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OX40 Ligand Regulates Inflammation and Mortality in the innate immune response to sepsis

Matthew Karulf,* Ann Kelly,* Andrew D. Weinberg,† and Jeffrey A. Gold*

The initial phase of sepsis is characterized by massive inflammatory cytokine production that contributes to multisystem organ failure and death. Costimulatory molecules are a class of receptors capable of regulating cytokine production in adaptive immunity. Recent studies described their presence on neutrophils and monocytes, suggesting a potential role in the regulation of cytokine production in innate immunity. The purpose of this study was to determine the role for OX40–OX40 ligand (OX40L) interaction in the innate immune response to polymicrobial sepsis. Humans with sepsis demonstrated upregulation of OX40L on monocytes and neutrophils, with mortality and intensive care unit stay correlating with expression levels. In an animal model of polymicrobial sepsis, a direct role for OX40L in regulating inflammation was indicated by improved survival, decreased cytokine production, and a decrease in remote organ damage in OX40L−/− mice. The finding of similar results with an OX40L Ab suggests a potential therapeutic role for OX40L blockade in sepsis. The inability of anti-OX40L to provide significant protection in macrophage-depleted mice establishes macrophages as an indispensable cell type within the OX40/OX40L axis that helps to mediate the clinical signs of disease in sepsis. Conversely, the protective effect of anti-OX40L Ab in RAG1−/− mice further confirms a T cell-independent role for OX40L stimulation in sepsis. In conclusion, our data provide an in vivo role for the OX40/OX40L system in the innate immune response during polymicrobial sepsis and suggests a potential beneficial role for therapeutic blockade of OX40L in this devastating disorder. The Journal of Immunology, 2010, 185: 4856–4862.

Sepsis, defined as the systemic inflammatory response to infection, is a devastating condition with high morbidity and mortality. It remains a significant burden on the health care system, affecting >700,000 people/yr in the United States, resulting in 250,000 deaths (1, 2). This results in a net cost of $17 billion/yr. Over the past decade, mortality from sepsis has remained >25%, despite adequate antimicrobial therapy (3). This highlights the need for newer adjuvant therapies.

The early phases of sepsis are characterized by activation of the innate immune response, with production of multiple pro- and anti-inflammatory cytokines. However, numerous attempts have been made to inhibit individual cytokines in sepsis, including TNF-α and IL-1β. These strategies, although effective in limiting LPS-mediated inflammation, failed to improve mortality in phase III clinical trials or in a mouse model of polymicrobial sepsis, such as cecal ligation and puncture (CLP) (4–6). One explanation for their failure is the redundant and overlapping actions of the individual cytokines. Consequently, investigators have begun to focus on costimulatory surface receptors capable of simultaneously regulating multiple inflammatory cascades.

Recent studies attribute a potentially important role to costimulatory molecules in regulating cytokine production and mortality in animal models of polymicrobial sepsis (7, 8). The OX40 (CD134)/OX40 ligand (OX40L; CD252) system has been well described in the regulation of T cell proliferation, effector cell function, and survival within the adaptive immune response (9). Growing evidence suggests that OX40L is present on activated macrophages and mononuclear cells, and OX40L is capable of directly activating intracellular signaling cascades and cytokine production (10, 11). Furthermore, OX40 has been described on activated neutrophils (polymorphonuclear leukocytes [PMNs]), as well as existing as a shed soluble form; the latter is also capable of binding to, and activating, OX40L, with subsequent upregulation of IL-6 on airways smooth muscle and c-fos and c-jun in transfected HUVECs (12, 13). Together, these data suggest that OX40/OX40L signaling within innate immune cells could play a role in the innate immune response to sepsis. In this report, we describe a pivotal role for OX40/OX40L in the regulation of mortality and inflammation in the innate immune response to mouse polymicrobial sepsis.

