NK-DC crosstalk controls the autopathogenic Th17 response through an innate IFN-γ–IL-27 axis

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IFN-γ is a pathogenic cytokine involved in inflammation. Paradoxically, its deficiency exacerbates experimental autoimmune encephalomyelitis, uveitis, and arthritis. Here, we demonstrate using IFN-$\gamma^{-/-}$ mice repleted with IFN-$\gamma^{+/+}$ NK cells that innate production of IFN-γ from NK cells is necessary and sufficient to trigger an endogenous regulatory circuit that limits autoimmunity. After immunization, DCs recruited IFN-γ–producing NK cells to the draining lymph node and interacted with them in a CXCR3-dependent fashion. The interaction caused DCs to produce IL-27, which in turn enhanced IFN-γ production by NK cells, forming a self-amplifying positive feedback loop. IL-10, produced by the interacting cells themselves, was able to limit this process. The NK-DC–dependent IL-27 inhibited development of the adaptive pathogenic IL-17 response and induced IL-10–producing Tr1-like cells, which ameliorated disease in an IL-10-dependent manner. Our data reveal that an early NK-DC interaction controls the adaptive Th17 response and limits tissue-specific autoimmunity through an innate IFN-γ–IL-27 axis.

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Abbreviations used: EAE, experimental autoimmune encephalomyelitis; EAU, experimental autoimmune uveitis; IRBP, interphotoreceptor retinoid-binding protein; Tr1, T regulatory type 1.

Autoreactive $CD4^+$ T cells have been linked to the pathogenesis of several tissue-specific autoimmune diseases, including multiple sclerosis, arthritis and uveitis. Until recently, Th1 cells and their signature cytokine, IFN-γ, were thought to be the main pathogenic mediators in these types of diseases because (i) autoimmune Th1 cells transfer disease in different animal models; (ii) the IFN-γ level is associated with disease severity in experimental models, including experimental autoimmune uveitis (EAU) and experimental autoimmune encephalomyelitis (EAE; Luger et al., 2008; Haak et al., 2009; Axtell et al., 2010), as well as in human diseases (Link, 1998; Takase et al., 2006); and (iii) transgenic expression of interferon (IFN)-γ in the tissue precipitates inflammation and causes pathology in various organs, directly linking IFN-γ to pathology (Geiger et al., 1994; Horwitz et al., 1997; Link, 1998; Egwuagu et al., 1999; Takase et al., 2006).

However, the notion that IFN-γ is solely a pathogenic cytokine is challenged by multiple studies demonstrating that interference with IFN-γ signaling, either by genetic deficiency or by antibodies that target IFN-γ or its receptor, exacerbates disease development (Caspi et al., 1994; Ferber et al., 1996; Jones et al., 1997; Matthys et al., 1998). The discovery that Th17 cells are dominant contributors to tissue damage in the models listed above, and that the development of these effector T cells may be hindered by IFN-γ, led some to propose that IFN-γ was protective because it inhibited the (even more pathogenic) Th17 response.

EAU is induced in mice by immunization with the retinal autoantigen interphotoreceptor retinoid-binding protein (IRBP) in CFA and represents an accepted rodent model of human autoimmune uveitis. It is one of a group of autoimmune disease models in which both the protective and the pathogenic effects of IFN-γ can be observed. On the one hand, IFN-γ–producing

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Th1 cells adoptively transfer disease and locally produced IFN-γ elicits inflammatory pathology in retinal tissue (Geiger et al., 1994; Egwuagu et al., 1999). On the other hand, however, EAU-prone IFN-$\gamma^{-/-}$ (GKO) mice exhibit exacerbated disease accompanied by an elevated Th17 response (Jones et al., 1997; Luger et al., 2008). In the course of studies designed to unravel the pathogenic from protective effects of IFN-γ, we noted that acute elicitation of IFN-γ by systemic injections of IL-12 or by stimulation of iNKT cells at the time of uveitogenic immunization, inhibits EAU pathology and blunts development of adaptive immunity (Th17 as well as Th1) to IRBP (Tarrant et al., 1999; Grajewski et al., 2008). Only IFN-γ produced during the first week after disease induction, but not later, had an ameliorating effect. These observations suggested that the protective IFN-γ could be derived from cells within the early/innate, rather than the late/adaptive, compartment. However, the cellular source of IFN-γ that limits autoimmunity in the absence of acute pharmacological stimuli has not been identified.

Natural killer (NK) cells are innate effector cells that kill target cells and produce proinflammatory cytokines, including IFN-γ. Recent studies indicate that NK cells can also affect adaptive immunity. In vitro studies documented that interaction between NK cells and DCs leads to activation and maturation of both types of cells and in production of cytokines, including IFN-γ and TNF by NK cells and IL-12, 15 and 18 by DC (Walzer et al., 2005; Deguine and Bousso, 2013). In vivo, NK cells can modulate T cell priming involving antigen-bearing DCs by providing an early source of IFN-γ that promotes the Th1 effector response (Martín-Fontecha et al., 2004). We hypothesized that innate IFN-γ produced by NK cells might explain the IFN-γ-dependent inhibitory effects on development of the adaptive effector response in uveitis, thus explaining the ameliorating effects of early systemic elicitation of IFN-γ on disease.

Similar to other autoimmune disease models induced by immunization with a tissue antigen in CFA, EAU is strongly Th17 driven, and IFN-γ knockout (GKO) mice develop exacerbated disease (Luger et al., 2008). By using GKO mice repleted with IFN-γ–sufficient NK cells, we show that IFN-γ from NK cells is necessary to ameliorate disease development and to suppress the adaptive Th17 response, and that IFN-γ derived only from NK cells is sufficient for this activity. Our data show that after uveitogenic immunization, both NK cells and DCs are recruited to the draining LNs and that recruitment of NK cells is dependent on DCs. IFN-γ from NK cells rescues the production of CXCR3 ligands by DCs in GKO mice and expression of CXCR3 by NK cells is important for them to migrate to the draining LNs. Intravital two-photon microscopy revealed that NK cells interact with DCs in the LN in a CXCR3-dependent manner. Mechanistic studies show that this interaction results in production of IFN-γ by NK cells and IL-27 by DCs, with each cytokine promoting production of each other in a positive feedback loop, resulting in inhibited development of adaptive Th17 cells and enhanced development of IL-10–producing T regulatory type 1 (Tr1)-like cells. Our data thus identify an innate IFN-γ–IL-27 axis brought about by NK–DC interaction, which acts to control autoimmunity.

RESULTS

IFN-γ from NK cells ameliorates severe EAU in GKO mice

Our previous data suggested that early production of IFN-γ from innate cellular sources protected from EAU by inhibiting development of adaptive immunity to the uveitis target antigen IRBP (Tarrant et al., 1999; Grajewski et al., 2008). Although, when activated with α-C-Gal-Cer, iNKT cells could generate sufficient IFN-γ to provide the protective effect (Grajewski et al., 2008), the cellular sources of IFN-γ in the absence of pharmacological activation of iNKT cells were not identified. several cell types are potentially able to produce early IFN-γ upon activation, including NK cells, γδ T cells, and innate-like $CD8^+$ T cells (Skeen and Ziegler, 1995; Martín-Fontecha et al., 2004; Kastenmüller et al., 2012). We therefore examined IFN-γ production by cells isolated from the draining LN (DLN) at early time points after uveitogenic immunization after stimulation with high and low doses of PMA/Ionomycin. Among the cell populations purified from the DLN 3 and 5 d after immunization, $CD3^-NK1.1^+$ NK cells were the first and by far the largest IFN-γ producers, followed by $CD8^+$ T cells (Fig. 1 A). No IFN-γ was detected in γδ T cells (unpublished data). We next investigated the functional importance of NK and $CD8^+$ T cells in regulating disease, by depleting them in vivo with anti-NK1.1 or anti-CD8 antibodies, respectively. Efficiency of depletion was confirmed by flow cytometric analysis of randomly selected animals. Although NK cell depletion exacerbated EAU, $CD8^+$ T cell depletion had no effect on disease severity (Fig. 1 B).

These data suggested that NK cells are a necessary source of the IFN-γ that can ameliorate EAU. To investigate the effects of NK cell–derived IFN-γ more directly, we devised a reductionist model consisting of IFN-$\gamma^{-/-}$ (GKO) mice depleted of endogenous NK cells using a minimal dose of depleting anti-NK1.1 antibody and repleted with IFN-γ–sufficient WT NK cells (or with GKO NK cells as a control), and concurrently immunized for EAU. In this model, the only source of IFN-γ is from the NK cells. The minimal dose of anti-NK1.1 antibody (50 µg/mouse) depletes >90% of host NK cells without depleting the subsequently transferred donor NK cells, as confirmed by quantitating CD45.1 NK cells in $CD45.2^+$ recipients (Fig. 2 A). Depletion of host NK cells resulted in a higher proportion of donor-to-host NK cells being recruited to the DLN (Fig. 2 A).

