Regulation of IL-17 Family Members by Adrenal Hormones During Experimental Sepsis in Mice

Markus Bosmann,*† Fabien Meta,* Robert Ruemmler,* Mikel D. Haggadone,* J. Vidya Sarma,* Firas S. Zetoune,* and Peter A. Ward*

From the Department of Pathology,* University of Michigan Medical School, Ann Arbor, Michigan; and the Center of Thrombosis and Hemostasis† and the Department of Hematology and Oncology,* University Medical Center, Mainz, Germany

Severe sepsis is a life-threatening disease that causes major morbidity and mortality. Catecholamines and glucocorticoids often have been used for the treatment of sepsis. Several recent studies have suggested a potential role of IL-17 during the development and progression of sepsis in small animal models. In this study, the cross-talk of catecholamines and glucocorticoids with members of the IL-17 family was investigated during sepsis in C57BL/6 mice. The concentrations in plasma of IL-17A, IL-17F, and the IL-17AF heterodimer all were increased greatly in mice after endotoxemia or cecal ligation and puncture as compared with sham mice. Surprisingly, when compared with IL-17A (487 pg/mL), the concentrations of IL-17F (2361 pg/mL) and the heterodimer, IL-17AF (5116 pg/mL), were much higher 12 hours after endotoxemia. After surgical removal of the adrenal glands, mice had much higher mortality after endotoxemia or cecal ligation and puncture. The absence of endogenous adrenal gland hormones (cortical and medullary) was associated with 3- to 10-fold higher concentrations of IL-17A, IL-17F, IL-17AF, and IL-23. The addition of adrenaline, noradrenaline, hydrocortisone, or dexamethasone to lipopolysaccharide-activated peritoneal macrophages dose-dependently suppressed the expression and release of IL-17s. The production of IL-17s required activation of c-Jun-N-terminal kinase, which was antagonized by both catecholamines and glucocorticoids. These data provide novel insights into the molecular mechanisms of immune modulation by catecholamines and glucocorticoids during acute inflammation. (Am J Pathol 2013, 182: 1124–1130; http://dx.doi.org/10.1016/j.ajpath.2013.01.005)
IL-17A was protective after lethal endotoxemia in mice.\textsuperscript{7} In \textit{Bacteroides fragilis} infection, neutralization of IL-17A prevented the formation of abscesses.\textsuperscript{18} On the other hand, peritoneal clearance of \textit{Escherichia coli} was impaired by neutralization of IL-17A, indicating that certain levels of IL-17A are protective during the early phases of infection.\textsuperscript{9} Collectively, accumulating evidence suggests a role of IL-17 family members during sepsis and related diseases. However, it also is clear that these cytokines have important functions in mediating immune defenses against infections.

Currently, no Food and Drug Administration—approved drugs are available to improve the outcome of sepsis. Besides modern interventions of critical care medicine (low tidal volume mechanical ventilation, fluid resuscitation, and so forth), low-dose glucocorticoids and catecholamines have been used as supportive treatments for patients with sepsis.\textsuperscript{19} Here, we report on the immune modulatory functions of glucocorticoids and catecholamines related to the appearance of IL-17 isoforms during two experimental models of sepsis in mice.

Materials and Methods

Research Animals

The studies were performed in accordance with the guidelines of the US NIH and received approval from the University Committee on Use and Care of Animals of the University of Michigan. Male C57BL/6 mice (10-12 weeks old) were purchased from The Jackson Laboratory (Bar Harbor, ME). Surgical adrenalectomy was performed by Taconic Farms, Inc. (Germantown, NY), with sham mice receiving anesthesia and surgical incisions without removal of adrenal glands. After adrenalectomy, mice were maintained on 0.9% NaCl as drinking water. Mice were allowed at least 10 days after adrenalectomy for recovery from the surgical procedure before use in further experiments. All animals were housed under specific pathogen-free conditions.

Endotoxemia

Mice received 10 mg/kg body weight intraperitoneal injection of lipopolysaccharide (7 mg/kg body weight for survival studies) from \textit{E. coli} (0111:B4; Sigma-Aldrich, St. Louis, MO) and EDTA blood was obtained as described previously.\textsuperscript{7} For survival studies, mice were monitored at least every 12 hours for a duration of 10 days.

Cecal Ligation and Puncture

Ketamine-/xylazine-induced anesthesia followed by ligation and bilateral puncture of the cecum using an 18-/23-gauge needle plus fluid resuscitation was performed as described elsewhere.\textsuperscript{20} Control mice (sham-OP) received anesthesia and a midline incision without manipulation of the cecum. Blood collection or monitoring of survival was performed as described in the paragraph above.

