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Cutting Edge: The Role of IFN-α Receptor and MyD88 Signaling in Induction of IL-15 Expression In Vivo

Sara L. Colpitts,*1 Thomas A. Stoklasek,*1,2 Courtney R. Plumlee,* Joshua J. Obar,*3 Caiying Guo,† and Leo Lefrançois*

IL-15 plays a multifaceted role in immune homeostasis, but the unreliability of IL-15 detection has stymied exploration of IL-15 regulation in vivo. To visualize IL-15 expression, we created a transgenic mouse expressing emerald-GFP (EmGFP) under IL-15 promoter control. EmGFP/IL-15 was prevalent in innate cells including dendritic cells (DCs), macrophages, and monocytes. However, DC subsets expressed varying levels of EmGFP/IL-15 with CD8+ DCs constitutively expressing EmGFP/IL-15 and CD8− DCs expressing low EmGFP/IL-15 levels. Virus infection resulted in IL-15 upregulation in both subsets. By crossing the transgenic mice to mice deficient in specific elements of innate signaling, we found a cell-intrinsic dependency of DCs and Ly6C+ monocytes on IFN-α receptor expression for EmGFP/IL-15 upregulation after vesicular stomatitis virus infection. In contrast, myeloid cells did not require the expression of MyD88 to upregulate EmGFP/IL-15 with CD8+ DCs constitutively expressing EmGFP/IL-15 and CD8− DCs expressing low EmGFP/IL-15 levels. These findings provide evidence of previously unappreciated regulation of IL-15 expression in myeloid lineages during homeostasis and following infection. The Journal of Immunology, 2012, 188: 000–000.

Dendritic cells (DCs) and other APCs such as macrophages help maintain the homeostasis of immune cells. DCs produce the pleiotropic cytokine IL-15, which binds the IL-15Rα chain intracellularly (1, 2). Using a unique mechanism termed transpresentation, the two proteins form a cell–surface complex through which IL-15 is presented in trans to apposing cells that express CD122 (the IL-2/15Rβ-chain) and CD132 (the common γ-chain) (1). Genetic deletion of IL-15 or IL-15Rα results in a lack of NK cells and the loss of most CD8+ memory T cells and subsets of intestinal intraepithelial lymphocytes (IELs) (3, 4). Specific deletion of IL-15Rα within the CD11c+ DC population or the LysM+ macrophage population has a negative impact on some populations of IL-15/IL-15Rα-dependent cells, but does not reduce any one population to the levels of complete knockout (KO) mice (5). Alternatively, limiting IL-15 expression to DCs can partially restore the defect within the NK cell and CD8+ memory T cell populations but fails to rescue IEL subsets (6) that require IL-15 production specifically from intestinal epithelial cells (IECs) (7).

The mechanisms controlling the regulation of IL-15 remain largely unknown. Considering the severe lymphoproliferation and immune activation that occurs in IL-15 transgenic mice and mice treated with IL-15/IL-15Rα complex (8, 9), it is perhaps not surprising that IL-15 expression is under tight transcriptional and translational control (10). Current methods for detecting IL-15 directly ex vivo are limited and further complicated by the very short in vivo half-life of soluble IL-15 (9, 11), as well as the unique mechanism of IL-15 trans-presentation. IL-15 expression is often measured by PCR detection of mRNA. Alternatively, we and others (2, 9) have used ELISA to measure low levels of IL-15 in the sera of mice and to detect the formation of IL-15–IL-15Rα complexes within the cell. Detection of IL-15 by Western blot or flow cytometry has proven difficult, and robust stimulation with TLR ligands is required to upregulate IL-15 to detectable levels (5, 6). Moreover, direct detection of IL-15 production by isolated cells ex vivo or in vivo has not been documented in the literature, thus heralding the need for a fresh approach. Therefore, we used bacterial artificial chromosome (BAC) technology to generate a transgenic mouse line in which emerald GFP (EmGFp) is expressed under the control of endogenous IL-15 regulatory elements. This system has allowed us to identify cellular subsets with the potential to...
produce IL-15 and visualize IL-15 regulation during the generation of an immune response.

Materials and Methods

Mice
C57BL/6 mice were purchased from Charles River-National Cancer Institute. Chimeric mice were generated by i.v. injection of \(2 \times 10^6\) bone marrow cells from donor mice into lethally irradiated (1000 rad) recipients. All mice were housed in accordance with the Animal Care Committee at the University of Connecticut Health Center.

Generation of EmGFP/IL-15 reporter mice

The details regarding construction of these mice can be found in the legend for Supplemental Fig. 1. EmGFP/IL-15 mice were further crossed to MyD88 KO mice (12) and IFN-\(\alpha\) receptor (IFNAR) 1 KO mice (13).

