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Coordination between TLR9 Signaling in Macrophages and CD3 Signaling in T Cells Induces Robust Expression of IL-30

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IL-30, the p28 subunit of IL-27, interacts with EBV-induced gene 3 (EBI3) to form IL-27, which modulates both proinflammatory and anti-inflammatory responses during autoimmune or infectious disease. It also acts as a natural antagonist of gp130, thereby attenuating the signals of other gp130-associated cytokines. IL-30 regulation via LPS has been reported by others, but the intercellular communication that induces IL-30 expression is unknown. In this study, we show that treatment with anti-CD3/CD28 Abs plus CpG oligodeoxynucleotides induces robust expression of IL-30, whereas either treatment alone induces only low expression of IL-30. This observation in vitro mirrors the murine model in which administration of CpG under inflammatory conditions in vivo induces IL-30 expression. This robust induction of IL-30 occurs through the coordination of helper CD4+ T cells and innate immune cells (e.g., macrophages) and, to a lesser degree, B cells via the CD40/CD154 signaling pathway. These findings reveal a previously unrecognized mechanism that integrates signaling pathways from T cells and macrophages at the cellular level to induce IL-30 expression. The Journal of Immunology, 2012, 188: 000–000.

Materials and Methods

Reagents

Vendors for all reagents were as follows: thiol-modified CpG oligodeoxynucleotide (ODN) 1668 or control ODN (Sigma), anti-mouse CD3 (eBioscience), anti-mouse CD28 (Biolegend), anti-OX86 (Andrew Weinberg, Robert W. Franz Cancer Research Center, Earle A. Chiles Research Institute, Providence Cancer Center, Providence Portland Medical Center), anti-mouse 4-1BB (Shu-Hsia Chen, Mount Sinai Medical Center), activating anti-CD40 (Novus, NBIF-06657), anti-mouse TRL9-Pe (Imgenex) recombinant mouse IL-12 and IFN-γ (R&D systems), LPS (Sigma), lipoteichoic acid (Invivogen), polyinosinic-polycytidylic acid (Invivogen), concanamycin A (Sigma), and rat IgG (eBioscience).

Cell separation and coinoculations

Splenocytes were prepared as described previously (7). Purification of DCs, B cells, NK cells, and CD4+ T cells from splenocytes was performed using magnetic beads according to the manufacturer’s instructions (StemSep). Peritoneal exudate macrophages were obtained 3 d after i.p. injection of 3% sodium thioglycollate medium (2 ml per mouse; Sigma). Cells were seeded into 24-well plates, and after 3 h the cells were washed and fresh RPMI 1640 medium was added. Splenocytes (5 \times 10^5) were seeded in 0.75 ml of heat-inactivated RPMI 1640 media, activated with CD3 (2.0 µg/ml) and CD28 (0.5 µg/ml; CD3/CD28) in the presence or absence of CpG ODN 1668 (1 µg/ml; CPG) for 72 h, and the IL-30 levels in the supernatant were measured via IL-27p28 (R&D Systems) and EBI3 ELISA (USCN Life Science). When appropriate, splenocytes were treated with anti-CD40 (10 µg/ml), LPS (1 µg/ml on days 0 and 2), polyinosinic-polycytidylic acid (50 µg/ml), lipoteichoic acid (5 µg/ml), rll-12 (50 ng/ml), IFN-γ (50 ng/ml), OX86 (2 µg/ml), or 4-1BB (2 µg/ml). Splenocytes depleted of various cell subsets were seeded as mentioned above. For the
coincubation assay of CD4 T cells, B cells, DCs, whole T cells (CD3+), NK cells, and macrophages. 2 × 10^5 cells of each type were seeded in 300 μl heat-inactivated RPMI 1640 for 72 h.

**Mice**

MyD88^-/- splenocytes were harvested from mice provided by Dr. Samithamby Jeyaseelan (Louisiana State University). CD40^-/-, CD154^-/-, and IFNγR1^-/- mice were obtained from Jackson Laboratories. C57BL/6, nude, and SCID mice were purchased from Harlan Laboratories. All experiments were performed using 6–8-wk old mice. C7Bl/6 mice were immunized with injections into each foot pad with 50 μg OVA (duck egg; MP Biomedicals) in 12.5 μl saline emulsified 1:1 in CFA containing 1.0 mg/ml of Mycobacterium tuberculosis (H37RA; heat killed and dried; Sigma). Twenty-four hours after immunization, 10 μg CpG or control nucleotide (GpC) ODNs were administered via i.p. injections. Serum was collected 72 h after immunization and was analyzed for IL-30 production via ELISA. All animal procedures were approved by the Institutional Animal Care and Use Committee.

