IL-15Rα chaperones IL-15 to stable dendritic cell membrane complexes that activate NK cells via trans presentation

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Natural killer (NK) cells are innate immune effectors that mediate rapid responses to viral antigens. Interleukin (IL)-15 and its high affinity IL-15 receptor, IL-15Rα, support NK cell homeostasis in resting animals via a novel trans presentation mechanism. To better understand how IL-15 and IL-15Rα support NK cell activation during immune responses, we have used sensitive assays for detecting native IL-15 and IL-15Rα proteins and developed an assay for detecting complexes of these proteins. We find that IL-15 and IL-15Rα are preassembled in complexes within the endoplasmic reticulum/Golgi of stimulated dendritic cells (DCs) before being released from cells. IL-15Rα is required for IL-15 production by DCs, and IL-15 that emerges onto the cell surface of matured DCs does not bind to neighboring cells expressing IL-15Rα. We also find that soluble IL-15–IL-15Rα complexes are induced during inflammation, but membrane-bound IL-15–IL-15Rα complexes, rather than soluble complexes, support NK cell activation in vitro and in vivo. Finally, we provide in vivo evidence that expression of IL-15Rα specifically on DCs is critical for trans presenting IL-15 and activating NK cells. These studies define an unprecedented cytokine–receptor biosynthetic pathway in which IL-15Rα serves as a chaperone for IL-15, after which membrane-bound IL-15Rα–IL-15 complexes activate NK cells via direct cell–cell contact.

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Abbreviations used: BMDC, bone marrow–derived DC; DT, diphtheria toxin; HSC, hematopoietic stem cell; poly I:C, poly inosinic–polycytidylic acid; TLR, Toll-like receptor.

NK cells are innate immune effector cells that secrete cytokines and acquire cytolytic function within hours of viral infections. The activation of NK cells is regulated by the balance of inhibitory and activating signals and is dependent on DCs, cytokines, and co-stimulatory molecules (1–3). NK cell responses to microbes are triggered by Toll-like receptor (TLR) ligands, which stimulate secretion of type I IFNs that in turn support IL-15 and IL-15Rα production (4–6).

IL-15 and its proprietary receptor IL-15Rα are both essential for supporting NK cell homeostasis (7). An unusual aspect of IL-15 and IL-15Rα binding is the extraordinarily high affinity with which these proteins interact ($\sim 5 \times 10^{-11}$ M) (8, 9). Another notable observation is that the phenotypes of IL-15 (IL-15$^{-/-}$) and IL-15Rα (IL-15Rα$^{-/-}$) –deficient mice are indistinguishable, suggesting that physiologically relevant IL-15 signals require IL-15Rα and that these molecules function in close cooperation (10, 11). On the other hand, some studies have reported secretion of soluble IL-15, and the ability of recombinant IL-15 to stimulate cells bearing IL-2/15Rβ and γc but lacking IL-15Rα raises questions about whether and when IL-15 may perform IL-15Rα–independent functions. Additional questions surrounding IL-15 and IL-15Rα biology relate to the observations that IL-15 cDNAs are difficult to express heterologously unless leader sequences, ATG sites, and 3′ mRNA sequences are altered (12). These findings suggest that complex intracellular processing regulates the bioavailability of IL-15.

Prior studies from our laboratory showed that IL-15Rα functions in an unexpected non–cell-autonomous fashion in vivo (13–15). IL-15Rα expression on responding cells does not enhance the responsiveness of IL-15–dependent cells in vivo (13–16). Soluble IL-15 can bind to IL-15Rα on accessory cells that "trans present" IL-15 to responding cells in vitro (17).

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Trans presentation in vivo requires expression of IL-15 and IL-15Rα by hematopoietic accessory cells and expression of IL-2/15Rβ by IL-15–responsive NK cells and memory $CD8^+$ T cells (18). Trans presentation appears to be the dominant mechanism by which IL-15 signals are delivered in vivo (13–16, 18).

Why IL-15 signals should be delivered via this somewhat complicated trans presentation mechanism is unclear. Some clues have been provided by recent studies revealing that coordinate expression of IL-15 and IL-15Rα by the same accessory cells such as DCs is required for supporting both NK and $CD8^+$ memory T cell homeostasis (18, 19). These results are not consistent with a mechanism in which IL-15 is secreted by DCs and subsequently bound to IL-15Rα on the surface of other DCs to be trans presented to IL-15–responsive cells. Instead, they suggest a more complicated model of trans presentation.

The studies above focused on the functions of IL-15 and IL-15Rα during noninflamed, homeostatic conditions. TLRs induce the production of both IL-15 and IL-15Rα by myeloid cells (4–6, 20–22). Hence, to understand how endogenous IL-15 and IL-15Rα proteins function during inflammatory responses, we have developed sensitive assays for detecting endogenous IL-15 and IL-15Rα proteins. We have used these assays to further examine how these proteins are induced in DCs and how they support NK cell activation.

RESULTS

IL-15, IL-15Rα, and complexes of IL-15–IL-15Rα proteins are induced in DCs after TLR stimulation

Analyses of the biology of IL-15 and IL-15Rα have been challenging because the potent physiological effects of these proteins occur at relatively low levels of expression. To understand how IL-15 and IL-15Rα function under physiological circumstances, we first optimized ELISAs for these proteins using recombinant IL-15 and IL-15Rα proteins and found that we were able to detect picomolar levels of these proteins (unpublished data). Because IL-15 and IL-15Rα bind to each other with high affinity and may function as a complex in physiological settings, we investigated whether the presence of either protein affects the detection of the other. Detection of IL-15Rα protein was not affected by the presence of IL-15 (Fig. S1 A, available at http://www.jem.org/cgi/content/full/jem.20071913/DC1). In contrast, the detection of IL-15 protein was progressively diminished by the presence of increasing amounts of IL-15Rα protein (Fig. S1 B). These results suggest that at least one of the anti–IL-15 antibodies used in our capture ELISA binds to an epitope that is masked when IL-15 binds to IL-15Rα. Several commercially available antibodies to IL-15 shared this capacity for interference with IL-15Rα binding (unpublished data).

mRNA transcripts for IL-15 and IL-15Rα are induced by TLR ligands in DCs, but the translation of these proteins may not follow mRNA levels. We thus measured the levels of IL-15 and IL-15Rα proteins before and after stimulation with LPS or poly inosinic–polycytidylic acid (poly I:C). Negligible levels of IL-15Rα were detected in cell lysates from nonstimulated bone marrow–derived DCs (BMDCs), and IL-15Rα protein levels were similarly induced in lysates from WT or *IL-15*$^{-/-}$ cell BMDCs (but not *IL-15Rα*$^{-/-}$ cells) between 2 and 12 h (Fig. 1 A). Hence, IL-15Rα is induced in DCs by TLR stimulation, and IL-15 does not influence the production of IL-15Rα.

Somewhat surprisingly, IL-15 protein was only transiently detected at modest levels within *IL-15Rα*$^{-/-}$ BMDCs and not at all in WT cells (Fig. 1 B). This occurred despite the fact that IL-15 mRNA levels, detected by real-time PCR, were markedly induced by poly I:C in both *IL-15Rα*$^{-/-}$ and WT BMDCs (unpublished data). To understand why IL-15 protein was not detected in WT cells, we hypothesized that IL-15 in WT cells might be complexed with IL-15Rα protein. As noted above, epitopes of IL-15 detected by several anti–IL-15 antibodies are blocked by high affinity interactions between IL-15 and IL-15Rα. To investigate whether IL-15 is complexed with IL-15Rα in poly I:C–stimulated BMDCs, we established conditions to dissociate these proteins without denaturing them before ELISA analyses. We boiled lysates from poly I:C–stimulated BMDCs in the presence of 0.01% SDS before analyzing IL-15 protein levels by ELISA. These experiments showed that IL-15 protein was indeed induced in WT cells at nanomolar levels in parallel with the induction of IL-15Rα (Fig. 1 C). Similar results were obtained with LPS stimulation of DCs (unpublished data). Thus, IL-15 is induced by TLR stimulation and appears to be predominantly bound to another protein (e.g., IL-15Rα) inside DCs.

To further examine the possibility that endogenous IL-15 and IL-15Rα proteins exist in complexes in normal BMDCs, we developed a "complex" ELISA in which an anti–IL-15 antibody that detects IL-15 in the presence of IL-15Rα was

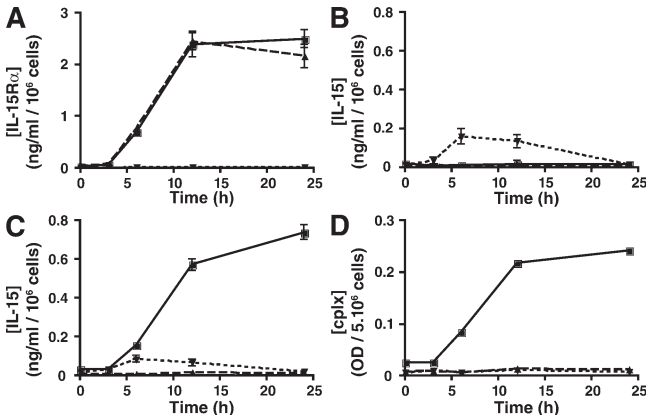

Figure 1. Detection of IL-15 and IL-15Rα proteins and IL-15–IL-15Rα complex expression in lysates of TLR-stimulated DCs. ELISA measurement of IL-15Rα (A) and IL-15 (B) proteins and IL-15–IL-15Rα complex (D) expression in lysates of poly I:C–stimulated BMDCs at the indicated time points. (C) ELISA determination of IL-15 protein levels after dissociation of IL-15–IL-15Rα protein complexes with 0.01% SDS and boiling. Lines represent data from WT (■), *IL-15*$^{-/-}$ (▲), and *IL-15Rα*$^{-/-}$ (▼) BMDCs. All data are representative of at least three separate experiments.

used as a "capture" antibody and an anti–IL-15Rα antibody was used as a "detection" reagent (Fig. S1 C). We used this complex ELISA to measure the levels of IL-15–IL-15Rα complexes in cell lysates from BMDCs. IL-15–IL-15Rα complexes were induced by poly I:C in WT BMDCs but not in *IL-15*$^{-/-}$ BMDCs or *IL-15Rα*$^{-/-}$ BMDCs (Fig. 1 D). The kinetics of IL-15–IL-15Rα complex induction was very similar to the induction of IL-15 and IL-15Rα proteins. Collectively, these studies suggest that IL-15 and IL-15Rα proteins are induced in BMDCs by TLR ligands and exist largely in IL-15–IL-15Rα complexes.

IL-15–IL-15Rα complexes are not detected unless IL-15 and IL-15Rα are coordinately expressed by the same DCs

Exposure of DCs to TLR ligands is thought to stimulate IL-15 secretion from DCs, after which IL-15 may be subsequently bound to IL-15Rα on the cell surface of DCs. The identification of IL-15–IL-15Rα complexes in the lysates of stimulated DCs could thus reflect this sequence of events. Alternatively, our prior studies suggest that IL-15 and IL-15Rα must be coordinately expressed on the same hematopoietic cells to support IL-15–dependent NK cells and memory CD8$^+$ T cells under homeostatic (non-inflamed) conditions (18). Thus, it is possible that IL-15Rα and IL-15 must be coordinately expressed during TLR-induced inflammatory conditions as well. To test this hypothesis, we stimulated a 1:1 mixture of *IL-15*$^{-/-}$ and *IL-15Rα*$^{-/-}$ BMDCs as well as uniform cultures of WT, *IL-15*$^{-/-}$, and *IL-15Rα*$^{-/-}$ BMDCs with poly I:C and measured the production of IL-15–IL-15Rα complexes in BMDC lysates by ELISA. As these cells were plated at concentrations at which *IL-15*$^{-/-}$ and *IL-15Rα*$^{-/-}$ DCs were in direct contact, the production of freely soluble IL-15 protein from *IL-15Rα*$^{-/-}$ DCs should allow binding of IL-15 to IL-15Rα on the surface of neighboring *IL-15*$^{-/-}$ DCs and potential recycling of these complexes. These experiments revealed that IL-15–IL-15Rα complexes are detected only in cultures of poly I:C–stimulated WT BMDCs and not in 1:1 mixtures of *IL-15*$^{-/-}$ and *IL-15Rα*$^{-/-}$ BMDCs (Fig. 2 A). IL-15–IL-15Rα complexes were predictably absent from stimulated *IL-15*$^{-/-}$ cells and *IL-15Rα*$^{-/-}$ BMDCs (Fig. 2 A). Thus, IL-15 may not be secreted from *IL-15Rα*$^{-/-}$ cells. Moreover, IL-15 and IL-15Rα proteins must be coordinately expressed within the same DCs to form IL-15–IL-15Rα complexes—even after TLR stimulation.

To independently confirm that IL-15 and IL-15Rα proteins must be coordinately expressed to form IL-15–IL-15Rα complexes, we developed an immunoblotting assay to detect endogenous IL-15 bound to endogenous IL-15Rα protein. We stimulated cultures of WT, *IL-15*$^{-/-}$, *IL-15Rα*$^{-/-}$, or a mixture of *IL-15*$^{-/-}$ and *IL-15Rα*$^{-/-}$ DCs with LPS, lysed cells, immunoprecipitated endogenous IL-15Rα, and immunoblotted for IL-15. As IL-15 is dissociated from IL-15Rα by boiling in sample buffer (containing 1% SDS) before immunoblotting with anti–IL-15 antibody, this approach to detecting IL-15 is not compromised by potential masking of IL-15 epitopes by IL-15Rα. Immunoprecipitation of IL-15Rα from WT DCs co-precipitated significant amounts of IL-15 protein, confirming that IL-15–IL-15Rα complexes are induced in DCs by TLR stimulation (Fig. 2 B). In contrast, immunoprecipitation of IL-15Rα yielded negligible amounts of co-precipitated IL-15 in resting WT DCs, or LPS-stimulated *IL-15*$^{-/-}$ DCs and *IL-15Rα*$^{-/-}$ DCs (Fig. 2 B and unpublished data). Importantly, IL-15 was not co-precipitated with IL-15Rα from 1:1 mixtures of LPS-stimulated *IL-15*$^{-/-}$ and *IL-15Rα*$^{-/-}$ DCs (Fig. 2 B). Therefore, IL-15 and IL-15Rα must be coordinately expressed by the same cells to form IL-15–IL-15Rα complexes—even under inflammatory conditions during which both proteins are induced.

IL-15–IL-15Rα complexes are preassembled in DCs in the absence of protein secretion

IL-15 protein is not efficiently produced in *IL-15Rα*$^{-/-}$ cells (Fig. 1 B). One mechanism by which IL-15Rα could facilitate the production of IL-15 would be if IL-15Rα directly bound newly synthesized IL-15 before being secreted (e.g.,

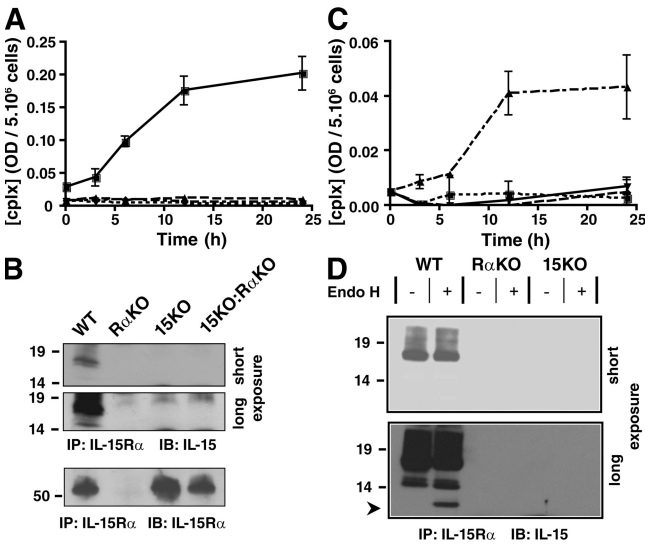

Figure 2. Intracellular formation of IL-15–IL-15Rα complexes within TLR-stimulated DCs before protein secretion. (A) ELISA detection of IL-15–IL-15Rα complexes in lysates from poly I:C–stimulated BMDCs of the indicated genotypes: WT (■), *IL-15*$^{-/-}$ (15KO; ▲), and *IL-15Rα*$^{-/-}$ (RαKO; ▼), and a 1:1 mixture of *IL-15*$^{-/-}$ with *IL-15Rα*$^{-/-}$ (15KO:RαKO; ◆) BMDCs. (B) Immunoblotting detection of IL-15Rα co-precipitated IL-15 in LPS-stimulated BMDCs. Long and short exposures of IL-15–specific immunoblots are shown above. Note the absence of IL-15 protein in immunoprecipitates of mixed 15KO:RαKO BMDCs. Immunoblotting of immunoprecipitates for IL-15Rα are shown below as a control (bottom). (C) ELISA detection of IL-15–IL-15Rα complexes in the lysates of untreated WT DCs (◆), DCs treated with LPS plus brefeldin A (▲), DCs treated with LPS plus cycloheximide (■), and DCs treated with LPS plus brefeldin A plus cycloheximide (▼) for the indicated periods of time. (D) EndoH sensitivity of IL-15Rα co-precipitated IL-15 from LPS-stimulated DCs. Note the appearance of an ~12.7-kD band corresponding to unglycosylated IL-15 in EndoH-treated WT DCs (arrow), but not in other control lanes. Short (top) and long (bottom) exposures of IL-15 expression are shown in the top panels. Data are representative of three separate experiments.

within the ER or Golgi) and stabilized IL-15 in a complex. To test this possibility, we pretreated BMDCs with brefeldin A (which blocks protein trafficking through the Golgi) before stimulation with LPS, and then tested lysates of these cells for the presence of IL-15–IL-15Rα complexes. Significant levels of IL-15–IL-15Rα complexes were induced in WT cells even in the presence of brefeldin A (Fig. 2 C). No IL-15Rα protein was observed on the surface of these cells by flow cytometry, and no soluble IL-15Rα (sIL-15Rα) was detected in supernatants, confirming that brefeldin A blocked the emergence of IL-15Rα from the Golgi complex (Fig. S2, A and B, available at http://www.jem.org/cgi/content/full/jem.20071913/DC1). Thus, IL-15Rα appears to bind IL-15 during biogenesis. Brefeldin A may also block the trafficking of proteins from recycling and/or late endosomes to the plasma membrane. To eliminate the possibility that brefeldin A exclusively unveiled recycling complexes in these experiments, we treated DCs with LPS plus cycloheximide, which should block the formation of nascent IL-15–IL-15Rα complexes but should not block recycling complexes, and then asked whether IL-15–IL-15Rα complexes were induced. These experiments revealed that cycloheximide prevented the formation of IL-15–IL-15Rα complexes, suggesting that the complexes we detect in LPS plus brefeldin A–treated DCs are newly synthesized (Fig. 2 C).

Like many transmembrane and secreted proteins, IL-15 undergoes N-linked glycosylation in the ER, a form that is sensitive to EndoH digestion (23). N-linked sugars are removed from IL-15 in the Golgi during further processing so that mature forms of IL-15 are resistant to EndoH. To determine whether IL-15–IL-15Rα complexes may be assembled when IL-15 bears EndoH-sensitive N-linked sugars during biogenesis, we asked whether IL-15 that co-precipitated with IL-15Rα from LPS-stimulated DCs is EndoH sensitive. LPS-stimulated DCs were lysed in NP-40 lysis buffer, immunoprecipitated with anti–IL-15Rα antibodies, and treated with either EndoH or buffer alone before immunoblotting analysis for IL-15 expression. These experiments revealed that EndoH treatment of anti–IL-15Rα–immunoprecipitated lysates produces a novel IL-15–specific band at ∼12.7 kD, corresponding to un-glycosylated IL-15 (Fig. 2 D). Therefore, native IL-15–IL-15Rα complexes contain EndoH-sensitive IL-15, indicating that these complexes form in the ER or early Golgi before the completion of Golgi processing.

TLR-stimulated DCs release soluble IL-15–sIL-15Rα protein complexes

To follow the fate of IL-15–IL-15Rα complexes after Golgi processing, we studied the expression of these proteins on the cell surface of stimulated DCs. Emergence of IL-15Rα protein onto the surface of DCs occurs normally in the absence of IL-15, but emergence of IL-15 requires IL-15Rα (Fig. S3, available at http://www.jem.org/cgi/content/full/jem.20071913/DC1). IL-15 may be secreted from cells, and a soluble form of IL-15Rα (sIL-15Rα) can be released from cells (24, 25). Accordingly, we studied the levels of IL-15 and sIL-15Rα proteins in supernatants from poly I:C–stimulated BMDCs. These experiments revealed that minimal amounts of IL-15 were induced by poly I:C stimulation in the supernatants of WT BMDCs after 24 (Fig. 3 A, gray columns) and 48 (Fig. 3 A, black columns) h, but not in supernatants from *IL-15*$^{-/-}$ and *IL-15Rα*$^{-/-}$ cells. Given the interference of IL-15Rα with ELISA-mediated detection of IL-15 in cell lysates (Fig. 1 B), we suspected that IL-15 might be present in IL-15–sIL-15Rα protein complexes in these supernatants. We thus boiled these supernatants in the presence of 0.01% SDS before ELISA and found that IL-15 was indeed induced and progressively accumulated in the supernatants from WT but not *IL-15*$^{-/-}$ cells (Fig. 3 B). Thus, IL-15 is secreted from WT DCs predominantly in the form of IL-15–sIL-15Rα complexes. Importantly, no IL-15 was observed in the supernatants from IL-15Rα$^{-/-}$ DCs, even after boiling of the supernatants (Fig. 3 B). Thus, IL-15Rα is required for the emergence of IL-15 from cells.

Our assays of sIL-15Rα levels in supernatants from poly I:C–stimulated DCs show that soluble IL-15Rα accumulated in the supernatants from WT cells and was detected at similar levels in the supernatants from *IL-15*$^{-/-}$ cells (Fig. 3 C). Hence, IL-15 is not required for the production or release of sIL-15Rα. To directly examine whether coordinate expression of IL-15 and IL-15Rα was required for producing soluble IL-15–sIL-15Rα protein complexes in the supernatants of poly I:C–stimulated DCs, we assayed supernatants of 1:1 mixed cultures of *IL-15*$^{-/-}$ and *IL-15Rα*$^{-/-}$ DCs for the presence of these complexes. These experiments revealed that IL-15–sIL-15Rα complexes were detected only in the supernatants of WT cells, but not when IL-15 and IL-15Rα are produced by separate cells (Fig. 3 D). These results all mirror the findings we obtained in the cellular lysates of BMDCs. Thus, the

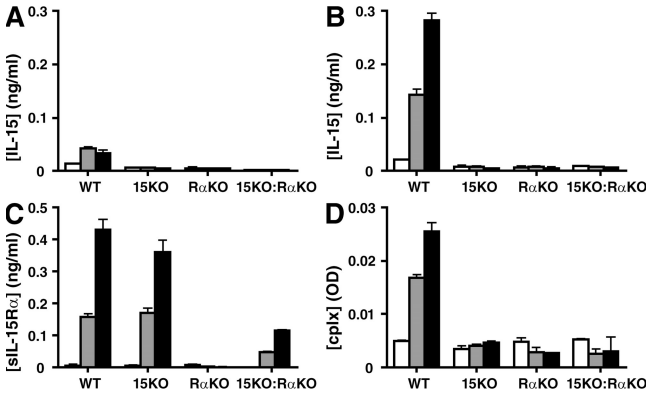

Figure 3. Release of IL-15 and sIL-15Rα proteins and IL-15–sIL-15Rα complexes from TLR-stimulated BMDCs. (A–D) ELISA of supernatants from stimulated BMDCs. WT, 15KO, RαKO, and mixed 15KO:RαKO BMDCs were stimulated with poly I:C for 0 (white bars), 24 (gray bars), and 48 h (black bars), after which supernatants were assayed for the production of (A) IL-15, (C) IL-15Rα, and (D) IL-15–sIL-15Rα complex expression. (B) Supernatants from TLR-stimulated DCs were supplemented with 0.01% SDS and boiled before ELISA for IL-15 expression. Data are representative of three independent experiments.

differences in IL-15, IL-15Rα, and IL-15–IL-15Rα protein levels are likely due to differences in protein production rather than differences in recycling or secretion from the cells.

Coordinate expression of IL-15 and IL-15Rα by DCs is required for supporting NK cell activation in vitro

Our prior studies indicated that IL-15Rα expression on DCs is required for NK cell activation (20). As IL-15 signaling in splenic DCs has been suggested to support their maturation, we first examined the activation of WT, *IL-15*$^{-/-}$, and *IL-15Rα*$^{-/-}$ BMDCs (26). Stimulation of BMDCs with poly I:C led to modestly reduced levels of IL-12 production and normal levels of CD40 and CD86 up-regulation (Fig. 4, A and B). Thus, TLR-triggered activation of BMDCs proceeds largely normally in the absence of either IL-15 or IL-15Rα.

To determine whether coordinated expression of IL-15 and IL-15Rα by DCs is required for activating NK cells, we stimulated WT, *IL-15*$^{-/-}$, *IL-15Rα*$^{-/-}$, or a 1:1 mixture of *IL-15*$^{-/-}$ and *IL-15Rα*$^{-/-}$ BMDCs with poly I:C and co-cultured these cells with NK cells. We then assayed NK cell activation in these cultures by testing the supernatants of these cultures for the production of IFN-γ by ELISA. These studies revealed that IFN-γ was secreted in significant amounts only in cultures containing WT BMDCs and not in cultures from *IL-15*$^{-/-}$, *IL-15Rα*$^{-/-}$, or a mixture of these DCs (Fig. 4 C). Flow cytometric analyses of these NK cells revealed that NK cells from all poly I:C–stimulated cultures up-regulated expression of the surface activation marker CD69 (Fig. 4 D). However, only NK cells co-cultured with WT DCs expressed significant levels of intracellular IFN-γ and intracellular granzyme B, indicating that these cells were fully activated and armed with cytotoxic granules (Fig. 4, D and E). Thus, coordinate expression of IL-15 and IL-15Rα in DCs is required for NK cell activation, and the failure of mixtures of *IL-15*$^{-/-}$ and *IL-15Rα*$^{-/-}$ BMDCs to form IL-15–IL-15Rα complexes leads to a failure of these cells to fully activate NK cells.

sIL-15Rα and IL-15–sIL-15Rα complexes are induced in the sera of stimulated mice

Multiple cell types may be able to respond to TLR ligands and elaborate IL-15 and IL-15Rα. To determine whether the mechanisms that we have elucidated in DCs apply to physiological responses in vivo, we studied the expression of IL-15, sIL-15Rα, and IL-15–sIL-15Rα complexes in the serum of resting and poly I:C–stimulated mice by ELISA. These experiments revealed that negligible levels of IL-15 and IL-15Rα are present in the sera of resting mice. 24 h after stimulation with poly I:C, sIL-15Rα was detected at similarly high levels in the serum of WT and *IL-15*$^{-/-}$ mice, but not in *IL-15Rα*$^{-/-}$ mice (Fig. 5 A). Similar results were obtained with LPS stimulation (unpublished data). Thus, sIL-15Rα is physiologically released into the sera from TLR-stimulated mice. Minimal levels of soluble IL-15 were detected in the serum of poly I:C–treated WT mice, and no appreciable IL-15 was found in the sera from *IL-15*$^{-/-}$ mice or *IL-15Rα*$^{-/-}$ mice (Fig. 5 B). It was not technically feasible to perform ELISA analyses on boiled sera samples and thereby quantitate IL-15 protein samples in complexes. However, ELISAs revealed that IL-15–sIL-15Rα complexes are induced only in WT mice (Fig. 5 C). Thus, similar to our findings with cultured BMDCs, soluble complexes of IL-15 and sIL-15Rα are induced by TLR stimulation in vivo.

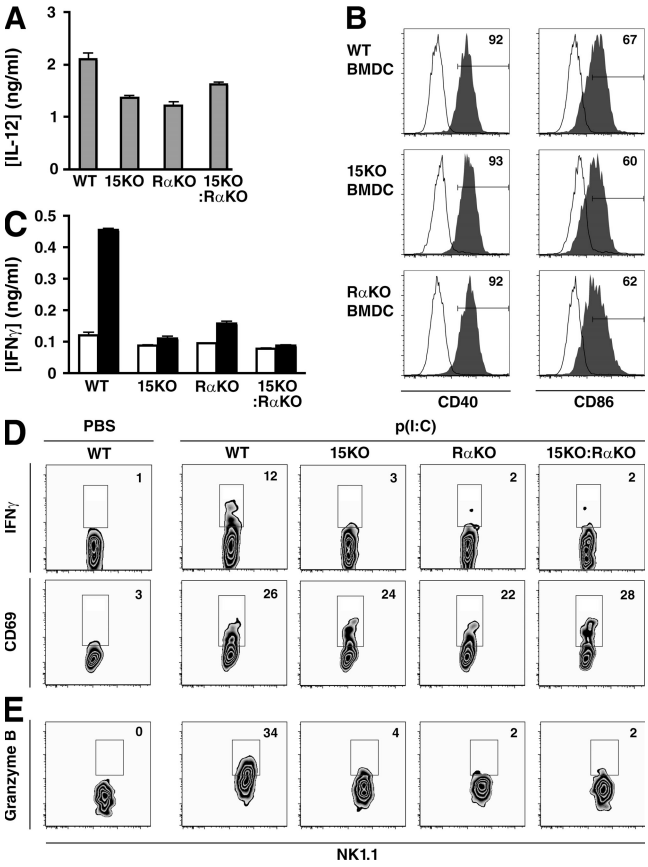

Figure 4. Coordinate expression of IL-15 and IL-15Rα is required for NK cell activation. (A) ELISA measurement of IL-12 secretion by poly I:C–stimulated BMDCs of the indicated genotypes. (B) Flow cytometric analysis of DC activation after poly I:C stimulation. Histograms of poly I:C–stimulated DCs (gray-filled histograms) of the indicated genotypes indicating surface expression levels of the surface activation markers CD40 and CD86. (C) ELISA determination of IFN-γ secretion by NK cells co-cultured with WT, 15KO, RαKO, or mixed 15KO:RαKO BMDCs. BMDCs were treated with PBS or poly I:C for 18 h, after which NK cells were co-cultured with activated DCs for an additional 6 h. Poly I:C–stimulated cultures are indicated by black bars, and PBS-stimulated control cultures are indicated by white bars. Note that significant poly I:C–induced NK cell activation occurs only in the presence of WT DCs. (D and E) Flow cytometric measurement of NK cell (NK1.1^{+}) activation by poly I:C (p(I:C)) –stimulated DC–NK cell co-cultures. Elevated CD69 surface staining reflects initial (TLR-dependent, IL-15–independent) activation of NK cells. IFN-γ and granzyme B expression by NK cells were performed by intracellular staining 6 and 12 h after stimulation, respectively. Numbers indicate the percentage of cells in the indicated gate. Note that NK cells express significant levels of both IFN-γ and granzyme B only when activated by WT DCs. Plots are representative of three separate experiments.

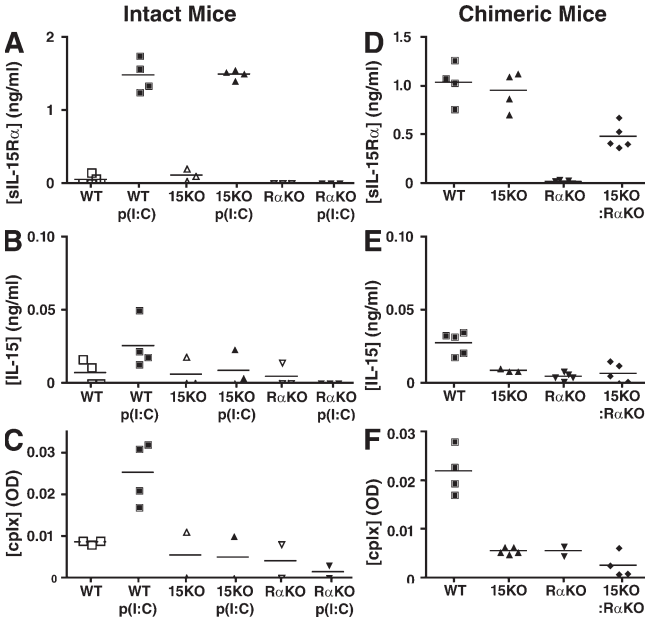

Figure 5. Soluble IL-15, IL-15Rα, and IL-15–sIL-15Rα complex detection in mouse sera. ELISA determination of sIL-15Rα, IL-15, and IL-15–sIL-15Rα proteins in sera from intact (A–C, on left) or chimeric (D–F, on right) mice stimulated with 25 µg/g poly I:C or PBS. Sera were collected 24 h after stimulation and IL-15 (B and E), sIL-15Rα (A and D), and IL-15–sIL-15Rα complex (C and F) protein levels were measured by ELISA. Each symbol reflects values obtained from individual mice.

We then asked whether a mixture of *IL-15*$^{-/-}$ and *IL-15Rα*$^{-/-}$ hematopoietic cells could generate IL-15–sIL-15Rα complexes in vivo after TLR stimulation. To address this question, we first interbred *IL-15*$^{-/-}$ and *IL-15Rα*$^{-/-}$ mice to generate double-mutant mice. These double-mutant mice phenotypically resemble both *IL-15*$^{-/-}$ and *IL-15Rα*$^{-/-}$ mice, reinforcing the idea that IL-15 and IL-15Rα function together and do not perform significant functions independently from each other (unpublished data). We irradiated these double-mutant mice and reconstituted them with WT, *IL-15*$^{-/-}$, *IL-15Rα*$^{-/-}$, or a 1:1 mixture of *IL-15*$^{-/-}$ and *IL-15Rα*$^{-/-}$ hematopoietic stem cells (HSCs). 6 wk after reconstitution, we stimulated these mice with poly I:C and assayed their sera for the presence of IL-15, IL-15Rα, and IL-15–sIL-15Rα complex proteins after 24 h by ELISA. These experiments revealed that modest levels of IL-15 were induced in chimeras reconstituted with WT cell HSCs, but not in the other chimera (Fig. 5 E). sIL-15Rα was detected at significant levels in the sera of both WT and *IL-15*$^{-/-}$ HSC-reconstituted chimera, and at roughly half these levels when *IL-15*$^{-/-}$ and *IL-15Rα*$^{-/-}$ HSCs were mixed at 1:1 (Fig. 5 D). Importantly, IL-15–sIL-15Rα complexes were detected only in the sera of poly I:C–stimulated chimera reconstituted with WT cells and not in chimera reconstituted with a mixture of *IL-15*$^{-/-}$ and *IL-15Rα*$^{-/-}$ HSCs (Fig. 5 F). Collectively, these results suggest that hematopoietic cells in intact mice regulate IL-15 and IL-15Rα production in a similar fashion to cultured BMDCs. Therefore, the cell biology we have defined in BMDCs is an accurate reflection of physiological IL-15–sIL-15Rα production in vivo.

Coordinate expression of IL-15 and IL-15Rα by hematopoeitic cells is required for supporting NK cell activation in vivo

To determine whether coordinated expression of IL-15 and IL-15Rα is also required for activating NK cells in vivo, we tested the ability of the radiation chimera mice described above to activate NK cells. Consistent with our prior studies, only mice reconstituted with WT HSCs possessed appreciable numbers of NK cells (14, 18, 19). To have comparable NK cells to assay, we purified NK cells from *RAG-1*$^{-/-}$ mice, labeled them with CFSE, and adoptively transferred these labeled NK cells into the radiation chimera before stimulating the mice with poly I:C (see experimental design, Fig. 6 A). Production of IFN-γ and granzyme B by the adoptively transferred NK cells was assayed by flow cytometry 6 and 12 h later, respectively. Importantly, although adoptively transferred (CFSE^{+}) NK cells were stimulated to express CD69 in all poly I:C–stimulated mice, NK cells only expressed significant amounts of IFN-γ and granzyme B in WT mice (Fig. 6, B and C). Thus, the failure of mixtures of *IL-15*$^{-/-}$ and *IL-15Rα*$^{-/-}$ hematopoietic cells to form IL-15–sIL-15Rα complexes correlates with a failure of these chimeric mice to activate NK cells in vivo.

Membrane-bound IL-15–IL-15Rα complexes, rather than soluble IL-15–sIL-15Rα complexes, support NK cell activation in vitro

The findings above suggest that membrane-bound IL-15–IL-15Rα complexes, soluble IL-15–sIL-15Rα complexes, or both stimulate NK cells. To determine the relative contributions of these forms of IL-15–IL-15Rα, we treated WT, *IL-15*$^{-/-}$, *IL-15Rα*$^{-/-}$, or a 1:1 mixture of *IL-15*$^{-/-}$ and *IL-15Rα*$^{-/-}$ BMDCs with poly I:C, after which supernatants of the four types of cultures were exchanged between cultures. NK cells were subsequently co-cultured with BMDCs and the exchanged supernatants, after which IFN-γ production was measured by ELISA. These studies revealed that poly I:C–stimulated WT DCs activated NK cells regardless of the type of supernatant present, whereas no other DCs or mixtures of DCs were able to activate NK cells (Fig. 7). In contrast, soluble IL-15–sIL-15Rα complexes found in WT supernatants did not augment NK cell activation any better than supernatants lacking these complexes (Fig. 7). Moreover, soluble IL-15–sIL-15Rα complexes in supernatants of poly I:C–stimulated WT BMDCs were unable to support NK cell activation in the presence of *IL-15*$^{-/-}$ or *IL-15Rα*$^{-/-}$ DCs (Fig. 7). Similar results were obtained with LPS stimulation (unpublished data). Thus, membrane-bound IL-15–IL-15Rα complexes are the critical mediators of IL-15–mediated NK cell activation. Meanwhile, soluble IL-15–sIL-15Rα complexes are unable to either augment membrane-bound complexes or compensate for the lack of membrane-bound complexes.

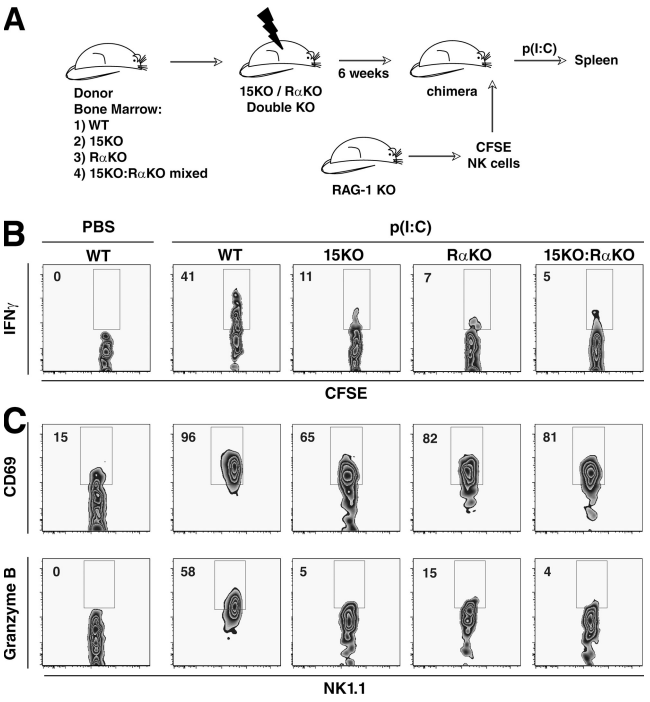

Figure 6. Full activation of NK cells requires coordinate expression of IL-15 and IL-15Rα in vivo. (A) Experimental design. Bone marrow HSCs of the indicated genotypes were used to reconstitute irradiated *IL-15*$^{-/-}$ *IL-15Rα*$^{-/-}$ double-mutant (15KO/RαKO DKO) mice. After 6 wk, NK cells from *RAG-1*$^{-/-}$ (RAG-1 KO) mice were CFSE labeled and transferred into the chimera. Mice were then activated with 25 µg/g poly I:C and studied for NK cell activation. (B and C) Flow cytometric analyses of (B) surface CD69 expression and intracellular IFN-γ after 6 h and (C) CD69 and intracellular granzyme B expression after 12 h. Note robust NK cell expression of intracellular IFN-γ and granzyme B in WT HSC–reconstituted chimera, but not in chimera reconstituted with 15KO, RαKO, or mixed 15KO/RαKO HSCs. Results are representative of two independent experiments.

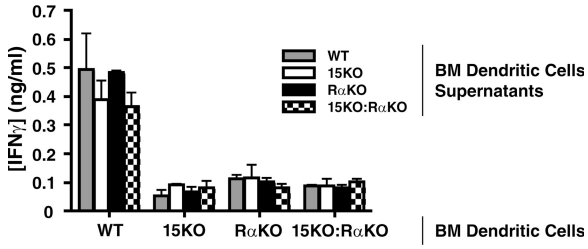

Figure 7. Soluble IL-15 complexed with sIL-15Rα fails to activate NK cells. Activation of IFN-γ secretion by NK cells co-cultured with various combinations of poly I:C–stimulated BMDCs and supernatants from these BMDCs. BMDCs from various genotypes (indicated along the x axis of graph) were activated with poly I:C and then supplemented with supernatants exchanged from similarly activated BMDCs. Genotypes of DCs from which supernatants were derived are indicated by individual columns (gray, WT; white, 15KO; black, RαKO; checkered, mixture of 15KO and RαKO). ELISA quantitation of NK cell IFN-γ secretion is indicated on the y axis. Note that WT DCs activate NK cells regardless of the type of supernatant added. Note also that WT DC–derived supernatants containing IL-15–sIL-15Rα complexes fail to support or augment NK cell activation.

Membrane-bound IL-15–IL-15Rα complexes, rather than soluble IL-15–sIL-15Rα complexes, support NK cell activation ex vivo

To determine whether membrane-bound or soluble IL-15–sIL-15Rα complexes support NK cell activation ex vivo, we tested whether serum containing these complexes could support NK cell activation. We generated radiation chimera in which *IL-15*$^{-/-}$ *IL-15Rα*$^{-/-}$ double-mutant mice were reconstituted with WT, *IL-15*$^{-/-}$, *IL-15Rα*$^{-/-}$, or a 1:1 mixture of *IL-15*$^{-/-}$ and *IL-15Rα*$^{-/-}$ HSCs. 24 h after stimulation with poly I:C, IL-15–sIL-15Rα complexes were present only in the sera of WT HSC–reconstituted chimera, consistent with our findings above (Fig. 5). We incubated this undiluted serum with purified NK cells in vitro for 6 h and assayed the NK cells for expression of CD69 and intracellular IFN-γ. These studies showed that serum from poly I:C–stimulated mice caused NK cells to express CD69 but did not induce IFN-γ expression (Fig. S4, available at http://www.jem.org/cgi/content/full/jem.20071913/DC1). Reinforcing our findings above with supernatants from activated BMDCs (Fig. 7), these studies indicate that membrane-bound IL-15–IL-15Rα complexes and not soluble IL-15–sIL-15Rα complexes provide the critical signal for stimulating IFN-γ production by NK cells in vitro and in vivo.

IL-15 that emerges from normal DCs does not bind to IL-15Rα on neighboring DCs

Free IL-15 has been reported in certain pathological conditions, and soluble recombinant IL-15 can be added to cells and bind to IL-15Rα. Hence, an additional question is whether IL-15–IL-15Rα complexes that emerge onto cell surfaces dissociate at an appreciable rate to release free soluble IL-15 that can bind to IL-15Rα on other cells. To address this question, we stimulated 1:1 mixtures of congenic CD45.1$^+$ WT DCs and either CD45.2$^+$ (CD45.1$^-$) *IL-15*$^{-/-}$ (15KO) DCs or CD45.2$^+$ (CD45.1$^-$) *IL-15Rα*$^{-/-}$ (RαKO) DCs with poly I:C. Cell surface expression levels of IL-15 and IL-15Rα were examined after 24 h. In mixtures of WT and *IL-15Rα*$^{-/-}$ DCs, both IL-15 and IL-15Rα proteins are readily induced by poly I:C on the surface of WT (CD45.1$^+$) cells, whereas neither protein is seen on the surface of *IL-15Rα*$^{-/-}$ (CD45.1$^-$) cells (Fig. 8 A, panels 1–4, and Fig. S5, which is available at http://www.jem.org/cgi/content/full/jem.20071913/DC1). In mixtures of WT (CD45.1$^+$) and *IL-15*$^{-/-}$ (CD45.1$^-$) DCs, IL-15Rα protein is induced to identical levels on the surfaces of both types of cells by poly I:C (Fig. 8 A, panels 7 and 8, and Fig. S5). Remarkably, IL-15 protein was induced only on the surface of WT (CD45.1$^+$) cells and was not found on *IL-15*$^{-/-}$ (CD45.1$^-$) cells, despite the fact that *IL-15*$^{-/-}$ cells express identical levels of surface IL-15Rα (Fig. 8 A, panels 5 and 6, and Fig. S5). Thus, IL-15 that emerges from WT cells is not released in appreciable amounts to bind to contiguous, neighboring cells.

To further examine whether IL-15–IL-15Rα complexes on the surface of DCs might release free soluble IL-15 protein, even if transiently, we tested the ability of an anti–IL-15

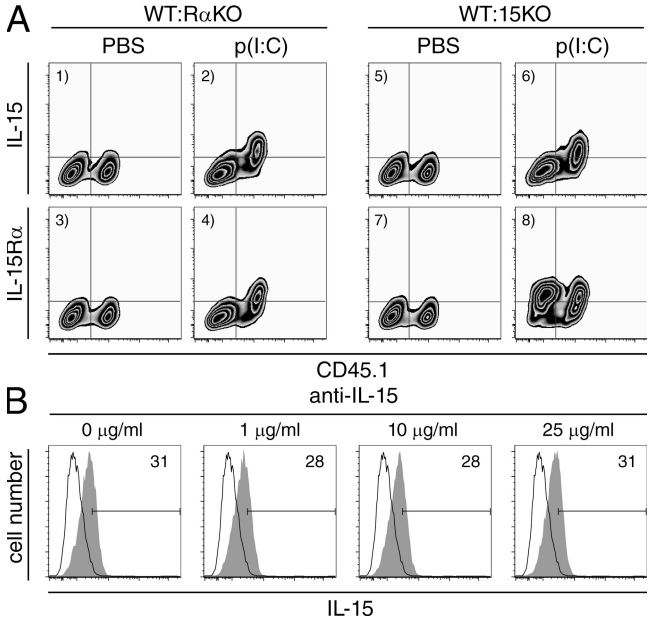

Figure 8. IL-15 that emerges from normal DCs does not bind to IL-15Rα on neighboring DCs. (A) Flow cytometric analyses of surface IL-15 and IL-15Rα expression in mixed DC cultures. 1:1 mixtures of congenic WT (CD45.1$^+$) and IL-15Rα$^{-/-}$ BMDCs (WT:RαKO; panels 1–4) or 1:1 mixtures of congenic WT (CD45.1$^+$) and IL-15$^{-/-}$ BMDCs (WT:15KO; panels 5–8) were stimulated in vitro with PBS or poly I:C for 24 h. Surface expression of IL-15 and IL-15Rα (and CD45.1) proteins was then analyzed by flow cytometry. Note that poly I:C–stimulated IL-15$^{-/-}$ BMDCs express normal levels of IL-15Rα (panel 8), but fail to capture or bind IL-15 even though virtually all of their neighboring WT DCs possess elevated levels of IL-15 in the same cultures (panel 6). (B) Flow cytometric analysis of surface IL-15 expression on normal BMDCs stimulated with poly I:C in the presence of the indicated doses of anti–IL-15 antibody. Note that WT BMDCs induce similar amounts of surface IL-15 protein (gray, filled histograms) regardless of the amount of inhibitor anti–IL-15 antibody in the cultures. Numbers indicate the percentage of cells whose fluorescence intensity lies beyond the unstimulated control cells. All data are representative of at least two independent experiments.

monoclonal antibody that binds only free IL-15 to compete with the formation of membrane IL-15–IL-15Rα complexes on the surface of poly I:C–stimulated DCs. We incubated progressively higher doses of anti–IL-15 antibody with WT DCs at the same time that they were stimulated with poly I:C and measured the amount of membrane-bound IL-15 after 24 h. These experiments revealed that the number of DCs expressing surface IL-15 and the intensity of surface staining were unchanged by doses of anti–IL-15 antibody of up to 25 μg/ml (Fig. 8 B). Hence, negligible amounts of membrane-bound IL-15 are released by membrane IL-15Rα. Therefore, physiologically induced IL-15–IL-15Rα complexes on DC surfaces do not dissociate at appreciable rates.

IL-15Rα expression on DCs is essential for NK cell activation in vivo

Our recent work showed that IL-15Rα expression by DCs is essential for DC-mediated NK cell activation in vitro, and recent studies have suggested that both IL-15 and DCs are critical for NK cell activation in vivo (20, 22). However, these studies leave open the possibility that IL-15 and DCs might independently support NK cell activation in vivo. For example, IL-15 might be produced by macrophages to stimulate DCs to activate NK cells in vivo. To more directly investigate whether IL-15Rα expression by DCs mediates NK cell activation in vivo, we reconstituted irradiated *IL-15*$^{-/-}$ *IL-15Rα*$^{-/-}$ double-mutant mice with a 1:1 mixture of HSCs from CD11c–diphtheria toxin (DT) receptor (CD11c-DTR) transgenic mice and HSCs from either WT or *IL-15Rα*$^{-/-}$ mice. After hematopoietic reconstitution, DT was injected into the chimera to transiently eliminate DCs. 24 h later, mice were injected with poly I:C. Finally, 6 h later, the status of CD11cHi DCs and the activation of NK cells were studied by flow cytometry (see experimental scheme, Fig. 9 A). These experiments revealed that DT eliminated approximately half of the CD11cHi DCs in chimera reconstituted with a 1:1 mixture of CD11c-DTR and other HSCs (Fig. 9 B). The remaining CD11cHi DCs expressed IL-15Rα after poly I:C stimulation in chimera reconstituted with WT HSCs and did not express IL-15Rα in chimera reconstituted with *IL-15Rα*$^{-/-}$ HSCs (Fig. 9 C). Thus, this experiment created chimeric mice in which *IL-15Rα*$^{-/-}$ DCs could be compared directly to IL-15Rα$^+$ DCs in their ability to activate NK cells.

Turning to NK cell activation in these chimera, we found that NK cells up-regulated CD69 in all poly I:C–stimulated mice, so both WT and *IL-15Rα*$^{-/-}$ DCs support this initial step of NK cell activation (Fig. 9 D). In contrast, WT DCs stimulated much greater numbers of NK cells to produce IFN-γ when compared with *IL-15Rα*$^{-/-}$ DCs (Fig. 9 D, compare second to third columns). Mice bearing a mixture of CD11c-DTR and *IL-15Rα*$^{-/-}$ DCs also supported NK cell production of IFN-γ in the absence of DT (Fig. 9 D, compare third to fourth columns). Parallel experiments performed with chimeric mice reconstituted with mixtures of CD11c-DTR and either WT or *IL-15*$^{-/-}$ HSCs revealed similar results, i.e., *IL-15*$^{-/-}$ DCs are also unable to activate NK cells in vivo (Fig. 9 E). Therefore, DCs must express both IL-15 and IL-15Rα to activate NK cells under physiological conditions.

DISCUSSION

Our current studies reveal a novel cell biological mechanism by which IL-15 is coexpressed with IL-15Rα in DCs after TLR stimulation and binds IL-15 within the ER–Golgi complex. Plasma membrane-bound IL-15–IL-15Rα complexes are highly stable complexes that do not release appreciable amounts of free IL-15 to neighboring IL-15Rα–bearing cells. These membrane-bound complexes trans present IL-15 to responsive cells during cell–cell contact, mediating the critical stimulatory signal to NK cells. Soluble IL-15–sIL-15Rα complexes are released from the cell surface, but these complexes fail to provide agonistic signals. We have coupled these cell biological studies with in vivo studies that have confirmed the physiological validity of our experiments with cultured DCs. Finally, we have demonstrated that IL-15Rα

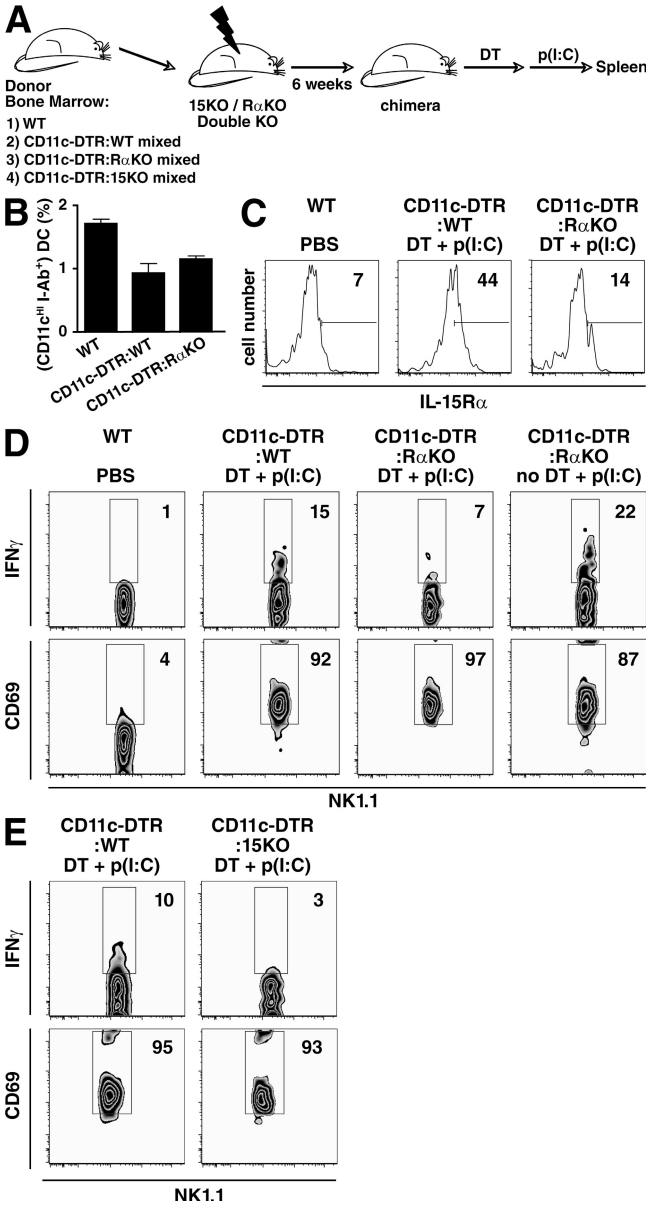

Figure 9. DC-specific expression of IL-15Rα is required for NK cell activation in vivo. (A) Experimental scheme. The indicated mixtures of HSCs were used to reconstitute double-mutant 15KO/RαKO mice. After 6 wk, chimeric mice were treated with DT and then activated with poly I:C. (B) Flow cytometric quantitation of splenic DCs ($CD11c^{HI}$ $I\text{-}Ab^{+}$) after DT treatment. The percentage of splenic DCs from the chimera reconstituted with the indicated HSCs is shown. (C) Selective loss of CD11c-DTR DCs in mixed radiation chimera. Surface expression of IL-15Rα after DT treatment and poly I:C was analyzed by flow cytometry. Note that elimination of CD11c-DTR DCs by DT treatment leaves elevated surface expression of IL-15Rα on the remaining WT DCs in CD11-DTR:WT mixed chimera (middle), whereas DT treatment of CD11c-DTR:RαKO mixed chimera leaves residual *IL-15Rα*$^{-/-}$ DCs (right). Splenic DCs from PBS-treated mice is shown as a control (left). (D) NK cells require IL-15Rα expression on DCs for full activation in vivo. Splenic NK cells from the mice above were analyzed for CD69 and IFN-γ expression by flow cytometry. Note that chimeras containing WT DCs (second column) or CD11c-DTR DCs (no DT treatment, fourth column) but not chimera containing RαKO DCs (third column) support NK cell production of IFN-γ (top panels). Note that mice containing significant residual DCs after DT treatment induce NK cell expression of CD69 after poly I:C (columns 2–4). WT HSC–reconstituted chimera treated with PBS (first column) are included as a control. Results are representative of two independent experiments, with eight mice used for each experiment. (E) NK cells require IL-15 expression on DCs for full activation in vivo. Chimera reconstituted with a 1:1 mixture of CD11-DTR HSCs with either WT HSCs (left) or *IL-15*$^{-/-}$ HSCs (right) were treated as in A and D above, and flow cytometric analyses of NK cell expression of CD69 and IFN-γ are shown.

expression specifically on DCs plays an essential role in supporting NK cell activation in mice. As membrane-bound IL-15Rα–IL-15 complexes on DCs appear to play a dominant role in supporting NK cell activation in vivo, these studies highlight the notion that cytokine signals may be delivered by specific cell types in cell contact–dependent fashion.

IL-15Rα, a chaperone for IL-15

We have found that EndoH-sensitive forms of IL-15 are bound to IL-15Rα in LPS-stimulated DCs, and that IL-15–IL-15Rα complexes can be found in brefeldin A–treated but not cycloheximide-treated cells. Considered together with our discovery that DCs must express IL-15Rα to secrete IL-15, it is likely that IL-15Rα binds to IL-15 and stabilizes this protein in the ER and/or Golgi apparatus. Such complexes probably prevent free IL-15 from undergoing ER-associated protein degradation, a well-established process by which "misfolded" proteins in the ER are proteolyzed in ubiquitin- and proteosome-dependent processes (27). Recycling and trans presentation of these membrane-bound complexes may then stimulate responding cells (14). Hence, our results suggest that IL-15Rα functions both as an intracellular chaperone for IL-15 as well as an extracellular scaffold that presents IL-15 to responsive cells.

We have shown that IL-15–IL-15Rα complexes that emerge onto the surface of DCs fail to release soluble IL-15 to neighboring IL-15Rα–expressing DCs. These surprising observations suggest that IL-15–IL-15Rα complexes on DC surfaces are highly stable, a finding consistent with the high affinity of IL-15Rα for IL-15 ($\sim 5 \times 10^{-11}$ M) (8). Thus, although heterologous IL-15 can be added to IL-15Rα–expressing cells, form cell surface complexes, and stimulate IL-15–dependent responses, the physiological production and trans presentation of IL-15 by DCs appears to predominantly involve IL-15–IL-15Rα complexes that form intracellularly during biosynthesis and remain intact for prolonged periods (e.g., 24 h) (17).

Preassembly of IL-15–IL-15Rα protein complexes in DCs explains a requirement for coordinate expression of IL-15 and IL-15Rα to support NK cell activation

Our finding that IL-15–IL-15Rα complexes are preassembled within DCs provides an explanation for why coordinate expression of these molecules is required for NK cell activation

in vitro and in vivo. Coordinate expression of IL-15 and IL-15Rα by DCs is required for the formation of IL-15–IL-15Rα complexes and the activation of NK cells in vitro and in vivo after TLR stimulation. This result parallels prior findings that coordinate expression of these molecules is necessary to perform IL-15–dependent homeostatic functions in resting mice (18, 19). Hence, the cell biology of IL-15 and IL-15Rα interactions during inflammatory conditions resembles the homeostatic condition.

In addition to identifying intracellular complexes of IL-15 and IL-15Rα, we have identified soluble extracellular IL-15–sIL-15Rα complexes that are released from DCs after TLR stimulation. A prior study described the presence of soluble IL-15Rα in the serum of resting mice, and proposed that IL-15Rα could be proteolytically cleaved from the cell surface (24). Our current experiments extend and contrast with the prior results in two respects. First, we find that the production and release of soluble sIL-15Rα is induced by TLR stimulation, rather than being constitutively present at high levels (e.g., 40 ng/ml) in resting C57BL/6J strain mice (24). Second, we find that the majority of soluble IL-15Rα is complexed with IL-15. Therefore, although free IL-15 is not released from DCs, soluble IL-15–sIL-15Rα complexes, which may represent IL-15 bound to a proteolytic product of IL-15Rα, are released into solution.

Our results with both cultured DC supernatants and mouse sera indicate that membrane-bound IL-15–IL-15Rα complexes on DCs and not soluble IL-15–sIL-15Rα complexes support NK cell production of IFN-γ and granzyme B. Although soluble IL-15–sIL-15Rα complexes might perform other functions, this important finding suggests that trans presentation of IL-15 requires cell–cell contact between IL-15–presenting and IL-15–responsive cells. The cell contact–dependent nature of IL-15 trans presentation also implies that IL-15 signals may be delivered in the context of other signals between the two cells, akin to antigen presentation between DCs and T cells. Finally, our findings imply that IL-15 signals are delivered via direct cell–cell contact rather than via soluble proteins. Hence, cytokine-mediated survival, growth, and activation signals may be delivered by specific cell types and in specific locations.

Although the physiological functions of soluble IL-15–sIL-15Rα complexes are unclear, it is important to note that these complexes are neither agonistic nor antagonistic in our DC–NK cell co-culture assays (Fig. 7 A). In this regard, nonagonsitic soluble IL-15–sIL-15Rα complexes contrast with agonistic soluble IL-6–IL-6R complexes (28). This observation provides important clues about their function. If soluble complexes bound to IL-2/15Rβ/γc receptors as well as membrane-bound complexes, one might expect them to display either agonist or antagonist activities (via competitive inhibition). Thus, soluble IL-15–sIL-15Rα complexes may not bind well to cell surface IL-2/15Rβ receptors on cell surfaces under physiological circumstances. This failure of IL-15–sIL-15Rα complexes to bind IL-2/15Rβ receptors could be due to differences between the structures of the soluble IL-15–sIL-15Rα complex and the membrane-bound IL-15–IL-15Rα complex. Alternatively, membrane-bound IL-15–IL-15Rα complexes may possess a competitive advantage over soluble IL-15–sIL-15Rα complexes due to the increased local concentration or valency of membrane-bound complexes during cell–cell contact.

The failure of endogenous soluble IL-15–sIL-15Rα complexes to activate NK cells can also be contrasted with recent studies indicating that certain cytokine-specific antibodies can form complexes with their cognate cytokines, stabilize cytokines, and amplify the immunological effects of these cytokines (29–31). There are multiple differences between antibody–cytokine complexes and receptor–cytokine complexes. For example, different anti–IL-15 antibodies or forms of IL-15Rα may interfere with IL-15 binding to IL-2/15Rβ and γc receptors. In addition, antibodies, but not soluble cytokine receptors, can also bind to Fc receptors on multiple cells. Recent structural studies of IL-15 and IL-15Rα proteins have revealed important insights into how these proteins interact and should facilitate future investigations into how such receptor–cytokine complexes differ from antibody–cytokine complexes (32). Combining these biochemical insights with the divergent biological properties of these complexes should facilitate future attempts to engineer these proteins for therapeutic benefit (33, 34).