Materials and Methods

Mice

Wild-type (WT) C57BL/6, MaFIA, and RAG1−/− mice were obtained from The Jackson Laboratory (Bar Harbor, ME). OX40L−/− mice on a C57BL/6 background were graciously provided by Dr. Naoto Ishii (Tohoku University, Sendai, Japan) and bred in the Oregon Health and Science University animal facility. All mice were sex- (female) and age-matched (6–8 wk) and allowed to acclimatize for 1 wk prior to use. All studies were performed in accordance with the Oregon Health and Science University Institutional Animal Care and Use Committee.

Cecal ligation and puncture

CLP was performed as previously described (8, 14). Briefly, mice were anesthetized with 2.5% isoflurane and underwent CLP with a 19-gauge needle. Mice received 1 ml 0.9% saline s.c. for resuscitation. At specified times, the mice were used to collect plasma, bronchoalveolar lavage (BAL), and peritoneal lavage (PL; 3 ml), as previously described (8). For survival experiments, mice were monitored for a total of 14 d. For Ab
inhibition, 250 μg anti-OX40L (hybridoma provided by Dr. Naoto Ishii) was injected i.p. 4 h prior to surgery. Rat IgG (BioLegend, San Diego, CA) was used as an Ab control, and PBS was used as a vehicle control. Because there was no difference between the two, these groups were combined where indicated. For experiments involving MaFIA mice, they were administered AP20187 i.p. for 5 d, which results in total macrophage depletion (15).

Cytokines and tissue myeloperoxidase (MPO) were determined by commercially available immunoassays (R&D Systems, Minneapolis, MN; Hycult Biotechnology, Uden, The Netherlands), which were performed according to the manufacturers’ specifications. NF-κB DNA binding was determined by DNA-binding ELISA (Active Motif, Carlsbad, CA). Pulmonary capillary leak was determined by Evans blue dye, as previously described (8).

Flow cytometry

Flow cytometry was performed as previously described (8). Splenocytes, whole blood, or PL was collected, and 1 × 10^6 cells were incubated with 100 μl Fc block (mouse cells only) for 15 min and then labeled with the following Abs: OX40, OX40L, CD4, CD8, LY6g (mouse), CD11b, F4/80 (mouse), and CD14 (human) at optimal concentrations for 45 min in the dark. RBCs were lysed with RBC lysis buffer, and the cells were fixed with 0.1% paraformaldehyde and analyzed on a BD LSRII 8-color analyzer with FlowJo software (Tree Star, Ashland, OR). All reagents were purchased from BD Pharmingen (San Jose, CA). BD compensation beads were used to calibrate the instrument before each use. PMNs were identified by forward light scatter (FSC)/side scatter (SSC) characteristics and Ly6G+ (mice only), mononuclear cells by FSC/SSC, CD11b+, and F4/80 (mice) or CD14+ (human). The T cells were identified by FSC/SSC and further subgrouped by CD4+ and CD8+ labeling. Isotype Ab-labeled cells were used to control for nonspecific staining.

Human studies

All studies were approved by the Oregon Health and Science University Institutional Review Board. All patients meeting Society of Critical Care Medicine/American College of Chest Physicians criteria for sepsis in the first 24 h of intensive care unit (ICU) stay were eligible for inclusion (8). ICU controls were selected at random from patients admitted to the medical ICU, and they did not have a suspected site of infection. Patients were excluded for the following reasons: presence of a do not resuscitate order or decision to institute comfort care measures, hemoglobin < 7g/dl, or the presence of active bleeding requiring >2 U packed RBCs. After obtaining informed consent, 25 ml blood was collected into glass (serum) or EDTA-coated tubes (platelet-poor plasma) on days 1 (defined as first 24 h of admission to ICU), 3–5, 7, and 14, or until death or hospital discharge. After obtaining preliminary data on soluble mediators and optimization of...
The experiment was performed three times. ["natants were collected and assayed for IL-6 via ELISA."
"OX40:Ig (10^[4] primed with LPS for 24 h, after which supernatant was performed three times."
"C[mallized for protein (75 [m]g/ml), or control Ig for 24 h. Supernatants were collected and assayed for IL-6 via ELISA."
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labeling protocols, all subsequent subjects had additional blood collected for flow cytometry. Clinical data, including Acute Physiology and Chronic Health Evaluation (APACHE) II scores, were recorded at the time of each blood draw. Patients were followed for 28 d or until death or hospital discharge. ICU and ventilator-free days were defined as the number of days alive outside the ICU or off the ventilator, respectively, during the 28-d observation period.

