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IL-1α and IL-1β Are Endogenous Mediators Linking Cell Injury to the Adaptive Alloimmune Response[1]

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Preoperative or perioperative ischemic injury of allografts predisposes to graft arteriosclerosis, the major cause of late graft failure. We hypothesize that injured tissues release mediators that increase the production of pathogenic cytokines by alloreactive T cells. We find that freeze-thaw lysates of human endothelial cells (EC) increase both IFN-γ and IL-17 production by human CD4$^+$ T cells activated by HLA-DR$^+$ allogeneic EC. Immunoadsorption of high-mobility group box 1 protein (HMGB1) reduces this activity in the lysates by about one-third, and recombinant HMGB1 increases T cell cytokine production. HMGB1 acts by inducing IL-1β secretion from contaminating monocytes via TLR4 and CD14. Upon removal of contaminating monocytes, the remaining stimulatory activity of EC lysates is largely attributable to IL-1α. Recombinant IL-1 directly augments IFN-γ and IL-17 production by activated memory CD4$^+$ T cells, which express IL-1R1. Furthermore, IL-1 increases the frequency of alloreactive memory CD4$^+$ T cells that produce IL-17, but not those that produce IFN-γ, in secondary cultures. Our results suggest that IL-1, released by injured EC or by HMGB1-stimulated monocytes, is a key link between injury and enhanced alloimmunity, offering a new therapeutic target for preventing late graft failure. *The Journal of Immunology,* **2007, 179: 6536–6546.**

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 (1). 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 (2). Affected arteries contain infiltrates of T cells subjacent to the luminal endothelium. Graft endothelial cells (EC)[3] 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 (3).

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 (4, 5). In humanized mouse models, IFN-γ alone is sufficient to induce arteriosclerosis in human artery grafts (6), whereas neutralization of IFN-γ protects human artery grafts from allogeneic T cell-mediated remodeling (7). Although the role of IL-17 in graft arteriosclerosis is less clear, IL-17 has been implicated in acute allograft rejection, as blockade of the IL-17R prolongs survival of murine heterotopic heart allografts (8, 9).

Nonimmune factors also contribute to the development of graft arteriosclerosis and dictate in part the lifespan of allografts (2). In human kidney transplantation, cadaver allografts fail at a faster rate than living donor grafts, often despite better MHC matching (10). 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 (11). 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 (12). 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 (13, 14). 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 (1, 15). 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 (16).

We hypothesize specifically that allograft injury decreases the overall life of an allograft by releasing mediators sometimes called damage-associated molecular patterns that, like pathogen-associated molecular patterns, direct the host adaptive immune response toward a more pathogenic response characterized by increased IFN-γ and IL-17 production. The mediators produced in injured allografts that could alter the host adaptive immune response in this way are unclear. Tissue injury can produce a number of substances with inflammatory or immunomodulatory properties, including matrix fragments, heat shock proteins, nucleic acids, uric acid, high-mobility group box 1 protein (HMGB1), and IL-1α (17–22). Heat

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[3] Abbreviations used in this paper: EC, endothelial cell; AGER, advanced glycosylation end product-specific receptor; HMGB1, high-mobility group box 1; ICS, intracellular cytokine staining.

shock proteins and nucleic acids may affect the adaptive immune response by acting through TLRs (23, 24). Recently, IL-1α was shown to be a signal released in response to dying cells that stimulates acute inflammation (25). Similarly, the effect of uric acid may be to enhance IL-1 secretion, as its actions also depend on the IL-1R (26).

HMGB1 is a nuclear, DNA-binding protein that is released passively from necrotic, but not apoptotic, cells and actively from stimulated APCs (27, 28). Upon release, HMGB1 induces a number of inflammatory responses, including monocyte inflammatory cytokine production, neutrophil activation, and dendritic cell maturation (29–31). Responses to HMGB1 have been attributed to signaling through at least three different receptors, including TLR2, TLR4, and the advanced glycosylation end product-specific receptor AGER (formerly RAGE) (28, 32). HMGB1 appears to alter adaptive immune responses, as both recombinant HMGB1 and HMGB1 secreted from LPS-matured dendritic cells promote production of Th1 cytokines in vitro, and blockade of HMGB1 slows rejection of murine heterotopic heart allografts in vivo (31, 33, 34).

IL-1α and IL-1β are evolutionarily ancient and prototypical proinflammatory cytokines (17). Products of two distinct genes, IL-1α and IL-1β bind the same IL-1 receptor 1 (IL-1R1) and elicit essentially indistinguishable responses mediated by the signaling adaptor protein MyD88. A third member of the IL-1 family, IL-1R antagonist, also binds IL-1R1, but does not transmit an activating signal and can inhibit the effects of IL-1α and IL-1β (17). Monocyte/macrophages are the predominant source of IL-1β, which is stored intracellularly as an inactive precursor that is cleaved by caspase-1 to the active form upon stimulation. In contrast, IL-1α is expressed in many cell types, including EC and vascular smooth muscle cells, and remains primarily cell-associated in an unprocessed form that is bioactive (17, 35–39). Effects on lymphocyte activation were some of the earliest actions recognized for IL-1. IL-1 costimulates proliferation of murine thymocytes and Th2, but not Th1 clones (40–42). IL-1 has also been shown to costimulate IFN-γ production and proliferation from activated murine T cells and to promote differentiation of naive murine T cells into IL-17-producing effector cells (43, 44). In addition, two recent reports demonstrate that IL-1β, in combination with IL-6, drives the differentiation of human naive CD4$^+$ T cells toward an IL-17-producing phenotype (45, 46).

In this study, we have used in vitro human EC-T cell cocultures as a model of the allogeneic memory T cell response to investigate the effects of mediators released by damaged EC on graft rejection. We report that injured EC release mediators that enhance alloreactive human T cell production of IFN-γ and IL-17. Using a candidate approach, we first identified HMGB1 as one of these mediators and demonstrated that HMGB1 enhances T cell IFN-γ production principally through an indirect pathway by inducing monocyte secretion of IL-1β. We subsequently identified IL-1α 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, we identify a molecular mechanism by which cell injury alters the adaptive alloimmune response through release of IL-1.

Materials and Methods

Reagents and Abs

PHA, PMA, and ionomycin were purchased from Sigma-Aldrich. 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. Mouse anti-human CD3 (OKT3), mouse anti-human 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. 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. FITC-conjugated anti-human ICAM-1 and an isotype control were purchased from Beckman Coulter. Anti-human HLA-DR (LB3.1) was a gift from J. Strominger (Harvard University, Cambridge, MA). Ultra-pure LPS was purchased from InvivoGen. Recombinant human IL-1α and IL-1β were purchased from PeproTech. IL-1Ra was purchased from Amgen. Recombinant human TNF was purchased from R&D Systems. Recombinant human IFN-γ was purchased from Invitrogen Life Technologies. Recombinant HMGB1 was prepared as previously described (47). Endotoxin content in the HMGB1 preparations was found to be 0.6 pg/μg of HMGB1 by the Chromogenic *Limulus* amebocyte lysate assay (BioWhittaker) (47).

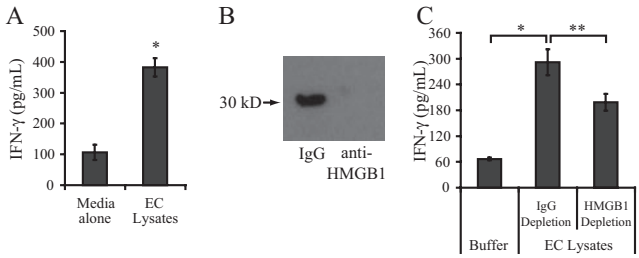

FIGURE 1. EC lysates contain mediators, including HMGB1, that enhance IFN-γ production in EC-T cell cocultures. *A*, EC lysates were added to cocultures of CD4$^+$ T cells with allogeneic HLA-DR$^+$ EC, and IFN-γ in the culture medium was assayed by ELISA at 24 h. Mean $\pm$ SD of triplicate samples is shown. *B*, After immunoadsorption by incubation with isotype control- or anti-HMGB1-coated beads, EC lysates were assayed for HMGB1 by immunoblotting. *C*, Control- or HMGB1-depleted EC lysates were added to cocultures of CD4$^+$ T cells with allogeneic HLA-DR$^+$ EC, and IFN-γ in the culture medium was assayed by ELISA at 24 h. Mean $\pm$ 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.

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). 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). 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 cocultures.