Typically, unmodified GKO mice develop severe disease and exhibit more IL-17–producing cells in their ocular inflammatory infiltrate than their WT littermates (Luger et al., 2008). GKO mice repleted with WT NK cells had significantly attenuated EAU compared with control GKO mice repleted with GKO NK cells (Fig. 2 B). In contrast, IFN-γ–sufficient T cells ($CD4^+CD62L^+$) were unable to suppress EAU development (unpublished data). Direct ex vivo analysis of eye-infiltrating cells revealed markedly fewer IL-17A– and

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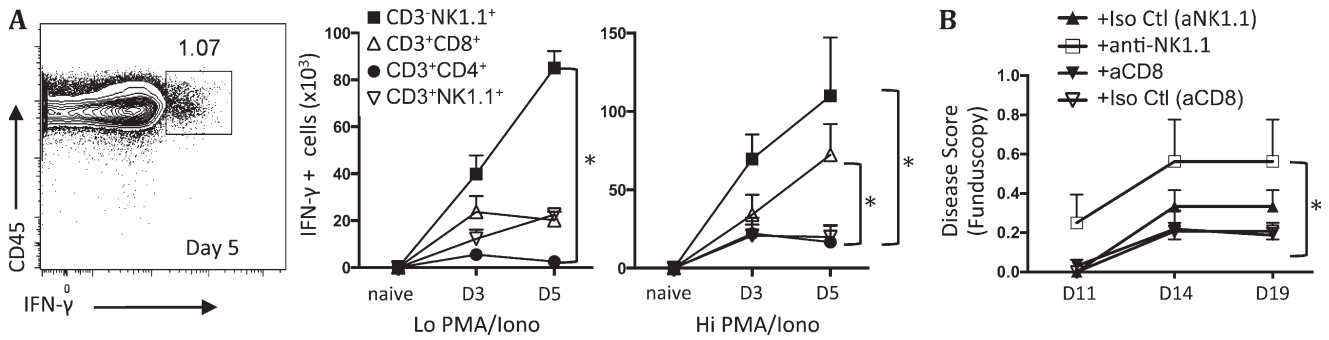

Figure 1. NK cells are the major source of innate IFN-γ in EAU. (A) DLN of WT mice immunized for EAU were harvested at the specified time and restimulated with low (10 ng/1 µg) or high dose (200 ng/5 µg) PMA/Ionomycin. Intracellular IFN-γ was analyzed in cells expressing NK1.1, CD3, CD4, CD8, and γδ TCR. Data are combined from two experiments with five mice at each time point. (B) WT mice were immunized for EAU and some animals received anti-NK1.1 or anti-CD8 to deplete NK or CD8 T cells. Disease was monitored at the indicated time points by fundus examination (n = 4–6 mice per group). Data are combined from two experiments totaling at least 15 mice per point. *, $P < 0.05$; linear regression in A and B.

GM-CSF–producing CD4⁺ T cells in recipients of WT NK cells (Fig. 2, C and D). Adoptive transfer of WT NK cells also suppressed EAU development in the GKO recipients without NK cell depletion, albeit to a lesser extent when compared with NK cell–depleted animals (Fig. 2 B). Although these cells were able to actively produce IFN-γ (Fig. 2 E). The lower protection efficiency in recipients whose endogenous NK cells had not been depleted may have been caused by a decrease in the ratio of donor-to-host NK cells in the draining LNs (Fig. 2 A). These data were consistent with the hypothesis that innate IFN-γ from NK cells was sufficient to attenuate tissue pathology and infiltration of pathogenic effector T cells. However, it was also possible that some other property of the NK cells from an IFN-γ –ufficient host rather than their IFN-γ production per se was responsible for their ability to ameliorate disease after transfer. We therefore treated the recipient GKO mice with an IFN-γ neutralizing antibody. Under these conditions, WT NK cells were not able to ameliorate EAU in GKO mice, showing that IFN-γ elaborated by the NK cells was responsible for the protective effect (Fig. 2 F). Transfer of WT NK cells 9 d after immunization, a time point before clinical disease onset but after the adaptive response has already developed, failed to affect disease (Fig. 2 G). This is in accord with our previous results, where only early, but not late, elicitation of IFN-γ could ameliorate disease (Tarrant et al., 1999; Grajewski et al., 2008), and suggests that IFN-γ from NK cells protects by inhibiting the priming, rather than the function, of adaptive effector T cells.

To address the question of whether NK-produced IFN-γ was responsible for the protective effect in IFN-γ–sufficient animals, we elicited EAU in IL-15 KO mice, which are deficient in NK cells (Kennedy et al., 2000) and infused them with two doses of WT or GKO NK cells 3 d apart (repeated infusion was done to compensate for the limited survival time of NK cells in an IL-15–deficient environment; Koka et al., 2003). IFN-γ–sufficient NK cells ameliorated EAU in IL-15 KO mice (Fig. 2 H). These data support the interpretation that IFN-γ from NK cells is functionally relevant in animals that have other cellular sources of IFN-γ. Although the NK cell deficiency is not complete in IL-15 KO mice, and there remains a theoretical possibility that such cells might expand in response to immunization and IFN-γ–sufficient donor NK reconstitution, this is made unlikely by lack of functional effect after reconstitution with IFN-γ–deficient donor NK.

After immunization, DCs recruit NK cells to the draining LN

After immunization, DCs activated with the innate stimuli from CFA will migrate to the draining LNs, which are the inguinal LNs in our EAU model. DCs produce various cytokines, including IL-12 and IL-23, which respectively contribute to Th1 or Th17 fate choice after antigen presentation to and activation of CD4⁺ T cells. NK cells are also involved in shaping T cell activation/polarization by interacting with DCs (Moretta, 2002). Martin-Fontecha et al. (2004) demonstrated that DCs, matured in vitro with LPS and injected into footpads of mice, migrated to the draining LNs, and recruited NK cells. NK cells in turn produced IFN-γ that promoted Th1 priming. We therefore wished to determine whether recruitment of NK cells to the draining LNs would occur when endogenous DCs are activated at the site of immunization with auto-Ag in CFA.

In WT C57BL/6 mice immunized with a uveitogenic regimen of IRBP in CFA, both CD3⁻NK1.1⁺ NK cells and CD169⁻CD11c⁺ DCs were recruited to the draining LNs within 5 d of immunization (Fig. 3 A). To investigate whether NK cell recruitment is dependent on DCs, we used CD11c.DTR mice, in which DC can be depleted by an injection of diphtheria toxin (DT; Jung et al., 2002). CD11c.DTR mice immunized for EAU and depleted of DCs 3 d after immunization recruited significantly fewer NK cells to the draining LNs compared with the nondepleted control (Fig. 3 B). These data indicate that both DCs and NK cells are recruited to the draining LNs after immunization, and that the recruitment of NK cells to the LN is, at least in part, dependent on CD11c⁺ DCs.

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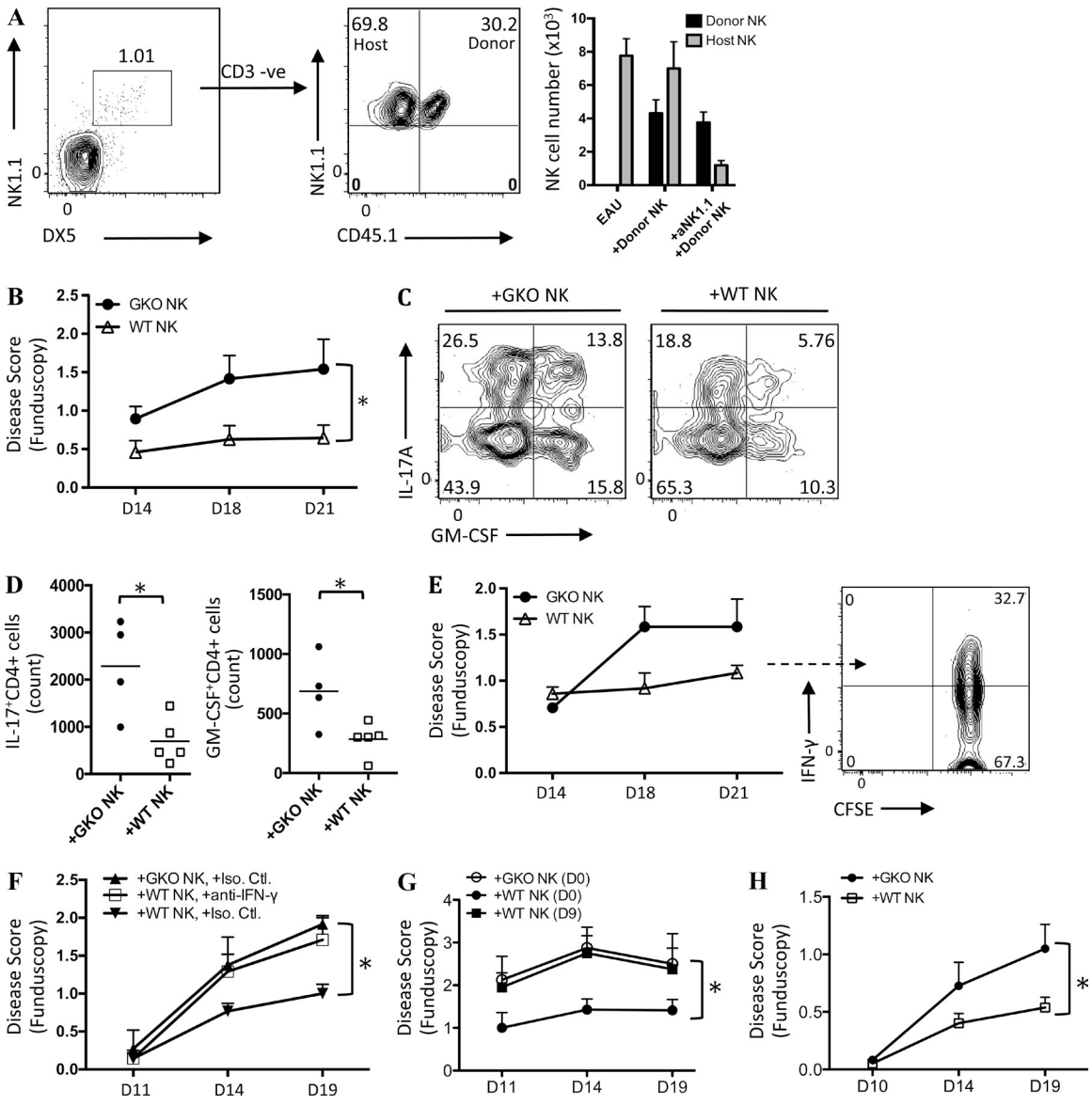