Cell culture

THP-1 cells were differentiated with PMA for 24 h. Cells were subsequently stimulated with LPS (100 ng/ml) for 24 h and harvested for immunoblot, as previously described (7). For stimulation studies, cells were blocked with CD16/32 for 1 h and then OX40:Ig (10 [m]g/ml), Fc control, or PBS was added for 24 h. Supernatants were collected for ELISA.

Statistics

Survival was analyzed by Kaplan–Meier analysis. All comparisons between groups were performed using the Mann–Whitney or Kruskal–Wallis (ANOVA) test, depending on the number of groups analyzed. For multiple-group analysis, intergroup comparisons were performed with the Dunn test. Correlations were made with the Spearman test. All statistics were done with GraphPad Prism 5.0 software (GraphPad, La Jolla, CA).

Results

Expression of OX40/OX40L in human sepsis

We first examined OX40 and OX40L expression in human sepsis. We obtained blood from subjects admitted to the ICU with sepsis, subjects admitted to the ICU for nonseptic conditions (ICU controls), and healthy controls. Overall, healthy controls were more likely to be younger than septic patients or ICU controls (Table I). In concert with prior studies documenting OX40L expression on dendritic cells and macrophages and increased expression of OX40L on circulating monocytes from patients with acute coronary syndromes, patients with sepsis exhibited upregulation of OX40L on circulating monocytes compared with healthy controls (Fig. 1B, 1C) (16). Of even greater interest was the expression of OX40L on circulating PMNs (Fig. 1D). The presence of OX40L on PMNs was specific, as evidenced by lack of staining for other costimulatory molecules, CD40 and CD80 (Fig. 1A). Overall, expression of monocyte and PMN OX40L was greater in septic subjects compared with healthy and ICU controls on day 1 in the ICU (Fig. 1C, 1D); it decreased over time with resolution of symptoms (Supplemental Fig. 1). Finally, among septic subjects there was no correlation between monocyte or PMN OX40L expression on day 1 and level of IL-6, IL-10, or IL-12. However, for the entire cohort (septic patients and controls), there was a significant correlation between day 1 OX40L expression and IL-6 ([r] = +0.35; p = 0.009) and IL-10 ([r] = +0.35; p = 0.02). This is consistent with the known ability of OX40L to regulate IL-6 in other disease systems (12).

We next sought to determine whether day-1 expression of OX40L could potentially serve as a biomarker for outcome among septic patients. Levels of monocyte OX40L were greater in non-survivors compared with survivors (Fig. 1E), and expression levels loosely correlated with severity of illness, as determined by APACHE II score ([r] = +0.35; p = 0.04). Further, PMN OX40L negatively correlated with ICU-free days (Fig. 1F). However, there was no association between monocyte or PMN OX40L expression levels on day 1 and the presence of bacteremia, renal failure, circulatory failure, or respiratory failure (data not shown).

FIGURE 2. sOX40 is expressed in human sepsis and activates OX40L. A, Expression of sOX40 in healthy controls, ICU controls, and septic patients within 24 h of admission to the ICU. B, THP-1 monocytes were treated with LPS (100 ng/ml) or PBS for 24 h, and cells were lysed for immunoblot (left panel) or stained for OX40L via flow cytometry (right panel). Lanes normalized for protein (75 [m]g/lane). Experiment was done with GraphPad Prism 5.0 software (GraphPad, La Jolla, CA).