HUVEC were isolated from umbilical cords by collagenase (Worthington Biochemical) digestion and cultured on gelatin-coated (Fisher Scientific) tissue culture plates in M199 supplemented with 20% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (all from Invitrogen Life Technologies), 0.1% endothelial cell growth supplement (Collaborative Biomedical Products), and 100 μg/ml porcine heparin (Sigma-Aldrich) as previously described (48). 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) for 1 min. 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) confirmed >90% cell lysis. Lysates were centrifuged at

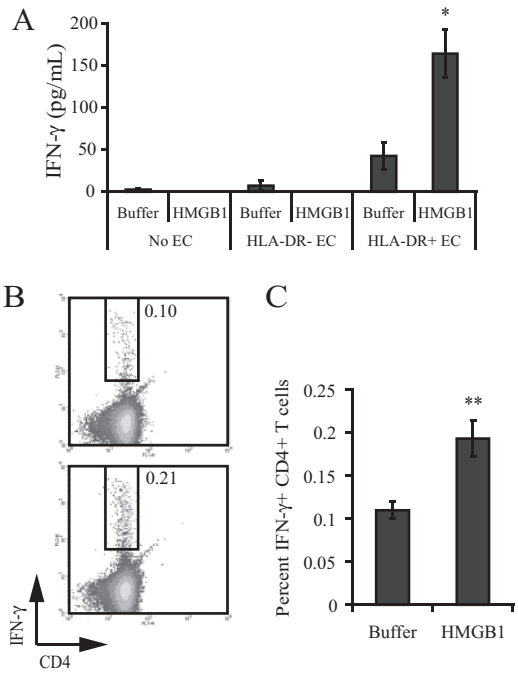

FIGURE 2. Recombinant HMGB1 increases $CD4^+$ T cell IFN-γ production. *A*, Recombinant HMGB1 (5 μg/ml) was added to cultures of $CD4^+$ T cells alone or to cocultures of $CD4^+$ T cells with allogeneic $HLA\text{-}DR^-$ or $HLA\text{-}DR^+$ EC. IFN-γ in the culture medium was assayed by ELISA at 24 h. Mean ± SD of quadruplicate samples is shown. *B*, $CD4^+$ T cells were cocultured with allogeneic EC and stimulated with 1 μg/ml PHA in the presence or absence of recombinant HMGB1 (5 μg/ml). Brefeldin A was added for the final 4 h of the 24-h culture, at which time cells were permeabilized and stained for IFN-γ and CD4. *C*, Mean ± SD of triplicate samples analyzed as in *B*. *, $p < 0.0005$; ** $p < 0.002$. One of eight (*A*) or one of three (*B*) independent experiments is shown using three different donors with similar results.

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 cocultures.

T cell activation in vitro

Where indicated, EC were treated with 50 ng/ml recombinant human IFN-γ for 72 h to restore HLA-DR expression lost during cell culture. EC were washed three times with HBSS before coculture 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) 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 h 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 cocultured 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 (49), were coated with 1 μg/ml anti-CD3 mAb for 30 min, washed three times with HBSS, and then cocultured with T cells. T cells were activated and rested as above and then restimulated for 6 h 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) were incubated with a mAb against HMGB1 or an isotype control Ab at a concentration of 1 mg/ml for 1 h at room temperature with continuous shaking. Beads were washed four times with PBS, blocked by incubation with PBS containing 1% BSA for 30 min, then washed another four times. EC lysates or recombinant HMGB1 stock solution was incubated with the coated beads for 1 h 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 cocultures.

Flow cytometric analysis

For cell surface staining of EC, cells were washed in HBSS and incubated with trypsin-EDTA (Invitrogen Life Technologies) for 1 min. 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 min on ice. For ICS, 10 μg/ml brefeldin A (Molecular Probes) was added for the last 4 of 24 h EC-T cell cocultures or at the start of 6 h 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 min 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 h 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).

Immunoblotting

To assess p38 and JNK phosphorylation, EC samples were prepared as previously described (50). These lysates and freeze-thaw lysates were

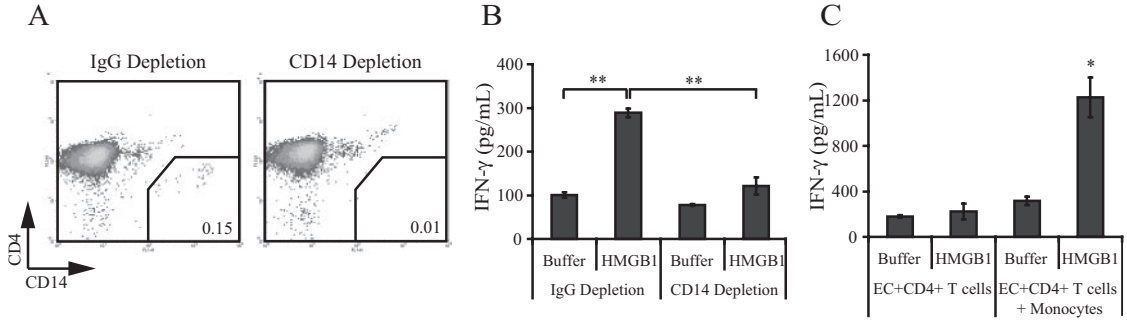

FIGURE 3. The effect of HMGB1 on alloreactive T cell IFN-γ production is markedly reduced in the absence of monocytes. *A*, Positively isolated $CD4^+$ T cells were depleted of monocytes using an anti-CD14 Ab and compared with depletion with a control Ab by flow cytometric analysis for CD4 and CD14. *B*, $CD4^+$ T cell populations either depleted of $CD14^+$ monocytes or not depleted of monocytes were cocultured with allogeneic $HLA\text{-}DR^+$ EC and treated with HMGB1 (5 μg/ml) or control buffer. IFN-γ in the culture medium was assayed by ELISA at 24 h. Mean ± SD of triplicate samples is shown. *C*, Monocytes were depleted from $CD4^+$ T cell isolates as in *A*, and negatively isolated autologous monocytes were added back to $CD4^+$ T cell isolates or not before coculture with $HLA\text{-}DR^+$ 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.

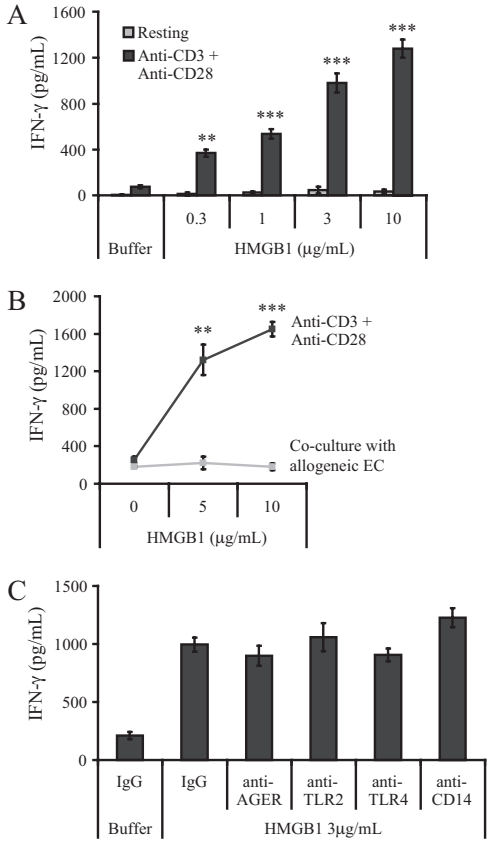

FIGURE 4. HMGB1 directly costimulates IFN-γ production by $CD4^+$ T cells activated by anti-CD3 and anti-CD28 mAbs. *A*, $CD4^+$ T cells depleted of monocytes were either activated with plate-bound anti-CD3 (5 μg/ml) plus anti-CD28 (1 μg/ml) or not activated. T cells were treated with increasing concentrations of HMGB1 as indicated, and IFN-γ in the culture medium was assayed by ELISA at 24 h. Mean ± SD of triplicate samples is shown. *B*, T cells were activated as in *A* or by coculture with allogeneic HLA-DR^+ EC. IFN-γ in the culture medium was assayed by ELISA at 24 h. Mean ± SD of triplicate samples is shown. *C*, T cells were activated as in *A* and preincubated with blocking Abs (20 μg/ml) to potential receptors before addition of HMGB1. IFN-γ in the culture medium was assayed by ELISA at 24 h. Mean ± SD of triplicate samples is shown. *, $p < 0.05$; **, $p < 0.0005$; ***, $p < 0.0001$. One of three independent experiments with similar results is shown.

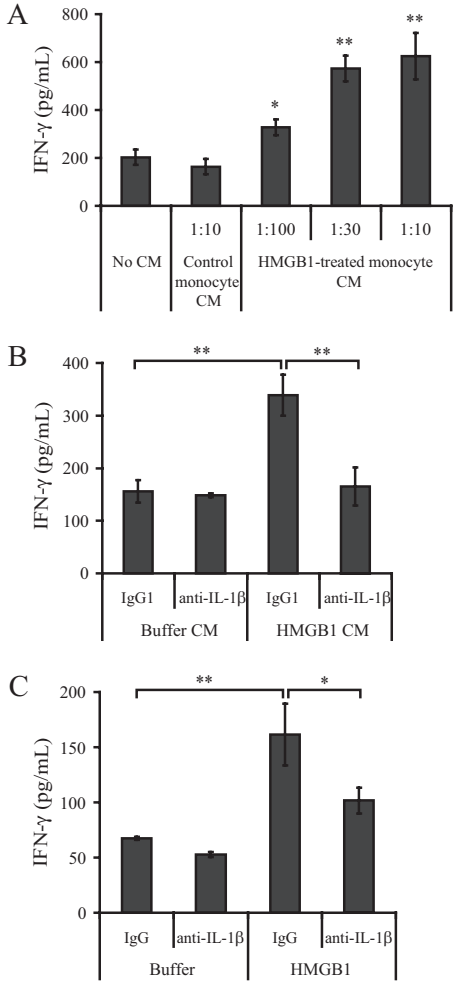

FIGURE 5. HMGB1-treated monocytes enhance alloreactive T cell IFN-γ production through secretion of IL-1β. *A*, Negatively isolated monocytes were treated with HMGB1 (5 μg/ml) or control buffer for 24 h. Conditioned medium (CM) from HMGB1- or control-treated monocytes was added to cocultures of $CD4^+$ T cells, thoroughly depleted of monocytes, with allogeneic HLA-DR^+ EC at the indicated dilutions, and IFN-γ in the culture medium was assayed by ELISA at 24 h. Mean ± SD of triplicate samples is shown. *B*, 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). IFN-γ in the culture medium was assayed by ELISA at 24 h. Mean ± SD of triplicate samples is shown. *C*, 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. IFN-γ in the culture medium was assayed by ELISA at 24 h. 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.

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 min. 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 h at 4°C. After blocking with TBST containing 5% nonfat milk for 1 h 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) and ECL substrate (Pierce).

Measurement of cytokine production

Supernatants from EC cultures, monocyte cultures, and EC-T cell cocultures were collected after 24 h. ELISA analysis for IL-1β, IL-17 (both R&D Systems), IFN-γ, IL-8 (both Invitrogen Life Technologies), and IL-5 (eBioscience) 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) according to the manufacturer's instructions. Quantitative RT-PCR was performed exactly as described (51) using the following primers: (5′-3′): IL-1R1 (forward) 5′-GTATCTACAGAACAAG CCTCC-3′, (reverse) 5′-GTTTGCAATCCTTATACCACTG-3′; GAPDH (forward) 5′-GAAGGTGAAGGTCGGAGTC-3′, (reverse) 5′-GAAGAT GGTGATGGGATTTC-3′; AGER (forward) 5′-AGATTCTGCCTCTGAA CTCAC-3′, (reverse) 5′-CCTTCACAGATACTCCCTTCTC-3′; TLR2 (forward) 5′-CTTTCACTGCTTTCAACTGGTAGTT-3′, (reverse) 5′-TGA GGGAATGGAGTTTAAAGATCCT-3′; and TLR4 (forward) 5′-AGAA CTGCAGGTGCTGGATTTAT-3′, (reverse) 5′-GTTCTCTAGAGATGC TAGATTTGT-3′.

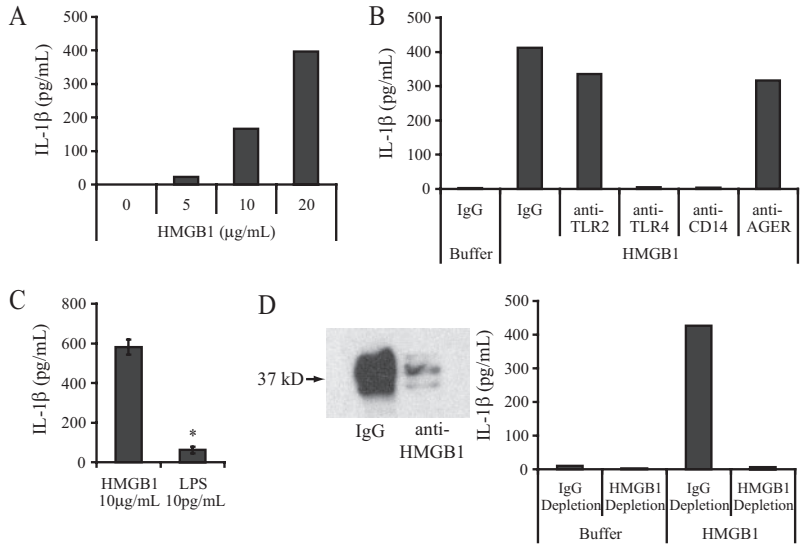

FIGURE 6. HMGB1 induces monocyte secretion of IL-1β via TLR4 and CD14. *A*, Monocytes isolated by negative selection were treated with increasing concentrations of HMGB1. IL-1β in the culture medium was assayed by ELISA at 24 h. *B*, Monocytes were pretreated with blocking Abs to TLR2, TLR4, CD14, or AGER or an isotype control Ab (20 μg/ml) for 30 min before addition of HMGB1 (10 μg/ml). IL-1β in the culture medium was assayed by ELISA at 24 h. *C*, Monocytes were treated with HMGB1 or LPS. IL-1β in the culture medium was assayed by ELISA at 24 h. Mean $\pm$ SD of triplicate samples is shown. *, $p < 0.00005$. *D*, The HMGB1 stock solution was incubated with beads coated with a control Ab or an anti-HMGB1 Ab, and then an aliquot of each was analyzed by immunoblotting for HMGB1. Both the HMGB1 stock solution and the control buffer were subjected to immunoadsorption and then added to monocytes as in *A*, and IL-1β in the culture medium was assayed by ELISA at 24 h. One of three independent experiments is shown using two different donors with similar results.

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 $p < 0.05$.

Results

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 (52). We initially investigated whether damage to EC can release any 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-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 (Fig. 1*A*), consistent with the hypothesis that damaged EC can release mediators that modulate alloreactive T cell cytokine production. We hypothesized that one such mediator might be HMGB1, a NF released from necrotic cells. HMGB1 was readily detected in the lysates by immunoblotting (Fig. 1*B*). 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 (Fig. 1*C*).

Recombinant HMGB1 effects on IFN-γ production in EC-T cocultures depend on monocytes

To further assess how HMGB1 affects T cell cytokine production, we used recombinant HMGB1 rigorously depleted of endotoxin, as previously described (47). Addition of recombinant HMGB1 to cocultures of CD4$^+$ T cells with allogeneic HLA-DR$^+$ EC, like EC lysates, increases T cell IFN-γ production (Fig. 2*A*). 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 (Fig. 2*B*).

HMGB1 has been reported to induce activation of EC, which constitutively express both AGER and TLR4 (53–56). We therefore examined whether HMGB1 might affect IFN-γ production by enhancing the efficiency with which EC can activate allogeneic T cells. However, we did not observe evidence of EC activation by HMGB1 as measured by several parameters. 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 we consider it unlikely that EC are the target of HMGB1 action in our cocultures.

Because we could not detect responses from EC to explain the effect of HMGB1 on IFN-γ production in EC-T cell cocultures, we explored whether the target of this effect is provided by the T cell population. Recently, it has been asserted that T cell responses to allogeneic EC depend upon the presence of monocytes (57). We have found that serially passaged EC cultures do not contain CD45$^+$ leukocytes. However, our T cell preparations isolated solely by positive selection do contain a small (<1%) number of CD14$^+$ HLA-DR$^+$ monocytes (Fig. 3*A*). We depleted these cells 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, in our hands, 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 (Fig. 3*B*). 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 (Fig. 3*C*). HMGB1 induced much more IFN-γ production in cocultures to which monocytes were added back.

We also investigated whether HMGB1 acts directly on highly purified T cells to modulate cytokine production. 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 (Fig. 4*A*). 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 (Fig. 4*B*). We detect mRNA for AGER (2.2 $\pm$ 1.2% of GAPDH) and TLR2 (0.035 $\pm$ 0.018% of GAPDH), 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 (Fig. 4*C*). 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.

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, we 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 (Fig. 5*A*). 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 (Fig. 5*B*). 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 (Fig. 5*C*). Thus, in EC-T cell cocultures, HMGB1 enhances alloreactive $CD4^+$ T cell IFN-γ production indirectly by inducing IL-1β secretion from contaminating monocytes.

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 (Fig. 6*A*). HMGB1-induced IL-1β secretion could be blocked by blocking Abs against TLR4 and CD14, but not against TLR2 or AGER (Fig. 6*B*). 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 (47). 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 (Fig. 6*C*). 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 (Fig. 6*C*). 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

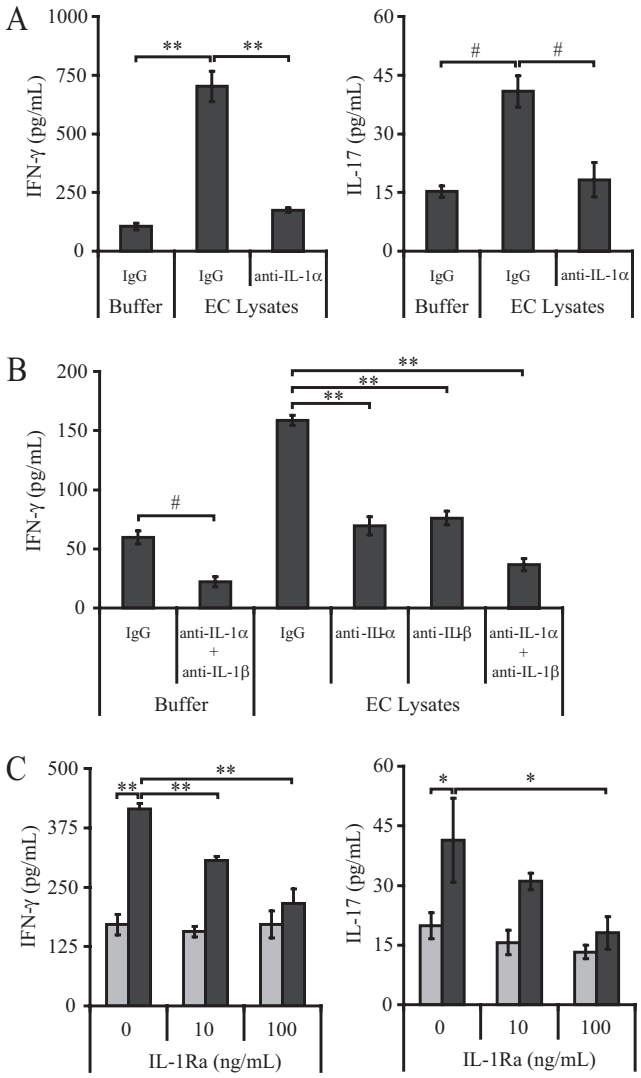

FIGURE 7. Neutralization of IL-1α and IL-1β blocks the effect of EC lysates on T cell IFN-γ and IL-17 production. *A*, EC lysates generated by freeze-thaw were added to cocultures of HLA-DR^+ EC with allogeneic $CD4^+$ T cells thoroughly depleted of monocytes. A neutralizing Ab to IL-1α or an isotype control was added at 10 μg/ml. IFN-γ and IL-17 in the culture medium were assayed by ELISA at 24 h. Mean $\pm$ SD of triplicate samples is shown. *B*, 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. IFN-γ in the culture medium were assayed by ELISA at 24 h. Mean $\pm$ SD of triplicate samples is shown. *C*, Increasing concentrations of IL-1R antagonist were added as indicated to cocultures treated with EC lysates (■) or control buffer (▨). IFN-γ and IL-17 in the culture medium were assayed by ELISA at 24 h. Mean $\pm$ 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.

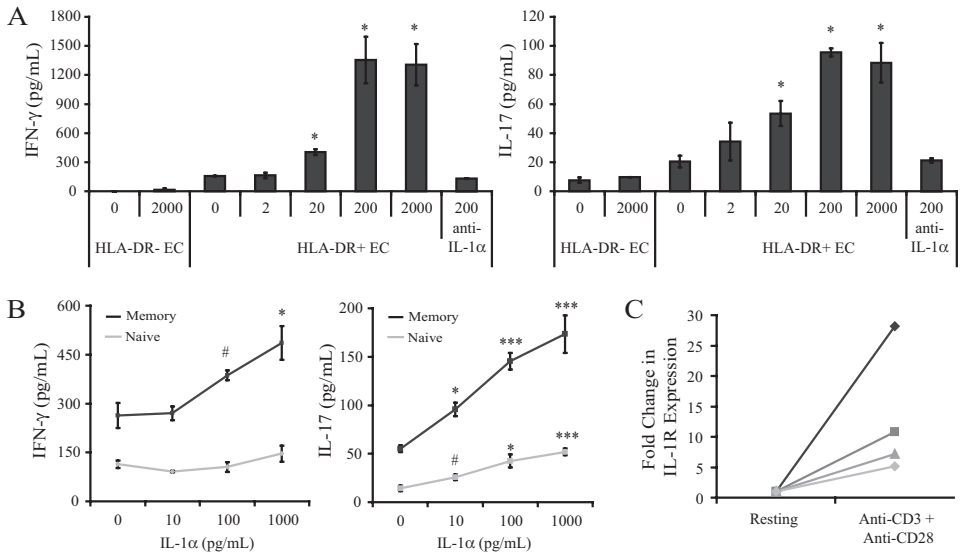

FIGURE 8. IL-1 increases IFN-γ and IL-17 production from memory CD4$^+$ T cells. *A*, Increasing concentrations of IL-1α were added to cocultures of CD4$^+$ T cells depleted of monocytes with allogeneic HLA-DR$^-$ or HLA-DR$^+$ EC. A neutralizing Ab to IL-1α (10 μg/ml) was also added where indicated. IFN-γ and IL-17 in the culture medium were assayed by ELISA at 24 h. Mean ± SD of triplicate samples is shown. *B*, 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. IFN-γ and IL-17 in the culture medium were assayed by ELISA at 24 h. Mean ± SD of triplicate samples is shown. #, $p < 0.01$; *, $p < 0.005$; **, $p < 0.001$; ***, $p < 0.0005$. One of three independent experiments using three different donors with similar results is shown. *C*, Quantitative RT-PCR for IL-1R1 expression in CD4$^+$ T cells activated by anti-CD3 and anti-CD28 mAbs. Each line 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.

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.

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 (17, 35). Because IL-1β strongly enhances IFN-γ production in EC-T cell cocultures (Fig. 5, *B* and *C*), we 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 (Fig. 7*A*). 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 (44, 58). Therefore, we also investigated the effect of IL-1α in EC lysates on alloreactive T cell IL-17 production. 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 (Fig. 7*B*). 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 (Fig. 7*C*).

IL-1 enhances cytokine production from human memory T cells

To directly examine the ability of IL-1 to modulate T cell cytokine production, we added recombinant IL-1α 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 (Fig. 8*A*). 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 (Fig. 8*B*). 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 naive 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 (Fig. 8*C*).

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. We also investigated 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. To investigate this, we performed restimulation experiments 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

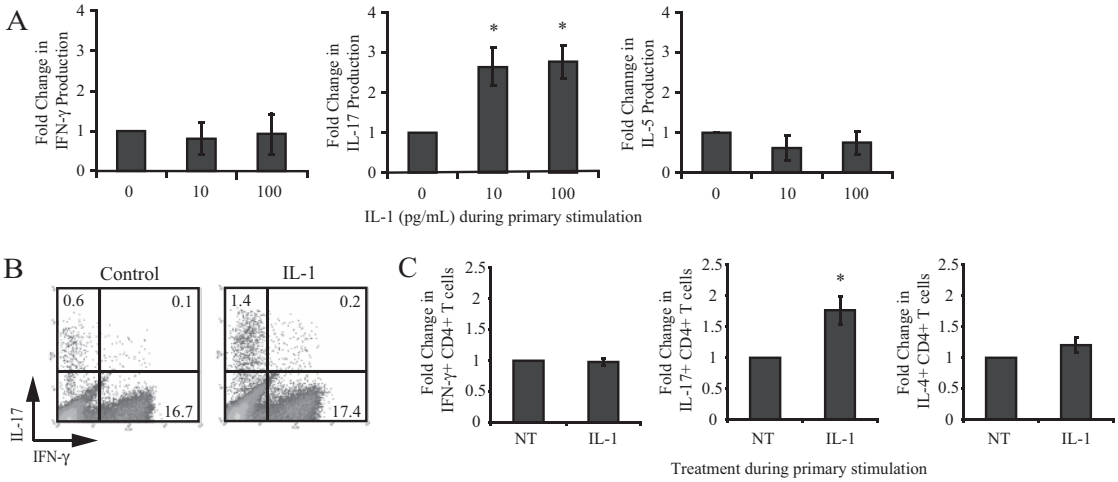

FIGURE 9. IL-1 promotes preferential expansion of alloreactive human memory CD4$^+$ T cells that produce IL-17. *A*, Purified memory CD4$^+$ T cells were activated by coculture with allogeneic EC with the indicated concentrations of IL-1α and then restimulated by coculture with EC from the same donor as the primary coculture without further addition of IL-1. IFN-γ, IL-17, and IL-5 in the culture medium were assayed by ELISA after 24 h of restimulation. Pooled data from three T cell donors are shown. *B*, 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. Representative dot plots of ICS for IFN-γ and IL-17 from one donor are shown. *C*, Pooled data from four T cell donors treated as in *B* are shown. *F*old change in the amount of cytokine produced (*A*) or the number of cytokine-positive T cells (*C*) upon restimulation between IL-1-treated cocultures and control cocultures was calculated for each donor. Data were analyzed by a one-sample *t* test and represent the mean $\pm$ SE in ($n = 3$ experiments) *A* or ($n = 4$ experiments) *C*. $*$, $p < 0.05$. The range of values for amount of cytokine measured in *A* 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%).

significant increase the amount of IL-17 produced by the alloreactive T cell population upon restimulation (Fig. 9*A*). 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, we used a model, developed in our laboratory, in which EC are transduced to express the FcγR 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 we adapted the model 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 (Fig. 9, *B* and *C*). Consistent with a recent report (59), we observed both IL-17$^+$ single producers and IL-17$^+$ IFN-γ^+ double producers within the memory CD4$^+$ T cell population, and both subpopulations were similarly increased by exposure to IL-1. Consistent with our 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.

Discussion

Injury to an allograft incurred before or during the transplantation process appears to enhance the immunogenicity of the allograft, but the specific molecules that mediate the effect remain unclear. In this study, we have used an in vitro coculture system to examine the effect of mediators released by damaged cells on human memory CD4$^+$ T cell responses to allogeneic EC. Although this is a reductionist model, it has been widely used to study human alloimmunity and offers two important advantages over the use of mouse systems. First, human EC, but not mouse EC, can activate resting allogeneic CD4$^+$ T cells (60, 61). Given that these two cell types are in frequent contact in an allograft, the EC-T cell interaction is likely to be important in human allogeneic responses in vivo, especially those that lead to graft arteriosclerosis (1). Second, this model produces selective activation of memory T cells (62). Alloreactive memory CD4$^+$ T cells, which are difficult to study in murine models, constitute a considerable portion of the total human alloreactive T cell pool and are important mediators of allograft rejection (63, 64). By examining human CD4$^+$ T cell responses to allogeneic EC, we specifically focus on this population.

Using this system, we identify two pathways by which EC injury can result in the release of IL-1, a potent modulator of T cell responses. First, IL-1α can be released directly by damaged EC. Second, EC release of HMGB1 can induce monocyte secretion of IL-1β. The combined actions of IL-1α and IL-1β account for essentially all of the ability of EC lysates to enhance T cell IFN-γ and IL-17 production in EC-T cell cocultures. We further demonstrate that exposure to IL-1 upon activation preferentially promotes expansion of human memory CD4$^+$ T cells that produce IL-17.

Although more commonly thought of as an inflammatory mediator of innate immunity, IL-1 is known to exert strong effects on adaptive immune responses (65). Both exogenous and endogenous IL-1 can function as adjuvants in promoting T cell-dependent Ab responses in vivo (66, 67). Originally described as a T cell coactivator, IL-1 acts directly on murine T cells to promote proliferation and cytokine production and can synergize with IL-12 to enhance human T cell cytokine production (41–43, 68). We find that human CD4$^+$ T cells up-regulate IL-1R1 expression following activation and that IL-1 acts directly on human CD4$^+$ T cells

activated by anti-CD3 and anti-CD28 mAbs or by allogeneic EC to augment IFN-γ and IL-17 production.

In cocultures of highly purified $CD4^+$ T cells with allogeneic EC, neutralization of IL-1α blocks almost all of the activity of EC lysates in enhancing IFN-γ and IL-17 production. IL-1α is expressed by both resting EC and vascular smooth muscle cells and remains primarily cell-associated in a bioactive, unprocessed form (17, 35, 69). Once released, it may act on neighboring cells to induce an initial inflammatory response following cell injury. In addition to mechanical injury, both hypoxia and sublytic complement deposition, which are stimuli relevant to solid organ transplantation, can induce EC release of IL-1α (70, 71). Sublytic complement deposition allows for IL-1α release, independent of cell death, through disruption of the plasma membrane, either by generating membrane pores or by inducing membrane vesiculation (72, 73). Also, inflammatory cytokines such as TNF and IL-1 itself cause EC to present bioactive IL-1α on their surface in a membrane-bound form (35). Thus, allograft injury may facilitate release or presentation of IL-1α to host immune cells by several mechanisms.

In addition to IL-1α, we identify HMGB1 as a second mediator released from damaged EC that modulates alloreactive T cell IFN-γ and IL-17 production. It has been previously shown that HMGB1 is the major factor released from freeze-thaw lysates of murine fibroblasts that induces monocyte TNF secretion (27). We now show that HMGB1 can be released from damaged cultured human EC and that HMGB1 contributes a significant fraction of the IFN-γ-stimulating activity of clarified EC freeze-thaw lysates. Interestingly, both IL-1α and HMGB1 are inflammatory molecules that also participate in gene regulation in the cell nucleus and have thus been labeled "dual-function" cytokines (74, 75). As both molecules lack signal peptides, it is tempting to speculate that detection of released nuclear mediators may be an ancient pathway of recognition of cell injury.

The effect of HMGB1 on cytokine production is not due to direct effects on EC, as HMGB1 does not activate EC in our studies. HMGB1-induced activation of a transformed microvascular EC cell line, HMEC-1, was shown to depend in large part on TNF production by HMEC-1 cells (53). We cannot detect TNF production from untransformed HUVEC, thus this may explain the lack of effect in our system. One other study did report HMGB1-induced activation of HUVEC (54). It is unclear why we have not observed such an effect. Our HUVEC are devoid of $CD45^+$ contaminants, mostly monocytes, present in some other culture systems (76). Thus a possible explanation is that HMGB1 effects on HUVEC cultures may depend on monocyte contamination. However, it is also possible that HUVEC grown under different conditions are different from each other and that some HUVEC cultures may respond to HMGB1, whereas others do not.

Rather than an effect on EC, we find that the effect of HMGB1 on EC-T cell cocultures requires the presence of small numbers of monocytes. We observe that HMGB1 induces monocyte secretion of IL-1β, as has been previously described (29, 77). HMGB1 may signal through multiple receptors, including TLR2, TLR4, and AGER. We find that HMGB1-induced IL-1β secretion is mediated through TLR4 and CD14, in accord with recent reports that HMGB1 signals through TLR4 in primary human macrophages and that the two proteins physically associate (32, 78). TLR4-dependent effects of endogenous HMGB1 have also been demonstrated in murine models in vivo, as neutralization of HMGB1 protects mice with intact TLR4 signaling, but not mice with defective TLR4 signaling, from hepatic ischemia-reperfusion injury (79). Although TLR4 is a well-characterized receptor for endotoxin, our controls strongly suggest that the IL-1β-stimulating activity is conferred by HMGB1 and not microbial contaminants. The HMGB1 preparations used in these studies contain insufficient endotoxin to account for the IL-1β-inducing activity of HMGB1. The cytokine-stimulating activity can be removed from HMGB1 stock solutions by immunoadsorption using a specific anti-HMGB1 Ab. In addition, the activity is fully heat-labile, though we also observe some decrease in the activity of low concentrations of ultra-pure LPS upon heating, as has been described (80). We cannot fully eliminate the possibility that recombinant HMGB1 preparations carry microbial components that may contribute to the cytokine-inducing activity. However, HMBG1 contained in lysates of primary human EC, which contain little if any microbial contamination, contributes a significant fraction of the activity of EC lysates in enhancing T cell cytokine production. We have not ruled out the possibility that HMGB1 contained in EC lysates carries other molecules, for example nucleic acids or lipids, that contribute to the cytokine-stimulating activity of the lysates. If additional cofactors are involved, our immunoadsorption results suggest that such cofactors must physically interact with HMGB1.

We have also observed that HMGB1 acts directly on $CD4^+$ T cells to enhance IFN-γ production following activation by anti-CD3 and anti-CD28 mAbs. This effect could not be inhibited by blocking Abs against TLR2, TLR4, or AGER. Thus there may be other, as yet unidentified, receptors for HMGB1, and the interesting possibility remains that HMGB1 may act through atypical mechanisms that involve entrance into the cytosol or nucleus of target cells (29). Interestingly, this effect is not seen when $CD4^+$ T cells are activated by coculture with allogeneic HLA-DR^+ EC, which may suggest that strong activation, as delivered by mAbs, may be required to sensitize T cells to HMGB1, e.g., by inducing a receptor. Alternatively, cocultured EC may inhibit the actions of HMGB1 on T cells, for example by producing a factor that either neutralizes HMGB1 or blocks the TCR for HMGB1. It appears unlikely that EC directly neutralize HMGB1, however, because EC do not appear to inhibit the ability of HMGB1 to induce monocyte secretion of IL-1β in EC-T cell-monocyte cocultures.

HMGB1 blockade has been reported to delay rejection of fully mismatched allografts in a murine heterotopic heart transplantation model, however, the specific mechanisms by which HMGB1 act remain unclear (34). We demonstrate here that in EC-T cell cocultures, the ability of HMGB1 to enhance alloreactive T cell cytokine production arises primarily from its ability to induce IL-1β secretion from monocytes, indicating a potential mechanism by which HMGB1 may modulate alloreactive T cell responses in vivo. In addition to HMGB1, other mediators that promote IL-1β secretion from monocytes may also be liberated following cell damage. Extracellular ATP activates purinergic P2X7 receptors, leading to activation of the inflammasome and IL-1β processing (81). Likewise, monosodium urate crystals, which can precipitate from high uric acid concentrations produced during cell death, also induce IL-1β secretion through activation of the inflammasome, and IL-1R knockout mice are protected from monosodium urate crystal-induced gouty inflammation (26, 82). As monocytes are capable of producing large quantities of IL-1β, this response may serve to amplify an initial signal of cell death, for example HMGB1 release from necrotic cells. A recent report demonstrated in a murine peritonitis model that heat-shocked, UV-irradiated EL4 cells induce secretion of IL-1α, rather than IL-1β, from peritoneal macrophages (25). This finding is in contrast with our data, which demonstrate that human monocytes produce primarily IL-1β in response to EC lysates, due at least in part to HMGB1. This discrepancy may reflect differences in the source of dead cells or the method used to induce cell death. Alternatively, it may reflect differences in the target cell responses or species differences.

In addition to augmenting early cytokine production from alloreactive $CD4^+$ T cells, we show that exposure of memory $CD4^+$ T cells to IL-1 during a primary coculture with allogeneic EC selectively increases the number of IL-17-producing cells assessed in a secondary culture. Our data do not distinguish whether the increase in the number of IL-17-producing cells induced by IL-1 is due to differentiation of primary uncommitted human memory $CD4^+$ T (Th0) cells toward the Th17 lineage or selective expansion of previously differentiated IL-17-producing cells. Interestingly, there appeared to be an expansion of IFN-γ/IL-17 double-positive T cells comparable to that of IL-17 single-positive cells. There is considerable plasticity in the phenotypes of differentiated human memory $CD4^+$ T cells; however, little is known about the signals that direct further differentiation of human memory T cells upon reactivation (83). While our manuscript was under review, two groups of investigators recently reported that the combination of IL-1β plus IL-6, but not TGF-β, strongly promotes the differentiation of naive human $CD4^+$ T cells toward an IL-17-producing phenotype (45, 46). As EC are prodigious producers of IL-6, the combination of IL-6 plus IL-1 in the T cell-EC cocultures reported in this study may promote differentiation of EC-activated memory $CD4^+$ T cells toward the Th17 lineage (84). Alternatively, IL-1 may selectively enhance the proliferation or survival of previously differentiated IL-17-producing cells. In either case, our results indicate that, in addition to the described effects on naive T cell polarization, IL-1 also skews the alloreactive memory T cell population toward IL-17 production. As T cell production of IL-17 fuels inflammation and tissue injury in a number of models, our results suggest that IL-1 release from injured allografts may have long-lasting detrimental effects on allograft survival by skewing the responding alloreactive T cell population toward production of IL-17-driven inflammation. It will be of considerable interest to more clearly define the role of IL-17 in human graft arteriosclerosis.

There is continued interest in developing novel strategies to inhibit the nonimmune signals that may contribute to allograft rejection, and particular attention has been focused on dampening the early inflammatory response to transplantation-associated injury. Using an in vitro model of an interaction that is likely to be important in the development of clinical graft arteriosclerosis, our studies suggest that IL-1 production may be an important convergence point for multiple signals of cell injury or death that may occur during transplantation. In addition to eliciting inflammatory responses, IL-1 released following cell injury appears likely to also enhance the host antigraft adaptive response, with the IL-1R on T cells serving in part as a sensor of tissue injury. Our results thus provide a rationale for testing the utility of perioperative treatment with IL-1R antagonist, a clinically approved therapeutic agent, to improve late allograft survival.

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Disclosures

K. J. Tracey is an inventor on technology related to HMGB1 and is a consultant to Critical Therapeutics, Inc. The other authors have no financial conflict of interest.

References

1. Libby, P., and J. S. Pober. 2001. Chronic rejection. *Immunity* 14: 387–397.
2. Tullius, S. G., and N. L. Tilney. 1995. Both alloantigen-dependent and -independent factors influence chronic allograft rejection. *Transplantation* 59: 313–318.
3. Choi, J., D. R. Enis, K. P. Koh, S. L. Shiao, and J. S. Pober. 2004. T lymphocyte-endothelial cell interactions. *Annu. Rev. Immunol.* 22: 683–709.
4. Wiseman, A. C., B. A. Pietra, B. P. Kelly, G. R. Rayat, M. Rizeq, and R. G. Gill. 2001. Donor IFN-γ receptors are critical for acute $CD4^+$ T cell-mediated cardiac allograft rejection. *J. Immunol.* 167: 5457–5463.
5. Nagano, H., R. N. Mitchell, M. K. Taylor, S. Hasegawa, N. L. Tilney, and P. Libby. 1997. Interferon-γ deficiency prevents coronary arteriosclerosis but not myocardial rejection in transplanted mouse hearts. *J. Clin. Invest.* 100: 550–557.
6. Tellides, G., D. A. Tereb, N. C. Kirkiles-Smith, R. W. Kim, J. H. Wilson, J. S. Schechner, M. I. Lorber, and J. S. Pober. 2000. Interferon-γ elicits arteriosclerosis in the absence of leukocytes. *Nature* 403: 207–211.
7. Wang, Y., W. R. Burns, P. C. Tang, T. Yi, J. S. Schechner, H. G. Zerwes, W. C. Sessa, M. I. Lorber, J. S. Pober, and G. Tellides. 2004. Interferon-γ plays a nonredundant role in mediating T cell-dependent outward vascular remodeling of allogeneic human coronary arteries. *FASEB J.* 18: 606–608.
8. Antonysamy, M. A., W. C. Fanslow, F. Fu, W. Li, S. Qian, A. B. Troutt, and A. W. Thomson. 1999. Evidence for a role of IL-17 in organ allograft rejection: IL-17 promotes the functional differentiation of dendritic cell progenitors. *J. Immunol.* 162: 577–584.
9. Tang, J. L., V. M. Subbotin, M. A. Antonysamy, A. B. Troutt, A. S. Rao, and A. W. Thomson. 2001. Interleukin-17 antagonism inhibits acute but not chronic vascular rejection. *Transplantation* 72: 348–350.
10. Terasaki, P. I., J. M. Cecka, D. W. Gjertson, and S. Takemoto. 1995. High survival rates of kidney transplants from spousal and living unrelated donors. *N. Engl. J. Med.* 333: 333–336.
11. Salahudeen, A. K., N. Haider, and W. May. 2004. Cold ischemia and the reduced long-term survival of cadaveric renal allografts. *Kidney Int.* 65: 713–718.
12. Perico, N., D. Cattaneo, M. H. Sayegh, and G. Remuzzi. 2004. Delayed graft function in kidney transplantation. *Lancet* 364: 1814–1827.
13. Weis, M., and W. von Scheidt. 1997. Cardiac allograft vasculopathy: a review. *Circulation* 96: 2069–2077.
14. Lietz, K., and L. W. Miller. 2004. Current understanding and management of allograft vasculopathy. *Semin Thorac Cardiovasc Surg.* 16: 386–394.
15. Halloran, P. F., J. Homik, N. Goes, S. L. Lui, J. Urmson, V. Ramassar, and S. M. Cockfield. 1997. The "injury response": a concept linking nonspecific injury, acute rejection, and long-term transplant outcomes. *Transplant Proc.* 29: 79–81.
16. Chalasani, G., Q. Li, B. T. Konieczny, L. Smith-Diggs, B. Wrobel, Z. Dai, D. L. Perkins, F. K. Baddoura, and F. G. Lakkis. 2004. The allograft defines the type of rejection (acute versus chronic) in the face of an established effector immune response. *J. Immunol.* 172: 7813–7820.
17. Dinarello, C. A. 1996. Biologic basis for interleukin-1 in disease. *Blood* 87: 2095–2147.
18. Tesar, B. M., D. Jiang, J. Liang, S. M. Palmer, P. W. Noble, and D. R. Goldstein. 2006. The role of hyaluronan degradation products as innate alloimmune agonists. *Am. J. Transplant.* 6: 2622–2635.
19. Andrade, C. F., T. K. Waddell, S. Keshavjee, and M. Liu. 2005. Innate immunity and organ transplantation: the potential role of toll-like receptors. *Am. J. Transplant.* 5: 969–975.
20. Rock, K. L., A. Hearn, C. J. Chen, and Y. Shi. 2005. Natural endogenous adjuvants. *Springer Semin. Immunopathol.* 26: 231–246.
21. Zeh, H. J., III, and M. T. Lotze. 2005. Addicted to death: invasive cancer and the immune response to unscheduled cell death. *J. Immunother.* 28: 1–9.
22. Shi, Y., J. E. Evans, and K. L. Rock. 2003. Molecular identification of a danger signal that alerts the immune system to dying cells. *Nature* 425: 516–521.
23. Rifkin, I. R., E. A. Leadbetter, L. Busconi, G. Viglianti, and A. Marshak-Rothstein. 2005. Toll-like receptors, endogenous ligands, and systemic autoimmune disease. *Immunol. Rev.* 204: 27–42.
24. Land, W. G. 2005. The role of postischemic reperfusion injury and other nonantigen-dependent inflammatory pathways in transplantation. *Transplantation* 79: 505–514.
25. Chen, C. J., H. Kono, D. Golenbock, G. Reed, S. Akira, and K. L. Rock. 2007. Identification of a key pathway required for the sterile inflammatory response triggered by dying cells. *Nat. Med.* 13: 851–856.
26. Chen, C. J., Y. Shi, A. Hearn, K. Fitzgerald, D. Golenbock, G. Reed, S. Akira, and K. L. Rock. 2006. MyD88-dependent IL-1 receptor signaling is essential for gouty inflammation stimulated by monosodium urate crystals. *J. Clin. Invest.* 116: 2262–2271.
27. Scaffidi, P., T. Misteli, and M. E. Bianchi. 2002. Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. *Nature* 418: 191–195.
28. Dumitriu, I. E., P. Baruah, A. A. Manfredi, M. E. Bianchi, and P. Rovere-Querini. 2005. HMGB1: guiding immunity from within. *Trends Immunol.* 26: 381–387.
29. Andersson, U., H. Wang, K. Palmblad, A. C. Aveberger, O. Bloom, H. Erlandsson-Harris, A. Janson, R. Kokkola, M. Zhang, H. Yang, and K. J. Tracey. 2000. High mobility group 1 protein (HMG-1) stimulates proinflammatory cytokine synthesis in human monocytes. *J. Exp. Med.* 192: 565–570.
30. Park, J. S., J. Arcaroli, H. K. Yum, H. Yang, H. Wang, K. Y. Yang, K. H. Choe, D. Strassheim, T. M. Pitts, K. J. Tracey, and E. Abraham. 2003. Activation of gene expression in human neutrophils by high mobility group box 1 protein. *Am. J. Physiol.* 284: C870–C879.
31. Messmer, D., H. Yang, G. Telusma, F. Knoll, J. Li, B. Messmer, K. J. Tracey, and N. Chiorazzi. 2004. High mobility group box protein 1: an endogenous signal for dendritic cell maturation and Th1 polarization. *J. Immunol.* 173: 307–313.
32. Yu, M., H. Wang, A. Ding, D. T. Golenbock, E. Latz, C. J. Czura, M. J. Fenton, K. J. Tracey, and H. Yang. 2006. HMGB1 signals through toll-like receptor (TLR) 4 and TLR2. *Shock* 26: 174–179.
33. Dumitriu, I. E., P. Baruah, B. Valentinis, R. E. Voll, M. Herrmann, P. P. Nawroth, B. Arnold, M. E. Bianchi, A. A. Manfredi, and P. Rovere-Querini. 2005. Release

of high mobility group box 1 by dendritic cells controls T cell activation via the receptor for advanced glycation end products. *J. Immunol.* 174: 7506–7515.
34. Huang, Y., H. Yin, J. Han, B. Huang, J. Xu, F. Zheng, Z. Tan, M. Fang, L. Rui, D. Chen, et al. 2007. Extracellular hmgb1 functions as an innate immune-mediator implicated in murine cardiac allograft acute rejection. *Am. J. Transplant.* 7: 799–808.
35. Kurt-Jones, E. A., W. Fiers, and J. S. Pober. 1987. Membrane interleukin 1 induction on human endothelial cells and dermal fibroblasts. *J. Immunol.* 139: 2317–2324.
36. Libby, P., J. M. Ordovas, K. R. Auger, A. H. Robbins, L. K. Birinyi, and C. A. Dinarello. 1986. Endotoxin and tumor necrosis factor induce interleukin-1 gene expression in adult human vascular endothelial cells. *Am. J. Pathol.* 124: 179–185.
37. Libby, P., J. M. Ordovas, L. K. Birinyi, K. R. Auger, and C. A. Dinarello. 1986. Inducible interleukin-1 gene expression in human vascular smooth muscle cells. *J. Clin. Invest.* 78: 1432–1438.
38. Mosley, B., D. L. Urdal, K. S. Prickett, A. Larsen, D. Cosman, P. J. Conlon, S. Gillis, and S. K. Dower. 1987. The interleukin-1 receptor binds the human interleukin-1 alpha precursor but not the interleukin-1β precursor. *J. Biol. Chem.* 262: 2941–2944.
39. Loppnow, H., and P. Libby. 1992. Functional significance of human vascular smooth muscle cell-derived interleukin 1 in paracrine and autocrine regulation pathways. *Exp. Cell Res.* 198: 283–290.
40. Kurt-Jones, E. A., S. Hamberg, J. Ohara, W. E. Paul, and A. K. Abbas. 1987. Heterogeneity of helper/inducer T lymphocytes. I. Lymphokine production and lymphokine responsiveness. *J. Exp. Med.* 166: 1774–1787.
41. Luqman, M., L. Greenbaum, D. Lu, and K. Bottomly. 1992. Differential effect of interleukin 1 on naive and memory CD4$^+$ T cells. *Eur. J. Immunol.* 22: 95–100.
42. Lichtman, A. H., J. Chin, J. A. Schmidt, and A. K. Abbas. 1988. Role of interleukin 1 in the activation of T lymphocytes. *Proc. Natl. Acad. Sci. USA* 85: 9699–9703.
43. O'Sullivan, B. J., H. E. Thomas, S. Pai, P. Santamaria, Y. Iwakura, R. J. Steptoe, T. W. Kay, and R. Thomas. 2006. IL-1β breaks tolerance through expansion of CD25$^+$ effector T cells. *J. Immunol.* 176: 7278–7287.
44. Sutton, C., C. Brereton, B. Keogh, K. H. Mills, and E. C. Lavelle. 2006. A crucial role for interleukin (IL)-1 in the induction of IL-17-producing T cells that mediate autoimmune encephalomyelitis. *J. Exp. Med.* 203: 1685–1691.
45. Wilson, N. J., K. Boniface, J. R. Chan, B. S. McKenzie, W. M. Blumenschein, J. D. Mattson, B. Basham, K. Smith, T. Chen, F. Morel, et al. 2007. Development, cytokine profile and function of human interleukin 17-producing helper T cells. *Nat. Immunol.* 8: 950–957.
46. Acosta-Rodriguez, E. V., G. Napolitani, A. Lanzavecchia, and F. Sallusto. 2007. Interleukins 1β and 6 but not transforming growth factor-β are essential for the differentiation of interleukin 17-producing human T helper cells. *Nat. Immunol.* 8: 942–949.
47. Li, J., H. Wang, J. M. Mason, J. Levine, M. Yu, L. Ulloa, C. J. Czura, K. J. Tracey, and H. Yang. 2004. Recombinant HMGB1 with cytokine-stimulating activity. *J. Immunol. Methods* 289: 211–223.
48. Pober, J. S., M. P. Bevilacqua, D. L. Mendrick, L. A. Lapierre, W. Fiers, and M. A. Gimbrone, Jr. 1986. Two distinct monokines, interleukin 1 and tumor necrosis factor, each independently induce biosynthesis and transient expression of the same antigen on the surface of cultured human vascular endothelial cells. *J. Immunol.* 136: 1680–1687.
49. Manes, T. D., S. L. Shiao, T. J. Dengler, and J. S. Pober. 2007. TCR signaling antagonizes rapid IP-10-mediated transendothelial migration of effector memory CD4$^+$ T cells. *J. Immunol.* 178: 3237–3243.
50. Mahboubi, K., and J. S. Pober. 2002. Activation of signal transducer and activator of transcription 1 (STAT1) is not sufficient for the induction of STAT1-dependent genes in endothelial cells: comparison of interferon-θ and oncostatin M. *J. Biol. Chem.* 277: 8012–8021.
51. Mahboubi, K., N. C. Kirkiles-Smith, J. Karras, and J. S. Pober. 2003. Desensitization of signaling by oncostatin M in human vascular cells involves cytoplasmic Tyr residue 759 in gp130 but is not mediated by either Src homology 2 domain-containing tyrosine phosphatase 2 or suppressor of cytokine signaling 3. *J. Biol. Chem.* 278: 25014–25023.
52. Valantine, H. A. 2003. Cardiac allograft vasculopathy: central role of endothelial injury leading to transplant "atheroma". *Transplantation* 76: 891–899.
53. Fiuza, C., M. Bustin, S. Talwar, M. Tropea, E. Gerstenberger, J. H. Shelhamer, and A. F. Suffredini. 2003. Inflammation-promoting activity of HMGB1 on human microvascular endothelial cells. *Blood* 101: 2652–2660.
54. Treutiger, C. J., G. E. Mullins, A. S. Johansson, A. Rouhiainen, H. M. Rauvala, H. Erlandsson-Harris, U. Andersson, H. Yang, K. J. Tracey, J. Andersson, and J. E. Palmblad. 2003. High mobility group 1 B-box mediates activation of human endothelium. *J. Intern. Med.* 254: 375–385.
55. Wautier, J. L., and A. M. Schmidt. 2004. Protein glycation: a firm link to endothelial cell dysfunction. *Circ. Res.* 95: 233–238.
56. Dauphinee, S. M., and A. Karsan. 2006. Lipopolysaccharide signaling in endothelial cells. *Lab. Invest.* 86: 9–22.
57. Xu, H., K. K. Dhanireddy, and A. D. Kirk. 2006. Human monocytes as intermediaries between allogeneic endothelial cells and allospecific T cells: a role for direct scavenger receptor-mediated endothelial membrane uptake in the initiation of alloimmunity. *J. Immunol.* 176: 750–761.
58. Dong, C. 2006. Diversification of T-helper-cell lineages: finding the family root of IL-17-producing cells. *Nat. Rev. Immunol.* 6: 329–333.
59. Acosta-Rodriguez, E. V., L. Rivino, J. Geginat, D. Jarrossay, M. Gattorno, A. Lanzavecchia, F. Sallusto, and G. Napolitani. 2007. Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells. *Nat. Immunol.* 8: 639–646.
60. Savage, C. O., C. C. Hughes, B. W. McIntyre, J. K. Picard, and J. S. Pober. 1993. Human CD4$^+$ T cells proliferate to HLA-DR$^+$ allogeneic vascular endothelium: identification of accessory interactions. *Transplantation* 56: 128–134.
61. Kreisel, D., A. M. Krasinskas, A. S. Krupnick, A. E. Gelman, K. R. Balsara, S. H. Popma, M. Riha, A. M. Rosengard, L. A. Turka, and B. R. Rosengard. 2004. Vascular endothelium does not activate CD4$^+$ direct allorecognition in graft rejection. *J. Immunol.* 173: 3027–3034.
62. Shiao, S. L., J. M. McNiff, and J. S. Pober. 2005. Memory T cells and their costimulators in human allograft injury. *J. Immunol.* 175: 4886–4896.
63. Lombardi, G., S. Sidhu, M. Daly, J. R. Batchelor, W. Makgoba, and R. I. Lechler. 1990. Are primary alloresponses truly primary? Int. *Immunol.* 2: 9–13.
64. Valujskikh, A. 2006. The challenge of inhibiting alloreactive T-cell memory. *Am. J. Transplant* 6: 647–651.
65. Durum, S. K., J. A. Schmidt, and J. J. Oppenheim. 1985. Interleukin 1: an immunological perspective. *Annu. Rev. Immunol.* 3: 263–287.
66. Staruch, M. J., and D. D. Wood. 1983. The adjuvanticity of interleukin 1 in vivo. *J. Immunol.* 130: 2191–2194.
67. Chen, Q., G. Sen, and C. M. Snapper. 2006. Endogenous IL-1R1 signaling is critical for cognate CD4$^+$ T cell help for induction of in vivo type 1 and type 2 antipolysaccharide and antiprotein Ig isotype responses to intact Streptococcus pneumoniae, but not to a soluble pneumococcal conjugate vaccine. *J. Immunol.* 177: 6044–6051.
68. Tominaga, K., T. Yoshimoto, K. Torigoe, M. Kurimoto, K. Matsui, T. Hada, H. Okamura, and K. Nakanishi. 2000. IL-12 synergizes with IL-18 or IL-1β for IFN-γ production from human T cells. *Int. Immunol.* 12: 151–160.
69. Cooper, A. L., and D. Beasley. 1999. Hypoxia stimulates proliferation and interleukin-1alpha production in human vascular smooth muscle cells. *Am. J. Physiol.* 277: H1326–H1337.
70. Shreeniwas, R., S. Koga, M. Karakurum, D. Pinsky, E. Kaiser, J. Brett, B. A. Wolitzky, C. Norton, J. Plocinski, W. Benjamin, et al. 1992. Hypoxia-mediated induction of endothelial cell interleukin-1 alpha: an autocrine mechanism promoting expression of leukocyte adhesion molecules on the vessel surface. *J. Clin. Invest.* 90: 2333–2339.
71. Saadi, S., R. A. Holzknecht, C. P. Patte, D. M. Stern, and J. L. Platt. 1995. Complement-mediated regulation of tissue factor activity in endothelium. *J. Exp. Med.* 182: 1807–1814.
72. Saadi, S., R. A. Holzknecht, C. P. Patte, and J. L. Platt. 2000. Endothelial cell activation by pore-forming structures: pivotal role for interleukin-1α. *Circulation* 101: 1867–1873.
73. Hamilton, K. K., R. Hattori, C. T. Esmon, and P. J. Sims. 1990. Complement proteins C5b-9 induce vesiculation of the endothelial plasma membrane and expose catalytic surface for assembly of the prothrombinase enzyme complex. *J. Biol. Chem.* 265: 3809–3814.
74. Thomas, J. O., and A. A. Travers. 2001. HMG1 and 2, and related 'architectural' DNA-binding proteins. *Trends Biochem. Sci.* 26: 167–174.
75. Werman, A., R. Werman-Venkert, R. White, J. K. Lee, B. Werman, Y. Krelin, E. Voronov, C. A. Dinarello, and R. N. Apte. 2004. The precursor form of IL-1α is an intracrine proinflammatory activator of transcription. *Proc. Natl. Acad. Sci. USA* 101: 2434–2439.
76. Watson, C. A., L. Camera-Benson, R. Palmer-Crocker, and J. S. Pober. 1995. Variability among human umbilical vein endothelial cultures. *Science* 268: 447–448.
77. Taniguchi, N., K. Kawahara, K. Yone, T. Hashiguchi, M. Yamakuchi, M. Goto, K. Inoue, S. Yamada, K. Ijiri, S. Matsunaga, et al. 2003. High mobility group box chromosomal protein 1 plays a role in the pathogenesis of rheumatoid arthritis as a novel cytokine. *Arthritis Rheum.* 48: 971–981.
78. Park, J. S., F. Gamboni-Robertson, Q. He, D. Svetkauskaite, J. Y. Kim, D. Strassheim, J. W. Sohn, S. Yamada, I. Maruyama, A. Banerjee, et al. 2006. High mobility group box 1 protein interacts with multiple Toll-like receptors. *Am. J. Physiol.* 290: C917–C924.
79. Tsung, A., R. Sahai, H. Tanaka, A. Nakao, M. P. Fink, M. T. Lotze, H. Yang, J. Li, K. J. Tracey, D. A. Geller, and T. R. Billiar. 2005. The nuclear factor HMGB1 mediates hepatic injury after murine liver ischemia-reperfusion. *J. Exp. Med.* 201: 1135–1143.
80. Gao, B., Y. Wang, and M. F. Tsan. 2006. The heat sensitivity of cytokine-inducing effect of lipopolysaccharide. *J. Leukocyte Biol.* 80: 359–366.
81. Mariathasan, S., D. S. Weiss, K. Newton, J. McBride, K. O'Rourke, M. Roose-Girma, W. P. Lee, Y. Weinrauch, D. M. Monack, and V. M. Dixit. 2006. Cryopyrin activates the inflammasome in response to toxins and ATP. *Nature* 440: 228–232.
82. Martinon, F., V. Petrilli, A. Mayor, A. Tardivel, and J. Tschopp. 2006. Gout-associated uric acid crystals activate the NALP3 inflammasome. *Nature* 440: 237–241.
83. Messi, M., I. Giacchetto, K. Nagata, A. Lanzavecchia, G. Natoli, and F. Sallusto. 2003. Memory and flexibility of cytokine gene expression as separable properties of human T_H1 and T_H2 lymphocytes. *Nat. Immunol.* 4: 78–86.
84. Jirik, F. R., T. J. Podor, T. Hirano, T. Kishimoto, D. J. Loskutoff, D. A. Carson, and M. Lotz. 1989. Bacterial lipopolysaccharide and inflammatory mediators augment IL-6 secretion by human endothelial cells. *J. Immunol.* 142: 144–147.