**Flow cytometric analysis**

Splenocytes were treated as indicated in Fig. 2B. At 48 or 72 h after incubation, TLR9 expression was analyzed with flow cytometry. Cells washed in PBS and permeabilized for 45 min at 4˚C, were blocked for FcRs via incubation with anti-CD16/32 (5 μg/ml) for 20 min at 4˚C. After blocking, cells were stained with anti–TLR9-Pe Ab for 30 min at 4˚C. Cells were washed and analyzed with flow cytometry using Attune (Invitrogen).

**Results**

Two studies laid the groundwork to understand and characterize the induction of IL-30 at the molecular level in macrophages (5, 6). However, those studies were based on a single type of immune cell, and the role of cell-to-cell communication or interaction in the induction of IL-30 was not reported, although intracellular interactions occur in every tissue and biologic system. We used splenocytes as a first step toward understanding IL-30 induction in biological systems similar to those found in vivo.

To test the coordination of different types of immune cells in inducing IL-30, two signals that stimulate different cell types were independently or simultaneously applied to the cell mixtures. One set of stimulation signals was CD3/CD28, which mimics the first and second signals that activate T cells. The other stimulation signal was CpG, which activates cells via TLR9 receptor, which is present on many cells but primarily on APCs such as macrophages, DCs, and B cells. To distinguish between IL-27 and free IL-30, we used the IL-27p28 ELISA kit from R&D Systems, which specifically binds IL-30 while showing only 7% cross-reactivity to rIL-27 according to the manufacturer’s data sheet.

Treatment of cells with the combination of two types of cell signals (CD3/CD28/CpG) induced synergistic expression of IL-30, whereas treatment of cells with either CD3/CD28 or CpG alone induced a relatively small increase in IL-30 production (Fig. 1A). The effects of CD3/CD28/CpG were at least 10-fold higher compared with either individual stimulation signal, whereas treatment with CD3/CD28 and control isogenic CpG (in which CpG was switched to GpC) was defective in inducing IL-30.

**FIGURE 1.** Coadministration of CD3/CD28 and CpG induce robust amounts of IL-30 expression in splenocytes compared with either signal alone. (A, B) Splenocytes were treated with CpG, CD3/CD28, or CD3/CD28/CpG, or left untreated for 72 h and analyzed for IL-30 and EBI3 production by ELISA. (C) Supernatants from WT or MyD88^-/- splenocytes treated with CD3/CD28/CpG for 72 h were analyzed for IL-30 production by ELISA. (D, E) Supernatants from splenocytes treated as indicated for 72 h were analyzed for IL-30 production by ELISA. (F) Supernatants from WT splenocytes treated with CD3/CD28/CpG were collected at various time points and analyzed for IL-30 production by ELISA. (G) Nonimmunized mice or mice were immunized with OVA/CFA were treated with CpG or GpC i.p. n = 5. Serum was collected 48 h after CpG treatment, and IL-30 was quantified in the serum. n = 3; data are representative of at least two independent experiments. *p < 0.05.
expression (Supplemental Fig.1). Others have also confirmed that a CpG signal alone is not effective in inducing IL-30 expression in monocyte-derived DC (8). To detect whether the combination treatment affects both subunits of IL-27 (IL-30 and EBI3) or IL-30 alone, the authors measured the levels of EBI3 protein via ELISA. Interestingly, the combination treatment induced IL-30 specifically, as EBI3 levels by CD3/CD28/CpG treatment were not altered when compared with either treatment alone (Fig. 1B).

It is well established that the TLR9 ligand CpG can activate downstream genes through MyD88-dependent pathways. Thus, to examine the role of MyD88 in CD3/CD28- and CpG-induced IL-30 expression, splenocytes from wild type (WT) and MyD88−/− mice were treated with CD3/CD28/CpG for 72 h, and IL-30 expression was measured using ELISA. IL-30 protein expression induced by CD3/CD28/CpG was completely abolished in the absence of MyD88 (Fig. 1C).