Figure 2. NK cells are necessary and sufficient to control EAU through production of IFN-γ. (A) CD45.2 WT recipients were or were not treated with anti–NK1.1. After 2 d, mice were challenged for EAU and repleted with CD45.1 WT NK cells. The gating strategy is shown in A and the numbers of CD45.2 (host) and CD45.1 (donor) NK cells were determined from the draining LNs 5 d after immunization (A). (B–D) GKO mice were depleted of endogenous NK cells by anti-NK1.1 antibody. 2 d later, mice were repleted with WT or GKO NK cells and immunized for EAU. (B) Disease was monitored at the indicated times. (C and D) Expression of IL-17A and GM-CSF in eye-infiltrating CD4⁺ cells of mice in B, as determined by intracellular staining on day 21. (E) GKO mice were immunized for EAU and WT or GKO donor NK cells were infused on the same day. Disease was monitored at the indicated times. IFN-γ expression in the draining LNs was determined in CFSE-labeled donor cells by intracellular cytokine staining on d 5. (F) Mice were given IFN-γ–neutralizing antibody or isotype control. (G) Mice received WT or GKO NK cells on day 0 or day 9 after immunization. (H) IL-15 KO mice received WT or GKO NK cells on day 0 after immunization. Data are combined from 2 (E–H) or 3 (B and C) independent experiments totaling at least 12 mice per point, or are representative of 2 independent experiments with at least 3 mice per group (A and D). *, $P < 0.05$; (B and F–H) linear regression or (D) Mann-Whitney *U* test.

CXCR3 is required for NK cells to migrate to draining LNs and to suppress EAU

In the GKO model, only repletion with IFN-γ–sufficient, but not with IFN-γ–deficient, NK cells was able to down-regulate disease (Fig. 2 B), implicating both NK cells and IFN-γ in amelioration of disease. Expression of the chemokine receptor CXCR3, as well as its ligands CXCL9, CXCL10, and CXCL11, is controlled by IFN-γ and recruitment of NK cells to LN of WT mice by in vitro-activated DC or in viral infection is CXCR3-dependent (Martín-Fontecha et al., 2004; Pak-Wittel et al., 2013). We therefore examined the ability of DCs in draining LNs of EAU-immunized GKO recipients repleted with IFN-γ–sufficient or –deficient NK cells to produce CXCR3 ligands, and (ii) the need for CXCR3 expression on

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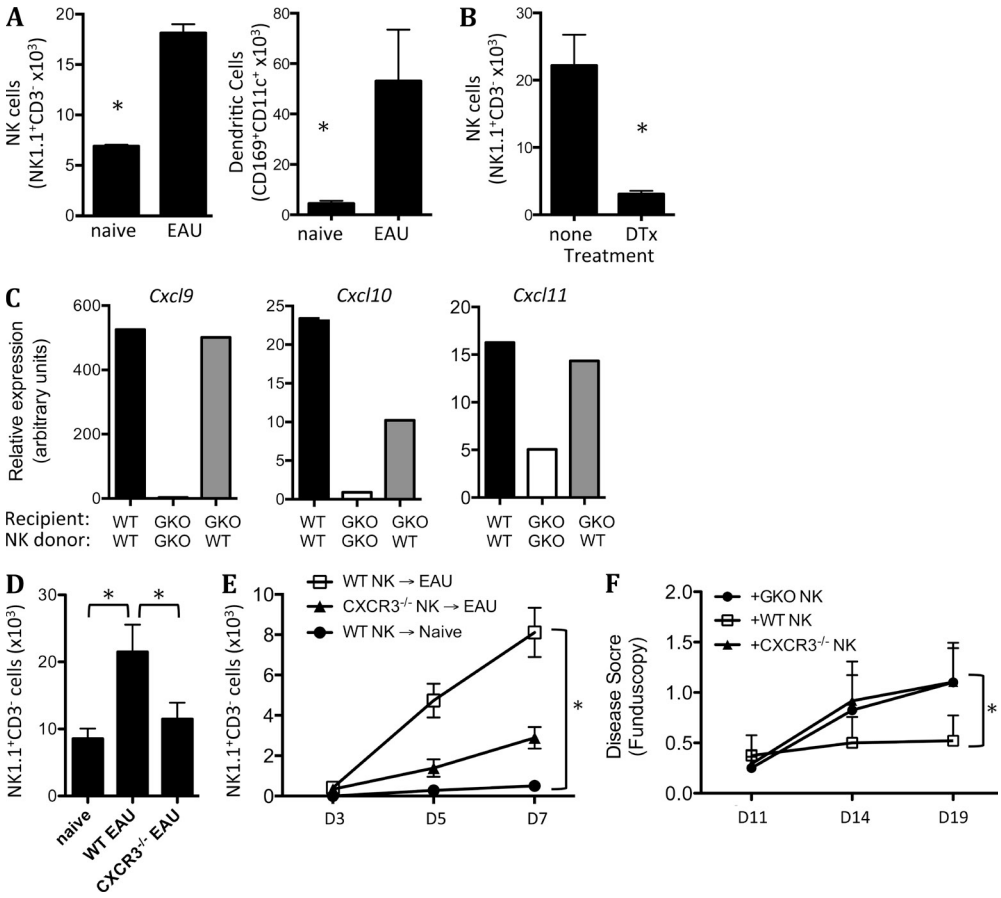

Figure 3. CXCR3⁺ NK cells are recruited by DC to draining LNs and control disease induction. (A) EAU was induced in C57BL/6 mice. The total number of NK cells and DCs in draining lymph nodes was determined at day 5. (B) CD11c⁺ DCs were depleted by DTX injection in CD11c.DTR mice on day 3. NK cells in draining LNs were enumerated on day 5. Data in A and B are the mean ± SEM from at least two combined experiments, totaling at least eight mice per group. *, $P < 0.05$, Student's *t* test. (C) WT or GKO NK cells were infused into EAU-immunized GKO mice. CD11c⁺ DCs were sorted from draining LNs on day 7 for gene expression analysis. (D) EAU was induced in WT and CXCR3$^{-/-}$ mice. NK cells in draining LNs were enumerated on D5. (E) CD45.2⁺ CXCR3$^{+/+}$ or CXCR3$^{-/-}$ NK cells were isolated and adoptively transferred to EAU immunized CD45.1 C57BL/6 mice. The number of CD45.2 NK cells in draining LNs was determined by flow cytometry at the indicated time points. (F) GKO mice depleted of NK mice were repleted after 2 d with WT, GKO, or CXCR3$^{-/-}$ NK cells and immunized for EAU. Data are representative of 2 independent experiments with 3 (A, B, and D) or 4–10 (C, E, and F) mice per group. *, $P < 0.05$; (D) 1-way ANOVA or (E and F) linear regression.

NK cells to be recruited to the draining LN of mice immunized for uveitis with IRBP/CFA.

