R antagonist, which binds the IL-1R but does not transmit a positive signal, also blocks the effect of EC lysates on T cell IFN-γ and IL-17 production (Figure 6C).

Example 6: IL-1 enhances cytokine production from human memory T cells

To directly examine the ability of IL-1 to modulate T cell cytokine production, recombinant IL-1α was added to EC-T cell cocultures depleted of monocytes. Addition of recombinant IL-1α increases both IFN-γ and IL-17 production in a dose-dependent manner (Figure 7A). IL-1 acts directly on T cells to enhance cytokine production, as T cells stimulated in the absence of APCs with plate

bound anti-CD3 plus anti-CD28 produce increasing amounts of IFN-γ and IL-17 upon treatment with IL-1 (Figure 7B). IL-1 enhances IFN-γ production more effectively from memory T cells compared with naive T cells, although IL-1 effectively enhances IL-17 production from both naive and memory cells. $CD4^+$ T cells express IL-1R1 mRNA, and three different donors demonstrated somewhat higher IL-1R1 mRNA expression in memory vs naïve $CD4^+$ T cell subsets (13-, 2.4-, and 2.6-fold greater); however, both naive and memory $CD4^+$ T cells up-regulate IL-1R1 expression following activation with anti-CD3 and anti-CD28 (Figure 7C).

Example 7: IL-1 promotes expansion of IL-17 secreting alloreactive human memory $CD4^+$ T cells

The experiments described above show that IL-1 released as a consequence of cell damage enhances both IFN-γ and IL-17 production from alloreactive $CD4^+$ T cells in primary EC-T cell cocultures. Whether IL-1 could affect the relative proliferation or differentiation of specific human alloreactive memory $CD4^+$ T cells, assessed by a change in frequency of T cells that produce specific cytokines was also investigated. To investigate this, restimulation experiments were performed in which purified memory $CD4^+$ T cells were cocultured with allogeneic EC, and increasing concentrations of IL-1 were added. After 3 days of coculture, T cells were rested for three days and then restimulated with fresh EC from the same donor as was used in the primary culture. Addition of IL-1 to primary EC-T memory cell cocultures causes a significant increase the amount of IL-17 produced by the alloreactive T cell population upon restimulation (Figure 8A). In contrast, IL-1 has little effect on the amount of IFN-γ produced upon restimulation and appears to slightly decrease the amount of IL-5 produced. These data are consistent with expansion of IL-17-secreting T cells.

To examine more directly whether addition of IL-1 to primary EC-T cell cocultures changes the frequency of cells that produce Th1, Th2, or Th17 cytokines upon restimulation, a model was used in which EC are transduced to express the Fc_R CD32, are coated with anti-CD3 mAb, and are then cocultured with memory $CD4^+$ T cells. In these cocultures, the bound anti-CD3 mAb provides a defined TCR signal and the EC provide costimulatory molecules, resulting in polyclonal activation of the cocultured T cells. In this experiment the model was

adapted to provide a primary stimulus, with or without IL-1, for 3 days. The activated memory T cells were then recovered, rested for 3 days, and then restimulated using PMA plus ionomycin in the presence of brefeldin A. The pattern of cytokine production was then assessed by ICS and flow cytometry. Addition of IL-1 during the primary cocultures in this system causes a significant increase in the number of T cells that produce IL-17 upon restimulation (Figure 8, B and C). Both IL-17$^+$ single producers and IL-17$^+$ IFN-γ^+ double producers were observed within the memory CD4$^+$ T cell population, and both subpopulations were similarly increased by exposure to IL-1. Consistent with the results in allogeneic cocultures, exposure to IL-1 during the primary activation did not substantially change the number of IFN- γ - or IL-4-producing CD4$^+$ T cells upon restimulation. These results suggest that IL-1 can skew the population of human memory CD4$^+$ T cells activated by EC toward IL-17 production.

Example 8: Under inflammatory conditions, EC enhance alloreactive CD4$^+$ T cell cytokine production via expression of IL-1α and skew the alloreactive memory CD4$^+$ T cell population towards IL-17 production via IL-1α

Human EC were pretreated with TNF or left untreated for 72 hours, washed extensively, and then co-cultured with purified allogeneic memory CD4$^+$ T cells. A neutralizing antibody to IL-1α or an isotype control was added as indicated. IFN-γ and IL-17 in the culture supernatant were measured by ELISA at 24 hours. TNF-treated EC are more potent inducers of allogeneic T cell cytokine production, and a significant portion of this activity is due to EC production of IL-1α. * $p < 0.05$ compared to untreated EC. ** $p < 0.05$ compared to TNF-treated EC with isotype control.

First, increases in IL-1α in cultured endothelial cells that are induced by treatment with TNF, are able to induce human memory CD4$^+$ T cells to increase their production of IL-17 and IFN-γ (Figure 9 A and B). The IL-17 effect (but not the IFN- γ effect) can still be seen when the activated T cells are rested and then restimulated, indicative of skewing the reaction towards IL-17 production.

In another set of experiments, human EC were pretreated with TNF or left untreated for 72 hrs, washed extensively, and then co-cultured with purified allogeneic memory CD4+ T cells for 3 days with or without addition of a neutralizing

antibody to IL-1α. T cells were then rested for three days and then restimulated with fresh, untreated EC from the same donor for 24 hours. IFN-γ, IL-17, and IL-5 in the culture supernatant were measured by ELISA as shown in Figure 10 A-C depicting the skewing of the alloreactive CD4+ populationg toward IL-17 production via IL-1α. This kind of skewing of the memory response is consistent with the hypothesis that IL-1 (α or β) present during the initial encounter with graft endothelial cells will produce long term changes in the T cell response that bode ill for the graft. Both in vitro effects are blocked by IL-1Ra. The data argue that endothelium need not be frankly injured to exert this effect, but merely "inflamed."

Example 9: Ischemia-reperfusion injury increases IL-1α expression in human coronary artery interposition grafts

1-2 mm segments of human coronary artery were interposed into the infrarenal aortas of immunodeficient SCID/beige mice and allowed to heal for >30 days. Grafts are then subjected to ischemia for 30 minutes or sham surgery and harvested 24 hours later. Staining for human IL-1α shows focally increased staining along the endothelium of ischemia-reperfused grafts. Although patterns of staining have varied, increased IL-1α staining has been observed in roughly half of the ischemia-reperfused arteries examined thus far.

Using an in vivo model of T cell-mediated human artery graft injury, segments of human arteries are interposed into the infrarenal aorta of an immunodeficient mouse (specifically, a C.B-17 SCID/beige recipient). Such arteries are healthy and functional and are completely human, i.e. no mouse cells are present within the graft. Figure 11 shows that a brief period (30 minutes) of ischemia (Figure 11B) followed by perfusion will upregulate IL-1α expression in the human endothelial cells lining the graft as copared to controls (Figure 11A).

Example 10: IL-1 blockade with IL-1 receptor antagonist (IL-1Ra) in vivo reduces IL-17 production and reduces intimal lesion size in rejecting human coronary artery interposition grafts

1-2 mm segments of human coronary artery were interposed into the infrarenal aortas of immunodeficient SCID/beige mice. Allogeneic human PBMC were adoptively transferred 2 days later, and mice were treated daily with 50mg/kg of

IL-1Ra or control PBS delivered subcutaneously. Three weeks after transfer of PBMCs, grafts were harvested, RNA isolated, and expression of IL-17 was evaluated by RT-PCR. Though not yet statistically significant, there appears to be a trend towards reduced total amount of IL-17 expression, reduced IL-17 expression per CD3+ T cell, and reduced intimal expansion in rejecting arteries with IL-1 blockade (n=7).

Interim analysis shows a reduction in intragraft expression of IL-17 mRNA when normalized to a control mRNA or to a T cell specific mRNA (Figure 12 A and Figure 12B). We also see reduced artery injury, measured as expansion of the intima (Figure 12C).

The disclosures of each and every patent, patent application, and publication cited herein are hereby incorporated herein by reference in their entirety. While this invention has been disclosed with reference to specific embodiments, it is apparent that other embodiments and variations of this invention may be devised by others skilled in the art without departing from the true spirit and scope of the invention. The appended claims are intended to be construed to include all such embodiments and equivalent variations.

CLAIMS

What is claimed:

1. A method of attenuating a host immune system's T cell response to an antigen present on an graft, said method comprising administering to said host a therapeutically effective amount of IL-1 receptor antagonist prior to establishing host circulation to said graft during transplantation of said graft into said host.

2. The method of claim 1, wherein said host is human.

3. The method of claim 2, wherein said graft is an allograft comprising a cell, a tissue, an organ, or any combination thereof, excluding a cell derived from bone marrow.

4. The method of claim 2, wherein said IL-1 receptor antagonist is administered systemically to said host up to at least 1 day but not more than 3 days after said host receives said graft.

5. The method of claim 2, wherein said IL-1 receptor antagonist is administered systemically to said host up to at least 1 day but not more than 7 days after said host receives said graft.

6. The method of claim 2, wherein said IL-1 receptor antagonistis administered systemically to said host up to at least 1 day but not more than 14 days after said host receives said graft.

7. The method of claim 2, wherein said IL-1 receptor antagonist is administered systemically to said host up to at least 1 day but not more than 21 days after said host receives said graft.

8. A method of enhancing the long-term survival of an allograft in a host, said method comprising administering to said host a therapeutically effective amount of IL-1 receptor antagonist prior to establishing host circulation to said allograft during transplantation of said allograft into said host.

9. The method of claim 8, wherein said host is human.

10. The method of claim 9, wherein said allograft comprises a cell, a tissue, an organ, or a combination thereof, excluding a cell derived from bone marrow.

11. The method of claim 9, wherein said IL-1 receptor antagonist is administered systemically to said host up to at least 1 day post-operatively, but not more than 3 days after said host receives said allograft.

12. The method of claim 9, wherein said IL-1 receptor antagonist is administered systemically to said host up to at least 1 day but not more than 7 days after said host receives said allograft.

13. The method of claim 9, wherein said IL-1 receptor antagonist is administered systemically to said host up to at least 1 day but not more than 14 days after said host receives said allograft.

14. The method of claim 9, wherein said IL-1 receptor antagonist is administered systemically to said host up to at least 1 day but not more than 21 days after said host receives said allograft.

15. A method of treating a patient experiencing a stroke, said method comprising administering to said patient a therapeutically effective amount of IL-1Ra within 24 hours of the onset of said stroke.

16. The method of 15, wherein said IL-1 receptor antagonist is administered systemically to said patient up to at least 1 day but not more than 3 days post-onset of said stroke.

17. The method of claim 15, wherein said IL-1 receptor antagonist is administered systemically to said patient up to at least 1 day but not more than 7 days post-onset of said stroke.

18. The method of claim 15, wherein said IL-1 receptor antagonist is administered systemically to said patient up to at least 1 day post-stroke, but not more than 14 days post-onset of said stroke.

19. The method of claim 15, wherein said IL-1 receptor antagonist is administered systemically to said patient up to at least 1 day but not more than 21 days post-onset of said stroke.