Others have shown that both IFN-γ and LPS can induce IL-30 in macrophages (5). We were interested in comparing the effects of CD3/CD28/CpG with those of other known tertiary signals (e.g., IFN-γ and the IFN-γ–inducing cytokine IL-12). The combination of two signals (CpG plus CD3/CD28) is much more effective than any of the other signal combinations, including the known IL-30 inducer LPS (Fig. 1D). Therefore, these data suggest that activation of both T cells and TLR9-positive cells are needed to synergistically induce IL-30 expression.

To determine whether this effect is TLR9 specific or other TLRs can induce similar effects, various TLR ligands were used in the presence of CD3/CD28 Abs. Fig 1E shows that the enhanced expression of IL-30 in coordination with T cell activation is specific only to TLR9, because stimulation with other TLR ligands did not induce expression of IL-30. Kinetic studies further elucidated the mechanism of this TLR9-dependent activation and showed that maximal induction of IL-30 via CD3/CD28/CpG occurs 48 to 72 h after treatment (Fig. 1F). However, LPS induction of IL-30 occurs during the initial 24 h—in agreement with Liu et al. (5)—and this induction is reduced over time (Supplemental Fig. 2). Importantly, the IL-30 induction by the TLR9 signal pathways is more robust (350–1000 pg/ml), whereas LPS induces only up to 100 pg/ml.

A relevant question to address is whether this TLR9 and T cell stimulation-coordinated induction of IL-30 occurs in vivo. Therefore, mice were immunized with OVA/CFA in the hind footpad and treated with either CpG or control nucleotides (GpC). Mice immunized with OVA/CFA plus CpG produced high IL-30 expression in the serum (Fig. 1G). Meanwhile, IL-30 was not expressed at detectable levels in mice immunized with OVA/CFA plus CpG produced high IL-30 expression in the serum (Fig. 1G). Although administration of CpG in the absence of immunization induced small amounts of IL-30 in vivo, the levels induced were 3-fold lower than those from the administration of CpG plus T cell stimulation. Indeed, the in vivo data mirror the in vitro data, supporting the observation
that TLR9 engagement via CpG in the presence of activated T cells induces high levels of IL-30.

Finding the mechanism responsible for IL-30 induction by CD3/CD28/CpG is important. In concert with other studies, we have previously discovered that IFN-γ is a strong inducer of IL-30 (5, 9), which led to the hypothesis that IFN-γ is the mediator that effectively induces IL-30 from this combination treatment. However, the absence of IFN-γ signaling reduces this upregulation only by 30%, suggesting that other mechanisms also contribute significantly to the induction of IL30 (Fig. 2A). The peak stimulation of IL-30 did not occur until 48-72 h after administration; therefore, another possibility was that the costimulation treatment increases the levels of TLR9, which promotes the IL-30 induction. However, we found that CpG alone is a better inducer of the TLR9 receptor than CD3/CD28/CpG treatment at 72 h. Therefore, it is unlikely that the combination treatment induces IL-30 via up-regulation of TLR9 expression (Fig. 2B).

The data above show that either CD3/CD28 or CpG alone induce a small amount of IL-30 expression, but the combination of these two treatments synergistically induces much higher IL-30 expression. This result suggests that simultaneous activation of both T cells and TLR9+ cell signaling is crucial for inducing robust IL-30 expression. To determine whether the T cell costimulation signal (CD28 stimulation) or the primary stimulation signal alone (CD3 stimulation) plus CpG is sufficient to induce robust IL-30 expression, splenocytes were activated in the presence of CpG with various T cell activators, such as 4-1BB, OX40, CD28, CD3, or mixed CD3/CD28 agonist Abs. Only in the presence of a primary T cell activation signal, anti-CD3 Abs, can CpG induce a high level of IL-30 production (Fig. 2C).

Immunocompromised nude mice were used to further confirm the role of T cells in high levels of IL-30 induction. As predicted, the absence of functional T cells eliminated the CD3/CD28/CpG-mediated induction of IL-30 (Fig. 2D). Similar results were also seen in immunodeficient SCID mice (Fig. 2E). These data suggest that T cell activation via CD3 is essential for maximal induction of IL-30. To further confirm the crucial role of T cells in inducing IL-30, the presence of concanamycin A, a known inhibitor of T cells (10), completely inhibited the CD3/CD28/CpG-induced IL-30 induction (Fig. 2F).

T cells are not an IL-30–expressing cell type and have low levels of TLR9 expression, which suggests that another cell type with effective TLR9-mediated activation is needed to coordinate with T cells to produce high levels of IL-30. To understand the primary cell type in which TLR9 signaling was activated, we performed a series of cell mixture studies. Because APCs are normally enriched for TLR9 expression, the purified CD4+ T cells were cocultured with various types of APCs, such as B cells, macrophages, and DCs, and the resulting supernatants were tested for IL-30 expression.