DCs require IL-15Rα to trans present IL-15 to NK cells during NK cell activation

We have used mixed radiation chimera to selectively unveil the role of IL-15Rα expression by DCs during NK cell activation in vivo. Our findings extend our prior work that IL-15Rα on BMDCs is critical for activating NK cells in vitro (20). Our current findings are also consistent with a recent study using both IL-15$^{-/-}$ mice and CD11c-DTR mice to show that both IL-15 and DCs are required for NK cell activation in vivo (22). By using mixed radiation chimera generated from both *IL-15Rα*$^{-/-}$ and CD11c-DTR HSCs, we have extended the prior work to show that IL-15Rα expression specifically on DCs mediates NK cell activation in vivo. Further elucidation of these interactions should be facilitated by the generation of mice bearing lineage-specific deletions of IL-15 or IL-15Rα. Considered together with our data showing that DCs trans present IL-15 via cell–cell contact, and with recent work showing the localization of NK cells to lymphoid structures, a model of NK cell activation emerges in which NK cells are recruited to DCs and receive critical IL-15–IL-15Rα–dependent activation signals during DC–NK cell contact (35, 36). In this regard, DC activation of NK cells broadly resembles DC activation of naive T cells, during which an immunological synapse constitutes a sophisticated cell contact–dependent activation mechanism. Indeed, direct evidence for a DC–NK cell synapse directing IL-15 signaling to NK cells has recently been reported (37).

Recent structural studies have revived the notion that IL-2Rα may trans present IL-2 (38–40). Although IL-2 signals clearly perform distinct biological functions from IL-15

signals in vivo, and although IL-2Rα has not shown the capacity to bind and trans present IL-2 in vivo, directed cytokine presentation may nevertheless be a feature of IL-15, IL-2, and perhaps other cytokines (7, 10, 38, 41, 42). This mode of cell contact–dependent delivery of cytokine signals would greatly restrict the cells that receive cytokine signals. Finally, given the rapidity with which memory $CD8^+$ T cells are reactivated in vivo, the requirement for DCs to reactivate memory $CD8^+$ T cells, the selective expression of IL-2/15Rβ receptors by these cells, and the IL-15 dependence of these cells, it is possible that memory $CD8^+$ T cell reactivation involves a similar IL-15–dependent activation event mediated by DCs (43).

In summary, we have shown that IL-15 and IL-15Rα are preformed as complexes in DCs in response to TLR stimulation. IL-15–IL-15Rα complexes progress to the DC surface where they deliver critical activating signals to NK cells during cell–cell contact. IL-15–IL-15Rα complexes that are cleaved from the surface of DCs may be byproducts of a termination mechanism for this potent stimulus. This novel series of cell biological events refines our understanding of IL-15Rα–mediated trans presentation, and highlights how the immune system delivers cytokine signals in a highly regulated and cell type– and geographically restricted fashion.

MATERIALS AND METHODS

Mice, radiation chimera, adoptive transfers, and in vivo DC ablation. *IL-15Rα*$^{-/-}$ (RαKO) mice were described previously (10). All strains were backbred to a C57B1/6J background for at least 12 generations. *IL-15*$^{-/-}$ (15KO) and *RAG-1*$^{-/-}$ mice on a C57BL/6J background were purchased from Taconic Laboratories. CD11c-DTR transgenic mice were purchased from The Jackson Laboratory (44). Double-mutant *IL-15*$^{-/-}$ *IL-15Rα*$^{-/-}$ (15KO/RαKO) mice were generated by interbreeding in our facility. Radiation bone marrow chimeras were produced as described previously, except that mixed 15KO:RαKO, CD11c-DTR:WT, and CD11c-DTR:RαKO radiation chimera were generated by using mixtures (1:1) of bone marrow cells from congenic donors of the indicated genotypes of mice (18). All mice were used between the ages of 6 and 12 wk of age. All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of California, San Francisco.

For in vivo stimulation experiments, mice were injected intraperitoneally with 10 g/kg LPS (Sigma-Aldrich) or 25 g/kg poly I:C (GE Healthcare). For adoptive transfer experiments, NK cells were purified from the spleens of *RAG-1*$^{-/-}$ mice by adherence and magnetic bead depletion of myeloid cells, and in some experiments, labeled with CFSE before intravenous injection into recipient mice as described previously (18). In vivo depletion of $CD11c^-DTR^+$ DCs was performed using a single injection of 2–4 μg/kg body weight of DT (EMD) (44).

In vitro DC preparations and assays. BMDCs were generated in culture as described previously (18). The purity of BMDC cultures was checked by analyzing an aliquot of each culture for surface expression of CD11c and CD11b by flow cytometry (BD Biosciences). Splenic DCs were enriched by digesting spleens with 2.5 mg/ml collagenase B (Boehringer Mannheim) and 0.3 mg/ml DNase I (Sigma-Aldrich), followed by isolation on percoll gradients as described previously (18). TLR stimulation of DCs was performed with either 1 μg/ml LPS or 25 μg/ml poly I:C. To inhibit protein secretion, BMDCs were treated with Golgi Plug (brefeldin A; BD Biosciences) during the LPS stimulation per the manufacturer's instructions. To inhibit protein synthesis, BMDCs were treated with 10 μg/ml cycloheximide during LPS stimulation.

For DC-mediated activation of NK cells in vitro, 2×10^5/ml BMDCs were cultured for 24 h in the presence of 1 μg/ml LPS or 25 μg/ml poly I:C, followed by incubation with 10^5/ml NK cells as described previously (20).

Detection of endogenous IL-15 and IL-15Rα proteins. For ELISA-based quantitation of proteins, IL-15Rα was quantified using the Duo-set kit (R&D Systems) according to the manufacturer's instructions. IL-15 was quantified by using anti–IL-15 AIO3 antibody (eBioscience) as a capture reagent and biotinylated BAF447 antibody (R&D Systems) as the detection reagent. For IL-15–IL-15Rα complex detection, anti–IL-15 AF447 antibody (R&D Systems) was used as the capture antibody and anti–IL-15Rα (from the Duo-set kit) as the detection antibody. For ELISA-based analyses of intracellular IL-15 and IL-15Rα proteins, $5–10 \times 10^6$ BMDCs per condition were lysed in NP-40 lysate buffer (50 mM Hepes, 120 mM NaCl, 1 mM EDTA, 0.1% NP-40, and protease inhibitor cocktail [Roche]). IFN-γ and IL-12 were quantified using a commercial assay kit according to the manufacturer's instructions (BD Biosciences).

For immunoblotting detection of intracellular IL-15 and IL-15Rα, $5–10 \times 10^6$ BMDCs were stimulated for 12 or 24 h with 0.1 μg/ml LPS washed in PBS and lysed in NP-40 or RIPA lysis buffer (0.5% SDS, 0.1% deoxycholic acid and 1% NP-40 in PBS, 150 mM NaCl, 50 mM Tris, pH 7.5, 1 mM NaVO4, 1 mM NaF, 1 mM PMSF, and protease inhibitors [Roche]), cleared by centrifugation (14,000 *g* for 20 min at 4°C), and immunoprecipitated with a mixture of anti–IL-15Rα AF 551 (R&D Systems) and anti–IL-15Rα N19 (Santa Cruz Biotechnology, Inc.) with a mixture of protein A– and protein G–coupled Sepharose beads. Immunoprecipitates were then washed in lysis buffer and subjected to SDS-PAGE. Immunoblotting of transferred proteins was performed with either rat anti–IL-15 antibody (Amgen) and goat anti–rat IgG horseradish peroxidase (Jackson ImmunoResearch Laboratories) or biotin-conjugated anti–IL-15Rα antibody BAF 551 (R&D Systems) and streptavidin–horseradish peroxidase (BD Biosciences). EndoH sensitivity assays were performed by treating immunoprecipitates with EndoH per the manufacturer's instructions (New England Biolabs, Inc.).

Surface IL-15 expression was detected using biotinylated anti–IL-15 antibody (PeproTech). IL-15Rα expression on cell surfaces was detected by using biotinylated anti–IL-15Rα antibody BAF551 (R&D Systems) as described previously (15). IFN-γ and granzyme B production by NK cells was determined by intracellular cytokine staining as described previously (Caltag and eBioscience) (12). Cell surface expression of NK1.1, CD69, CD40, CD86, CD11b, CD11c, and $I\text{-}A^b$ was performed using commercial antibodies (BD Biosciences). Cells were analyzed with an LSR II cytometer (Becton Dickinson) and FloJo software.

Online supplemental material. Fig. S1 shows the detection of IL-15 and IL-15Rα proteins and the IL-15–IL-15Rα complex. In Fig. S2, brefeldin A treatment blocks the emergence of IL-15Rα onto the surface of cells or into the supernatants of DCs. Fig. S3 shows the expression of IL-15 and sIL-15Rα proteins on the cell surface of TLR-stimulated BMDCs. Fig. S4 shows that soluble IL-15 complexed with sIL-15Rα fails to activate NK cells ex vivo. In Fig. S5, IL-15 that emerges from normal DCs does not bind to IL-15Rα on neighboring DCs. The online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20071913/DC1.

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