Quantitative PCR for CXCR3 ligands performed on DCs isolated 7 d after immunization from LN of control WT mice repleted with autologous NK cells expressed ample message for CXCL9, CXCL10 and CXCL11 (note that CXCL11 protein is not produced by C57BL/6 mice due to a frame-shift mutation; Sierro et al., 2007). In contrast, DC from GKO recipients of autologous NK cells produced little or no message for CXCR3 ligands. Notably, repletion of GKO mice with IFN-γ–sufficient NK cells from WT donors restored the ability of GKO DC to express CXCR3 ligands (Fig. 3 C).

To investigate dependence of NK cell recruitment on CXCR3 expression, we performed two types of experiments: first, we quantitated NK cells in the draining LNs of WT and CXCR3$^{-/-}$ C57BL/6 mice after uveitogenic immunization with IRBP/CFA. Recruitment of NK cells in CXCR3$^{-/-}$ mice immunized for uveitis was significantly reduced, as judged by the total number of NK1.1⁺CD3⁻ cells present in the draining LNs on day 5 after immunization (Fig. 3 D). Second, we adoptively transferred purified NK cells from CD45.2⁺ WT or CXCR3$^{-/-}$ donors intravenously into CD45.1⁺ WT recipients and quantitated donor-type (CD45.2⁺) cells recruited to the draining LNs at the indicated time points after immunization. CXCR3$^{+/+}$ NK cells were recruited into the draining LN much more efficiently than CXCR3$^{-/-}$ NK cells (Fig. 3 E).

Finally, we investigated whether expression of CXCR3 by NK cells is functionally important for suppression of EAU in GKO mice. GKO mice were immunized for EAU and received WT or CXCR3$^{-/-}$ NK cells. Disease scores on day 21 after immunization revealed that CXCR3$^{-/-}$ NK cells (despite

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being IFN-γ sufficient) were just as ineffective in suppressing EAU as GKO NK cells (Fig. 3 F), suggesting that recruitment of NK cells to the draining LNs is necessary for them to control autoimmunity.

NK cells recruited to the LN interact with DC in a CXCR3-dependent fashion

Although CXCR3 is important for NK cell recruitment to the draining LNs (Fig. 3, D and E) as had also been reported by others (Martín-Fontecha et al., 2004; Pak-Wittel et al., 2013) little is known about its role in NK-DC interaction after they arrive. It is possible that CXCR3 might also play a role in guiding NK to relevant DCs in DLNs, thus affecting Th17 priming. If chemokine production guided CXCR3$^+$ NK cells to the most activated DCs in the LN, we might expect to see a CXCR3 dependence to effective interaction between the NK cells and DCs. We therefore performed intravital imaging to investigate whether CXCR3 affected cellular interactions between NK cells and DCs in the draining LNs of EAU-challenged mice. WT NK cells sorted from actin-eGFP reporter mice (green) and CMTMR-labeled NK cells sorted from CXCR3$^{-/-}$ mice (red) were co-injected intravenously at a 1:1 ratio into EAU-immunized CD11c^{YFP} recipients. After 24 h, the draining (inguinal) LNs of the recipients were exposed surgically and two-photon intravital imaging was performed (Video 1). Expression of CXCR3 by WT NK cells resulted in a greater fraction of cells interacting productively with CD11c$^+$ DC as compared with CXCR3$^-$ NK cells, based on changes in NK cell velocity upon contact with the DC (Fig. 4 A). Other measures of NK–DC dynamics agreed with this result, with WT NK cells showing lower cellular velocities (Fig. 4 B), higher arrest coefficients (Fig. 4 C) and longer interaction times (Fig. 4 D) as compared with CXCR3$^-$ NK cells. These findings all indicate a significant role for CXCR3 in facilitating NK–DC interactions. The lack of CXCR3 expression by NK cells may also alter the recruitment NK cells to the LN and potentially represents a selection bias with respect to the cells analyzed in this manner. However, this does not alter the conclusion reached from the data presented here that NK–DC interactions are significantly reduced in the absence of CXCR3 and that productive engagement and promotion of stable of NK–DC interactions is dependent on CXCR3 expression.

IFN-γ produced by NK cells causes DCs to produce IL-27 for disease suppression

In view of previous studies showing that IFN-γ can stimulate DCs to produce IL-27, and that IL-27 can inhibit development of the Th17 response (Murugaiyan et al., 2010; Hunter and Kastelein, 2012), we decided to examine whether, as a result of the interaction between IFN-γ–producing NK cells and DC, the latter would produce IL-27. Toward that end, we immunized GKO mice repleted with WT or GKO NK cells for EAU. After 5 d, we harvested their draining LNs and determined the IL-27 production level from CD11c$^+$ DCs by intracellular staining. DCs from mice that received WT

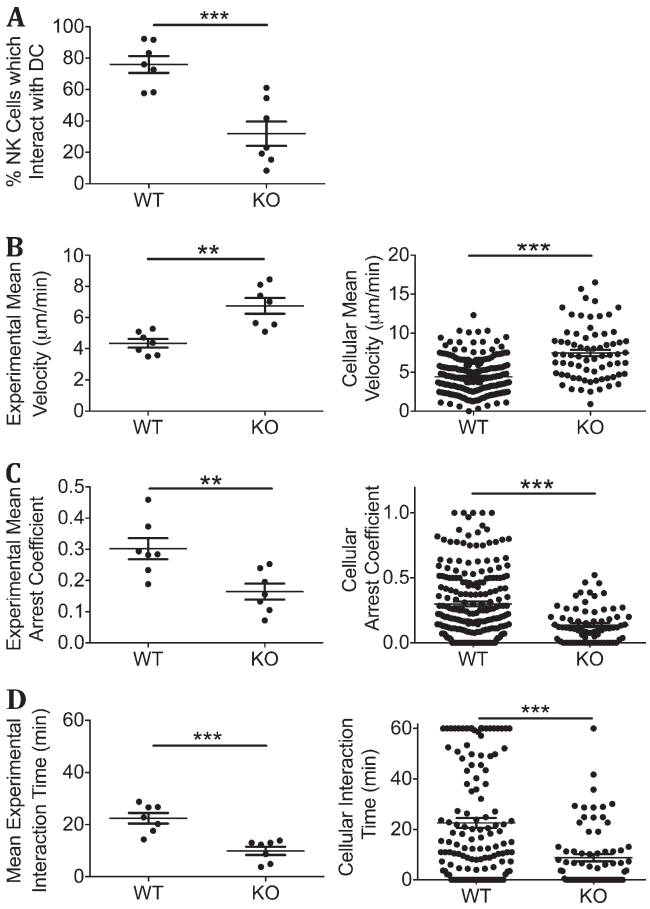

Figure 4. NK cells interact with DCs in draining LNs in a CXCR3-dependent fashion. GFP-CXCR3$^{+/+}$ (WT) and CMTMR-labeled CXCR3$^{-/-}$ (KO) NK cells were infused i.v., and cellular interactions in the draining LNs were imaged by 2P-IVM 24 h after transfer during 1 h. (A) Interaction incidence; (B) experimental velocities and individual cellular mean velocity distribution; (C) mean experimental arrest coefficients and cellular arrest coefficient distribution; (D) mean experimental interaction and individual interaction times between WT or CXCR3$^{-/-}$ NK cells and CD11c$^+$ DCs. Data represent mean of individual animal (n = 7) or are pooled from 4 separate experiments. **, P < 0.01; ***, P < 0.001, Student's t test.

NK cells had a higher expression of the *IL27p28* gene, which encodes the α subunit of IL-27, than DCs from recipients of GKO NK cells, and produced more IL-27p28 (Fig. 5, A and B). Finally, depletion of IL-27 by anti–IL-27 antibody abolished the protective effect of IFN-γ–sufficient NK cells in EAU-immunized GKO mice (Fig. 5 C). In the aggregate, these in vivo data support the conclusion that IFN-γ from NK cells induces IL-27 production from DCs, which diminishes the Th17 inflammatory response and ameliorates EAU in GKO mice.