We found that a mixture of macrophages and CD4+ T cells yields a high level of IL-30 expression upon exposure to CD3/CD28/CpG (Fig. 3A). We also found that B cells, and DCs to a lesser extent, have a role in IL-30 induction. These results clearly indicate that interaction between CD4+ T cells and APCs are needed for inducing a high level of IL-30 expression in the presence of the combination signals. Because macrophages induce the highest expression of IL-30 (1700 pg/ml in macrophages compared with 1000 pg/ml in B cells), these cells were used in additional studies. To confirm that CD4+ T cells and macrophages interact to induce high expression levels of IL-30, macrophages were reconstituted with CD4+ T cells, CD4+ T and NK cells, or whole T cells (CD3+ cells). As expected, reconstitution of macrophages with CD4+ T cells induces the highest level of IL-30 induction following CD3/CD28/CpG treatment (Fig. 3B). To determine whether CD8+ T cells also have an important role, purified CD8+ T cells, CD3+ T cells, or NK cells were cocultured with macrophages. The same combination treatment (CD3/CD28/CpG) was applied, and the levels of IL-30 induction were compared (Fig. 3C). Whereas both NK and CD8+ T cells boosted the level of CD3/CD28/CpG-induced IL-30 expression from macrophages, the overall expression levels were much lower compared with those of CD4+ T cells, suggesting that CD4+ T cells have a crucial role in IL-30 biology.

The role of CD4+ T cells in boosting IL-30 expression was further confirmed by cell depletion studies. Depletion of CD4+ T cells from splenocytes reduced IL-30 expression the most, suggesting that this cell type has an important role in upregulating IL-30 expression (Fig. 3D). Depletion of NK cells did not have any effect. This finding suggests that CD4+ T cells are not the only cell type responsible for inducing IL-30, and in absence of CD4+ T cells, other types or subtypes of cells (e.g., CD8+ T cells) might aid in the regulation of IL-30.

T cells can activate macrophages via cell contact (11). To determine whether macrophages and T cells need physical contact to induce IL-30, macrophages and CD4+ T cells were cocultivated in the presence or absence of a transwell barrier, treated with CD3/CD28/CpG, and analyzed for IL-30 protein expression. In the presence of a barrier, CD3/CD28/CpG failed to induce any IL-30 expression, suggesting that the expression of IL-30 in macrophages requires direct cell-to-cell contact with CD4+ T cells (Fig. 4A).
CD154 is one of the ligands on the T cell membrane that can bind to and activate the CD40 receptor on macrophages (12, 13). To demonstrate whether CD40/CD154 interaction between APCs and T cells accounts for the induction of IL-30, we compared IL-30 levels induced by CD3/CD28/CpG in WT, CD40−/−, and CD154−/− splenocytes in different combinations (Fig. 4C, 4D). Absence of either CD40 or CD154 reduced IL-30 expression by 60–70% compared with WT, indicating that the CD40/CD154 interaction between the two cell types has a major role in IL-30 induction and, to a lesser extent, other pathways can compensate for the absence of CD40. To further confirm the role of CD40 in IL-30 induction, we reconstituted the CD40 signaling in CD154−/− splenocytes using an anti-CD40 agonist Ab (Fig. 4D). Although the lack of CD154 inhibits IL-30 expression by 60%, agonist anti-CD40 rescues such inhibition and induces a 9-fold increase in IL-30 expression.

Our data have shown that maximal induction of IL-30 requires the activation of both T cells and macrophages and physical interaction between these two types of cells (Figs. 3, 4). The major mechanism accounting for a high level of IL-30 induction is most likely the CD40/CD154 pathway. To provide additional evidence to support this conclusion, purified CD4+ T cells and peritoneal macrophages lacking either CD154 or CD40 were treated with CD3/CD28/CpG, and IL-30 expression was analyzed 72 h later. As expected, the lack of CD154 in T cells or CD40 in macrophages greatly reduced IL-30 expression, which could be partially rescued by reconstitution with agonist anti-CD40 treatment (Fig. 4E). Anti-CD40 treatment rescued IL-30 expression more efficiently in CD154−/− splenocytes than in CD154−/− T cells (Fig. 4D, 4E). One explanation for this phenomenon is that CD40 engages both splenic macrophages and B cells, resulting in higher expression of IL-30 when compared with macrophages alone because the CD40/CD154 pathway is relevant in macrophages and B cells.

To determine whether CD40 stimulation alone can replace CD4+ T cells in inducing IL-30, macrophages were stimulated with CpG and an anti-CD40 agonist Ab in the absence of CD4+ T cells. Although others have shown that CpG or CD40 ligands are not inducers of IL-30 expression (8), stimulation with CpG and anti-CD40 induces higher IL-30 expression than CpG alone (Fig. 4F). However, this expression is less efficient than that in the presence of CD4+ cells, most likely because CD4+ T cells provide continuous stimulation of CD40 over time and may provide other tertiary signals.

Discussion

Understanding the induction of IL-30 has multiple implications in biology. Others have found that IL-30 has anti-inflammatory properties because it inhibits proinflammatory cytokines such as IL-6 in gp130 signaling (2). Supporting evidence for the anti-inflammatory role of this cytokine has been shown in other...
studies in which IL-30 expression in myoblasts inhibits allogeneic T cell responses and prolonged graft survival (14-17). Likewise, single nucleotide polymorphisms of IL-30 are associated with greater susceptibility to asthma and inflammatory bowel disease (14-17). Our group has also shown that IL-30 inhibits IL-12-, IFN-γ- and Con A-mediated liver toxicity (9).

This study has made a step forward in understanding how IL-30 is induced. Whereas our group and others have reported (5, 9) that IFN-γ and LPS are the two major inducers of IL-30, in this study we show that IFN-γ signaling is not required for inducing IL-30 via the combination treatment. In addition, the timing of induction is different between CpG/CD3/CD28 and LPS treatments. Liu et al. (5) showed that LPS induces maximal IL-30 levels between 12 and 24 h in macrophages. Conversely, IL-30 production in splenocytes is nearly absent 12–24 h after the combination treatment, but it is robustly increased after 48–72 h (Supplemental Fig. 2).

CpG has a strong capacity for inducing a Th1 response, but many studies have shown that CpG is proinflammatory in inflammatory conditions. For example, administration of CpGs prior to onset of dextran sulfate sodium-induced colitis ameliorates the colitis, inhibits release of proinflammatory cytokines, and induces regulatory properties in CD4+ T cells (18, 19). CpG has protective properties in Th2-mediated pathologies (20, 21). Certain CpGs, such as CpG 1668, have already been shown to have protective properties in an arthritis model (22). In addition, CpG was able to inhibit autoimmune diabetes in NOD mice (23). These protective measures have been attributed to IL-10 expression. Results from this study show that CpG administration during an ongoing inflammatory condition (OVA/CFA) induces 3-fold higher levels of IL-30 than either CpG or OVA/CFA alone. In coordination with previous findings that IL-30 has a protective role in liver injury models and CpG is proinflammatory in a number of inflammatory conditions, this discovery suggests that the proinflammatory properties of CpG are due to induction of IL-10 and IL-30 (9). Therefore, understanding the in vivo significance of this IL-30 induction via CpG and whether such induction has anti-inflammatory properties must be addressed. Such a discovery is imperative because CpG could serve as a therapeutic modality in many chronic inflammatory disease models.

Previous work showed that MyD88, cRel, IRF1, and IRF3 are necessary to induce IL-30 expression (5, 6). Although we have shown that MyD88 is critical in the induction of IL-30, additional work is necessary to understand whether the above-mentioned transcription factors are necessary to induce IL-30 expression via CD3/CD28/CpG treatment. Interestingly, only activation of TLR9, and no other TLRs, induces robust IL-30 expression at delayed time points. Epidemiologic study findings have suggested that low exposure to bacterial products in developing countries contributes to increased risk of developing inflammation of the gut (24, 25). It is tempting to speculate that because CpG is proinflammatory in these conditions, simultaneous activation of TLR9 and T cells induces anti-inflammatory cytokines, such as IL-30.

In summary, we have described a novel cellular mechanism that controls IL-30 production. Our data reveal that coordination between an innate immune cell-derived signal and a Th cell-derived signal is necessary to induce a high level of IL-30 expression. These findings lay the groundwork for future studies to investigate how to manipulate IL-30 production during inflammation, cancer, or autoimmune diseases.

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Inflammatory arthritis can be reined in by CpG-induced DC-NK cell cross talk. *J. Exp. Med.* 204: 1911–1922.