Flow cytometry and cell sorting

A total of 5–10 \(10^6\) cells were treated with Fc block followed by staining with the indicated Abs obtained from ebioscience, BioLegend, or BD Pharmingen and analysis on a BD LSRII (BD Bionsciences). Data were analyzed using FlowJo Software (Tree Star, San Carlos, CA).

Vesicular stomatitis virus infection and in vivo Ab treatment

Mice were infected i.v. with 1.5 \(\times 10^5\) PFU vesicular stomatitis virus (VSV). Where indicated, mice were treated with 0.5 mg anti-plasmacytoid DC Ag-1 (PDCA-1; Miltenyi Biotec) i.v. 24 h prior to infection.

Confocal microscopy

Spleens were sectioned using a scalpel and fixed in 2% PFA/PBS for 2 h at 4°C with agitation, followed by additional washing in PBS (three times). Tissue samples were then incubated with anti-B220 Alexa 647 (blue) in PBS/2% FBS/goat serum overnight at 4°C. Tissues were then washed three times, mounted on slides, and analyzed by confocal microscopy (Zeiss LSM 510 Meta; Carl Zeiss). Spleen images were taken with a 20\(\times\)0.75 numerical aperture lens, and images were analyzed using Imaris software (Bitplane).

Statistical analysis

Statistical significance was established by a one-way ANOVA test with a Tukey posttest (GraphPad Prism; GraphPad). A \(p\) value <0.05 was considered significant.

Results and Discussion

Generation of a BAC-EmGFP/IL-15 reporter mouse

To visualize cells with the potential to produce IL-15 in vivo, we generated a transgenic mouse line in which EmGFP was introduced into the IL-15 locus. EmGFP was inserted into exon 3 of the IL-15 gene contained in a BAC, which effectively deleted the start codon (Supplemental Fig. 1).

EmGFP/IL-15 expression differs between DC subsets

The ability of DCs to produce and present IL-15 has been documented (14). However, in all instances, additional sources of stimulation are required to enhance IL-15 production to detectable levels, and an analysis of IL-15 expression levels within different DC subsets has not been performed (5, 6). We initially characterized EmGFP/IL-15 expression in individual DC subsets at steady state. Two major subsets of conventional DCs (cDCs) can be identified in the spleens of naive mice: CD8\(^{+}\) and CD8\(^{-}\). CD8\(^{+}\) DCs were examined using confocal microscopy. In uninfected mice, EmGFP/IL-15 levels were generally low, but EmGFP/IL-15+ cells were observed in the red pulp and the T cell zone, the peritubular lymphoid sheath, of the white pulp (Fig. 2C). Twenty-four hours after VSV infection, a dramatic increase in the number of EmGFP/IL-15+ cells as well as in the intensity of EmGFP/IL-15 expression was observed in the spleen. EmGFP/IL-15+ cells appeared to be localized to the T cell.
zones, although EmGFP/IL-15+ cells were also present in the red pulp (Fig. 2C and T.A. Stoklasek, unpublished observations). Therefore, the EmGFP/IL-15 reporter system allowed the visualization of increased IL-15 promoter activity in myeloid subsets following virus infection by ex vivo as well as in situ analysis.

IL-15 induction in response to virus infection is through IFNAR-independent and MyD88-independent mechanisms

Both type I IFNs and TLR signaling through MyD88 are known to induce IL-15 (14). Furthermore, the ability of DCs to activate NK cells via IL-15 transpresentation occurs in an IFN-dependent fashion following stimulation with TLR ligands (16). VSV infection is also a potent inducer of type I IFN (17). To determine which factors were involved in EmGFP/IL-15 induction, we generated EmGFP/IL-15 mice lacking MyD88 or the type I IFN receptor, IFNAR1. We compared the MFI of EmGFP/IL-15 in VSV-infected KO mice to groups of littermate controls that were: 1) uninfected KO-EmGFP/IL-15 mice; or 2) VSV-infected mice heterozygous for expression of the gene in question. Between two independent experiments, MyD88 KO-EmGFP/IL-15 mice upregulated EmGFP/IL-15 to the level of heterozygous controls (naive only) were stained with anti-B220 Alexa 647 (blue) and analyzed by confocal microscopy. EmGFP/IL-15 is shown in green. Spleen images were obtained with a 20×/0.75 NA lens (14× magnification), and 40-μm merged z-stacks are shown.