NK–DC interaction elicits a positive feedback loop for IFN-γ and IL-27 production

In the draining LNs where NK cells and DCs interact, there are also many other cells and cytokines that can affect their interaction and the cytokines that are produced as a result. To

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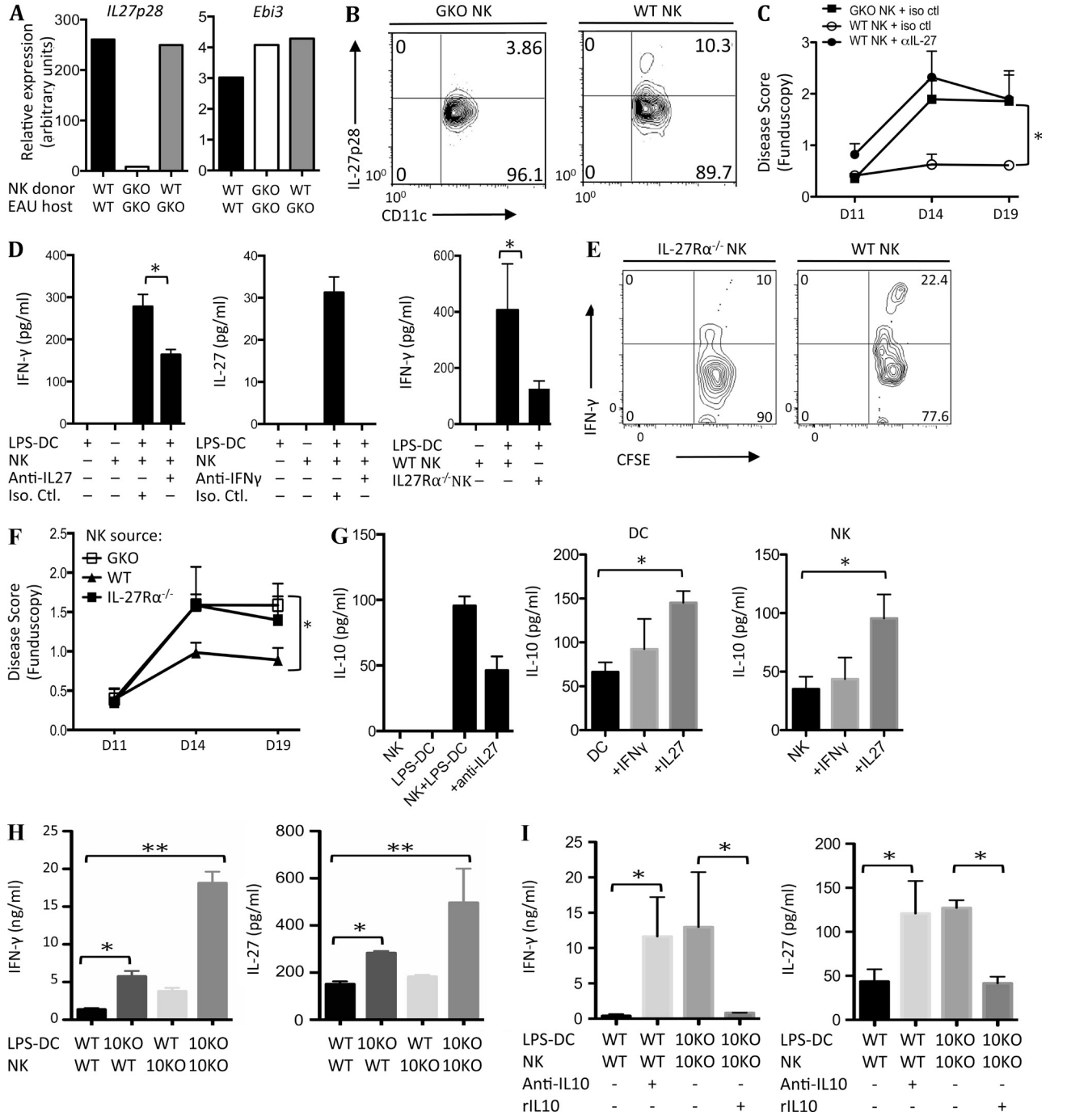

Figure 5. Interaction between NK cells and DC induces an innate IFN–γ–IL–27 amplification loop that limits disease and is controlled by IL–10. (A–C) GKO mice depleted of NK cells and repleted with autologous or WT NK cells were immunized for EAU. (A) *Il27* gene expression by RT PCR and (B) IL-27 protein expression by intracellular staining were determined in CD11c$^+$ DCs isolated from draining LNs on d 5. (C) GKO mice repleted with autologous or WT NK cells were immunized for EAU and treated with anti-IL27 or isotype control antibody. Disease was monitored by funduscopy. (D) DCs from WT mice were incubated with LPS overnight and washed. The LPS-DCs were then co-cultured with WT NK cells in the presence of anti–IL-27, anti–IFN-γ, or isotype control. IL-27 and IFN-γ were quantitated by ELISA in 48 h supernatants. (E) CFSE-labeled WT or IL-27rα$^{-/-}$ NK cells were transferred to the GKO mice at the day of immunization. Their IFN-γ expression in the draining LNs was determined by intracellular staining on day 5. (F) GKO mice were repleted with WT, GKO, or IL27Ra$^{-/-}$ NK cells and were immunized for EAU. A, B, D, and E are representative of at least two independent experiments. Data in C and F are combined from two independent experiments with total of at least seven mice per group (G) IL-10 was measured in co-culture supernatant of LPS-DCs and NK cells, prepared as in D, or NK cells and DCs stimulated with IFN-γ or IL-27. IL-10 was measured in 48-h culture supernatants. (H and I) IL-10–sufficient or –deficient LPS-DCs and NK cells were co-cultured as in D in all possible permutations. Data in G–I are representative of at least three independent experiments. *, $P < 0.05$; **, $P < 0.01$, by linear regression (D–F).

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eliminate such exogenous influences, we co-cultured sorted NK cells with sorted and LPS-matured DCs and examined 48 h co-culture supernatants for IFN-γ and IL-27. Each cell type by itself did not produce detectable levels of either cytokine, but co-culture supernatants contained high levels of both IFN-γ and IL-27 (Fig. 5 D). It should be pointed out that, although formally we cannot distinguish which cytokine was secreted by which cell type into the co-culture supernatants, current knowledge supports the contention that IFN-γ is a product of NK cells and IL-27 is a product of DCs. Notably, inclusion in the co-culture of neutralizing antibodies to IL-27 resulted in significantly diminished IFN-γ production, and neutralization of IFN-γ completely abrogated production of IL-27. Conversely, sorted NK cells from IL-27Rα$^{-/-}$ NK cells co-cultured with WT DCs produced less IFN-γ than WT NK cells (Fig. 5 D). These data indicate that IFN-γ and IL-27 promote secretion of each other and suggest the existence of a positive feedback loop between NK cells and DCs for IFN-γ and IL-27 production.

To examine whether interruption of this putative amplification loop would have functional consequences in vivo, we transferred CFSE-labeled WT or IL-27Rα$^{-/-}$ NK cells, which are unable to respond to IL-27, to EAU-immunized GKO mice and determined their IFN-γ production in the draining LNs. As supported by our in vitro data (Fig. 5 C), IL-27Rα$^{-/-}$ NK cells produced less IFN-γ compared with WT NK cells, suggesting that NK cells required IL-27 to boost their IFN-γ production in the draining LNs (Fig. 5 E). Moreover, in contrast to WT NK cells, IL-27ra$^{-/-}$ NK cells were unable to dampen development of disease (Fig. 5 F). This result demonstrates that NK cells must be able to respond to IL-27 to produce sufficient IFN-γ for ameliorating EAU and supports the importance of this positive feedback mechanism in regulation of autoimmunity.

An uncontrolled positive feedback loop has the potential to cause immune imbalance, or even outright pathology. We therefore investigated how this mutual amplification loop between NK cells and DCs for IFN-γ and IL-27 production can be regulated. IL-10 can be produced by NK cells and DCs upon activation (Mehrotra et al., 1998; Akbari et al., 2001; Corinti et al., 2001; Laroni et al., 2011). In the co-cultures of LPS–DC and NK cells, not only IFN-γ and IL-27 but also IL-10 was secreted into the supernatant, and its levels were reduced by neutralization of IL-27 (Fig. 5 G). Addition of IL-27, but not of IFN-γ, to NK or DC cultures was able to stimulate IL-10 production from both (Fig. 5 G, middle and right), indicating that induction of IL-10 is a direct effect of IL-27, whereas IFN-γ exerts its effect by stimulating IL-27 production. The notion that this IL-10 was produced by both cell types, and was functionally important in dampening the IFN-γ–IL-27 positive feedback loop, is supported by the finding that IL-10 deficiency in NK cells and DCs augmented levels of both IFN-γ and IL-27 (Fig. 5 H). By far, the greatest increase occurred when both NK and DCs were IL-10$^{-/-}$, whereas IL-10 deficiency in only one cell type had a partial effect. The functional importance of IL-10 in dampening the IFN-γ–IL-27 feedback loop was further confirmed by blocking IL-10 in the WT NK–DC co-cultures or repleting IL-10 in IL-10 KO NK–DC co-cultures. Whereas blocking IL-10 enhanced the production of IFN-γ and IL-27, its addition suppressed their production (Fig. 5 I). These data indicate that NK and DC can regulate their own IFN-γ–IL-27 positive feedback loop by producing IL-10.

The NK–DC driven IFN-γ–IL-27 axis induces generation of Tr1-like cells that suppress EAU in an IL-10–dependent manner

In Fig. 5 (A and B), we showed that IFN-γ from NK cells is necessary and sufficient to induce production of IL-27 from DCs of GKO mice. IL-27 was reported to suppress the Th17 response in part by promoting IL-10–producing Tr1 cells (Awasthi et al., 2007; Fitzgerald et al., 2007). We therefore looked for Tr1-like cells in LNs of GKO mice repleted with IFN-γ–sufficient NK cells. Direct ex vivo analysis of draining LNs cells from EAU-immunized GKO mice that had been repleted with WT or GKO NK cells revealed that recipients of WT NK cells had an increased number of IL-10/Tbet double-positive Tr1-like CD4 T cells (Fig. 6 A). When isolated and analyzed by RT-PCR, these cells displayed a gene expression profile consistent with the one described for Tr1 cells, with higher gene expression for IL-10, IL-21, Tbet, Ahr, and cMaf (Apetoh et al., 2010; Gandhi et al., 2010; Fig. 6 B). In contrast, numbers of Foxp3$^+$ T reg cells in the DLNs did not change (Fig. 6 C).