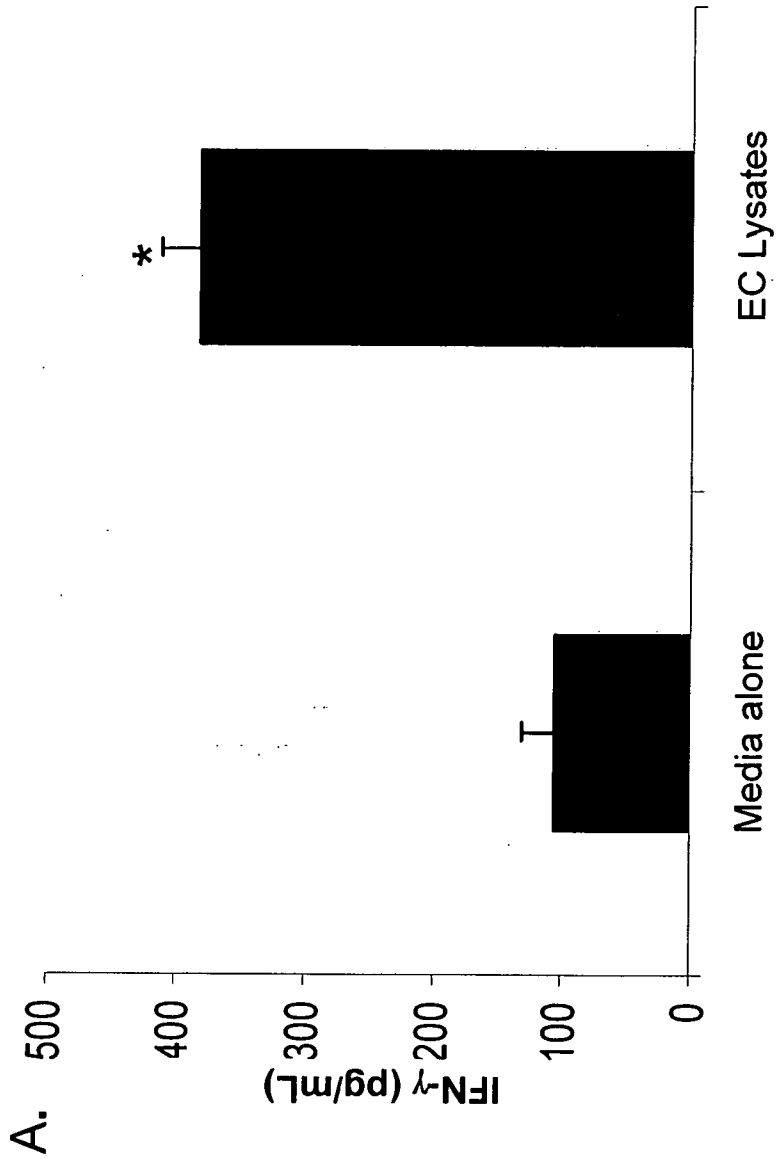

Figure 1A

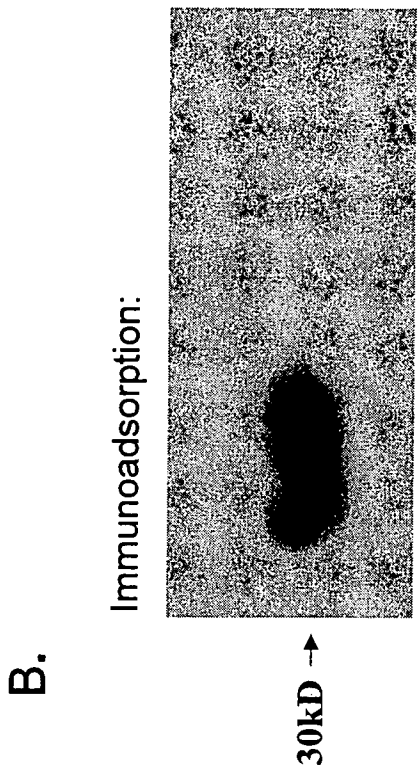

Figure 1B

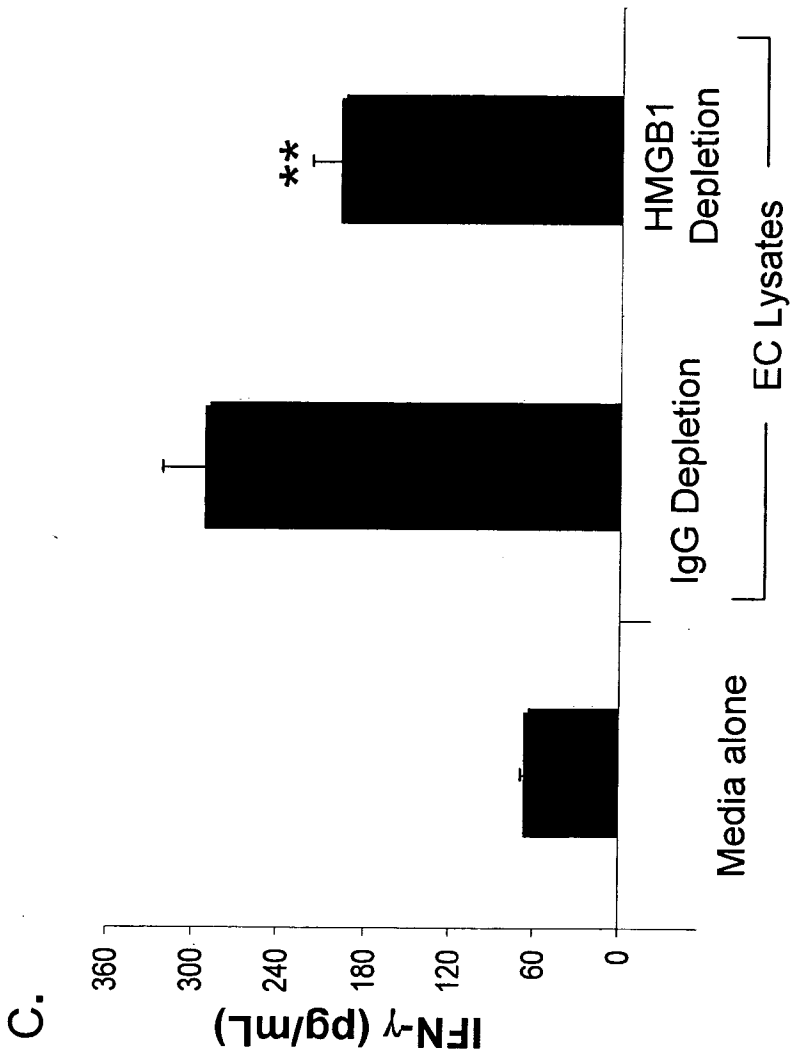
C.
IFN-γ (pg/mL)
360
300
240
180
120
60
0
**
Media alone
IgG Depletion
HMGB1 Depletion
EC Lysates

Figure 1C

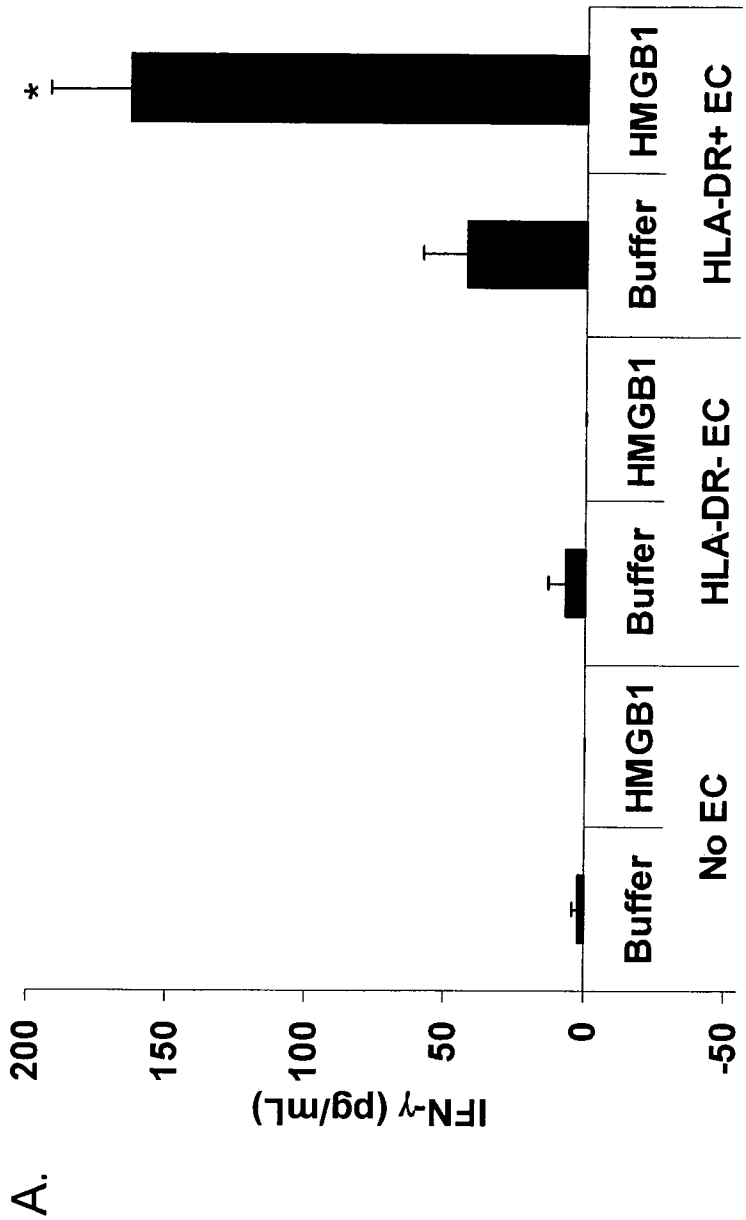
A.
IFN-γ (pg/mL)
200
150
100
50
0
-50
*
Buffer
HMGB1
No EC
Buffer
HMGB1
HLA-DR- EC
Buffer
HMGB1
HLA-DR+ EC

