Eosinophil-derived neurotoxin acts as an alarmin to activate the TLR2–MyD88 signal pathway in dendritic cells and enhances Th2 immune responses

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Eosinophil-derived neurotoxin (EDN) is an eosinophil granule–derived secretory protein with ribonuclease and antiviral activity. We have previously shown that EDN can induce the migration and maturation of dendritic cells (DCs). Here, we report that EDN can activate myeloid DCs by triggering the Toll-like receptor (TLR)2–myeloid differentiation factor 88 signaling pathway, thus establishing EDN as an endogenous ligand of TLR2. EDN activates TLR2 independently of TLR1 or TLR6. When mice were immunized with ovalbumin (OVA) together with EDN or with EDN-treated OVA-loaded DCs, EDN enhanced OVA-specific T helper (Th)2-biased immune responses as indicated by predominant production of OVA-specific interleukin (IL)-5, IL-6, IL-10, and IL-13, as well as higher levels of immunoglobulin (Ig)G1 than IgG2a. Based on its ability to serve as a chemoattractant and activator of DCs, as well as the capacity to enhance antigen-specific immune responses, we consider EDN to have the properties of an endogenous alarmin that alerts the adaptive immune system for preferential enhancement of antigen-specific Th2 immune responses.

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Abbreviations used: AMP, antimicrobial peptide or protein; EDN, eosinophil-derived neurotoxin; FSL, fibroblast-stimulating lipopeptide; hAng, human angiogenin; HMGB1, high mobility group box 1; HPC, hematopoietic progenitor cell; iDC, immature DC; MAPK, mitogen-activated protein kinase; MyD88, myeloid differentiation factor 88; PGN, peptidoglycan; RANTES, regulated upon activation, normal T cell–expressed, and secreted; RLU, relative luciferase unit; SDF, stromal cell–derived factor; SLC, secondary lymphoid tissue chemokine; TLR, Toll-like receptor.

In response to microbial infection or tissue injury, cells of the innate immune system, such as neutrophils, eosinophils, and basophils, monocytes/macrophages, NK cells, and epithelial cells (including keratinocytes), produce antimicrobial peptides and proteins (AMPs), such as defensins, cathelicidins, and high mobility group box 1 (HMGB1) proteins (1–3). Recent studies have revealed that these AMPs, although structurally distinct, share some properties in addition to their direct antimicrobial effect, including direct chemoattracting and activating activities for various subpopulations of leukocytes, including DCs in vitro, and the capacity to enhance antigen-specific immune responses to a coadministered antigen in vivo (3, 4). These AMPs are rapidly released by leukocytes and/or various epithelial cells through cellular degranulation, necrosis, or immediate induction and secretion in response to danger signals, such as infection, tissue injury, and inflammatory cytokines. Based on their rapid release in response to infection or tissue injury, their dual roles as both chemoattractants and activators of antigen-presenting cells, as well as their capacity to enhance antigen-specific immune responses, we have classified these structurally distinct AMPs as immune alarmins, which are defined as endogenous mediators that rapidly galvanize host defenses against exogenous danger signals (5, 6).

Eosinophil-derived neurotoxin (EDN), a member of the RNase A superfamily, is a mediator produced by human eosinophils and placental epithelial cells (7). In addition to its ribonuclease activity (7), EDN reduces the infectivity of respiratory syncytial virus for target cells in vitro (8) and is also responsible in part for the anti–HIV-1 activity found in the supernatants of mixed lymphocyte cultures (9), thus identifying EDN as an AMP. We have previously

shown that both EDN and mouse eosinophil–associated RNase 2, one of a cluster of divergent orthologs of human EDN, act as selective chemoattractants for DCs (10). We have also reported that EDN has the ability to stimulate human DCs to produce a variety of proinflammatory cytokines and to undergo phenotypic maturation (11). In addition to its release by degranulation of eosinophils (7, 12), EDN expression can also be induced in macrophages by treatment with TNF-α and *Escherichia coli* LPS (11).

In this study, we sought to investigate the mechanism of EDN-induced maturation of DCs and its capacity to enhance antigen-specific immune responses in vivo. We have established that EDN can fully activate DCs in a myeloid differentiation factor 88 (MyD88)- and Toll-like receptor (TLR)2-dependent manner, and demonstrated the capacity of EDN to enhance antigen-specific Th2-polarized immune responses in vivo. Consequently, our results demonstrate that EDN has the properties of an alarmin.

RESULTS

EDN induces full activation of DCs

DC activation is characterized by the development of a mature phenotype (including increased expression of surface costimulatory and MHC molecules, production of proinflammatory cytokines, and conversion to a $CCR7^+$ phenotype) and the acquisition of the capacity for antigen presentation (13, 14). We have previously reported that EDN, either purified from a natural source or recombinant, has the same capacity to upregulate CD80, CD83, and CD86 expression and production of a variety of cytokines by DCs (11). To further determine its effect on DCs, we performed a thorough analysis of the phenotype and function of human monocyte–derived DCs upon treatment with recombinant EDN. The recombinant EDN used in this study displayed a single band on SDS-PAGE gel (not depicted) and did not contain a detectable level of LPS or peptidoglycan (PGN) as measured by the Cambrex QCL-1000 Chromogenic LAL Assay kit (Fig. 1 A) and the Wako SLP Reagent Set (Fig. 1 B), attesting to its purity. The capacity of the same batch of EDN to induce DC IL-6 production was previously shown to be destroyed by boiling (11). In addition, in comparison with EDN, human angiogenin (hAng) expressed in the same system did not induce DC IL-6 production (11). Therefore, the effect of EDN on DCs was unlikely to be due to LPS contamination. As shown by Fig. 1 C, human DCs incubated in the absence of any stimulant (sham-treated) for 48 h were positive for CD11c ($CD11c^+$), negative for CD83 ($CD83^-$), and expressed a very low level of CD80 ($CD80^{low}$) and an intermediate level of CD86 ($CD86^{int}$); these features are characteristic of an immature phenotype. DCs treated with EDN, similar to those treated with *E. coli* LPS, became $CD83^+$, $CD80^{int}$, and $CD86^{high}$, characteristic of surface marker expression of mature DCs. Additionally, treatment of monocyte-derived DCs with 200~2,000 ng/ml EDN for 48 h (Fig. 1 D, hatched bars) stimulated DC production of IL-6, IL-8, IL-12p70, and TNF-α in a dose-dependent fashion as compared with DCs incubated without a stimulant (Fig. 1 D, open bars). Of note, EDN at 2 μg/ml stimulated similar levels of IL-6, IL-8, and TNF-α, but much less IL-12p70 than 1 μg/ml of LPS (Fig. 1 D). Furthermore, we also examined whether EDN-treated DCs could migrate in response to an agonist for CCR5 (regulated upon activation, normal T cell–expressed, and secreted [RANTES]) or CCR7 (secondary lymphoid tissue chemokine [SLC]). As expected, sham-treated DCs migrated in response to RANTES, but not SLC (Fig. 1 E, white bars), whereas LPS-treated DCs migrated in response to SLC, but not RANTES (Fig. 1 E, hatched bars). DCs incubated with 1 μg/ml EDN for 24 h lost the capacity to migrate to RANTES, but gained the capacity to migrate to SLC, indicative of a switch in functional chemokine receptor expression from $CCR5^+$ to $CCR7^+$ (Fig. 1 E, solid bars). The ability of stromal cell–derived factor (SDF)-1α to induce the migration of all types of DCs provides a positive control and is in agreement with the reported expression of its receptor, CXCR4, by both immature and mature DCs (14). Thus, EDN induced phenotypic maturation of DCs.