Peritoneal-Elicited Cells

Peritoneal-elicited cells (PECs) (>80% F4/80\textsuperscript{-}CD11b\textsuperscript{+}) were collected 4 days after injection of 2.4% thioglycollate and cultured in RPMI 1640 media containing 100 U/mL penicillin-streptomycin, 0.1% bovine serum albumin, and 25 mmol/L HEPES.\textsuperscript{7,14} Cell-free supernatant fluids were stored at −80°C until further analysis.

ELISA

Detection of mouse IL-17A, IL-17F, IL-17AF, and IL-23 was accomplished by sandwich enzyme-linked immuno-sorbent assay (ELISA) (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions. We found that there was no cross-reactivity between the ELISA from IL-17 family members, further validating the manufacturer’s assurance (data not shown).

Real-Time PCR

Total RNA was isolated, reverse-transcribed, and amplified as described earlier.\textsuperscript{14,21} The following primers were used: mouse IL-17A (forward) 5’-CTCCAGAAGGCCCTCAG-CTTACTAC-3’, mouse IL-17A (reverse) 5’-AGCTTTTCCTC-CCGATTGACACAG-3’, mouse glyceraldehyde-3-phosphate dehydrogenase (forward) 5’-TACCCCAATGTGTCCGTCTG-3’, mouse glyceraldehyde-3-phosphate dehydrogenase (reverse) 5’-CTTTGATGGGGCTCACATGC-3’ (all from Invitrogen, Carlsbad, CA), and mouse IL-17F (QT00144347; Qiagen, Valencia, CA). Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

Bead-Based Assays

The cells were lysed (Bio-Plex Cell Lysis Kit; Bio-Rad, Hercules, CA) and incubated with beads coated with antibodies specific for c-Jun-N-terminal kinase (JNK) phosphorylated at Thr183/Tyr185 according to the manufacturer’s instructions. Samples were analyzed on a Luminex xMAP/Bio-Plex-200 System with Bio-Plex Manager Software 5.0 (Bio-Rad) as described previously.\textsuperscript{20} For data analysis, fluorescence intensities were normalized to signals from lysates of untreated cells.

Reagents

The following reagents were used for the studies: lipopolysaccharide (LPS) from \textit{E. coli} (0111:B4; Sigma-Aldrich), adrenaline (Hospira, Inc., Lake Forest, IL), noradrenaline (Hospira, Inc.), hydrocortisone (Sigma-Aldrich), dexamethasone (APP Pharmaceuticals, LLC, Schaumburg, IL), SP600125 (InvivoGen, San Diego, CA), and AEG3482 (Tocris Bioscience, Park Ellisville, MO). Neutralizing polyclonal goat anti-mouse IL-23p19 IgG and normal goat IgG were from R&D Systems.
In vitro experiments were performed independently at least three times and in vivo experiments were performed with the number of mice indicated in Figure 1 and 2. All values are expressed as the mean, and error bars represent the SEM.

Statistical Analysis

Data shown in the figures were analyzed by an unpaired, two-tailed Student’s t-test, and survival curves were analyzed by the log-rank (Mantel-Cox) test (GraphPad Prism version 5.04; GraphPad Software Inc., La Jolla, CA). Differences were considered significant when the P value was less than 0.05. The cytokine concentrations were
determined from log-transformed standard concentrations on a standard curve and then subjected to statistical analysis.

Results
Hyperproduction of IL-17 Family Members during Endotoxemia and Polymicrobial Sepsis after Adrenalectomy

We recently reported on the production of IL-17A and IL-17F isoforms in experimental sepsis models.\textsuperscript{7,8,14,20} After CLP, the levels of mRNA for IL-17A and IL-17F were increased in spleen homogenates 8 hours after CLP, when compared with mice after sham-OP for the CLP procedure (Figure 1, A and B). The relative increases in mRNA for IL-17F were much lower compared with IL-17A, which might be explained by higher baseline mRNA expression in spleens from mice after sham-OP (Figure 1, A and B). The plasma levels of IL-17A, IL-17F, and IL-17AF were compared at various time points after endotoxemia in C57BL/6 mice (Figure 1C). All three IL-17 isoforms reached a plateau between 6 and 12 hours. The plasma concentrations for IL-17A were found to be much lower than those for IL-17F (fivefold) and IL-17AF (10-fold). The data in Figure 1C suggest that the IL-17AF heterodimer in plasma after endotoxemia is the predominant species.

To investigate the roles of catecholamines and glucocorticoids in the production of IL-17 family members during sepsis, we used mice in which the adrenal glands had been surgically removed several weeks before sepsis induction. We confirmed that after adrenalectomy, mice had much higher mortality rates in the models of endotoxemia and CLP (Figure 2, A and G). After endotoxemia, all adrenalectomized mice died in fewer than 2 days, whereas all mice after sham surgery for the adrenalectomy procedure survived (Figure 2A). In the CLP model, the survival of adrenalectomized mice was 0% (day 3) compared with 80% survival (at 160 h) for mice with intact adrenal glands (Figure 2G). The increased susceptibility of adrenalectomized mice after endotoxemia correlated with much higher plasma concentrations of IL-17A, IL-17F, and IL-17AF after 8 hours (Figure 2B–D). Likewise, adrenalectomy resulted in much higher concentrations of IL-23 after endotoxemia (Figure 2E). Blockade of IL-23 using neutralizing antibody reduced the amounts of IL-17A in endotoxemia by approximately 50% (Figure 2F). We previously reported that in endotoxemia the appearance of IL-23 in plasma precedes production of IL-17A.\textsuperscript{7} Hence, the hyperproduction of IL-23
in adrenalectomized mice may contribute to the higher concentrations of IL-17A seen in such mice.

In CLP mice, plasma levels of IL-17A, IL-17F, and IL-17AF were all more than fivefold higher when compared with mice with functional adrenal glands (Figure 2, H–J). In control mice that underwent sham procedures for both adrenalectomy and CLP, the IL-17 family members were almost undetectable in plasma (Figure 2, H–J). These data suggest that endogenous adrenal hormones suppressed the appearance of IL-17 family members during the acute inflammatory response in the two septic models used.

Catecholamines and Glucocorticoids Suppress the in Vitro Production of IL-17 Family Members

We sought to investigate which factors known to be secreted by the adrenal glands might regulate the production of IL-17 family members. We used in vitro cultures of mouse PECs, which are predominantly macrophages. We recently described PECs as a source for either IL-17A or IL-17F. Quantitative comparison of concentrations for IL-17A, IL-17F, and IL-17AF released from PECs stimulated with LPS revealed the IL-17AF isoform to be most abundant 10 hours after the addition of LPS (Figure 3A). This was in accordance with the findings obtained with the CLP and endotoxemia models (Figures 1 and 2). Cultures of PECs were incubated with 1 μg/mL LPS alone or in combination with different doses of adrenaline, noradrenaline, hydrocortisone, or dexamethasone (dose range, 10^{-9} to 10^{-6} mol/L). The presence of 10^{-6} mol/L of adrenaline or noradrenaline in cultures of LPS-activated PECs reduced the release of IL-17A to 20% (Figure 3B). As expected, dexamethasone showed higher potency than hydrocortisone in suppressing IL-17A (Figure 3C). Adrenaline and noradrenaline only modestly suppressed IL-17F (Figure 3D), whereas IL-17F was strongly reduced by the presence of glucocorticoids (hydrocortisone, dexamethasone) (Figure 3E). With higher concentrations of catecholamines, IL-17AF production decreased by 40% when values after LPS activation alone were used as 100% (Figure 3F). Glucocorticoids nearly completely inhibited IL-17AF at 10^{-6} mol/L (Figure 3G). At lower concentrations (10^{-8} mol/L, 10^{-7} mol/L), the effects seen with noradrenaline were less when compared with those in the presence of adrenaline (Figure 3, B, D, and F). When mRNA levels for the IL-17A and IL-17F subunits were studied, these mRNAs also were reduced by catecholamines and glucocorticoids (10^{-6} mol/L) (Figure 3H). This suggests that catecholamines and glucocorticoids regulate the release of IL-17 family members at the transcriptional level (or affect mRNA stability).

Role of the JNK Pathway for Production of IL-17 Family Members

To characterize what signaling mechanisms may be targeted by adrenal gland hormones to suppress IL-17 family members, we turned our attention to the JNK pathway. We used bead-based assays specific for relative quantitative detection of JNK when phosphorylated at Thr183/Tyr185. Incubation of PECs (C57BL/6 mice) with LPS for 60 minutes increased the levels of phospho-JNK 2.5-fold above untreated controls (Figure 4A). Pretreatment of PECs for 60 minutes with 1 μmol/L of either adrenaline or noradrenaline as well as hydrocortisone, or dexamethasone substantially diminished JNK phosphorylation (Figure 4A). To assess if activation of JNK was required for the release of IL-17 family members after LPS, we used specific small-molecule inhibitors to block JNK1/JNK2 isoforms (Figure 4B). SP600125 and AEG3482 (each at 10 μmol/L) significantly antagonized the production of IL-17A, IL-17F, and IL-17AF in cultures of LPS-activated PECs (Figure 4B). Collectively, these data suggest that catecholamines and glucocorticoids may both target the JNK pathway to regulate the acute release of IL-17 family members.

Figure 4  Catecholamines and glucocorticoids reduce activation of JNK to mediate inhibition of IL-17 family members. A: Bead-based assay of phosphorylated JNK in lysates of PECs (C57BL/6) activated for 60 minutes with 1 μg/mL LPS alone or after pretreatment (60 minutes) with 1 μmol/L adrenaline (A), 1 μmol/L noradrenaline (NA), 1 μmol/L hydrocortisone (HC), or 1 μmol/L dexamethasone (Dexa). B: Dependency of secretion of IL-17A, IL-17F, and IL-17AF on JNK activation. PECs (C57BL/6) were pre-treated with 10 μmol/L JNK inhibitors for 60 minutes (SP600125 or AEG3482) followed by 1 μg/mL LPS for 10 hours before detection of IL-17 isoforms. *P < 0.05, **P < 0.01, and ***P < 0.001.
Discussion

Our findings indicate that catecholamines and glucocorticoids suppress gene expression and release of IL-17A, IL-17F, and IL-17AF during sepsis (CLP and endotoxemia) and in cultures of LPS-activated PECs (macrophages). Endogenous IL-23 appears to enhance IL-17A plasma concentrations after adrenalectomy/endotoxemia. It has long been known that adrenalectomized animals are much more sensitive to the lethal effects of endotoxin, IL-1, and tumor necrosis factor (TNF).22–24 We have shown in earlier reports that neutralization of IL-17A was protective in defined settings of lethal endotoxemia and severe polymicrobial sepsis by reduction of TNFα, IL-1β, IL-6, and HMGB1 release and up-regulation of IL-10 and transforming growth factor-β.7,8 In addition, we found that IL-17A by itself has minimal effects on TNFα, IL-1β, and IL-6 production from peritoneal macrophages; whereas in the co-presence of LPS there was significant augmentation of mediator production.8 These findings may suggest that the hyperproduction of IL-17 family members in adrenalectomized mice during sepsis may have contributed to the impaired survival of these mice. On the other hand, when nonsevere polymicrobial sepsis was studied, IL-17R-deficient mice showed reduced recruitment of polymorphonuclear leukocytes and defective bacterial clearance together with increased mortality.25

Secretion of catecholamines and glucocorticoids from the adrenal glands is an integral part of the stress response during critical illness.26 In addition to their metabolic effects, glucocorticoids are potent regulators of transcriptional activity (transactivation or transrepression) of genes with immunologic functions. Multiple proinflammatory cytokines are antagonized by glucocorticoids including IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-11, IL-13, IL-16, granulocyte-macrophage colony-stimulating factor, TNFα, and the chemokines IL-8, regulated on activation normal T-cell expressed and secreted, eotaxin, macrophage inflammatory protein 1α, and monocyte chemoattractant protein-1.27 Production of IL-17 is inhibited by methylprednisolone in cultures of peripheral blood mononuclear cells from healthy humans.28 In addition, the biological effects of IL-17 are sensitive to the lethal effects of endotoxin, IL-1, and tumor necrosis factor (TNF).22

Notably, catecholamines are produced not only in the adrenal medulla but to some extent by innate immune cells facilitating autocrine and paracrine feedback loops.6,40–42 In some experimental settings, catecholamines at very low concentrations may promote the inflammatory response.41,42 Similar to the findings in this report, the phosphorylation of JNK in PECs after LPS activation was antagonized by β2 adrenergic-receptor agonists.36 In conclusion, adrenal hormones appear to interfere with the release of IL-17 family members. Such observations may be of importance for understanding the immune modulatory effects of catecholamines and glucocorticoids during sepsis and other states of acute inflammation.

Acknowledgments

We thank Rachel Voight and Vinay R. Patel for technical assistance, as well as Beverly Schumann, Sue Scott, and Robin Kunkel for excellent staff support.

References