Figure 2A

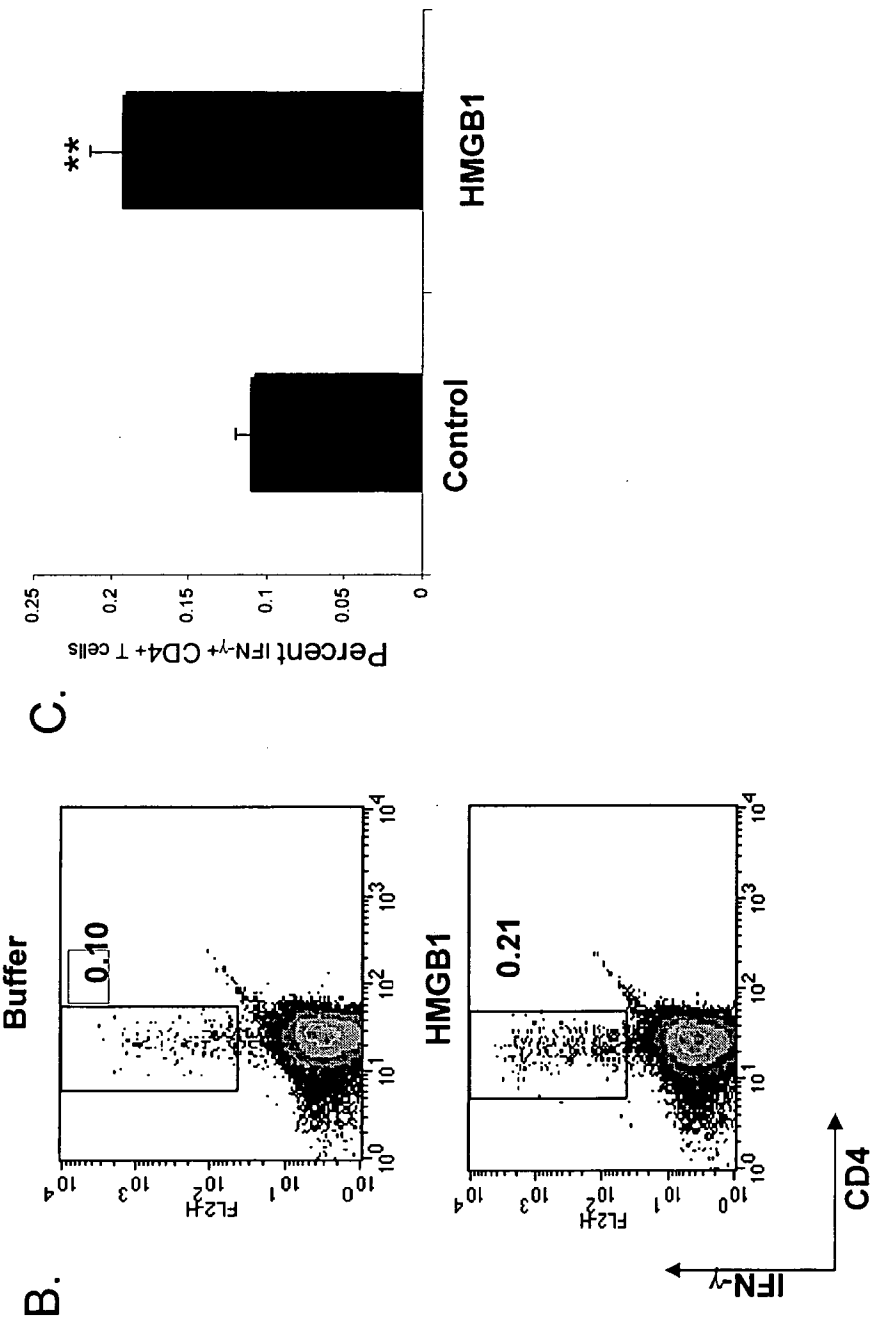

Figure 2B and Figure 2C

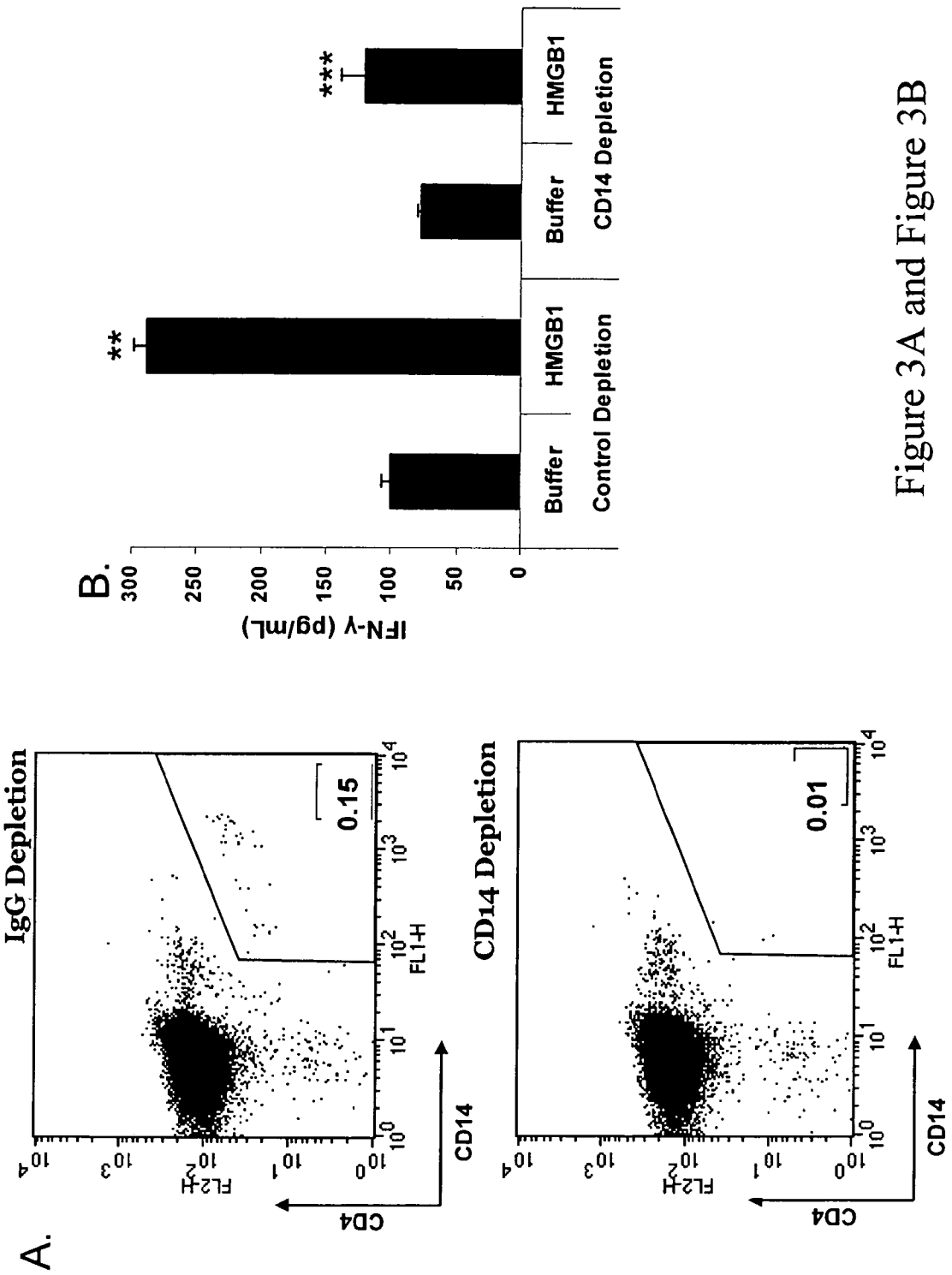

Figure 3A and Figure 3B

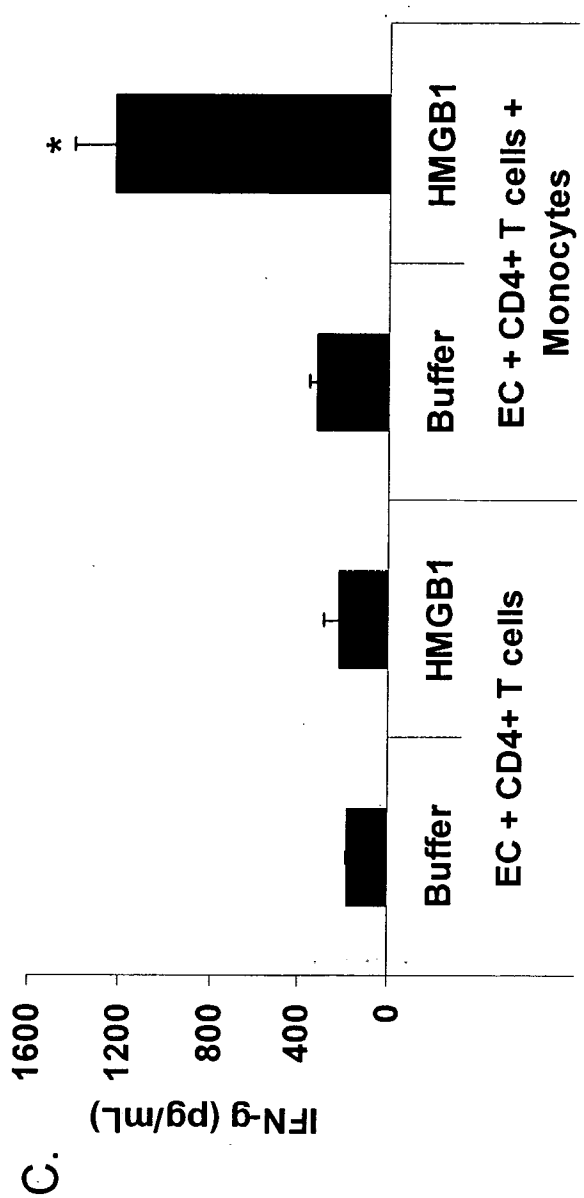
C.
IFN-g (pg/mL)
1600
1200
800
400
0
*
Buffer
HMGB1
Buffer
HMGB1
EC + CD4+ T cells
EC + CD4+ T cells + Monocytes

Figure 3C

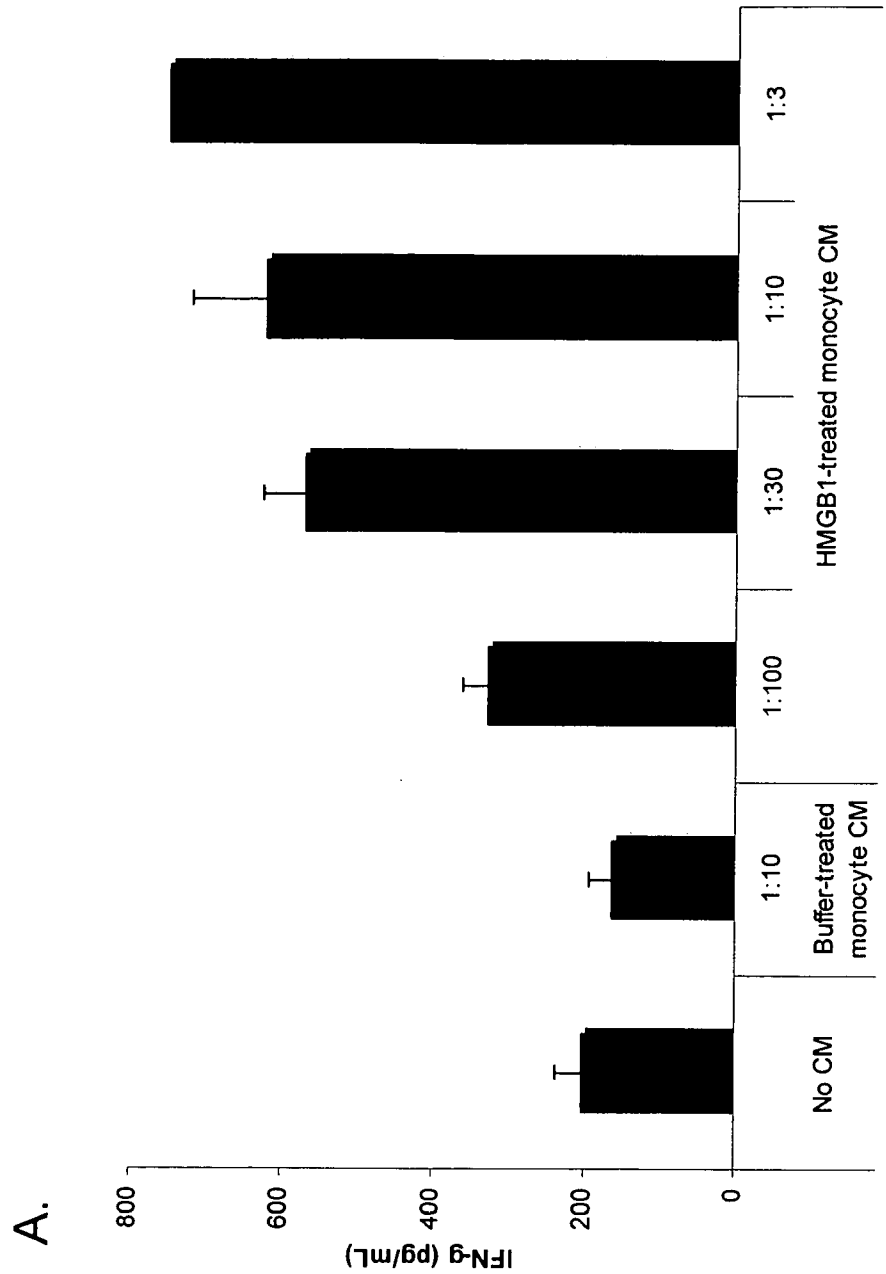
A.
IFN-g (pg/mL)
800
600
400
200
0
No CM
1:10
Buffer-treated monocyte CM
1:100
1:30
1:10
1:3
HMGB1-treated monocyte CM

Figure 4A

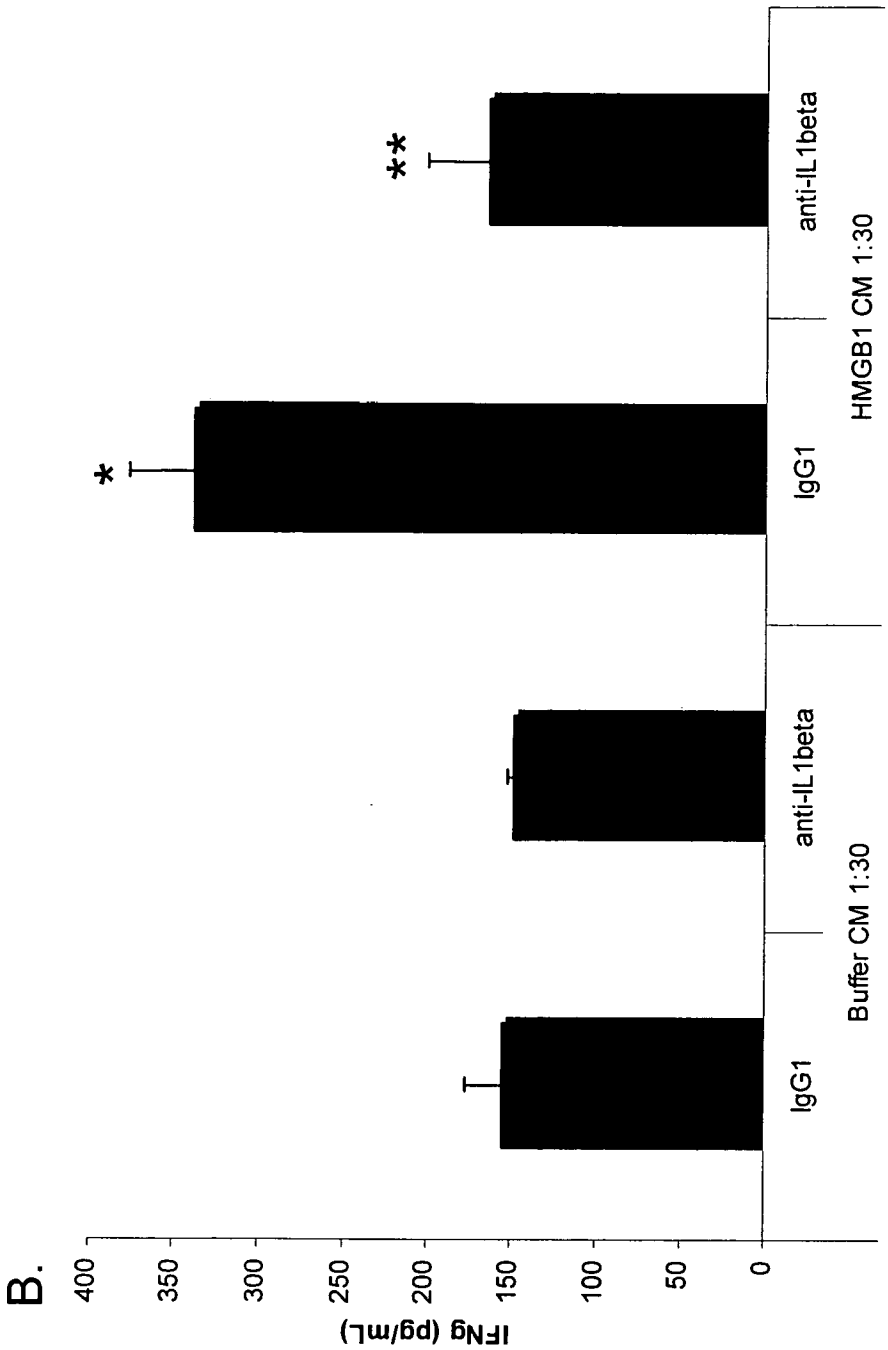
B.
IFNg (pg/mL)
400
350
300
250
200
150
100
50
0
*
**
IgG1
anti-IL1beta
IgG1
anti-IL1beta
Buffer CM 1:30
HMGB1 CM 1:30

Figure 4B

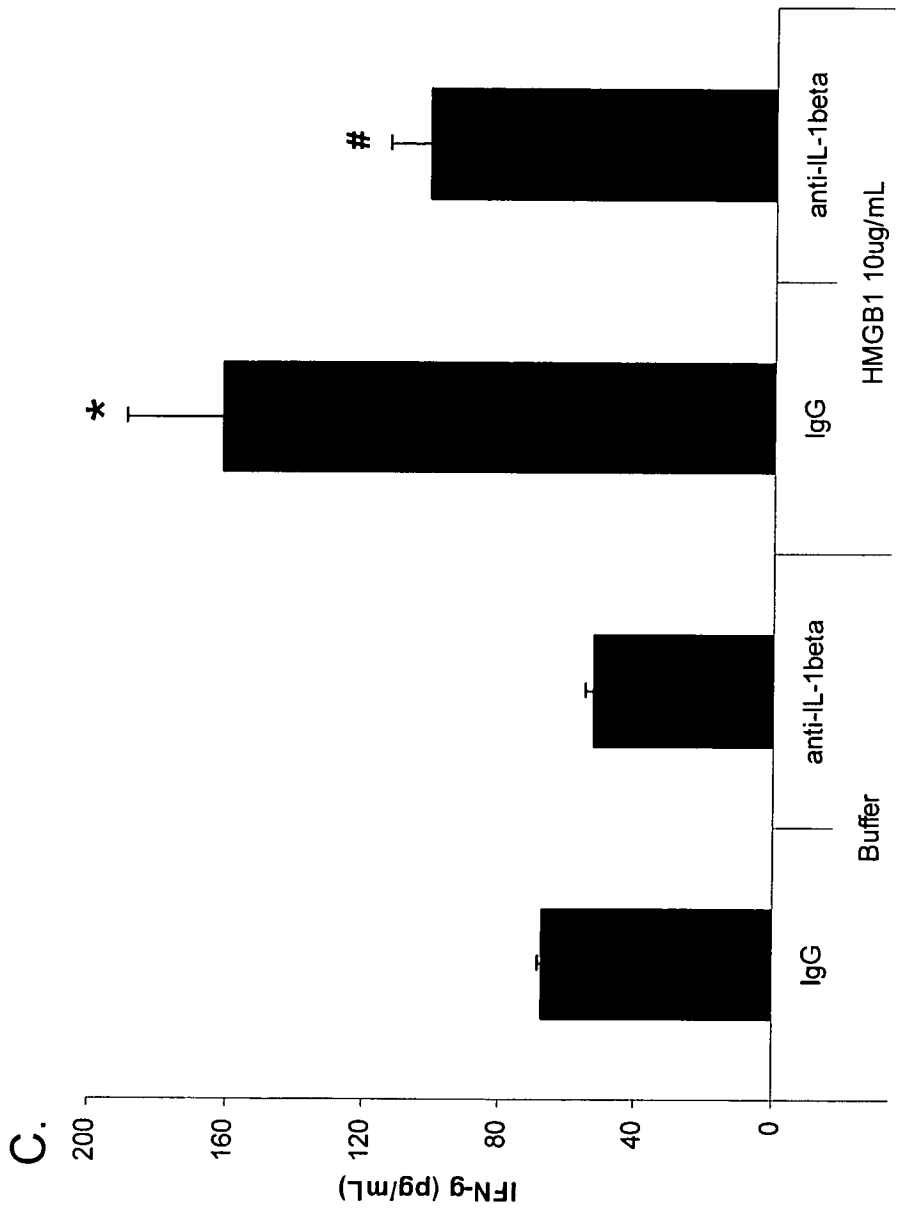
C.
IFN-g (pg/mL)
200
160
120
80
40
0
IgG
anti-IL-1beta
Buffer
IgG
anti-IL-1beta
HMGB1 10ug/mL
*
#

Figure 4C

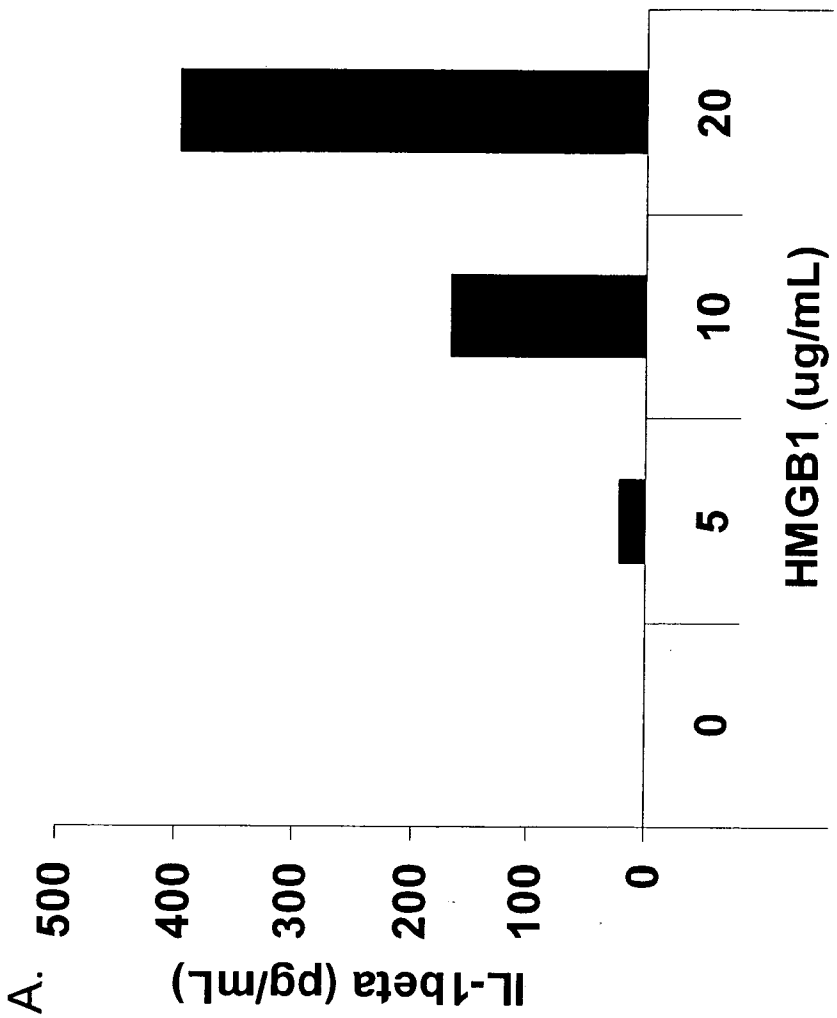

Figure 5A

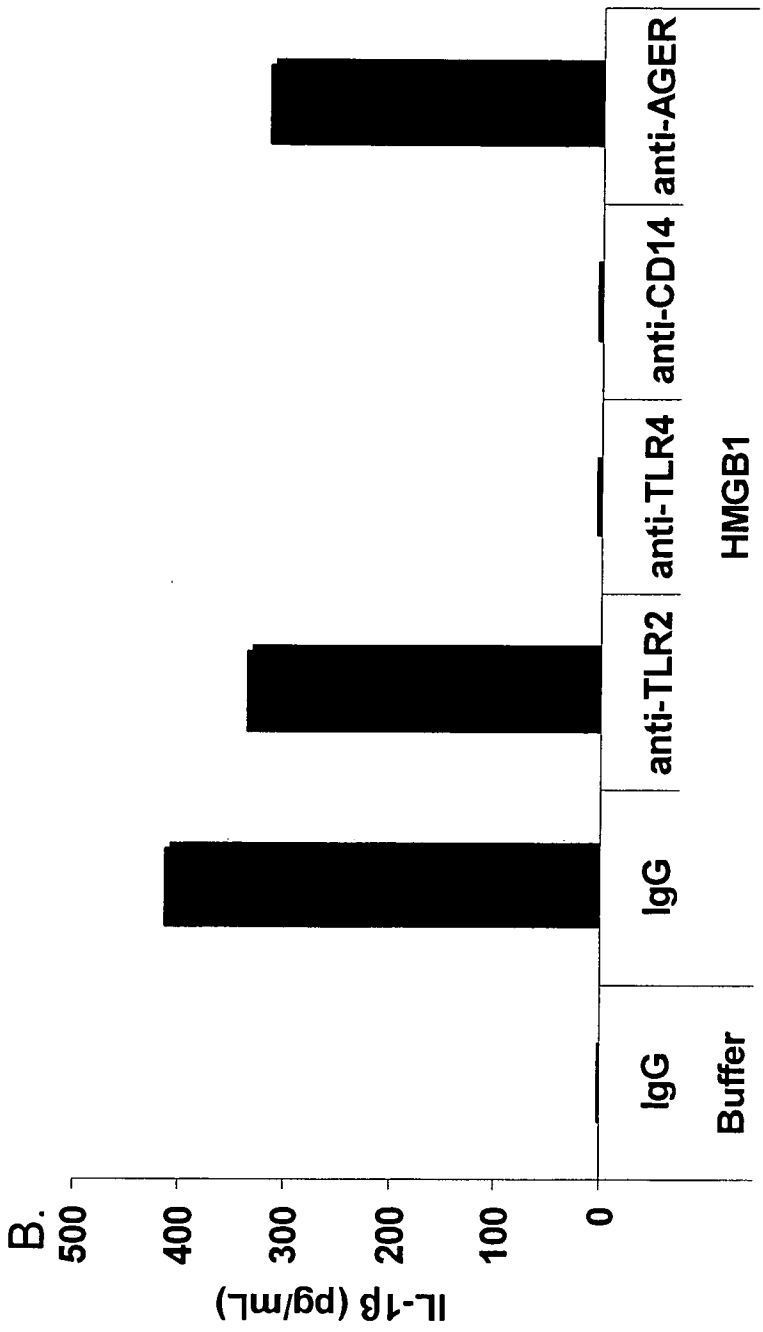
B.
IL-1β (pg/mL)
500
400
300
200
100
0
Buffer
IgG
IgG
anti-TLR2
anti-TLR4
anti-CD14
anti-AGER
HMGB1

Figure 5B

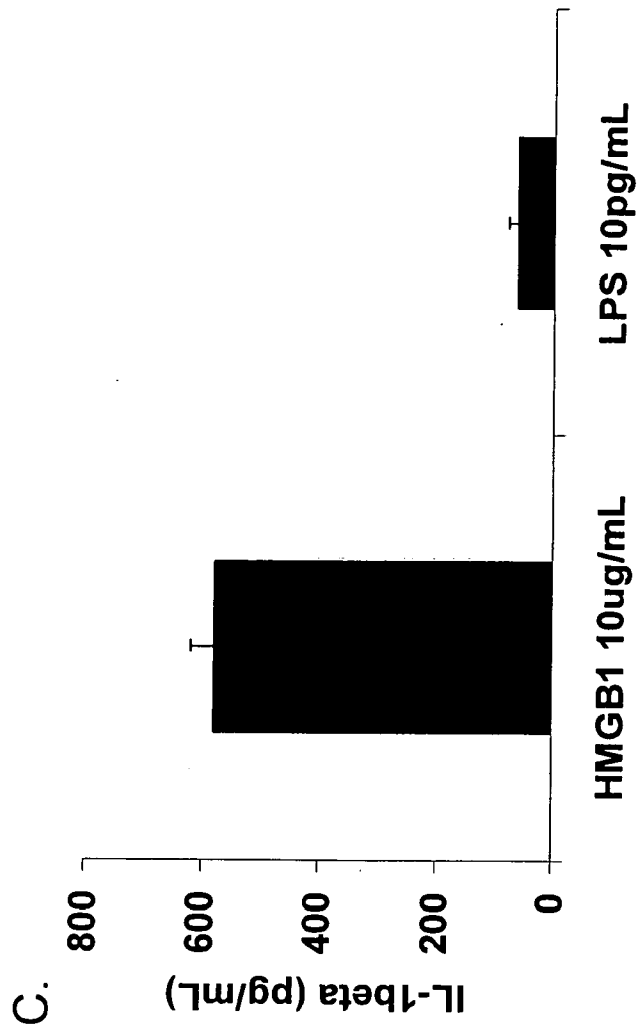

Figure 5C

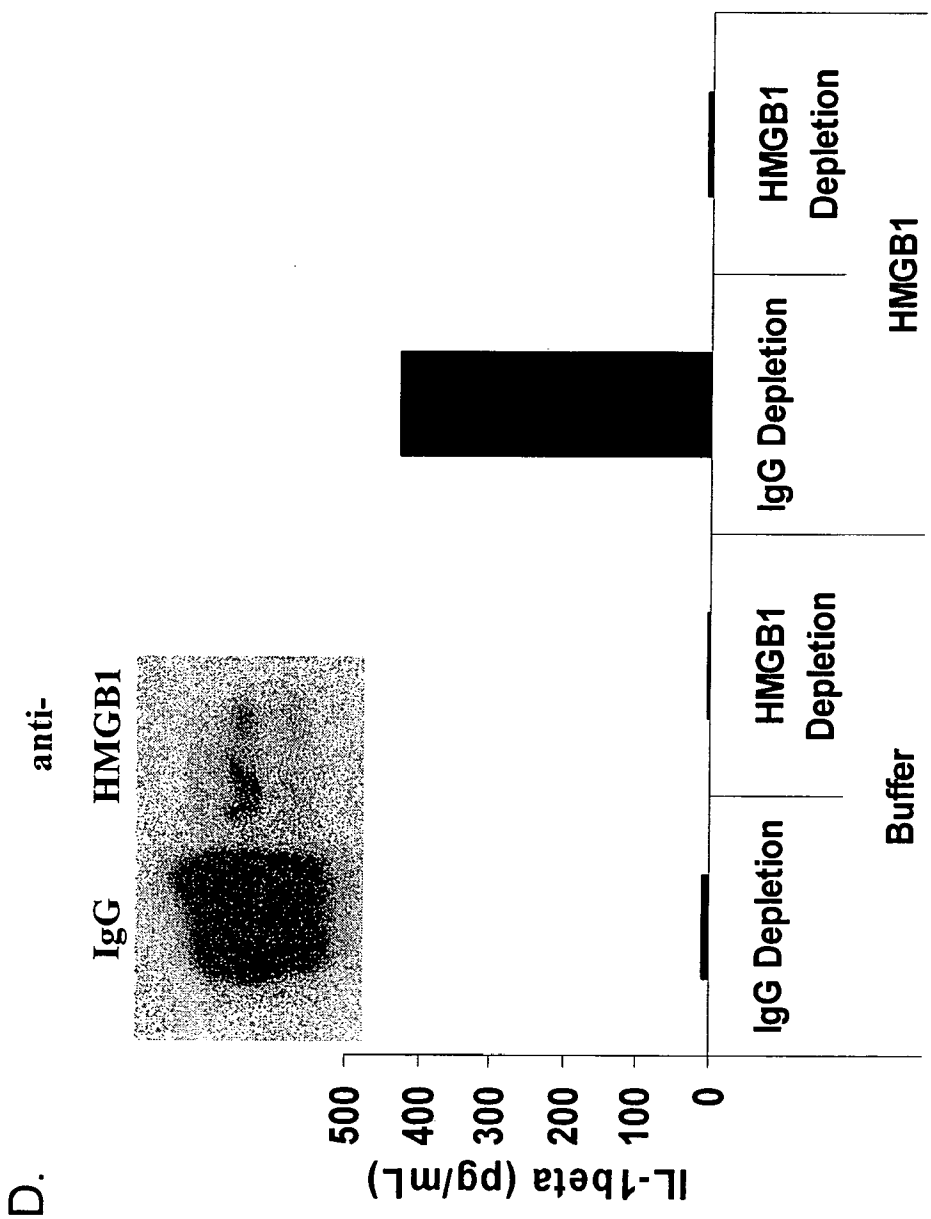
D.
anti-
HMGB1
IgG
IL-1beta (pg/mL)
500
400
300
200
100
0
IgG Depletion
HMGB1 Depletion
IgG Depletion
HMGB1 Depletion
Buffer
HMGB1

Figure 5D

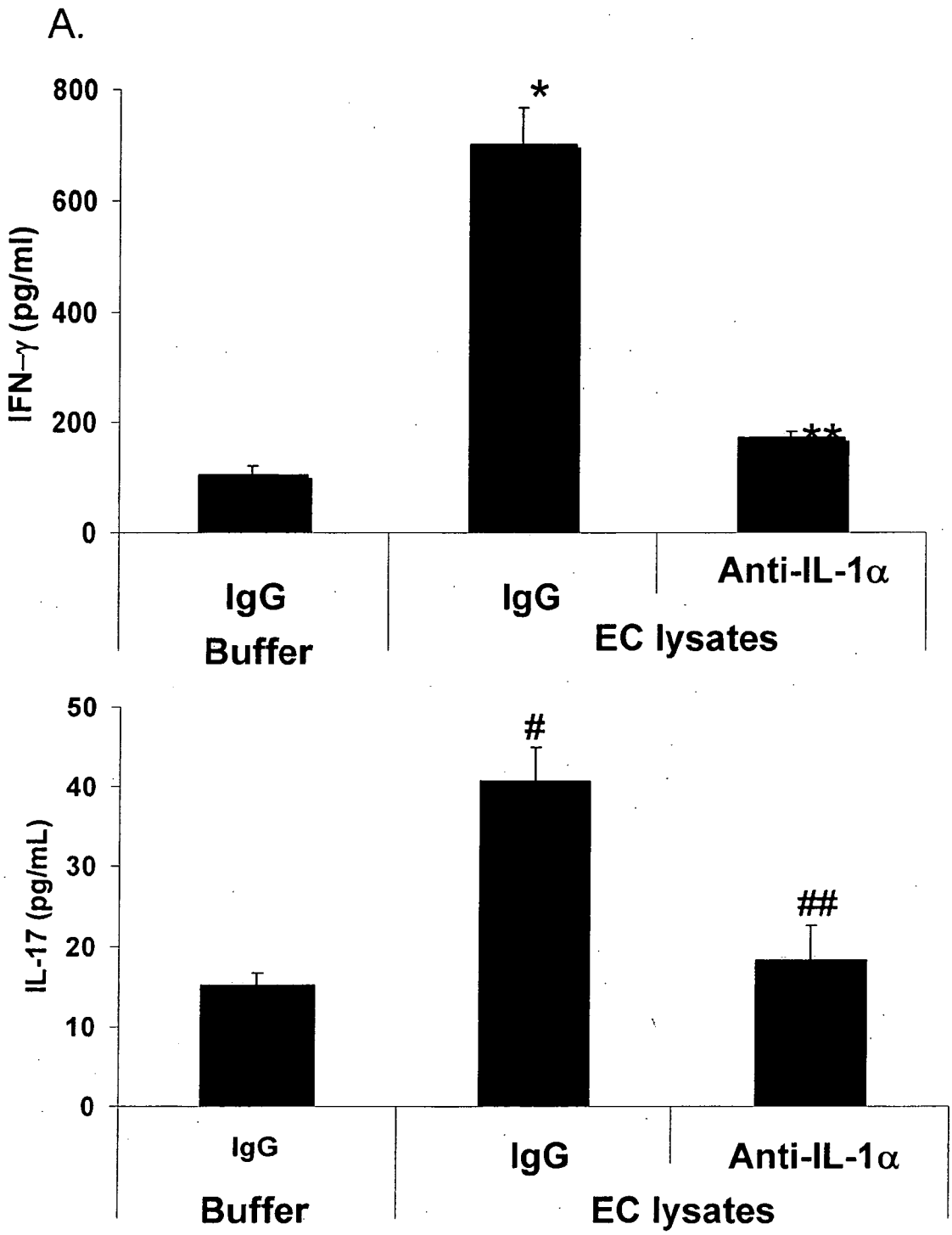
A.
800
600
400
200
0
IFN-γ (pg/ml)
*
**
IgG
Buffer
IgG
Anti-IL-1α
EC lysates
50
40
30
20
10
0
IL-17 (pg/mL)
#
##
IgG
Buffer
IgG
Anti-IL-1α
EC lysates

Figure 6A

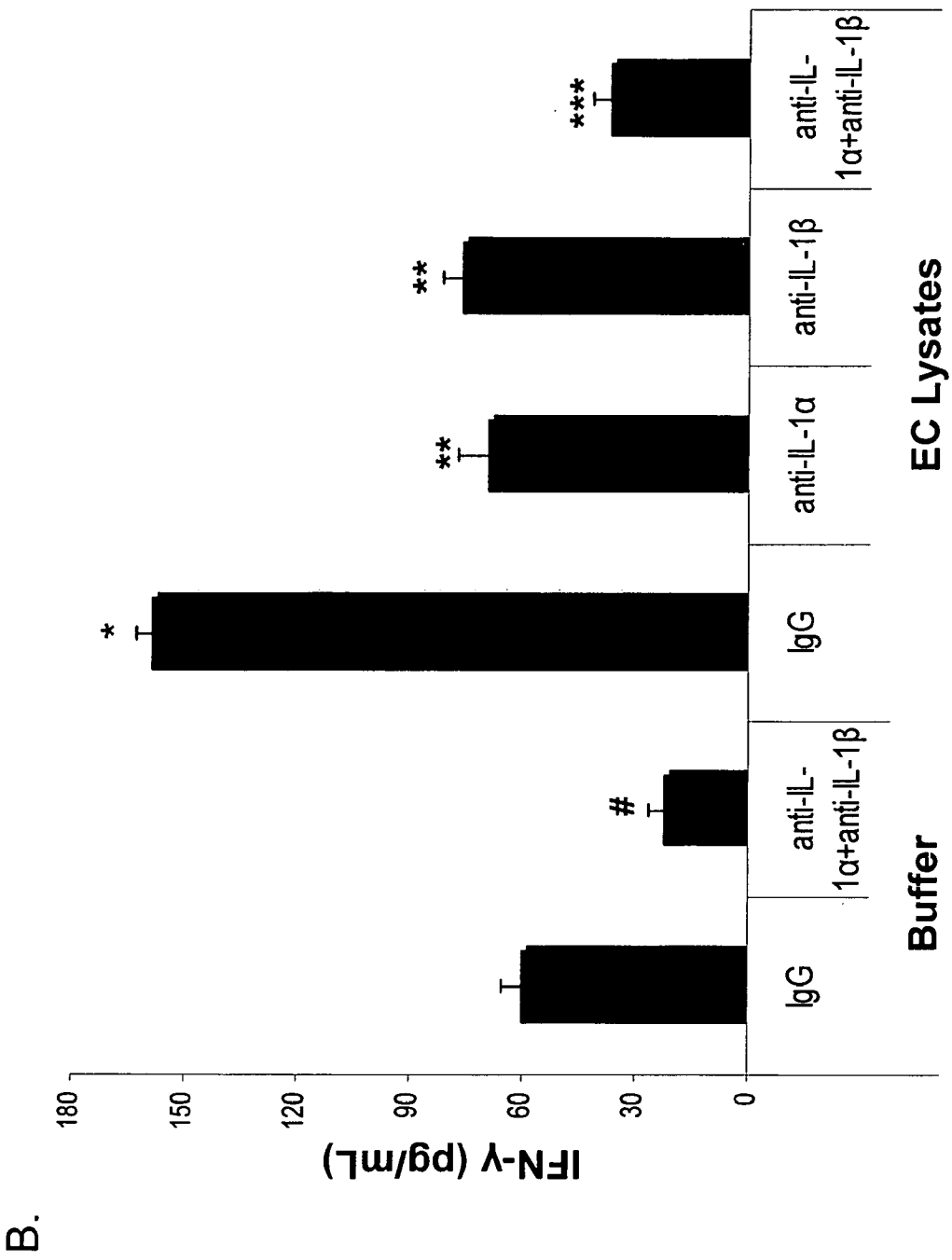
B.
IFN-γ (pg/mL)
180
150
120
90
60
30
0
IgG
anti-IL-1α+anti-IL-1β
IgG
anti-IL-1α
anti-IL-1β
anti-IL-1α+anti-IL-1β
Buffer
EC Lysates
*
#
**
**
***

Figure 6B

C.

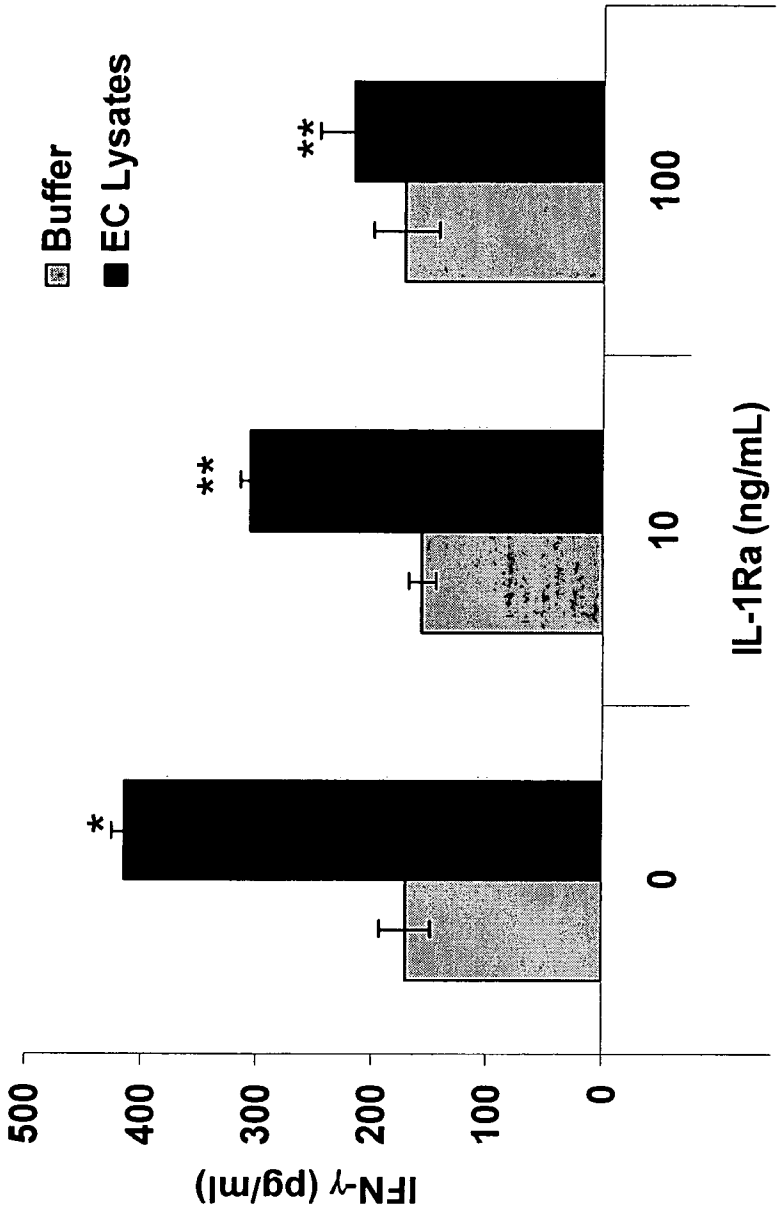
Buffer
EC Lysates
IFN-γ (pg/ml)
500
400
300
200
100
0
*
**
**
0
10
100
IL-1Ra (ng/mL)

Figure 6C

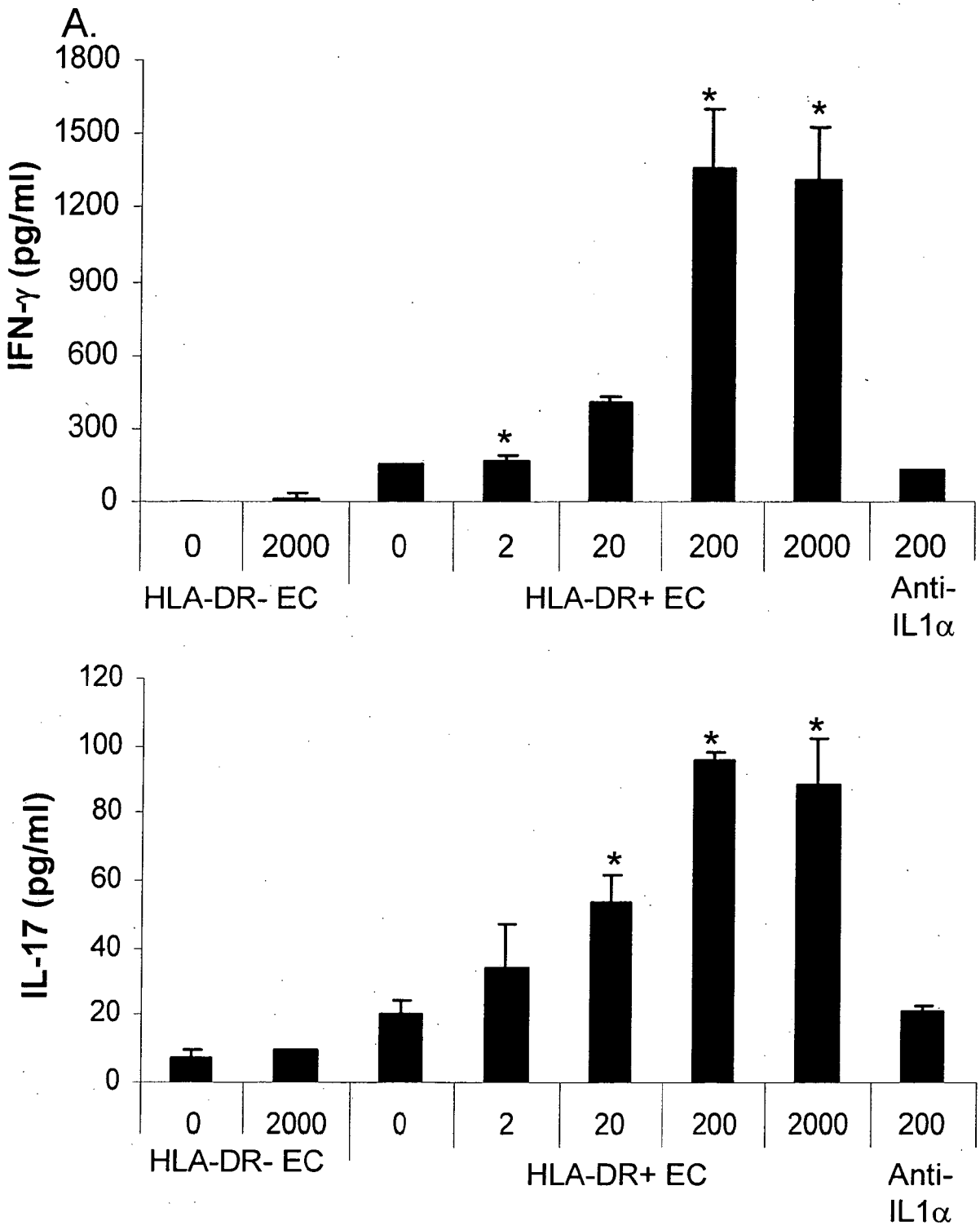
A.
IFN-γ (pg/ml)
1800
1500
1200
900
600
300
0
*
*
*
0
2000
0
2
20
200
2000
200
HLA-DR- EC
HLA-DR+ EC
Anti-IL1α
IL-17 (pg/ml)
120
100
80
60
40
20
0
*
*
*
0
2000
0
2
20
200
2000
200
HLA-DR- EC
HLA-DR+ EC
Anti-IL1α

Figure 7A

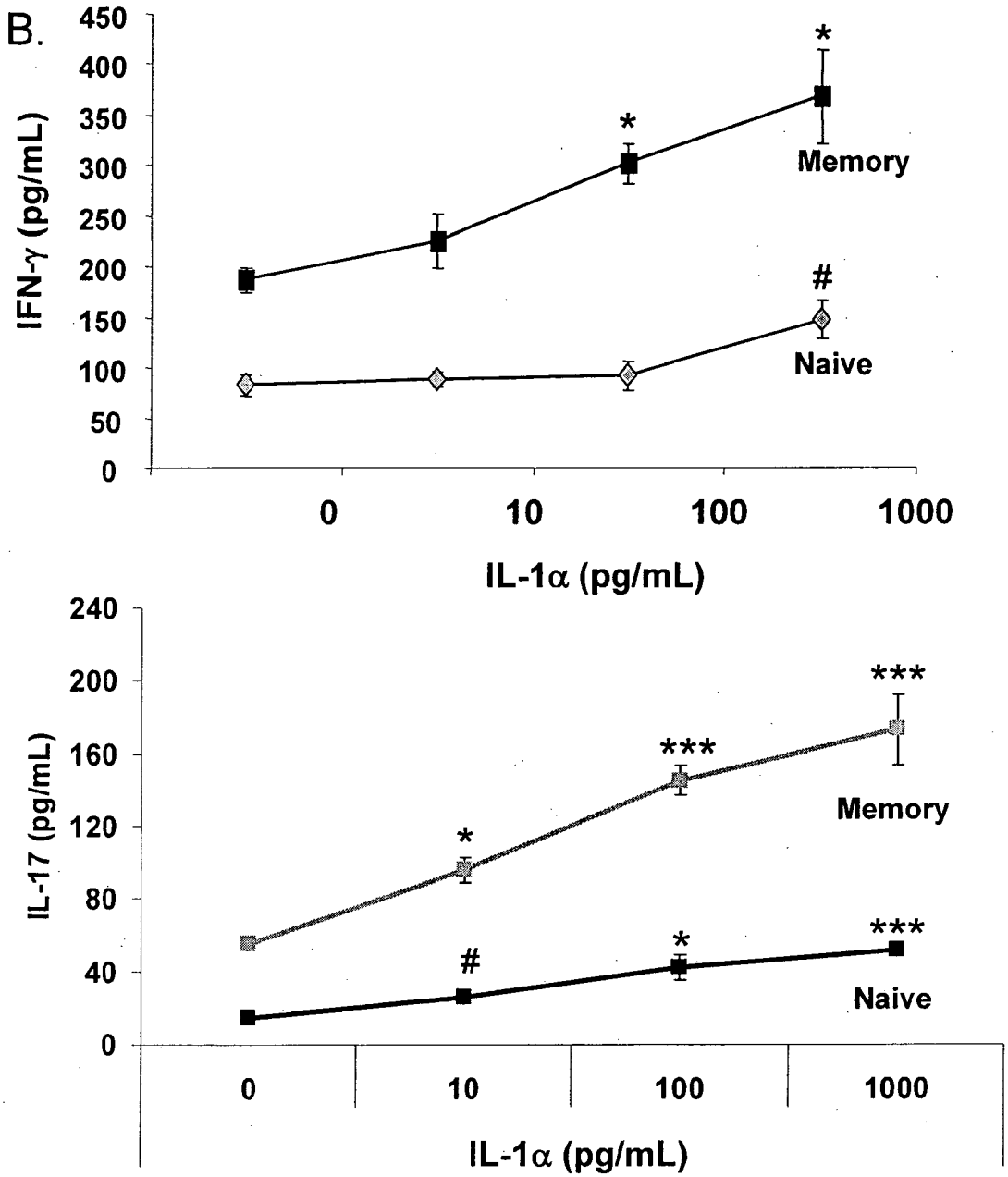
B.
IFN-γ (pg/mL)
450
400
350
300
250
200
150
100
50
0
*
*
Memory
#
Naive
0
10
100
1000
IL-1α (pg/mL)
IL-17 (pg/mL)
240
200
160
120
80
40
0
***
***
Memory
*
#
*
***
Naive
0
10
100
1000
IL-1α (pg/mL)