To ensure that EDN-induced maturation of DCs was reflected at the functional level, EDN-treated human DCs were analyzed for their capacity to stimulate the proliferation of T cells in an allogeneic mixed lymphocyte reaction using sham-treated DCs as a control (Fig. 1 F). At the DC/T ratios tested (1:6,250~1:250), sham-treated DCs did not stimulate proliferation of allogeneic T cells as judged by the failure to increase [^{3}H]–TdR incorporation. In contrast, DCs treated with 1 μg/ml EDN, similarly to LPS (1 μg/ml) -treated DCs, stimulated the proliferation of allogeneic T cells when used at a DC/T ratio higher than 1:6,250, indicating that EDN treatment enhanced the capacity of DCs to stimulate T cell proliferation. Thus, EDN treatment results in full activation of DCs that not only acquire a mature phenotype (Fig. 1, C–E), but also become capable of antigen presentation and T cell stimulation (Fig. 1 F).

EDN activates NF-κB and multiple mitogen-activated protein kinases (MAPKs) in DCs

Activation of DCs in response to many stimuli, such as cytokines, CD40 ligand, and microbial products, converges in activating intracellular signaling transducers, such as NF-κB and MAPKs (15–18). To determine the effect of EDN, the levels of I-κBα and MAPKs in DCs treated with EDN were determined by Western blot using *E. coli LPS* as a positive control (Fig. 2). LPS caused down-regulation of I-κBα and activation of JNK, p38, and Erk MAPKs with distinct kinetics (Fig. 2, right), similarly to previous reports (17–19). EDN reduced the level of I-κBα in DCs in a time-dependent manner similarly to LPS, which began to decrease after 5~10 min of treatment and became evident after 20 min of treatment, indicating the activation of NF-κB by EDN (Fig. 2, left). EDN treatment also activated all three MAPKs, including JNK, p38, and Erks, as shown by an increase in the levels of phosphorylated JNK, p38, and Erks (Fig. 2, left). Of note, EDN-induced activation of DC MAPKs showed different kinetics from those induced by LPS. EDN-induced phosphorylation of

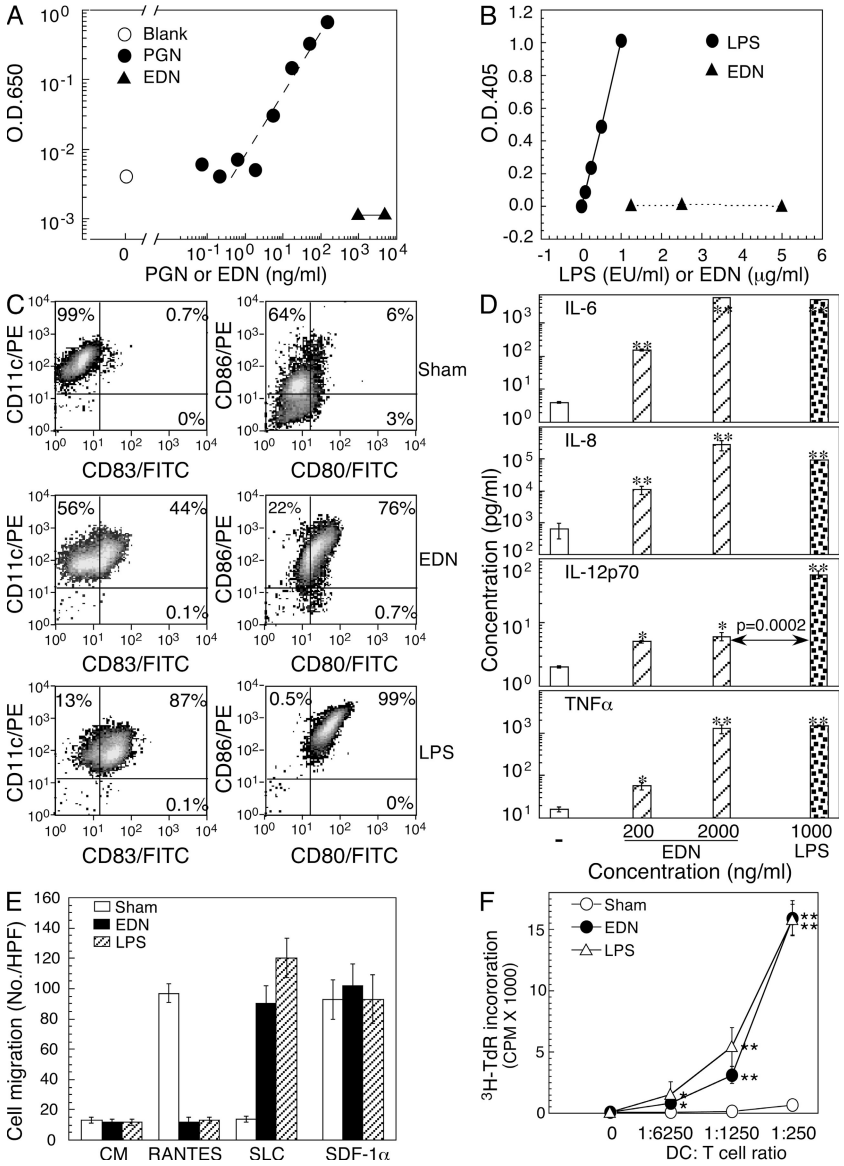

Figure 1. EDN activation of DCs. (A and B) Measurement of EDN preparation by the Wako SLP Reagent Set or Cambrex QCL-1000 Chromogenic LAL Assay kit for potential contamination with PGN and LPS, respectively. EDN preparation at the concentration tested (1~5 μg/ml) did not generate an optical density (O.D.) higher than that generated by the lowest concentration of standard PGN or LPS. (C) Flow cytometric analysis of the expression of DC surface markers. Monocyte-derived DCs were incubated in the absence (sham-treated) or presence of EDN at 1 μg/ml (EDN-treated) and LPS at 1 μg/ml (LPS-treated) for 48 h before immunostaining and flow cytometric analysis. The position of quadrants was determined by DCs stained with FITC- and PE-conjugated isotype-matched control antibodies. The density plots of the results of one of three representative experiments are shown. (D) Production of cytokines by DCs in response to EDN. Monocyte-derived iDCs (5×10^5 cells/ml) were incubated in triplicate in the absence or presence of EDN and LPS at the specified concentrations for 48 h before the supernatant was harvested for the measurement of IL-6, IL-8, IL-12p70, and TNF-α levels by SearchLight microarray. The average (mean ± SD) of two experiments using independent donors is shown. *, $P < 0.05$; **, $P < 0.001$ when compared with the corresponding sham-treated DC supernatant (open bars) using the unpaired *t* test. (E) Migration of EDN-treated DCs in response to selected chemokines. Monocyte-derived DCs were cultured in the absence (sham) or presence of 1 μg/ml EDN or LPS for 24 h before the measurement of their migration to the indicated chemokines using chemotaxis assay. RANTES, SLC, and SDF-1α were all used at 100 ng/ml. The migration of DCs is depicted as the average number of DCs per high-powered field (mean ± SD) of triplicate wells. (F) The proliferative response of human peripheral blood T lymphocytes to EDN-treated allogeneic DCs. Monocyte-derived DCs were treated with or without (sham) 1 μg/ml EDN or LPS for 48 h at 37°C in humidified air with 5% CO_2 for 48 h. Subsequently, the treated DCs were added together with T cells (10^5/well) at the indicated DC/T ratio into 96-well plates and incubated at 37°C in humidified air with 5% CO_2 for 6 d with the addition of [^{3}H]-TdR (0.5 μCi/well) for the last 18 h of incubation. The cells were harvested and measured for the incorporation of [^{3}H]-TdR, illustrated as the average CPM (mean ± SD) of triplicate wells. *, $P < 0.05$; **, $P < 0.001$ when compared with the corresponding sham group (unpaired *t* test).

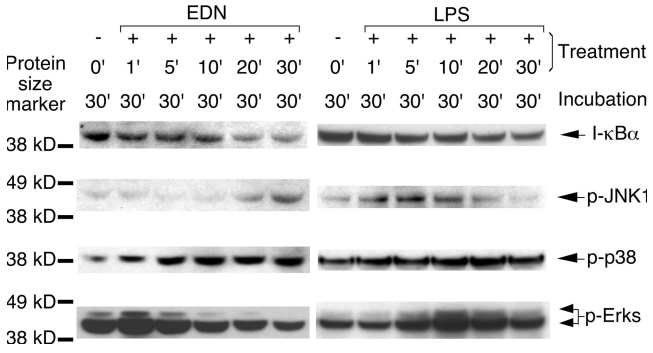

Figure 2. EDN induction of I-κBα degradation and phosphorylation of MAPKs in DCs. Serum-starved DCs (4×10^6 cells/tube in serum-free RPMI 1640) were incubated at 37°C in the absence or presence of EDN or *E. coli* LPS (final concentration, 1 μg/ml) for 30 min. EDN or LPS was added into corresponding tubes at the beginning and 10, 20, 25, and 29 min of the incubation to ensure treatment for 30, 20, 10, 5, and 1 min, respectively. At the end of incubation, the cells were solubilized in SDS-PAGE sample buffer. Identical amounts of cell lysate (15 μl) were run on a gradient SDS-PAGE gel and transferred onto a piece of PVDF membrane and sequentially Western blotted for I-κBα, phosphorylated JNK (p-JNK), p-p38, and p-Erks as described in the Materials and methods. Similar results were obtained in three independent experiments.

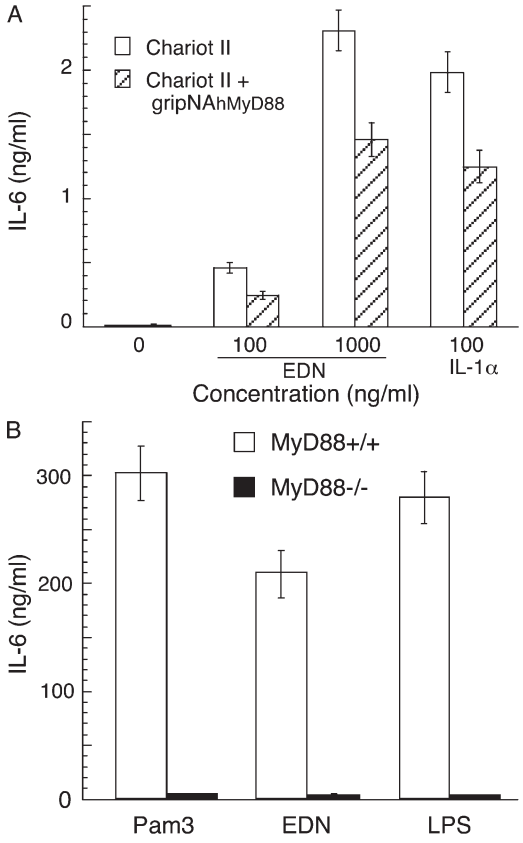

Figure 3. EDN-induced IL-6 production was dependent on MyD88. (A) Human monocyte–derived DCs were transfected with or without (Chariot II alone) gripNAhMyD88. After 24 h of culture, the DCs (4×10^5 cells/ml) were incubated with EDN at the indicated concentrations for 40 h, and the concentration of IL-6 in the culture supernatant was measured by ELISA. Shown are the data of one experiment representative of two. (B) DCs generated from the bone marrow progenitors of WT (MyD88$^{+/+}$) and MyD88 knockout (MyD88$^{-/-}$) mice were incubated at 10^6/ml in the presence of 1 μg/ml Pam3, EDN, or LPS for 48 h, and the production of IL-6 in the culture supernatants was measured by ELISA. The results of one experiment representative of two are presented as the average (mean ± SD) of triplicate wells.

JNK only became evident after 20 min of incubation; however, LPS-induced JNK phosphorylation peaked at 5~10 min and began to decrease thereafter. In addition, EDN induced more rapid phosphorylation of DC Erks (peaked at 1 min) than LPS (peaked at 10 min). Furthermore, the ratio of phosphorylated p44/42/p38 in EDN-treated DCs was relatively higher than that of LPS-treated DCs (Fig. 2).

EDN induction of IL-6 production by DCs requires MyD88

To identify the mechanism of EDN-induced DC activation, we applied the gripNA transient gene-silencing technique (20) to investigate if blockade of MyD88 in human monocyte–derived DCs would inhibit EDN-induced IL-6 production. To this end, gripNA-hMyD88 was delivered into human monocyte–derived DCs, and the effect of EDN or IL-1α on IL-6 production by the resulting DCs was measured. Compared with DCs treated with Chariot II alone, DCs treated with Chariot II and gripNA^{MyD88} produced much less IL-6 in response to EDN, suggesting that MyD88 might be involved in EDN-mediated DC activation (Fig. 3 A). IL-1α–induced IL-6 production in DCs was partially (~37%) inhibited by gripNA^{MyD88} treatment, suggesting incomplete blockade of DC MyD88 by gripNA^{MyD88} transfection (Fig. 3 A). To determine whether MyD88 was required for EDN-induced DC activation, DCs derived from MyD88$^{+/+}$ and MyD88$^{-/-}$ mice were used. When DCs generated from the bone marrow hematopoietic progenitor cells (HPCs) of littermate-matched MyD88$^{+/+}$ and MyD88$^{-/-}$ mice were tested for their capacity to produce IL-6 in response to EDN, EDN, like Pam3 and LPS, induced IL-6 production by MyD88$^{+/+}$ DCs, but failed to induce IL-6 production by MyD88$^{-/-}$ DCs, indicating that MyD88 plays a critical role in EDN-induced DC cytokine production (Fig. 3 B).

EDN signals through TLR2

Among the many diverse DC-activating stimulants, only the IL-1 family of cytokines (including IL-18) and many TLR ligands activate DCs in a MyD88-dependent manner (15, 21). There is no obvious structural or functional resemblance between EDN and cytokines. On the other hand, certain AMPs have previously been reported to act as endogenous TLR ligands, such as the activation of TLR4 by mouse β-defensin-2 (22) and of TLR2 and TLR4 by HMGB1 (23, 24). Therefore, we investigated whether EDN could act as an endogenous ligand for a TLR by determining if EDN could stimulate NF-κB activation in HEK293 cells transiently transfected with a combination of individual TLR and an NF-κB–reporting

luciferase construct. Human monocyte–derived DCs in our study expressed TLR1, 2, 3, 4, 5, 6, 7, 8, and 10 mRNAs, suggesting that TLR9 was unlikely to be involved (not depicted). In addition, the HEK293 cells used in our study, although expressing high levels of TLR5 and 6 mRNAs, and low levels of TLR1, 7, 8, and 10 mRNAs, did not show NF-κB activation in response to EDN (not depicted), suggesting that EDN did not activate TLR1, 5, 6, 7, 8, and 10. When HEK293 cells transiently transfected with a NF-κB–reporting luciferase construct together with plasmids expressing TLR2, TLR3, or a combination of TLR4 and MD-2 were measured for the luciferase activity in response to EDN, EDN induced an increase in luciferase activity only in cells expressing TLR2, but not in those expressing either TLR3 or TLR4, indicating that EDN interacted with TLR2 to activate NF-κB (Fig. 4 A). As expected, HEK293 cells expressing a particular TLR increased their luciferase activity in response to the corresponding agonists.

To confirm the requirement of TLR2 for EDN-induced activation of DCs, we compared EDN-induced production of IL-6 by DCs derived from the bone marrow progenitors of TLR2$^{-/-}$, TLR2$^{+/+}$, C3H/HeN, and C3H/HeJ (harboring a point mutation that renders TLR4 nonfunctional) mice (Fig. 4 B). TLR2$^{+/+}$ DCs produced considerable levels of IL-6 in response to EDN, Pam3, and LPS. However, TLR2$^{-/-}$ DCs failed to produce IL-6 in response to either EDN or Pam3, a known TLR2 ligand, but did produce IL-6 in response to *E. coli* LPS, a TLR4 ligand. Additionally, EDN induced a similar amount of IL-6 production by both C3H/HeN and C3H/HeJ DCs, suggesting that EDN does not trigger TLR4. *E. coli* LPS induction of similar levels of IL-6 from all but C3H/HeJ DCs ensured that C3H/HeJ DCs used in these experiments had no functional TLR4. These data clearly demonstrate that EDN is an endogenous ligand of TLR2. This conclusion is supported by the dependence of EDN-induced DC IL-6 production on MyD88 (Fig. 3) because MyD88 is the exclusive signal transducer for TLR2 (15). Furthermore, the observation that EDN induced much less IL-12p70 than LPS (Fig. 1 B) activated Erks MAPK more than p38 MAPK in DCs (Fig. 2) is also consistent with the usage of TLR2 by EDN, as other TLR2 ligands have also been reported to have these properties (19).

TLR2 often heterodimerizes with TLR1 or TLR6 to respond to many TLR2 ligands (21, 25–27). To determine whether EDN activation of DCs was also dependent on TLR1 or TLR6, we assessed whether EDN-induced production of TNF-α and IL-6 by DCs derived from the bone marrow progenitors of TLR1$^{-/-}$, TLR2$^{-/-}$, TLR6$^{-/-}$, and WT C57BL/6 mice using Pam3, a ligand for TLR2/1 (25), fibroblast-stimulating lipopeptide (FSL)-1, a ligand for TLR2/6 (27), and *E. coli* LPS as controls (Fig. 5). As expected, LPS stimulated the production of similar amounts of TNF-α and IL-6 by all types of DCs. EDN, although capable of inducing comparable amounts of cytokines in WT, TLR1$^{-/-}$, and

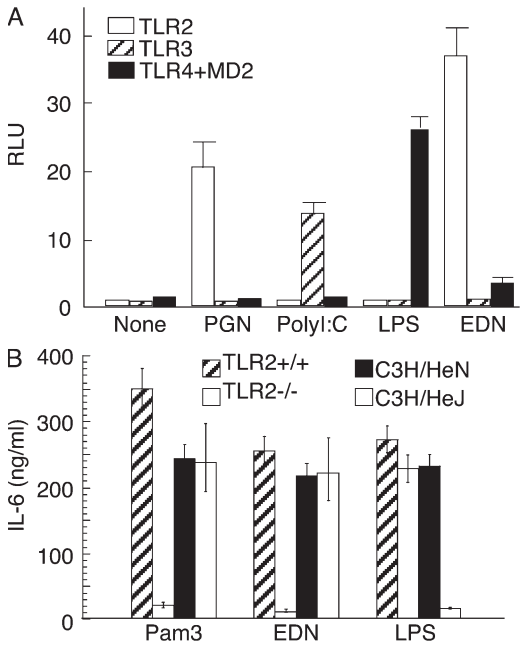

Figure 4. EDN activates TLR2. (A) HEK293 cells in a 96-well tissue culture plate were transfected with IgκB-luciferase and pSV-β-galactosidase in the presence of human TLR2, TLR3, or TLR4 plus MD2. After overnight culture, the cells were treated with of 20 μg/ml PGN, 10 μg/ml polyI:C, 1 μg/ml LPS, or 1 μg/ml EDN for 24 h. The relative luciferase activity was measure and illustrated as the average RLU (mean ± SD) of triplicate wells. (B) DCs generated from the bone marrow progenitors of WT (TLR$^{+/+}$), TLR2 knockout (TLR$^{-/-}$), C3H/HeN, and C3H/HeJ mice were incubated at 10^6/ml in the presence of 1 μg/ml Pam3, EDN, or LPS for 48 h. The production of IL-6 in the culture supernatants was measured by ELISA and presented as the average (mean ± SD) of triplicate wells. The results of one experiment representative of three are shown.

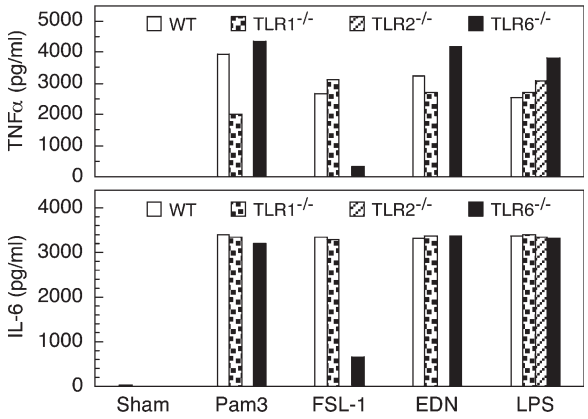

Figure 5. EDN induction of DC cytokine production does not depend on TLR1 or TLR6. DCs were generated from the bone marrow HPCs of WT, TLR1$^{-/-}$, TLR2$^{-/-}$, and TLR6$^{-/-}$ C57BL/6 mice (10~12 wk old) as described in Materials and methods. Subsequently, DCs were plated at 2×10^5/ml and treated in the absence (sham) or presence of 300 ng/ml Pam3, 1 μg/ml FSL-1, 1 μg/ml EDN, or 100 ng/ml *E. coli* (K12) LPS for 48 h at 37°C in humidified air with 5% CO_2 before the supernatants were harvested and assayed for TNF-α (top) and IL-6 (bottom) by ELISA. Similar results were obtained in two separate experiments.

TLR6$^{-/-}$ DCs, did not induce TNF-α and IL-6 in TLR2$^{-/-}$ DCs. The TLR1$^{-/-}$, TLR2$^{-/-}$, and TLR6$^{-/-}$ DCs showed the expected responses because (a) Pam3 and FSL-1 did not induce TNF-α and IL-6 production in TLR2$^{-/-}$ DCs, (b) Pam3 induction of TNF-α was partially reduced in TLR1$^{-/-}$ DCs, and (c) FSL-1–induced TNF-α and IL-6 were greatly reduced in TLR6$^{-/-}$ DCs. These data demonstrate that EDN acts only on TLR2, not on a heterodimer of TLR2/1 or TLR2/6.

EDN enhances antigen-specific Th2 immune response in a TLR2-dependent manner

To determine if EDN could enhance antigen-specific immune responses, C57BL/6 mice were immunized with OVA in the absence or presence of EDN on day 1 and boosted on day 14 with OVA alone. The levels of anti-OVA IgG antibody in the serum of mice taken on days 10 and 21 were measured for primary and secondary anti-OVA immune response. EDN dramatically enhanced both the primary (Fig. 6 A) and secondary (Fig. 6 B) anti-OVA antibody responses at both doses, demonstrating the capacity of EDN to promote an antigen-specific immune response. Alum, as a positive control, also enhanced anti-OVA antibody responses as expected (Fig. 6, A and B). hAng, another member of the RNase superfamily, which is unable to induce either migration or maturation of DCs (10, 11), did not enhance the primary or secondary anti-OVA antibody responses (Fig. 6, A and B). Assaying the subclass of anti-OVA IgG antibodies revealed that coimmunization with EDN selectively enhanced the production of OVA-specific IgG1, but not IgG2 or IgG3, during the primary immune response (Fig. 6 C). During the secondary immune response, booster with OVA caused an enhancement of all subclasses of anti-OVA IgG antibodies in EDN-treated mice; however, the OVA-specific IgG1 level was still higher than that of OVA-specific IgG2a, IgG2b, or IgG3 (Fig. 6 D). Alum, as expected, promoted the generation of more anti-OVA IgG1 than IgG2a in both primary and secondary antibody responses (Fig. 6, C and D). Because predominant generation of IgG1 antibody is an indicator of Th2 response, the data suggest that EDN may stimulate Th2 polarization.

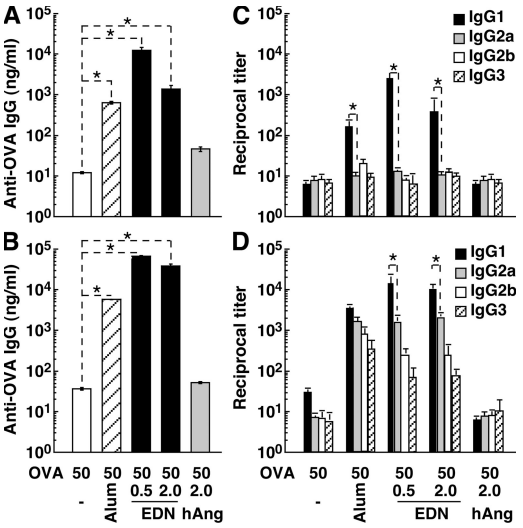

Figure 6. Enhancement of OVA-specific immune responses by EDN. C57BL/6 mice (female, 8-wk old, $n = 10$) were immunized by i.p. injection of 50 μg OVA in the presence or absence of alum (3 mg/mouse), EDN, or hAng on day 1 and boosted by i.p. injection of 50 μg OVA alone on day 14. Serum samples were taken on days 10 (A and C) and 21 (B and D) from each mouse for the measurement of total (A and B) and subclass (C and D) anti-OVA IgG antibodies using ELISA. The average OVA-specific IgG antibody titers (mean ± SEM) of each group is shown. *, $P < 0.001$ compared with the group immunized with OVA alone (Mann-Whitney test). Similar results were obtained in three independent experiments.

To determine whether EDN enhancement of antigen-specific immune response depends on its capacity to activate TLR2 and to evaluate the nature of EDN-enhanced antigen-specific immune response, TLR2 knockout (TLR2$^{-/-}$) and littermate-matched control (TLR2$^{+/+}$) mice were immunized with OVA in the absence or presence of EDN or LPS, and the subsequent immune responses were measured. Splenocytes from TLR2$^{+/+}$ mice immunized with OVA in the presence EDN or LPS incorporated significantly more [^{3}H]-TdR than cells of mice immunized with OVA alone, indicating the enhancement of OVA-specific T cell–proliferative response by EDN or LPS (Fig. 7 A). The splenocytes of TLR2$^{+/+}$ mice immunized with OVA in the presence of EDN produced predominantly IL-5, IL-6, IL-10, and IL-13, whereas splenocytes of TLR2$^{+/+}$ mice immunized with OVA in the presence of LPS produced predominantly IFN-γ, suggesting that EDN and LPS polarized in vivo OVA-specific Th2 and Th1 T cell responses, respectively (Fig. 7 B). The production of OVA-specific IL-4 was not significantly enhanced by simultaneous in vivo administration of either EDN or LPS (Fig. 7 B). The anti-OVA IgG responses in TLR2$^{+/+}$ mice immunized with OVA in the presence of EDN were very similar to those in C57/BL6 mice (Fig. 6), with significant enhancement of total anti-OVA IgG (Fig. 7 C, a and b) and higher levels of IgG1 than IgG2a (Fig. 7 C, c and d) in both primary (Fig. 7 C, a and c) and secondary (Fig. 7 C, b and d) antibody responses. TLR2$^{+/+}$ mice immunized with OVA in the presence of LPS also showed enhanced production of anti-OVA IgG (Fig. 7 C, a and b); however, the predominant subclass was IgG2a (Fig. 7 C, c and d), echoing the capacity of LPS to polarize Th1 immune response (Fig. 7 B). Importantly, there was no enhancement of OVA-specific T cell and antibody responses by EDN in TLR2$^{-/-}$ mice, whereas LPS similarly enhanced OVA-specific T cell and antibody responses in both TLR2$^{-/-}$ and TLR2$^{+/+}$ mice (Fig. 7, A–C). Thus, the capacity of EDN to enhance antigen-specific Th2-polarized immune response was dependent on TLR2.

EDN-activated DCs enhance antigen-specific Th2 immune response

To determine the role of DC activation in EDN enhancement of antigen-specific Th2 immune response, we investigated whether OVA-loaded DCs that were activated by EDN in vitro could promote OVA-specific Th2 immune response in vivo. To this end, DCs of WT or TLR2$^{-/-}$ mice were pulsed

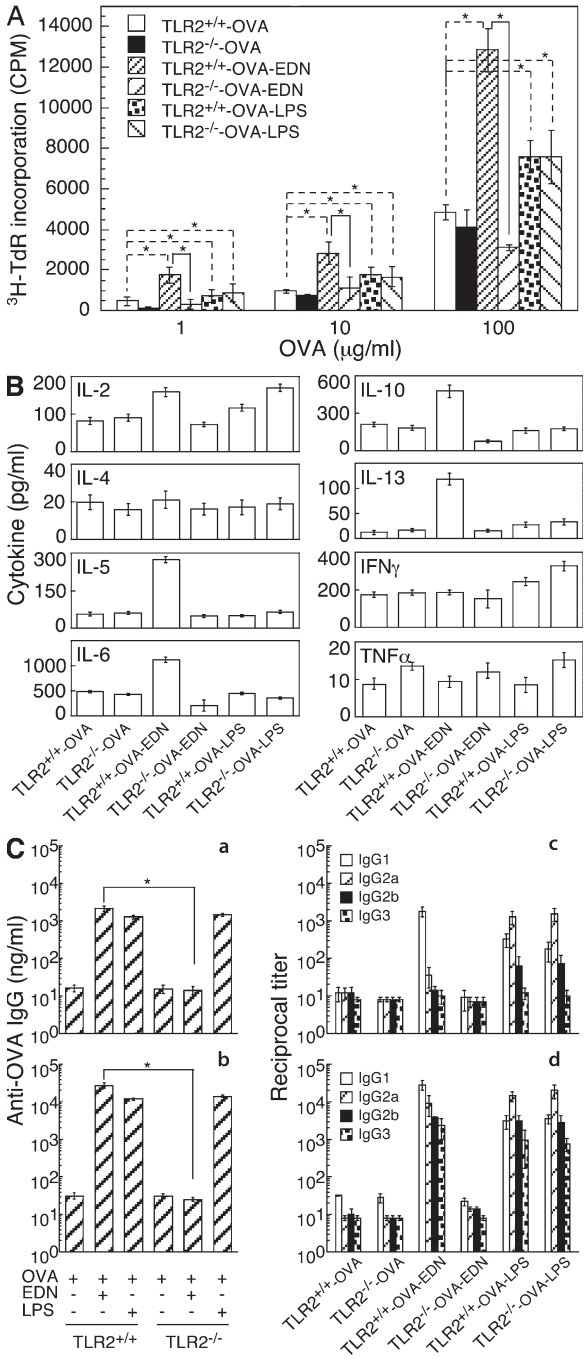

Figure 7. EDN enhancement of OVA-specific Th2-biased immune responses is ablated in TLR2 knockout (TLR2$^{2/2}$) mice. TLR2$^{-/-}$ and littermate-matched TLR2$^{+/+}$ mice (female, 8-wk old, $n = 4$) were immunized by i.p. injection of 50 μg OVA in the presence or absence of EDN (1 μg/mouse) or LPS (1 μg/mouse) on day 1 and boosted by i.p. injection of 50 μg OVA alone on day 14. Serum samples were taken on days 10 and 20 for the measurement of anti-OVA IgG antibodies using ELISA. On day 20, mice were killed and their spleens removed for the determination of OVA-specific proliferation and cytokine production. (A) Pooled splenocytes (5×10^5/0.2 ml/well) were stimulated with OVA at concentrations specified for 60 h with [^{3}H]-TdR pulse (1 μCi/well) for the last 18 h. Splenocyte proliferation was shown as the average (mean ± SD) [^{3}H]-TdR incorporation (CPM) of triplicate wells of one experiment representative of two. *, $P < 0.001$ by Student's t test. (B) Pooled splenocytes (5×10^6/1 ml/well) of each group were stimulated with 100 μg/ml OVA for 48 h, and the cytokines in the supernatants were measured by multiplex ELISA. Shown are the averages (mean ± SD) of individual cytokines of two independent experiments. (C) The average (mean ± SEM) of OVA-specific IgG antibody titers (a and b) and subclass (c and d) of each group ($n = 4$) on days 10 (a and c) and 21 (b and d) are shown. *, $P < 0.001$ by Mann-Whitney test. Similar results were obtained in two independent experiments.

with OVA, treated with EDN (or *E. coli* LPS as a control), and injected into the peritoneal cavity of C57BL/6 mice. The mice were immunized i.p. with OVA on day 5 and killed on day 12 for the determination of OVA-specific splenocyte production of cytokines. WT DCs in response to EDN produced a substantial amount of IL-1β, IL-6, IL-10, and TNF-α, a low (nevertheless statistically significant) amount of IL-12p70, and a little IL-23 (Fig. 8 A). LPS enhanced DC production of all cytokines measured, with much less induction of IL-10 and much greater induction of IL-6, IL-12p70, and IL-23 in comparison with EDN treatment (Fig. 8 A). TLR2$^{-/-}$ DCs did not produce any significant cytokine in response to EDN (Fig. 8 A). The splenocytes of C57BL/6 mice immunized with OVA-loaded and EDN-treated WT DCs generated predominantly IL-5, IL-6, IL-10, and IL-13, but no IFN-γ when stimulated with OVA (Fig. 8 B). In contrast, the splenocytes of mice immunized with LPS-treated DCs predominantly generated IFN-γ, some IL-6 and IL-10, but no IL-5 or IL-13 upon OVA stimulation (Fig. 8 B). The splenocytes of mice immunized with EDN-treated TLR2$^{-/-}$ DCs did not produce any Th1 or Th2 cytokines (Fig. 8 B). These data, in addition to further strengthening the conclusion that EDN activates DCs by triggering TLR2, also demonstrate that DCs contribute to EDN enhancement of OVA-specific Th2 immune response.

DISCUSSION

It has been well established that TLRs can recognize infectious "nonself" by sensing pathogen-associated molecular patterns and triggering antimicrobial immune responses of the host (28, 29); however, emerging evidence indicates that certain endogenous molecules can also activate TLRs. Several endogenous ligands have been reported to activate TLR4, such as fibronectin extra domain A (30), fibrinogen (31), lung surfactant protein A (32), heparan sulfate (33), hyaluronan fragments (34, 35), and mouse β-defensin-2 (22). Mammalian RNA and DNA in the form of immune complexes can act as endogenous ligands for TLR7 and TLR9, respectively (36, 37). Although heat shock proteins and HMGB1 have been reported to trigger both TLR2 and TLR4 (23, 24, 38–40), no endogenous ligand acting exclusively on TLR2 has been reported previously. We have shown that EDN is capable of inducing DC maturation, cytokine production, and activation of NF-κB and multiple MAPKs in DCs (Figs. 1 and 2). In addition, EDN induction of NF-κB activation in HEK293 cells is mediated by TLR2, and EDN-induced DC cytokine production is abrogated in TLR2$^{-/-}$ DCs, but not in TLR1$^{-/-}$,

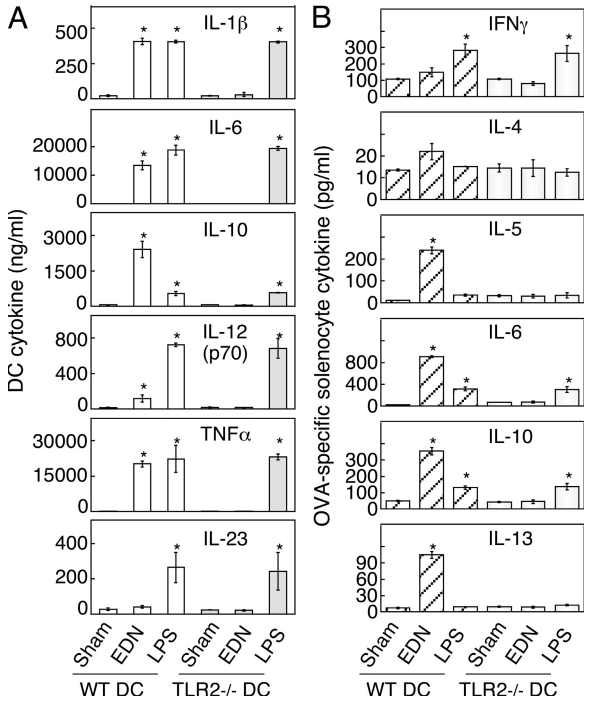

Figure 8. DCs treated with EDN promote Th2 immune response. DCs generated from the bone marrow progenitors of WT or TLR2 knockout mice were incubated with 100 μg/ml OVA for 24 h, and then cultured at 2×10^5/ml in the presence or absence of 1 μg/ml EDN or *E. coli* LPS for another 24 h. After harvesting supernatants for cytokine measurement, DCs were washed with PBS three times and suspended in PBS at 2.5×10^6/ml. C57BL/6 mice (three mice/group) were injected i.p. with 0.2 ml DC suspension on day 0, immunized i.p. with OVA (50 μg/0.2 ml PBS/mouse) on day 4, and killed on day 11 for the removal of spleens. Single splenocyte suspensions in complete RPMI 1640 were prepared from pooled spleens of each group and stimulated in vitro with 100 μg/ml OVA for 48 h for the measurement of OVA-specific cytokine production by splenocytes. (A) The production of cytokines in the supernatants of differently treated DCs was shown as the average (mean ± SD) of two independent experiments. (B) The generation of OVA-specific cytokines by splenocytes of various groups of mice was shown as the average (mean ± SD) of two independent experiments. *, $P < 0.05$ compared with sham-treated group (Mann-Whitney test).

$TLR4^{-/-}$, or $TLR6^{-/-}$ DCs (Figs. 4, 5, and 8). Furthermore, EDN enhancement of OVA-specific immune responses also relies on TLR2 (Figs. 7 and 8). Thus, EDN appears to be a TLR2-specific endogenous ligand with the capacity to enhance antigen-specific immune responses.

EDN enhancement of OVA-specific immune responses can be achieved by immunizing mice either with OVA in the presence of EDN (Figs. 6 and 7) or with EDN-activated OVA-loaded DCs (Fig. 8). The antigen-specific immune response selectively promoted by EDN is predominantly of the Th2 type, which is indicated by preferential promotion of antigen-specific IgG1 production in both the primary and secondary antibody responses (Figs. 6 and 7 C), as well as generation of T cells that are capable of secreting elevated levels of IL-5, IL-6, IL-10, and IL-13, but not of IFN-γ or TNF-α (Figs. 7 B and 8 B). This notion is in good agreement with data demonstrating that EDN, in comparison with *E. coli* LPS, induces much less IL-12p70 and considerably more IL-10 production by both human and mouse DCs (Figs. 1 D and 8 B). Conventional Th2 T cells, such as those generated in response to *Schistosoma* infection or DCs conditioned with *Schistosoma mansoni* egg antigen treatment, are characterized by simultaneous production of IL-4, IL-5, IL-10, and IL-13 (41, 42). In contrast, OVA-specific Th2 cells promoted by EDN differ from conventional Th2 cells in lacking production of IL-4 (Figs. 7 B and 8 B). It has also been documented that thymic stromal lymphopoietin can also condition DCs for the induction of Th2 cells that are distinct from conventional Th2 cells in producing IL-4, IL-5, IL-13, and TNF-α, but not IL-10 (43, 44). It thus appears that different stimulants can induce the generation of Th2 with a distinct cytokine profile. It remains to be determined why EDN leads to the generation of Th2 cells with this particular profile of cytokine production.

EDN stimulates DCs to generate an abundant amount of IL-6 and IL-1β (Figs. 1 and 8), and eosinophil can produce TGF-β (45). Because IL-6, IL-1β, and TGF-β are known to contribute to the differentiation of CD4 T cells into Th17 lineage (46), it is conceivable that eosinophils and/or EDN may promote the generation of antigen-specific Th17 cells. However, the splenocytes of mice immunized with EDN and OVA did not produce a significant amount of IL-17 (not depicted). Therefore, EDN could not promote the generation of Th17 cells, at least in the mouse models used in the present study.

EDN uses the TLR2–MyD88 signal transduction pathway for the activation of DCs and enhances OVA-specific Th2-polarized immune responses in a TLR2-dependent manner. Our data are consistent with several previous reports showing that DCs activated by TLR2 agonists exhibit more pronounced Erk activation, produce less IL-12p70, and preferentially induce antigen-specific Th2-polarized immune responses (19, 47–49). Is MyD88 or TLR2 crucial for Th2 polarization? Ample evidence indicates that signaling through MyD88 does not determine Th1/Th2 polarization. For example, MyD88 is not required for *Schistosoma mansoni* egg antigen–induced Th2 polarization (50). Moreover, although both TLR2 and TLR9 transduce signals exclusively through MyD88 (21, 28, 29), their agonists, PGN and synthetic stimulatory oligodeoxynucleotide, enhance Th2- and Th1-type immune responses, respectively (48). The TLR2 ligands, such as Pam3, PGN, and EDN, upon coadministration with antigen in the primary immunization, promote distinct Th2-type antigen-specific immune responses (19, 47–49, and this study), suggesting that signaling through TLR2 has the potential to promote Th2 polarization under certain conditions. However, TLR2 agonists, such as Pam3, lipoteichic acid, and macrophage-activating lipopeptide 2, are capable of suppressing previously established allergic Th2 responses (51–53). *Propionebacterium acnes*, albeit activating DCs via TLR2, not only induce a Pa-specific Th1 immune response (54), but also stimulate DCs to generate a negative signal for Th2 development in a MyD88-dependent manner (55). Compared with most other TLR2 agonists,

zymosan is reported to induce IL-10–producing antigen-specific Tr1 without generation of either Th1 or Th2 T cells (56). Thus, the effect of TLR2 activation on the polarization of Th responses may be influenced by multiple factors, such as the type of TLR2 ligands and the means and timing of administration, and it is still in need of further investigation.

The recruitment and activation of eosinophils are most often seen in tissues subject to parasitic infection and allergy (7, 41, 57). EDN is likely to be generated and present at sites of inflammation associated with such disorders. EDN is present in the serum and nasal lavage fluid of healthy individuals at ~20 ng/ml (58, 59), but its concentration in the nasal lavage fluid of patients with allergic rhinitis can reach as high as 880 ng/ml (60), suggesting that EDN in tissues subject to allergic reaction or parasitic infection can reach a concentration (100~1,000 ng/ml) necessary for the activation of DCs and enhancement of immune response described in the present study. Therefore, the capacity of EDN to activate DCs and to enhance a Th2-polarized immune response may provide a basis for the induction and/or maintenance of a Th2-polarized immune response during parasitic infection, allergy, or atopic reaction. However, a recent report showing that there is no deficit in the generation of IL-4 and IL-5 in eosinophil-ablated mice in response to helminth infection (61) suggests that eosinophil, and consequently eosinophil-generated EDN, may not be required for the generation of Th2-type immune response. How can this report be reconciled with data of the present study? Because EDN can also be produced by cells other than eosinophils, such as neutrophils, macrophages, and certain placental epithelial cells (7, 11, 62), it is possible for EDN to be present at sites of infection even in the absence of eosinophils. It is also possible that factors other than EDN provide the Th2-polarizing signal in the absence of eosinophils.

Alarmins are defined as endogenous mediators rapidly released by cells of the host innate immune system in response to infection and/or tissue injury, which possess the dual activities of recruiting and activating DCs and consequently are capable of enhancing antigen-specific adaptive immune responses (5, 6). EDN is an endogenous mediator rapidly released from eosinophils, neutrophils, and by activated macrophages (7, 11, 12, 62). In addition, EDN can selectively induce the recruitment of DCs to the site of inflammation (10). Furthermore, EDN has DC-activating activity and enhances antigen-specific immune response in vivo (this study). Thus, EDN can be considered a bona fide alarmin that favors an alternative type of Th2 immune response.

MATERIALS AND METHODS

Reagents and mice. Recombinant human and mouse GM-CSF, IL-4, SDF-1α, RANTES, and SLC were purchased from PeproTech. Recombinant EDN was generated and purified as described previously (11). The purity of this batch of EDN was analyzed by SDS-PAGE. The potential levels of contaminating LPS and PGN in the sample were measured using QCL-1000 Chromogenic LAL Assay kit (Cambrex) and SLP Reagent Set (Wako) according to the manufacturers' protocols. hAng was purchased from R&D Systems.

MyD88$^{-/-}$, TLR1$^{-/-}$, TLR2$^{-/-}$, and TLR6$^{-/-}$ mice generated by S. Akira (Osaka University, Osaka, Japan) (21, 25, 26, 63, 64) were backcrossed eight or more generations onto the C57BL/6 background, and then intercrossed to obtain the knockout genotypes. C57BL/6, C3H/HeN, and C3H/HeJ mice were obtained from Charles River Laboratories. All mice were kept under specific pathogen-free conditions, with water and food given ad libitum. All experiments with mice were performed in compliance with the principles and procedures outlined in the NIH Guide for the Care and Use of Animals and were approved by the NCI-Frederick Animal Care and Use Committee.

Cell isolation and purification. Human PBMCs were isolated by Ficoll density gradient centrifugation as described previously (65). Monocytes were purified (>95%) from human PBMCs with MACS CD14 monocyte isolation kit (Miltenyi Biotec). Human T cells were isolated from human PBMCs by using CD3 Enrichment Columns (R&D Systems). Mouse HPCs were isolated as reported previously (66) from the bone marrow of knockout and littermate-matched WT mice.

DC generation and treatment. Human and mouse DCs were generated as described previously (66). In brief, monocytes or mouse bone marrow progenitors were incubated at 2~5 × 10^5/ml in G4 medium (RPMI 1640 containing 10% FBS [Hyclone], 2 mM glutamine, 25 mM Hepes, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 ng/ml of human or mouse GM-CSF, and 50 ng/ml of human or mouse IL-4) at 37°C in a CO_2 (5%) incubator for 5~7 d to generate immature DCs (iDCs), with 50% of the culture medium replaced with prewarmed fresh G4 medium on days 3 and 5. Subsequently, iDCs were incubated in fresh G4 medium in the absence or presence of EDN, Pam3 (*N*-palmitoyl-S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-[R]-cys-[S]-serl-[S]-lys [reference 4] trihydrochloride; InvivoGen), FSL-1 (InvivoGen), or LPS (*E. coli* O55:B5; Sigma-Aldrich) at specified concentrations for 24~48 h in a CO_2 (5%) incubator before analyzing their phenotype and function. In some experiments, mouse DCs were pulsed with antigen by incubating with 100 μg/ml OVA for 24 h before treatment with EDN or *E. coli* LPS.

For the transient silencing of DC MyD88, monocyte-derived iDCs were transduced without or with gripNA^{MyD88} (5′-CCTGCAGCCAYDD-CGGGC-3′; Active Motif) at 10 nmol/10^6 cells using Chariot II (Active Motif) according to the manufacturer's instructions. After overnight incubation, both sham-transfected and gripNA^{MyD88}-transfected DCs were harvested, counted, and treated with EDN or IL-1α for 24 h before the collection of the culture supernatants.

Measurement of DC phenotype and function. Multiple cytokines in the culture supernatants of human DCs were measured using SearchLight microassay (Thermo Fisher Scientific). Mouse IL-6 and TNF-α in the supernatants of mouse DC culture were measured using ELISA kits (R&D Systems). DC migration was assessed using a 48-well microchemotaxis chamber assay, as described previously (67). The expression of surface molecules was determined by flow cytometry using a FACScan (Becton Dickinson) after staining DCs (5 × 10^5/sample) with FITC- or PE-conjugated mouse monoclonal antibodies (all from BD Biosciences) against human CD11c (IgG1, κ, clone B-Ly6), CD80 (IgG1, κ, clone L307.4), CD83 (IgG1, κ, clone HB15e), CD86 (IgG1, κ, clone 2331), or isotype-matched control antibodies. The capacity of DCs to stimulate the proliferation of allogeneic T cells was evaluated by mixed lymphocyte reaction (68) with minor modifications. In brief, purified allogeneic T cells (10^5/well) were cultured with different numbers of DCs treated in the absence (sham) or presence of EDN in a 96-well flat-bottom plate for 6 d at 37°C in humidified air with 5% CO_2. The proliferative response of T cells was examined by pulsing the culture with 0.5 μCi/well of [^{3}H]-TdR (New England Nuclear) for the last 18 h before harvesting. [^{3}H]-TdR incorporation was measured with a microbeta counter (Wallac).

Western blot. DCs starved in serum-free G4 medium for 6 h were incubated at 37°C in the absence or presence of 1 μg/ml EDN or LPS for a period of time as specified. Treated cells were lysed in SDS sample buffer (62.5 mM

Tris-HCl, pH 6.8, at 25°C, 2% wt/vol SDS, 10% glycerol, 50 mM dithiothretol, 0.01% bromophenol blue) at 10^7/ml and loaded (20 μl/lane) on a 4–12% NuPAGE Bis-Tris Gel using 1× NuPAGE MES SDS Running Buffer as the electrode buffer and using SeeBlue Plus2 (Invitrogen) as molecular size markers. After transfer onto a piece of Immobilon membrane (Millipore), I-κBα was detected by Western blotting using rabbit anti–I-κBα (Cell Signaling Technology) as the first antibody and horseradish peroxidase–conjugated anti–rabbit IgG (Cell Signaling Technology) as the second antibody. The blot was developed using ECL Plus Western Blotting Detection System (GE Healthcare) and exposed to a piece of BioMax x-ray film (Kodak). Subsequently, the same membrane was stripped and probed consecutively for activated MAPKs in the same manner using rabbit anti-phosphorylated JNK, anti-phosphorylated p38, and anti–phospho-p44/42 (all from Cell Signaling Technology) as the first antibodies.

Transfection and measurement of NF-κB activation. For the measurement of NF-κB activation, HEK293 cells (10^4/well) were transfected using the calcium phosphate technique for 3 h in 96-well plates, with 12 ng IgκB-luciferase, 12 ng pSV-β-galactosidase, 2.5 ng pcDNA-hTLR2 or pcDNA-hTLR3, a combination of 2.5 ng pcDNA-hTLR4 and 6 ng pEF-boss-hMD2, or control plasmids (22). 24 h after transfection, the cells were stimulated with the indicated concentrations of PGN (Sigma-Aldrich), polyI:C (Sigma-Aldrich), LPS, or EDN at 37°C for a 24-h lysis of cells for the measurement of firefly and *Renilla* luciferase activities using the DualLuciferase Reporter Assay System (Promega) and for β-galactosidase (Tropix) on a Lumat LB9501 (Berthold). Firefly luciferase activity of individual transfections was normalized against Renilla luciferase activity. Data reflect the luciferase relative luciferase unit (RLU) divided by the control β-galactosidase RLU.

Immunization and detection of OVA-specific IgG antibody, splenocyte proliferation, and cytokine production. 8-wk-old C57BL/6, $TLR2^{+/+}$, or $TLR^{-/-}$ mice (3~5 mice/group) were injected i.p. on day 1 with 0.2 ml PBS containing 50 μg OVA (Sigma-Aldrich) in the presence or absence of alum (Sigma-Aldrich), EDN, hAng, or LPS. On Day 14, all mice were booster immunized by i.p. injection of 0.2 ml PBS containing 50 μg OVA. Blood samples were taken on days 10 and 21 for the preparation of serum samples used for the measurement of anti-OVA antibody. On day 21, immunized mice were killed to remove spleens. Alternatively, 8-wk-old C57BL/6 mice (three mice/group) were injected i.p. with OVA-loaded DCs (5×10^6/0.2 ml PBS/mouse) that had been treated in the absence or presence of 1 μg/ml EDN or LPS for 24 h. Mice were immunized by i.p. injection of OVA (50 μg/0.2 ml PBS/mouse) on day 5 and killed for the removal of spleens.

Total and subclass OVA-specific IgG antibodies were measured by ELISA as described previously (67). For the detection of total anti-OVA IgG, mouse anti-OVA IgG antibody (AntibodyShop) was used as the standard, and the concentration of total OVA-specific IgG in the serum was presented as ng/ml. For the measurement of subclass anti-OVA antibody, rabbit anti–mouse IgG1, IgG2a, IgG2b, or IgG3 (BD Biosciences) was used as the secondary antibody, and the level of anti-OVA IgG1, IgG2a, IgG2b, or IgG3 was illustrated as the reciprocal logarithmic value of the highest dilution that gave an absorbance ≥0.1 above the negative control.

Spleens of each group of mice were collected and pooled to make single splenocyte suspension. OVA-specific splenocyte proliferation and/or cytokine production were measured as described previously with minor modifications (69). In brief, splenocytes (5×10^5/well) were seeded in triplicate in round-bottomed 96-well plates in complete RPMI 1640 medium (0.2 ml/well) and incubated in the presence or absence of the indicated concentration of OVA at 37°C in a CO_2 incubator for 60 h. The cells were pulsed with [^{3}H]-TdR (1 μCi/well) for the last 18 h for the measurement of splenocyte proliferation. Alternatively, pooled splenocytes of each group were cultured in complete RPMI 1640 in 24-well plates (5×10^6/1 ml/well) with the indicated concentrations of OVA for 48 h before the culture supernatants were harvested for the measurement of cytokines (SearchLight multiplex ELISA; Thermo Fisher Scientific).

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