FIGURE 3. Expression and function of OX40L in mouse sepsis. A, WT mice underwent no surgery ([n] = 3) or CLP ([n] = 3) and harvested at 18 h. PL analyzed by flow cytometry for OX40L expression on macrophages (F4/80^+; left panel) or PMNs (Ly6G^+; middle panel). Representative dot plot of PMN expression (right panel). B, Immunoblot for OX40L from TG PMNs. C, Expression of OX40 on peritoneal PMNs (left panel) and representative dot plot (right panel).
In contrast with our findings with OX40L, the typical reservoir of OX40, CD4+ T cells, expressed only low levels of OX40 (<10% of cells stained positive), with no difference between septic subjects and either control group at any time point (data not shown). However, levels of the soluble isoform of OX40 (sOX40) were increased in septic patients compared with controls on day 1 (Fig. 2A). We further demonstrated a proinflammatory role for sOX40 on macrophages. We first established that OX40L could be upregulated on human macrophages in vitro with stimuli consistent with that observed with human sepsis. LPS stimulation of THP-1 cells significantly upregulated OX40L, by immunoblot and flow cytometry, compared with vehicle controls (Fig. 2B). Further stimulation of LPS-primed cells with an OX40:1g fusion protein significantly upregulated IL-6 compared with vehicle- or Ig-treated controls, confirming that OX40L is capable of reverse signaling in macrophages via stimulation with soluble ligands (Fig. 2C).

**OX40L regulates lethality of polymicrobial sepsis**

To better understand the biologic role of the OX40/OX40L system in sepsis, we used a mouse model of polymicrobial sepsis: CLP. We first wished to confirm expression of OX40 and OX40L on mouse innate immune effector cells in polymicrobial sepsis, thereby confirming our human observations and further validating the mouse model as a useful preclinical model for OX40L-targeted therapeutic interventions. Eighteen hours after CLP, OX40L was upregulated on peritoneal macrophages and PMNs compared with controls (Fig. 3A). We confirmed PMN expression of OX40L via immunoblot (Fig. 3B). PMNs were also the predominant source of IL-6 compared with vehicle- or Ig-treated controls, confirming that OX40L is capable of reverse signaling in macrophages via stimulation with soluble ligands (Fig. 2C).

The upregulation of OX40L on PMNs and mononuclear cells suggests a pivotal role for myeloid cells in mediating OX40L inflammation in vivo; however, numerous other reservoirs for OX40L have been described, including vascular endothelium and smooth muscle (17, 18). To confirm the requirement of macrophages in our system, we used MaFIA mice, which contain a FasR (CD95) expressing transgene driven by the CSF1 promoter (19). These mice, when treated with the dimerizing compound AP20187, get targeted FasR dimerization and subsequent systemic macrophage apoptosis and total animal macrophage depletion by day 5 (15, 19). Unlike our observations with WT mice or macrophage-intact MaFIA mice (data not shown), anti-OX40L failed to provide any meaningful protection in macrophage-depleted MaFIA mice (Fig. 7A), suggesting macrophages as a central effector cell type of OX40/OX40L-mediated inflammation in sepsis.

We next sought to establish that OX40L stimulation was indeed T cell independent (either through PMN-expressed OX40 or generation of sOX40), further establishing its role in innate immunity. To establish a T cell-independent role for OX40L activation in CLP, we repeated the experiments in RAG1−/− mice. Surprisingly, similar to our observations in WT mice, anti-OX40L significantly improved sepsis-specific survival in RAG1−/− mice compared with controls (Fig. 7B). Improvement in survival was associated with attenuation in cytokine production was associated with a decrease in remote organ injury, as evidenced by pulmonary capillary leak (Fig. 5A), hepatic NF-κB induction (Fig. 5B), and hepatic PMN accumulation assessed by MPO 18 h after CLP (Fig. 5C).

We next wished to establish whether physiologic blockade of OX40L would have a similar effect, to confirm the genetic model, as well as to provide important preclinical data. Administration of an anti-OX40L mAb significantly improved survival compared with isotype-treated controls (Fig. 6A). Similar to results with OX40L−/− mice, mice treated with anti-OX40L showed a reduction in plasma IL-6, IL-10, and IL-12 18 h after CLP compared with controls (Fig. 6B). Finally, use of anti-OX40L as a rescue therapy, administered 4 h after the onset of sepsis, resulted in a significant improvement in survival (Fig. 6C).

**OX40L activation is macrophage dependent and T cell independent in vivo**

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of IL-6, IL-10, and IL-12 in plasma (Fig. 7C) and BAL (data not shown). To the best of our knowledge, these data strongly suggest, for the first time, a T cell-independent mechanism of OX40L activation in vivo.

**Discussion**

Sepsis is a devastating disorder for which there are limited adjuvant therapies. Numerous attempts at anticytokine therapy have failed in animal models and humans. The redundant and overlapping effects of many proinflammatory cytokines suggest that strategies targeting transcription factors or upstream receptors that control multiple inflammatory pathways may be more effective, such as observations with macrophage migration inhibitory factor and high mobility group box 1 (20–22). Costimulatory molecules represent another potential target, based on their presence on macrophages, a potent source of cytokine production in sepsis, and their well-documented ability to regulate numerous transcriptional pathways in a bidirectional manner (7, 8, 23).

One of the major findings of this study is the improved survival in OX40L−/− mice subjected to polymicrobial sepsis. The ability to recapitulate this with pharmacological inhibition of OX40L implies that this is not an epiphenomenon due to congenital absence of OX40L in the knockout mice. However, it remains unclear why such a large discrepancy exists between OX40L−/− and anti-OX40L–treated mice. Whether this represents incomplete blockade and/or compensatory dysregulation of other pathways with congenital OX40L deficiency remains to be determined. More importantly, the ability of an OX40L Ab to provide protection after the onset of disease further established OX40L inhibition as a potential therapeutic intervention in human sepsis. Enthusiasm for the potential of OX40L blockade in human sepsis is bolstered by the finding of increased OX40L expression in our cohort of septic patients. This seemed to be relatively specific for sepsis in a medical ICU population, because similar findings were not observed in healthy or ICU controls, and the increase in expression levels returned to that of healthy controls with resolution of illness. The association between OX40L expression levels and mortality, severity of illness, and ICU-free days further supports the hypothesis that OX40L contributes to the lethality of polymicrobial sepsis. Although numerous studies investigated the role of the OX40/OX40L system in graft rejection, autoimmune disease, and antitumor immunity, to our knowledge, this is the first description of OX40L blockade in the innate immune response to an acute bacterial infection (sepsis) (24–26).
The mechanism by which OX40L regulates lethality lies in its ability to regulate multiple inflammatory pathways. The OX40/ OX40L system has been well described to control T cell proliferation in the adaptive immune response. However, a growing literature describes a putative receptor function for OX40L as well. Stimulation of OX40L on dendritic cells and airway smooth muscle cells results in JNK activation and subsequent IL-6 production (12, 13). We now show that macrophages are capable of similar OX40L-mediated IL-6 production in vitro. This correlates with the attenuation of IL-6, IL-10, and IL-12 observed in the OX40L−/− and anti-OX40L–treated mice after CLP. Our in vitro findings and the correlation between OX40L expression levels and circulating IL-6 and IL-10 in our human cohort suggest that this is valid in humans as well. Clinically, the attenuation of inflammatory cytokines improves survival through a reduction in remote organ injury. In our mice, this was represented by a reduction in pulmonary capillary leak and hepatic NF-κB and PMN accumulation in the OX40L−/− mice compared with controls.