Figure 7B

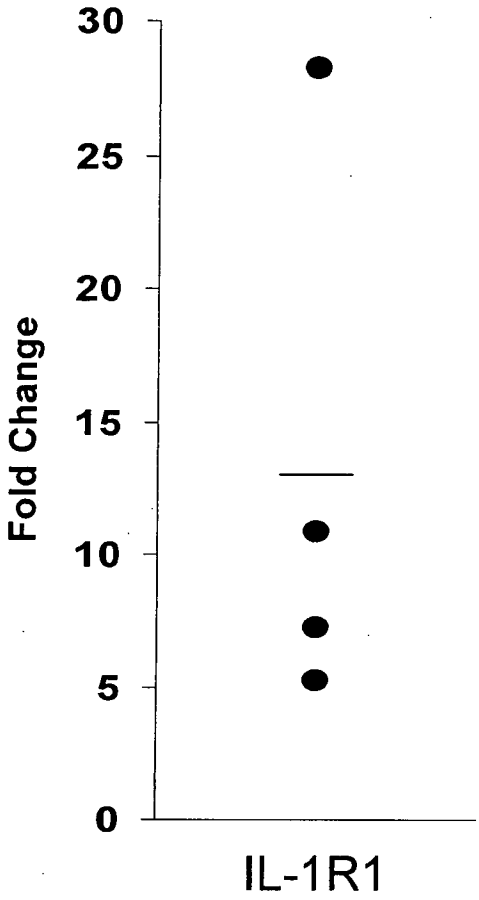

Figure 7C

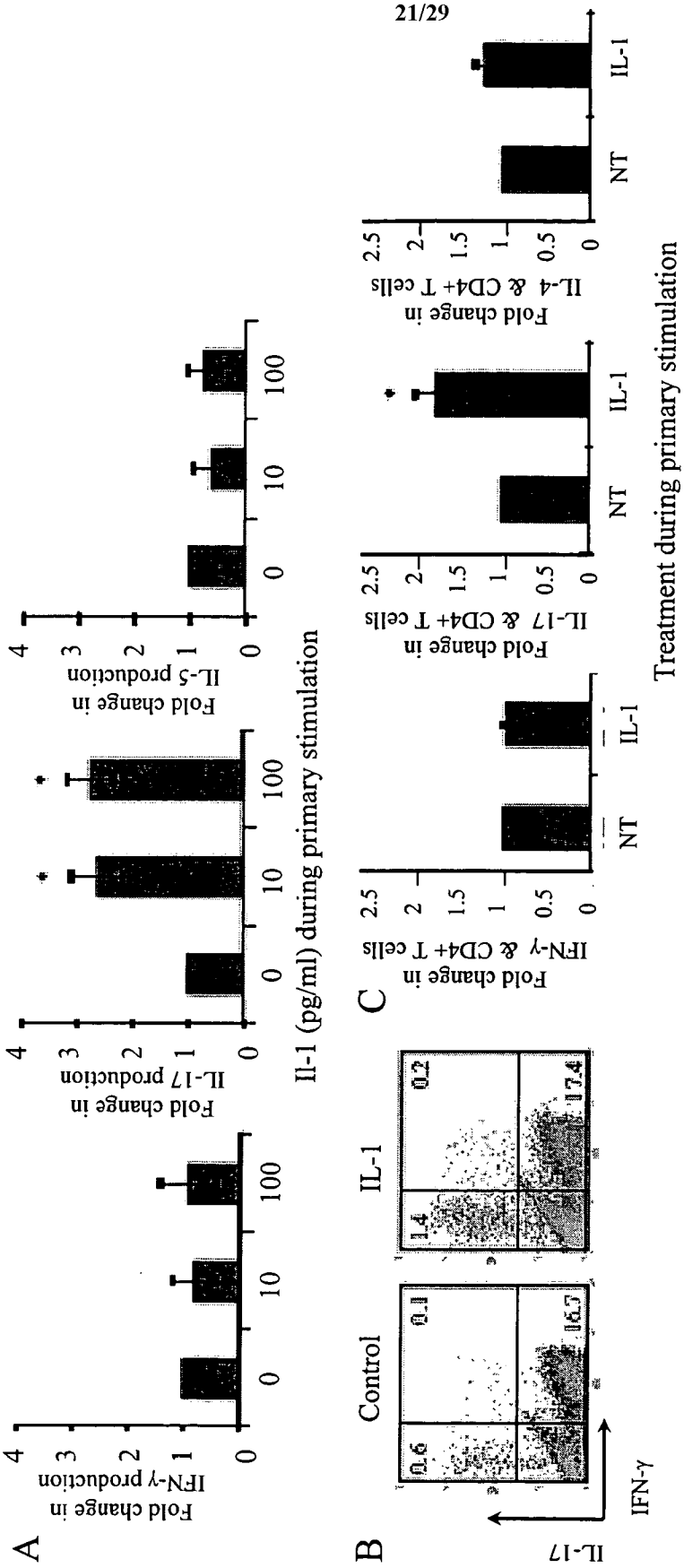
A
Fold change in IFN-γ production
Fold change in IL-17 production
Fold change in IL-5 production
Il-1 (pg/ml) during primary stimulation
B
Control
IL-1
IL-17
IFN-γ
0.6
0.1
16.7
1.4
0.2
17.4
C
Fold change in IFN-γ & CD4+ T cells
Fold change in IL-17 & CD4+ T cells
Fold change in IL-4 & CD4+ T cells
NT
IL-1
Treatment during primary stimulation

Figure 8

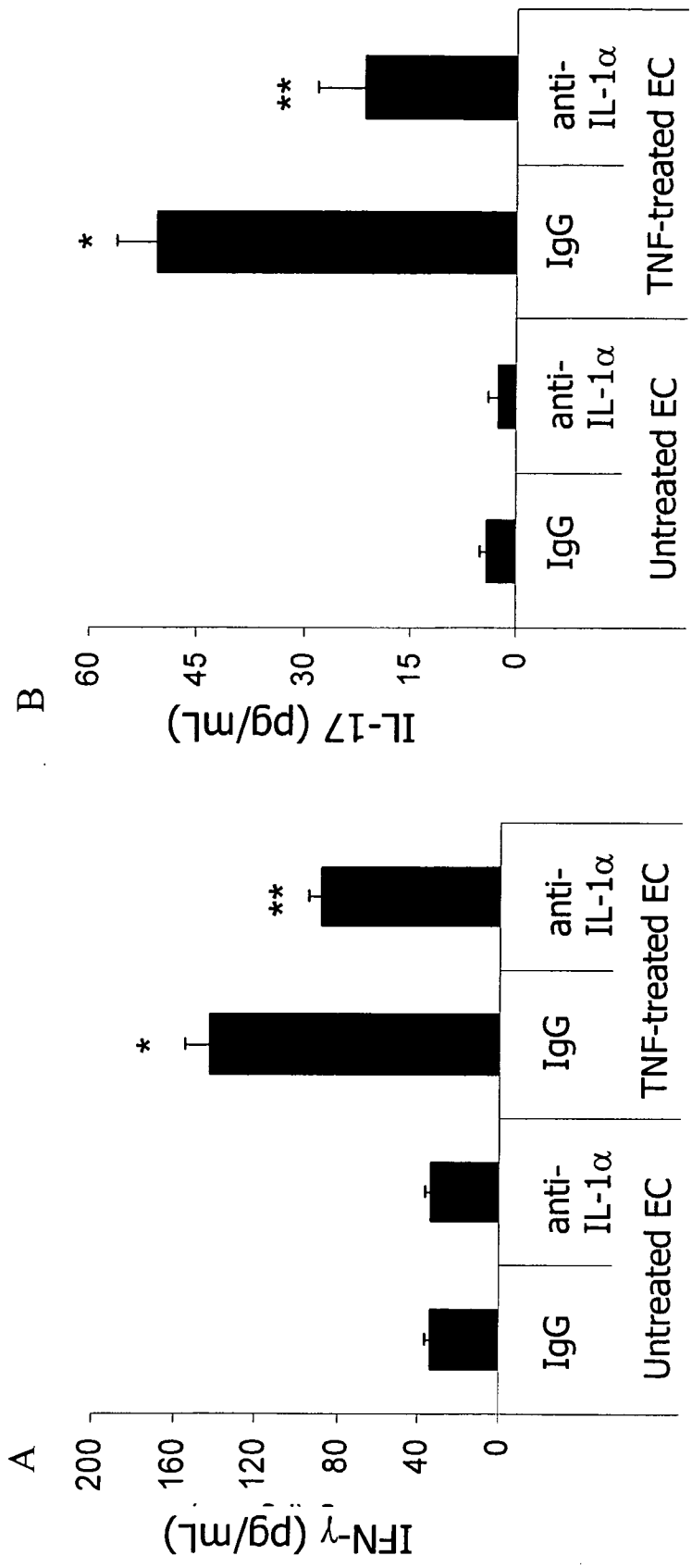
A
IFN-γ (pg/mL)
200
160
120
80
40
0
IgG
anti-IL-1α
IgG
anti-IL-1α
Untreated EC
TNF-treated EC
*
**
B
IL-17 (pg/mL)
60
45
30
15
0
IgG
anti-IL-1α
IgG
anti-IL-1α
Untreated EC
TNF-treated EC
*
**

Figure 9

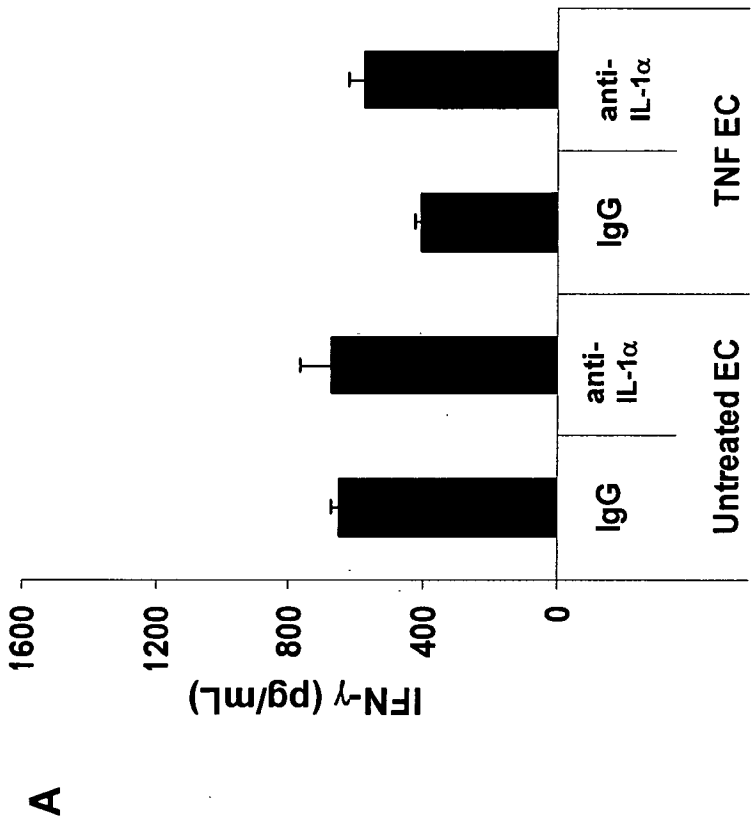
A
IFN-γ (pg/mL)
1600
1200
800
400
0
IgG
anti-IL-1α
IgG
anti-IL-1α
Untreated EC
TNF EC

Figure 10A

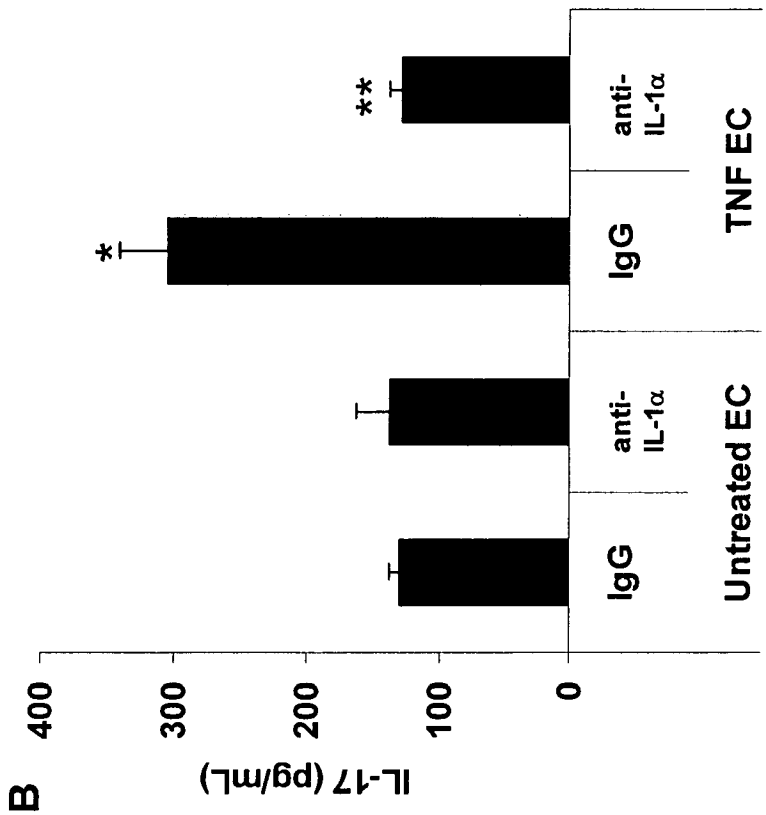
B
IL-17 (pg/mL)
400
300
200
100
0
Untreated EC
TNF EC
IgG
anti-IL-1α
IgG
anti-IL-1α
*
**

Figure 10B

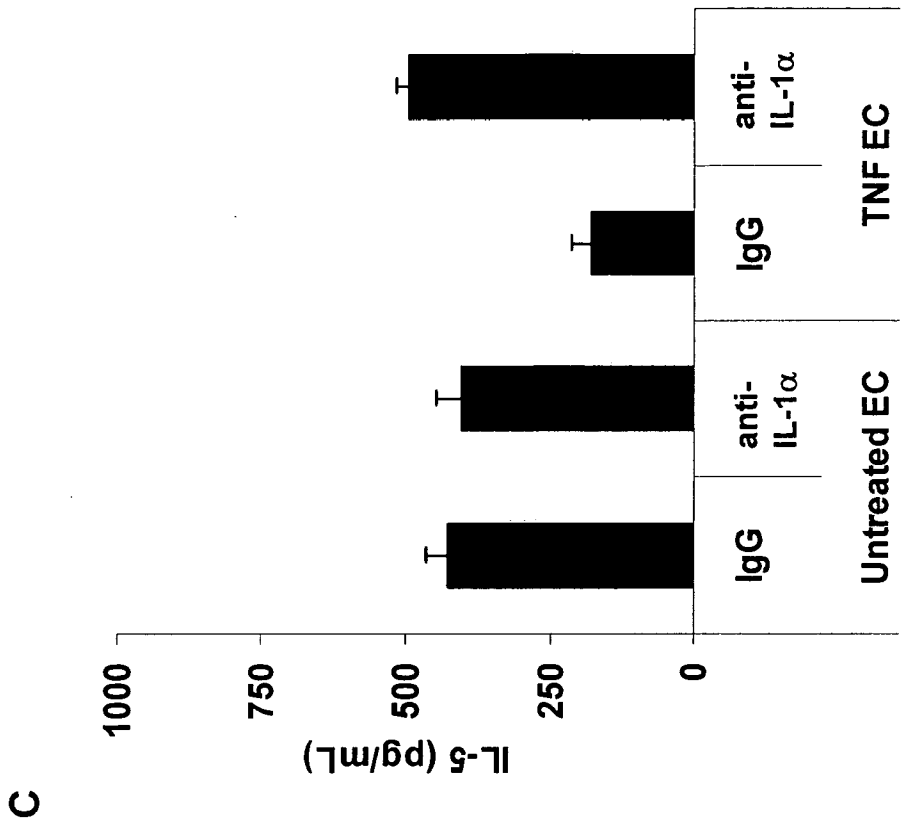
C
IL-5 (pg/mL)
1000
750
500
250
0
IgG
anti-IL-1α
IgG
anti-IL-1α
Untreated EC
TNF EC

Figure 10C

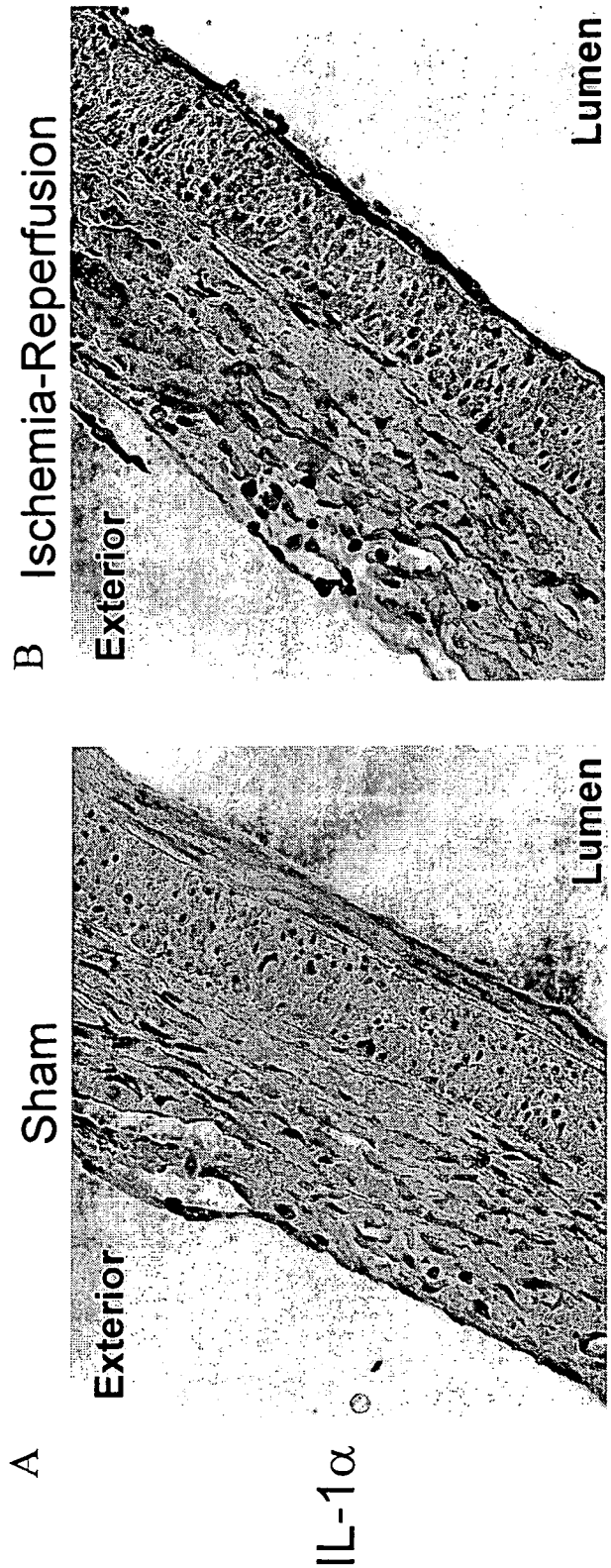
A
Sham
Exterior
Lumen
B
Ischemia-Reperfusion
Exterior
Lumen
IL-1α

Figure 11

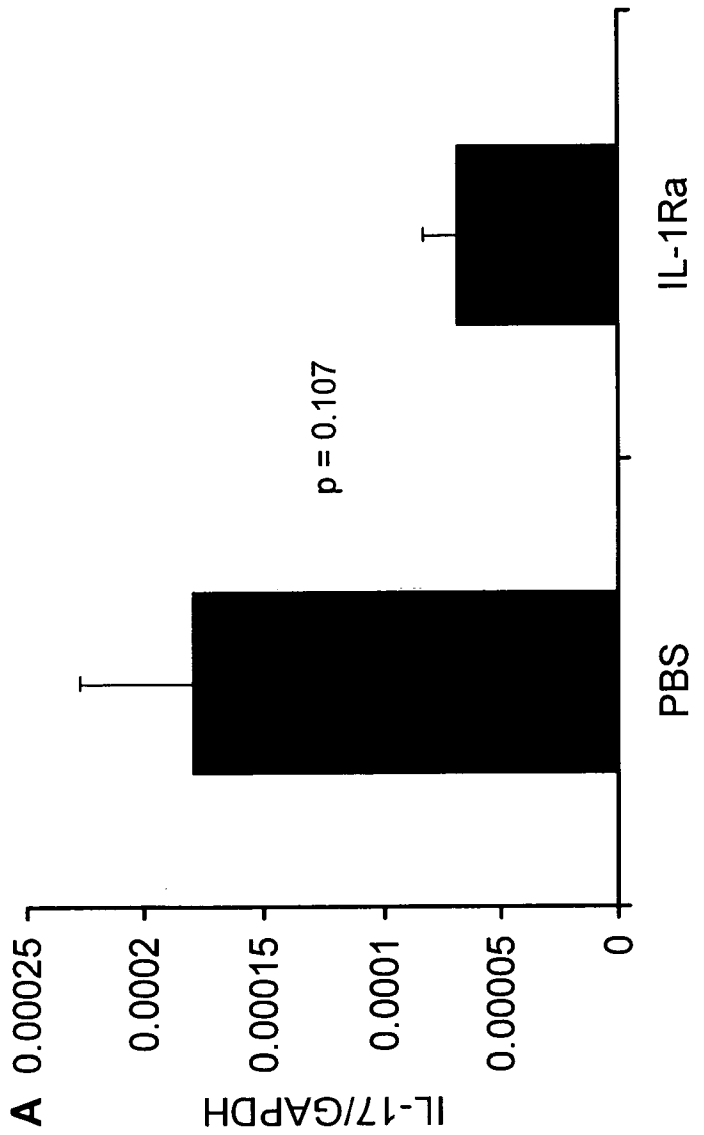
A
IL-17/GAPDH
0.00025
0.0002
0.00015
0.0001
0.00005
0
p = 0.107
PBS
IL-1Ra

Figure 12A

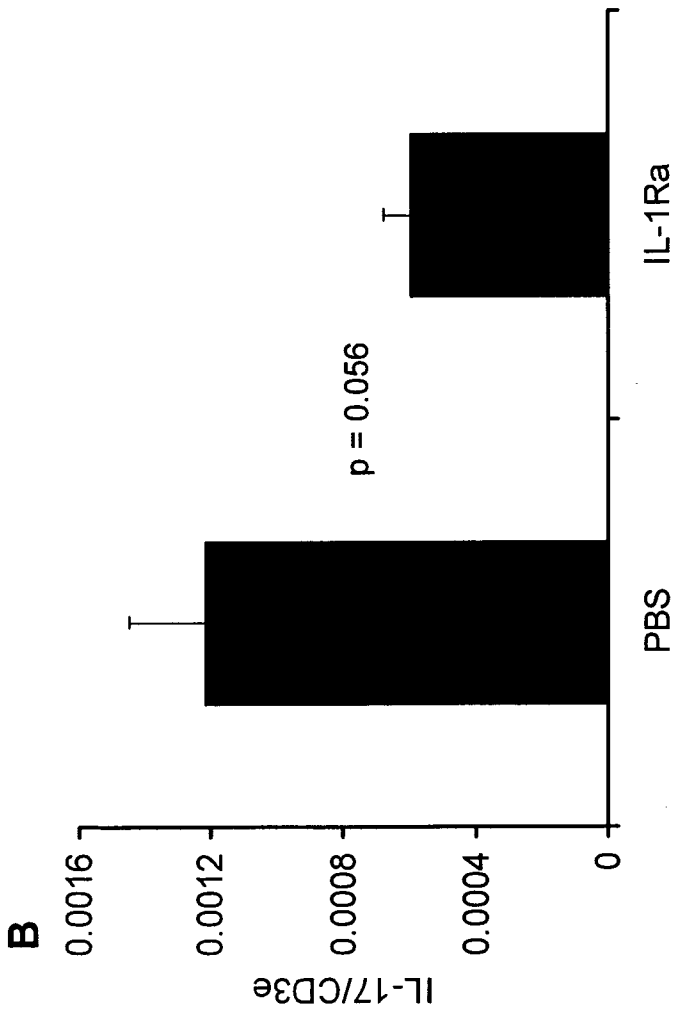

Figure 12B

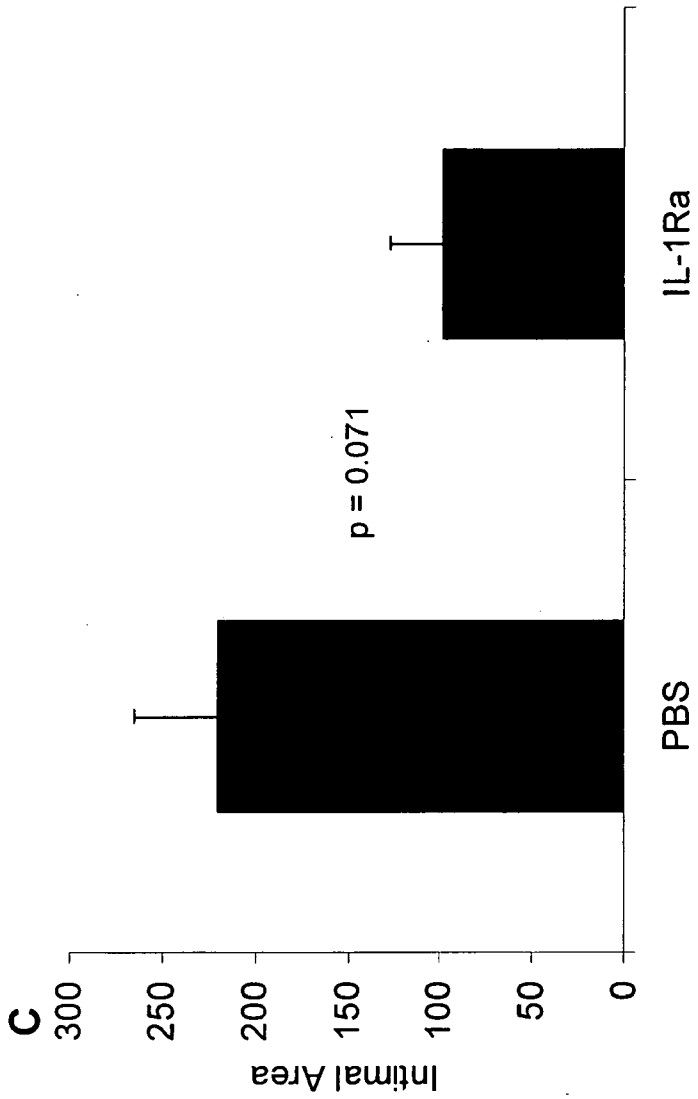

Figure 12C