To investigate requirements for induction of Tr1-like cells through the NK–DC driven IFN-γ–IL-27 axis, we resorted again to co-cultures of NK cells with LPS-matured DC, similar to Fig. 4 (D and G). After allowing the NK cells and DCs to interact, we added naive CD62L$^+$ CD4$^+$ T cells and anti-CD3 to the cultures. 4 d later, T cells from co-cultures of DC + WT NK produced more IL-10 than T cells from control co-cultures of DC + GKO NK cells (Fig. 7 A), indicating that IFN-γ from NK cells is required for T cells to produce IL-10 in this system. Notably, this IL-10 production by T cells was also dependent on IL-27 signaling, as IL-27Rα$^{-/-}$ T cells produced significantly less IL-10 (Fig. 7 B). Analysis of the gene expression profile confirmed that the T cells acquired a Tr1-like phenotype, with higher gene expression for *Il10*, *Il21*, *Tbx21*, *cMaf*, and *Ahr*, but not Foxp3 (Fig. 7 C).

Finally, to examine whether the Tr1-like cells induced as a result of the milieu created by interaction of NK cells and DC had in vivo significance in terms of disease suppression, Tr1-like cells generated in the co-cultures were sorted and transferred to GKO recipients that had been immunized for EAU on day 0 or on day 9 relative to immunization. The data showed that recipients of cells sorted from co-cultures of NK with DC ameliorated the disease development, but T cells co-cultured with DC alone, without NK cells, were unable to do so (Fig. 7, D and E). Notably, suppression was IL-10-dependent, as IL-10$^{-/-}$ T cells isolated from parallel T–NK–DC cultures failed to significantly ameliorate disease (Fig. 7, D and E)

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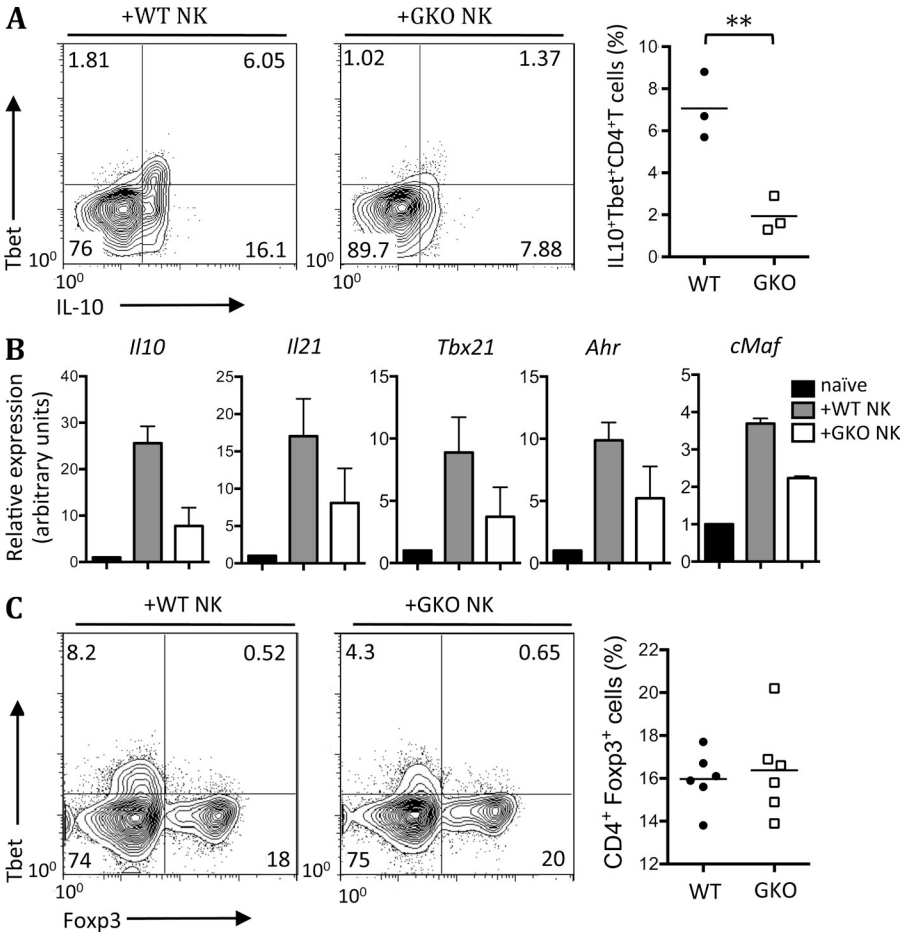

Figure 6. NK cells induce IL-10–producing Tr1-like cells in GKO mice. GKO mice depleted of NK cells and repleted with autologous or WT NK were immunized for EAU. (A) On day 11, $CD4^+$ T cells were isolated from draining LNs and pulsed with PMA/ionomycin. Expression of Tbet and IL-10 in $CD4^+$ T cells was studied by intracellular staining. (left) Representative staining; (right) mean values of three combined experiments; (B) Expression of Tr1-related genes: *Il10*, *Il21*, *Tbx21*, *cMaf*, and *Ahr* of cells in A by real-time PCR. (C) Expression of Tbet and Foxp3 in $CD4^+$ T cells was quantitated by intracellular staining. Data in B and C are from three independent experiments. **, $P < 0.01$ by Mann-Whitney *U* test.

and failed to suppress Th17 response in the uveitic eyes (Fig. 7 F). To examine whether IL-27Rα on Tr1 cells was required for their function in vivo, we infused cultured IL-27Rα$^{-/-}$ Tr1 cells into immunized GKO mice. IL-27Rα$^{-/-}$ Tr1 cells did not suppress either the disease or the Th17 response (Fig. 7 G). In the aggregate, these data suggest that NK–DC interaction, though production of IFN-γ and IL-27 induces naive T cells to commit to a Tr1 phenotype able to inhibit disease by producing IL-10.

DISCUSSION

IFN-γ has long been recognized to have pleiotropic effects in autoimmunity and the dichotomy between the pathogenic and protective roles of IFN-γ has generated considerable debate. Our previous studies demonstrated that pharmacologic elicitation of IFN-γ at the time of priming, but not later, inhibited development of autoimmunity and dampened the subsequent adaptive effector response (Tarrant et al., 1999; Grajewski et al., 2008). Although this pointed toward innate origins of the protective IFN-γ, its natural cellular source (absent pharmacological stimulation) and its mode of action remained poorly defined. In the present study, we identify NK cells as a necessary and sufficient cellular source of the IFN-γ that limits autoimmunity, and uncover their CXCR3-dependent interaction with DC and operation of an innate IFN-γ–IL-27 feedback loop leading to IL-10 production, as the underlying mechanism.

Published studies indicate that NK cells migrate to, and interact with DCs in, LNs draining the site of immunization (Bajénoff et al., 2006; Beuneu et al., 2009), but in vivo evidence for NK cells as active modulators of the adaptive immune response is still sparse. Martín-Fontecha et al. (2004) reported that NK cells provide the initial source of IFN-γ for Th1 polarization. We demonstrate that NK–DCs interaction results in inhibition of the adaptive Th17 response through a positive feedback mechanism that elicits an innate IFN-γ–IL-27 axis. In these studies, we use a reductionist model of EAU in GKO mice repleted with WT NK cells as the only source of IFN-γ. Although in the normal situation (WT background) NK cells are only one of several potential sources of innate IFN-γ, their central importance is upheld by the finding that only the depletion of NK cells, but not of innate-like $CD8^+$ T cells (the next largest source of innate IFN-γ, Fig. 1), affected disease scores in the WT host. That said, we are not excluding that IFN-γ from other innate cells, e.g., innate-like $CD8^+$ T cells, iNKT cells and γδ T cells, can contribute to regulation of the Th17 response in WT animals.

CXCR3 expression is required for NK cell to efficiently migrate to the draining LNs (Fig. 3; Martín-Fontecha et al., 2004; Pak-Wittel et al., 2013). Our data point out a novel role

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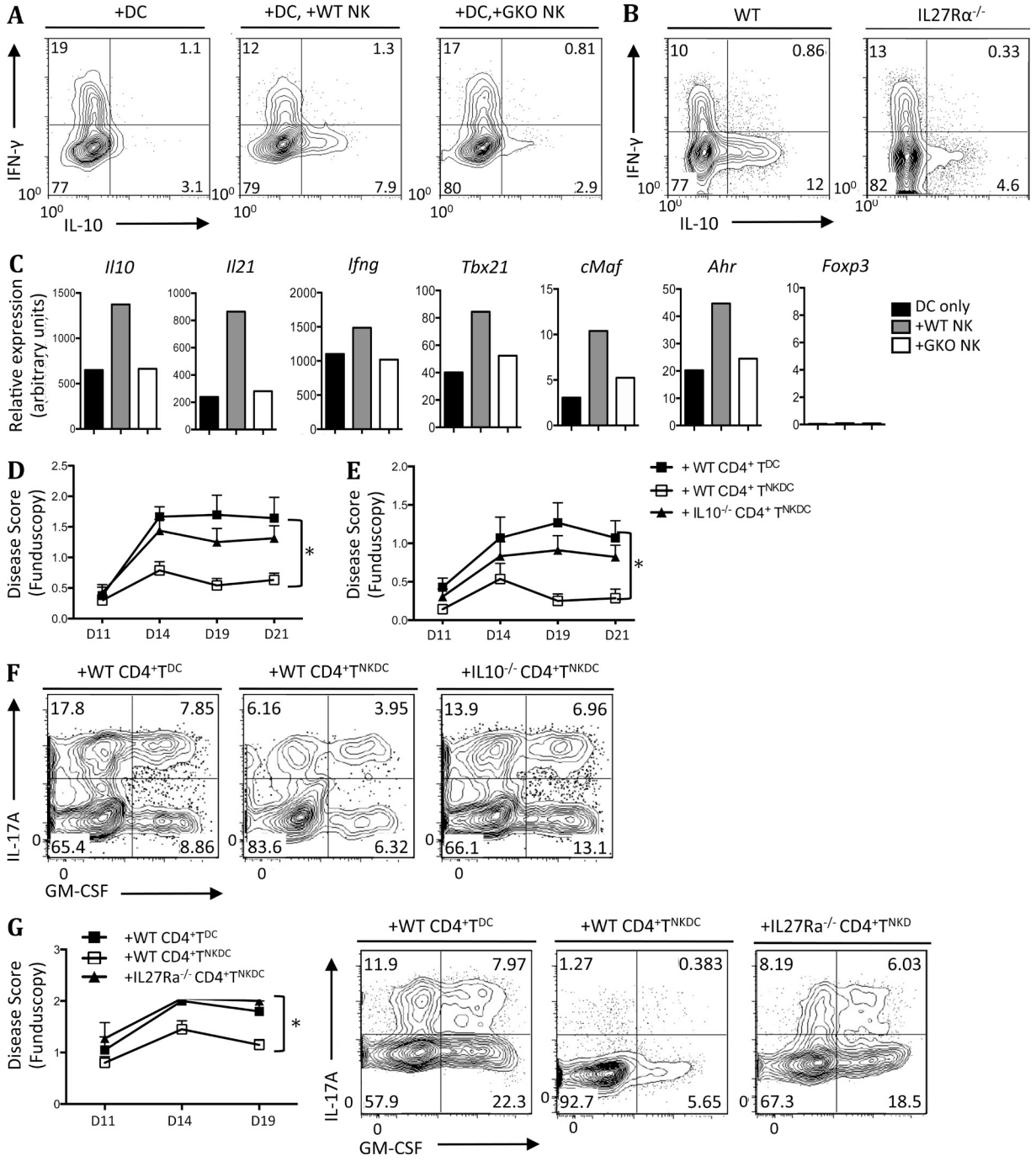

Figure 7. NK–DC interaction, through IFN-γ and IL-27, directs differentiation of Tr1-like cells, which suppress EAU in an IL-10–dependent fashion. (A–C) CD11c+ DCs were precultured with LPS for 24 h and added to cultures of fresh NK cells for another 24 h. Naive CD62L+CD4+ T cells + anti CD3 were then added for 4 d. T cells sorted from the co-cultures were restimulated with anti-CD3/CD28 for 24 h. (A) Production of IFN-γ and IL-10 was examined by intracellular staining. (B) CD62L+CD4+ T cells purified from WT or IL-27Rα$^{-/-}$ mice were co-cultured with DCs and NK cells as in A. (C) mRNA expression of Tr1-related genes, i.e., *Il10*, *Il21*, *Ifng*, *Tbx21*, *cMaf*, and *Ahr*, by real time PCR. (D–G) CD62L+CD4+ T cells were collected from WT, IL10$^{-/-}$ (D–F) or IL27Rα$^{-/-}$ (G) mice and were co-cultured with DCs and NK cells, as above. CD4+ T cells were then isolated from the co-culture and infused into EAU-immunized GKO mice on the day of immunization (D and G) or 9 d after immunization (E). (F and G) Expression of IL-17A and GM-CSF in eye-infiltrating CD4+ cells of mice was determined by intracellular staining on day 21. Disease was quantitated by fundus examination on the indicated days. Data in D–G are combined from two independent experiments totaling at least six mice per group. *, $P < 0.05$, linear regression. A–C are representative of at least three independent experiments.

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for CXCR3 by showing that it not only promotes NK migration to the LN, but also directs their physical interaction with DCs once they get there. Pak-Wittel et al. (2013) recently reported in a viral infection model that lack of IFN-γ signaling leads to failure of NK cell recruitment secondary to insufficient expression of CXCR3 ligands. Our data demonstrate that the innate IFN-γ from NK cells alone is sufficient to largely restore expression of CXCR3 ligands by DCs and restore the biological effects dependent on them in IFN-$\gamma^{-/-}$ animals. These findings underscore the critical role of innate IFN-γ produced by NK cells in regulating their own trafficking and function.

IL-27, a member of the IL-12 cytokine family, is produced by DCs that have received signals through TLRs, IFN-α, or IFN-γ (Shinohara et al., 2008; Murugaiyan et al., 2010). IL-27 was reported to inhibit autoimmune disease by dampening the Th17 response (Pot et al., 2011). This was attributed to inhibition of RORγt expression, GM-CSF production, enhancement of PD-L1 in T cells and induction of Tr1 cells (Diveu et al., 2009; Murugaiyan et al., 2010; Codarri et al., 2011; Hirahara et al., 2012). Our data show that innate IFN-γ from NK cells is sufficient to trigger IL-27 production from DCs with amelioration of tissue pathology, reduction in IL-17– and GM-CSF–producing cells, and induction of IL-10–producing Tr1 cells in vivo. In vitro–induced Tr1 cells were reported to coproduce IL-10 and IFN-γ (Stumhofer et al., 2007; Batten et al., 2008; Spolski et al., 2009) and several mechanisms have been proposed for the switch of $CD4^+$ T cells from IFN-γ^+IL-10^- to IFN-γ^+IL-10^+ and finally to IFN-γ^-IL-10^+ (Cardone et al., 2010; Cope et al., 2011). In our hands, NK cells that interact with DCs in vitro promoted IL-10 single-producing cells, which have been speculated to be more suppressive than double IFN-γ–IL-10 producers (Cope et al., 2011). Mascanfroni et al. (2013) reported in the EAE model that IL-27 induces DCs to express CD39, a membrane-bound protein that degrades extracellular ATP and ADP, inhibiting the NLRP3 inflammasome. In these mice, EAE scores and the associated Th1 and Th17 responses were reduced. Because we did not address the effects on CD39 in our study, our data do not exclude this pathway as another possible route of immunoregulation.

In the aggregate, our data are compatible with the interpretation that, upon encountering innate stimuli (mycobacterial components in CFA), DCs activate and migrate to the draining LNs, where (by producing IL-12 and IL-18) they activate the small resident population of NK cells to produce IFN-γ, which in turn induces the DCs to produce CXCR3 ligands and IL-27. This recruits peripheral CXCR3-expressing NK cells and induces them to produce more IFN-γ, which in turn will induce more IL-27 from DCs, and so on, in a positive feedback loop. The interdependence of IFN-γ and IL-27 in this self-amplification mechanism is supported by the finding that IFN-γ from NK cells was required to induce IL-27 production by DCs, and abrogation of IL-27 signaling (IL-27R$\alpha^{-/-}$ NK or anti-IL-27 antibody) abolished the protective effect of IFN-γ–sufficient NK cells in EAU. These findings highlight the importance of the innate NK–DC interaction and the consequent IFN-γ–IL-27 amplification loop in modulating the priming of adaptive autopathogenic Th17 cells.

The question arises, what terminates the self-amplifying IFN-γ–IL-27 cycle? We propose that IL-10, which can be at least in part be produced by the interacting NK cells and DCs themselves, could constitute the brake on the IFN-γ–IL-27 positive feedback. In co-cultures of NK cells and DCs, IL-10 was expressed by both cell types and was promoted by IL-27. Conversely, deficiency of IL-10 in both cell types strongly enhanced their endogenous IFN-γ and IL-27 production, demonstrating a built-in control mechanism triggered by the IFN-γ–IL-17 amplification loop itself. Additional IL-10 could come from Tr1 cells that are also induced as a result of the NK–DC interaction. These data demonstrate that the NK–DC interaction can be essentially self-regulating and point out a new and important biological role for IL-10 in controlling the innate IFN-γ–IL-27 axis.

In our earlier studies, we showed that early elicitation of IFN-γ dampens both the Th1 and the Th17 effector responses. Although the Th17 response is dominant over Th1 in EAU, EAE, CIA, and similar tissue-specific autoimmune diseases, a common feature of all these models is that the Th1 response is also present, and Th1 is also a pathogenic effector phenotype. Our current study in GKO mice repleted with WT NK cells demonstrates that the IFN-γ–IL-27 axis regulates the autopathogenic Th17 response, but by its nature, the GKO model does not lend itself to study of the Th1 response. Because IL-27 is known to promote, not only inhibit, commitment to the Th1 lineage, it stands to reason that innate IFN-γ acts to dampen the Th1 response by other mechanisms. One such mechanism, as reported in our earlier work, can be through elicitation of inducible nitric oxide synthase (iNOS = NOS2) and production of NO, resulting in apoptosis of newly primed effector T cells. That said, Th1 responses could also be downregulated by Tr1 cells induced via the IFN-γ–IL-27 axis.

A diagram synthesizing the proposed mechanisms by which IFN-γ and IL-27 control the adaptive autopathogenic effector response is depicted in Movie S2.

In conclusion, we identified a novel role for NK cells in regulating the adaptive Th17 response by interacting with DCs in the draining LNs. This regulation involves an IFN-γ–IL-27 positive feedback loop and a built-in restraint system that controls it via IL-10. Our study thus sheds new light on the controversial and insufficiently understood effects of IFN-γ in limiting tissue-specific autoimmune disease.

MATERIALS AND METHODS

Mice. WT, IFN-$\gamma^{-/-}$, IL-$10^{-/-}$, CD11c.DTR, CXCR$3^{-/-}$, and GFP C57BL/6 mice were purchased from The Jackson Laboratory. IL27R$\alpha^{-/-}$ mice were obtained from Genentech; $CD11c^{YFP}$ reporter mice were purchased from Taconic. IL-15 KO mice were provided by T. Waldmann (National Cancer Institute, Baltimore, MD). All mice were kept in a specific pathogen–free facility. Animal care and the animal study protocols were approved by the Animal Care and Use Committees of the National Eye Institute (protocol # NEI-182) and of the National Institute of Allergy and Infectious Diseases (protocol LSB-1E).

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Induction of EAU and disease scoring. EAU was induced as described previously (Luger et al., 2008), with 150 µg IRBP emulsified in an equal volume of CFA containing 2.5 mg/ml *Mycobacterium tuberculosis* strain 37RA (Sigma-Aldrich) and 0.5 µg of *Bordetella pertussis* toxin. Some mice were treated with 0.5 mg of anti–IL-27 antibody (provided by J. Van Snick and C. Uyttenhoeve, Ludwig Institute for Cancer Research, Brussels, Belgium) or with isotype control antibody on days 0, 7, and 14. For depletion of NK cells and $CD8^+$ cells, 0.2 mg of anti-NK1.1 antibody (PK136; Bioxcell) or 1 mg of anti-CD8 antibody (YTS169; Harlan), respectively, (or corresponding isotype controls) were injected on day 0, 3, and 6. Disease was evaluated by fundus examination on a scale of 0–4 based on the number, type, and size of lesions and extent of inflammation (Agarwal et al., 2012).

Ex vivo analysis of DCs and $CD4^+$ T cells from EAU mice. DCs and $CD4^+$ T cells were isolated from draining LNs of EAU immunized mice using CD11c and CD4 isolation kit (Miltenyi Biotec), respectively. mRNA was extracted for cDNA synthesis and gene expression profiles were determined by quantitative real time PCR.

Real-time PCR. RNA was extracted by using RNeasy mini kit (QIAGEN) and cDNA was synthesized with Superscript III First Strand Synthesis System (Invitrogen). Real-time PCR was performed with a TaqMan 7500 (Applied Biosystems) using gene-specific primer and TaqMan probe (Applied Biosystems). Data were normalized to GAPDH expression, and results are expressed relative to resting/naive T cells or DCs.

Isolation and adoptive transfer of NK cells. NK cells were isolated from donor mice with the NK Cell Isolation kit II (Miltenyi Biotec) with 88.6 ± 3.8% (mean ± SD) of the isolated cells were $NK1.1^+$ $DX5^+$ $CD3^-$ NK cells. Recipient mice were infused intravenously 3×10^6 NK cells and were immediately immunized for EAU. Endogenous NK cells of the recipients had been depleted by an injection of anti-NK 1.1 antibody (50 µg) 2–3 d before immunization.

Isolation and analysis of eye-infiltrating cells. Eyes were collected 21 d after immunization. External tissues and lens were removed. The remaining tissue was minced in cold RPMI medium. After centrifugation, the resultant cell pellet was resuspended in RPMI with 1 mg/ml collagenase D (Sigma-Aldrich) and incubated for 45 min at 37°C. Samples were then dispersed by trituration, washed, filtered, and resuspended in RPMI + 10% FBS. Cells were pulsed for 4 h with PMA (50 ng/ml), ionomycin (500 ng/ml), and Brefeldin A (GolgiPlug), followed by 4% paraformaldehyde fixation and 0.05% Triton X-100 permeabilization before intracellular cytokine staining for IFN-γ, IL-17A, and GM-CSF. All flow cytometry reagents were obtained from BD.

2P intravital imaging. Isoflurane was used to anesthetize mice before exposure of popliteal lymph nodes (Baxter; 2.5% for induction, 1∼1.5% for maintenance, vaporized in an 80:20 mixture of O2 and air), and subsequent 2P-IVM was performed using a protocol modified from a previous study (Bajénoff et al., 2006). Imaging was conducted on a 710 microscope (Carl Zeiss) equipped with a Chameleon laser (Coherent) tuned to 800 nm in combination with a 20× water-dipping lens (NA 1.0; Carl Zeiss) using Zen 2010 acquisition software. Imaging was conducted in enclosed environmental chambers in which anesthetized mice were warmed by heated air and the surgically exposed LN was kept at 36–37°C with warmed PBS.

To visualize cells, C57BL/6 CD11c-YFP hosts were used in combination with WT GFP-NK cells and CXCR3 KO NK cells labeled with 10.0 µM CMTMR (Molecular Probes) for 15 min at 37°C, followed by washing over a FCS gradient.

Co-culture of DCs and NK cells. DCs were isolated from spleens using a $CD11c^+$ isolation kit (Miltenyi Biotec) as described. In brief, spleen was minced and digested with collagenase D and DNase I at 37°C and filtered through a 70-µM nylon mesh. $CD11c^+$ DCs were then isolated using the manufacturer's protocol and were activated with 0.5 µg/ml of LPS overnight. After activation, cells were washed with PBS and 1×10^5 DCs were co-cultured with 1×10^5 isolated NK cells in 0.2 ml of RPMI with 10% FBS for 2 d with or without 10 µg/ml of anti–IL-27 or anti–IFN-γ, or the appropriate isotype control antibody. Supernatants were assayed by ELISA (R&D Systems) for IL-27, IFN-γ, or IL-10 production.

Induction and analysis of Tr1 cells. NK cells were co-cultured with LPS-activated DCs for 2 d as described above. $CD4^+CD62L^+$ T cells were purified by using $CD4^+CD62L^+$ T Cell Isolation kit II (Miltenyi Biotec) and 2×10^5 isolated T cells were added to the NK–DC co-culture together with 2 µg/ml of anti-CD3 antibody. After 3–4 d, T cells were isolated from the co-culture by using $CD4^+$ isolation kit (Miltenyi Biotec) and restimulated with plate-bound anti-CD3 and soluble anti-CD28 (2 µg/ml) antibodies. Cells were harvested after 24 h for cDNA synthesis, or pulsed for 6 h with 50 ng/ml PMA, 500 ng/ml ionomycin, and Brefeldin A (GolgiPlug), followed by fixation (4% paraformaldehyde), permeabilization (0.05% Triton X-100), and staining for intracellular IFN-γ and IL-10.

Statistical analysis. Student's *t* test (parametric data) or Mann-Whitney *U* test (non-parametric data) was used for two-group comparisons. One-way ANOVA with Bonferroni's multiple comparison test was used for multi-group analysis. Disease scores of EAU mice were compared by linear regression. A p-value of < 0.05 was considered statistically significant. Data are displayed as mean ± SEM. Experiments were repeated at least twice, and usually three or more times, with essentially the same result. Depending on the experiment, figures depict combined or representative data, as specified.

Online supplemental material. Video 1 shows intravital two-photon imaging of the interaction between NK and DC in the LN draining the site of uveitogenic immunization. Video 2 illustrates the interaction between NK cells and DC and depicts its functional consequences. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20141678/DC1.

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