Macrophages seem to be critical for mediating the lethal effects of OX40L in sepsis in vivo. The ability of LPS to upregulate OX40L on macrophages provides a mechanism for the specificity of OX40L upregulation in septal humans. However, it is likely that LPS is not the only mediator of OX40L upregulation in vivo. The known ability of CD154 and IL-12, both of which are upregulated in sepsis, to upregulate OX40L expression on dendritic cells and T cells, respectively, suggests multiple potential pathways toward the increased expression seen in our patients and mice (8, 10, 27). Finally, the failure of OX40L blockade to rescue macrophage-depleted mice suggests that macrophages are necessary for mediating the toxic effects of OX40L in sepsis in vivo. However, we acknowledge that the induction of systemic macrophage apoptosis as a method of macrophage depletion may result in dysregulation of other pathways that might have been OX40L dependent in our model. Our finding of OX40L on circulating PMNs in septic mice and humans provides another potential source of OX40L. The significance of this remains unclear and will be the subject of future investigations. Finally, the description of OX40L expression on endothelial cells provides another mechanism by which OX40L can regulate lethality (17).

The source of OX40 in our model remains less clear. The ability of anti-OX40L to rescue RAG1−/− mice suggests that there is a T cell-independent source of OX40 in sepsis and firmly places OX40 in the innate-immune response to polymicrobial sepsis. One potential source is cell-bound OX40 on circulating PMNs. Baumann et al. (13) described OX40 expression on human PMNs, and our finding of OX40 expression on mouse PMNs is consistent with this result. It is unclear why we did not observe high levels of OX40 expression on human PMNs. Soluble OX40 represents another potential means of OX40L activation in sepsis. Recently, studies documented that OX40 can also be shed as a soluble isoform (sOX40) that, similar to its cell-bound form, is able to activate and induce cytokine production from OX40L-expressing airway smooth muscle and, as described in this paper, macrophages as well (18). It is believed this is generated through alternative splicing, but the source(s) of it remain less clear (28). The presence of sOX40 also establishes its potential as a circulating biomarker of the presence and/or outcome in human sepsis, similar to its ability to discriminate between patients with systemic sclerosis and systemic lupus erythematosus (29). However, greater numbers of subjects will be required to validate this.

However, it is important to point out some limitations to our study. First, and most importantly, we examined the initial phases of sepsis and septic shock. Although these early phases are characterized by a robust proinflammatory response, recovery is associated with a compensatory immunoparalysis and lymphocyte apoptosis (2, 30). This is thought to be mediated, in part, by T cells; other costimulatory molecules were also shown to regulate this phase of sepsis (2, 31). We cannot exclude a role for the OX40 system in the transition to this phase because of the high early lethality of our animal model. In addition, our animal model and our human cohort had predominantly bacterial sepsis, with multiple bacterial species represented. It should be acknowledged that the contribution of OX40L to the host-inflammatory response may be pathogen dependent. This is even more of an issue for invasive fungal diseases, which were not represented in our studies and account for up to 5% of isolatable pathogens in human sepsis, and the incidence of fungal sepsis is continuing to increase (3). Finally, we acknowledge that the ability of OX40L and sOX40 expression to serve as a biomarker for the presence or outcome in sepsis is preliminary and must be validated in a larger cohort, with a multivariate analysis to better control for other confounders. A recent study suggested that monocytes’ OX40L upregulation may be present in patients with acute coronary syndromes, and other studies described sOX40 upregulation in patients with autoimmune disease (29, 32). Neither of these populations was represented in our cohort.

In conclusion, our data describe an integral role for the OX40/ OX40L system in the regulation of inflammation and mortality in the early stages of the innate immune response to polymicrobial sepsis. The results of this study provide important preclinical information in terms of OX40/OX40L specific biomarker development and therapeutic interventions for the treatment of sepsis and septic shock in the future.

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Disclosures
The authors have no financial conflicts of interest.

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