In IFNAR KO mice, one could predict that downstream inflammatory mediators might also be lacking following VSV infection when all cells are unable to signal via type I IFNs. Therefore, we determined if direct signaling through the IFNAR was responsible for the upregulation of IL-15 following VSV infection. To address this question, we generated mixed bone marrow chimeras by reconstituting lethally irradiated wild-type (WT) mice (CD45.2) with an equal mixture of bone marrow cells from IFNAR KO-EmGFP/IL-15 mice (CD45.1/CD45.2) and WT-EmGFP/IL-15 (CD45.1) mice. Mixed chimeras from MyD88 KO-EmGFP/IL-15 and WT-EmGFP/IL-15 bone marrow were also made to further support the results described above. Chimeras were infected with VSV or not, and spleens were harvested after 24 h. In agreement with our observations in MyD88 KO-EmGFP/IL-15 mice, no significant defect in EmGFP/IL-15 upregulation was noted in mixed MyD88 KO chimeras (Fig. 4A). In contrast, IFNAR KO-EmGFP/IL-15−derived cells were unable to upregulate EmGFP/IL-15 following infection (Fig. 4B). These findings demonstrated that cell-intrinsic IFNAR expression was required by DCs and monocytes for the upregulation of IL-15 following VSV infection.

Lastly, we examined the source of type I IFNs following infection with VSV. Plasmacytoid DCs (pDCs) produce large quantities of type I IFNs following viral infection, and previous work suggests that pDCs are required for efficient production of IL-15 by cDCs (CD8+ and CD8−) following CpG stimulation (18). To determine if pDCs functioned as the primary source of IL-15–inducing type I IFNs following VSV infection, we treated mice with anti–PDCA-1 24 h prior to infection to deplete pDC (19). In all cell subsets examined, pDC depletion significantly reduced EmGFP/IL-15 induction compared with controls (Fig. 5). However, these subsets continued to produce significantly increased levels of EmGFP/IL-15 over that of uninfected controls, suggesting an alternative source of type I IFN production in the absence of pDCs. Thus, both pDC-dependent and -independent...
effects appear to regulate IL-15 induction in response to virus infection.

In this study, we have described a new BAC-EmGFP/IL-15 reporter mouse to track IL-15 expression. This system represents a major improvement over currently available IL-15 detection methodology, which cannot accurately identify ex vivo or in situ IL-15 expression levels or the cell types expressing IL-15. Our results indicated that steady-state levels of EmGFP/IL-15 were distinct between unique DC subsets. For example, CD8+ DCs expressed increased levels of EmGFP/IL-15 in comparison with the CD8− subset, the majority of which lacked IL-15 at rest. Our findings differ from recent work by Stonier et al. (6), in which the authors used a polyclonal anti-murine IL-15 Ab to show low but comparable levels of IL-15 on the surface of splenic CD8+ and CD8− DCs. However, analysis was performed on splenocytes that were cultured overnight prior to analysis, which has the potential to activate cells and possibly upregulate IL-15. In our hands, consistent and convincing staining of cells directly ex vivo for IL-15 has not been achievable (data not shown). A higher level of steady-state IL-15 expression by CD8+ DCs suggested that this subset was involved in homeostatic regulation mediated by IL-15. Moreover, the localization of CD8+ DCs to the T cell area suggests they may be pivotal in maintaining, for example, memory CD8+ T cells following viral or bacterial infection under homeostatic conditions (20).

Previous studies have found that IL-15 can be induced by a number of inflammatory stimuli, including cytokines and TLR ligands (14). The IL-15 promoter region contains several known transcription factor consensus sites that could be activated via TLR pathways such as NF-κB, IFN-stimulated regulatory element, AP-1, IFN-γ activation site, and IFN regulatory factor element (21, 22). Although injections of polyinosinic-polycytidylic acid or IFN-α both induce IL-15 in splenic DCs (14), whether virus infection has similar effects was not known. In addition, which cell types might be induced to express IL-15 in response to infection had not been examined. Our findings indicated that VSV infection significantly upregulated IL-15 promoter activity as reported by EmGFP expression in both DC and monocyte subsets, and IL-15 upregulation in splenic myeloid cells occurred independently of MyD88 expression. These findings are in contrast to previous reports in which iECs required MyD88 for the expression of IL-15 and the maintenance of IL-15–dependent IELs (23). Such differences in MyD88 dependency could reflect the dramatic environmental differences experienced by iECs (parenchymal cells) and splenic APCs (which are hematopoietic derived). iECs are continuously exposed to commensal bacteria in the absence of overt inflammation, and MyD88-mediated signaling could contribute to constitutive IL-15 expression during steady state. Meanwhile, the inflammatory milieu of the spleen following viral infection is under the influence of type I IFNs, and our data indicate that cell-intrinsic IFNAR expression was required for the upregulation of IL-15 by myeloid cells. In addition, although pDCs are considered a potent source of type I IFNs, their expression of CD40L has been shown to support IL-15 upregulation in cDCs following CpG exposure. Our finding that pDC depletion did not entirely ablate the ability of DCs and monocytes to upregulate IL-15 suggests that reduced upregulation can occur independently of pDC–cDC cross talk following VSV infection. It is possible that: 1) other cell types have the ability to provide limited CD40−CD40L interactions; or 2) IL-15 upregulation can occur entirely independent of CD40−CD40L engagement. It is also interesting to note that, following VSV infection, the assistance of pDCs was provided independently of MyD88 expression. This is in contrast to the findings of Kato et al. (24), in which MyD88 KO pDCs were unable to produce IFN-α following in vitro infection with another negative sense ssRNA virus, Newcastle Disease virus. Thus, our findings suggest that in the absence of MyD88, sufficient type I IFNs to upregulate IL-15 can be produced by other cell types, likely of both parenchymal and hematopoietic origin, possibly through a RIG-I–dependent mechanism (24).

In summary, our novel BAC-EmGFP/IL-15 transgenic reporter system has allowed us to track endogenous IL-15 promoter activity during steady-state conditions and inflammation. Analysis of the reporter refined our understanding.
of constitutive and induced IL-15 expression in myeloid subsets. Overall, the results suggested that IL-15 production is an important and tightly regulated characteristic of innate immune cells.

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Disclosures
The authors have no financial conflicts of interest.

References
Supplemental Information

Supplemental Figure 1. Generation of EmGFP/IL-15 transgenic mice. A mini targeting vector consisting of DNA sequence homologous to 581 base pairs (bp) in intron 2 and exon 3 of the murine IL-15 genomic locus, EmGFP sequence, frt-PGKneo-frt sequence, and DNA homologous to 510 bp in intro 3 of the murine IL-15 locus was engineered by homologous recombination into BAC construct RP24-275P1 containing the murine IL-15 genomic locus. The transgenic construct was then randomly integrated into ES cells (129svev X C57BL6) by electroporation. The EmGFP/frt-PGKneo-frt sequence was situated to delete the first 4 codons of the IL-15 gene as well as a portion of intron 3, replacing a total of 56 bp of the endogenous sequence. The BAC IL-15 transgenic construct is approximately 180kb in length and contains the whole IL-15 gene and 42kb of the upstream sequence. The PGKneo sequence was removed by breeding the BAC-IL-15-EmGFP frt-PGKneo-frt line to the FLP recombinase transgenics. Presence of the BAC transgene was determined by PCR using primers specific for GFP (Primer 1: 5’-TCATCTGCACCACCACGGCAAGC-3’; Primer 2: 5’-AGCAGGACCATGTGATCGCCTGCATTGTA TCGC-3’). All mice used for these experiments were backcrossed to C57BL/6 mice (Charles River-NCI) for at least three generations. After three generations, the process was accelerated using speed congenic technology (Speed Congenics Facility, Dartmouth Medical School, Hanover, NH). No differences in the levels of EmGFP/IL-15 expression were observed amongst subsequent generations throughout the back-crossing process.

Supplemental Figure 2. EmGFP expression correlates with endogenous IL-15 mRNA levels. Splenocytes were harvested from transgenic mice that were naïve or infected with VSV for 24
hrs. Prior to FACS sorting, cells were enriched by staining with CD11c-APC followed by anti-
APC MACS beads. The cells were then sorted based on CD8 and EmGFP/IL-15 expression as
shown by the gates. Histograms compare the levels of EmGFP/IL-15 on each of the
corresponding three purified populations gated on in the dot plots. RNA was isolated from
sorted cells with the RNeasy Mini Kit following cell disruption using QIAshredder (Qiagen).
cDNA was made with SuperScript (Invitrogen). SYBR Green (Invitrogen) and the following
primers (Integrated DNA Technologies) were used for quantitative PCR: IL-15 #1, 5’-
GCTCTTACCTGGGCATTAATGAA-3’; IL-15 #2, 5’-
TGACACAGCCCAAAATGAAC-3’; β-actin #1, 5’-AGAGGGAAATCGTGCGTGAC-3’;
β-actin #2, 5’-CAATAGTGATGACCTGGCCGT-3’. qPCR was performed on a Bio-Rad
iCycler. IL-15 mRNA expression was normalized to β-actin expression (2^ΔCT). Graph depicts
fold increase in IL-15 mRNA expression relative to the EmGFP/IL-15^low CD8^- DC population
(value of 1).
Supplemental Figure 1

IL-15 genomic Locus:

Mini targeting vector:

BAC transgenic construct:

After FLP excision: