Hirosaki Med. J. 59 (Suppl.) : S35—S42, 2007

IL-15-DEPENDENT CROSS-TALK BETWEEN CONVENTIONAL AND PLASMACYTOID DENDRITIC CELLS IS ESSENTIAL FOR CPG-INDUCED IMMUNE ACTIVATION

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Abstract Unmethylated CpG oligodeoxynucleotides (CpG) prevalent in bacteria and DNA viruses bind Toll-like receptor (TLR) 9 and directly stimulate DCs, thereby activating the innate and adaptive immune responses. CpG is potentially a powerful reagent for protective immunity against infection by a wide variety of pathogens, for cancer and allergy therapies, and for the development of prophylactic and therapeutic vaccines. Here we investigated the role of interleukin (IL)-15 in the activation of CpG-induced immune responses. We show that upon CpG-priming, both wild-type (WT) and NK cell-depleted WT mice produce interleukin (IL)-12 p70 and become resistant to a lethal dose of *Listeria monocytogenes* (LM), whereas IL-15$^{-/-}$ mice impair IL-12 p70 production and succumb to the infection. Notably, CpG-stimulated conventional dendritic cells (cDCs) are the major producer of both IL-15 and IL-12 p70, but cDCs do not produce IL-12 in the absence of plasmacytoid DCs (pDCs) in vivo. Importantly, cDC-derived IL-15 induces CD40 expression on cDCs, which interacts with CD40 ligand on pDCs, leading to CD40 cross-linking and IL-12 production. Collectively, these findings show that IL-15-dependent cross-talk between cDCs and pDCs is essential for CpG-induced immune activation (recently published in Nat Immunol 2006;7:740-6).

Hirosaki Med. J. **59, Supplement** : S35—S42, 2007

Key words: innate immunity; IL-12; TLR9; *Listeria monocytogenes*

Introduction

Under physiologic conditions, Toll-like receptor 9 (TLR9) is located intracellularly in vesicles, and CpG is transported into these vesicles by endocytosis[1-3]. CpG binds TLR9 inside these vesicles, and the subsequent recruitment of the myeloid differentiation primary response gene 88 (MyD88) adaptor protein results in initiation of the TLR signaling cascade[4]. Ultimately, activating protein 1 (AP-1) and NF-κB transcription factors enter the nucleus and activate a variety of inflammatory genes. The end result is that CpG stimulates antigen presentation, co-stimulatory molecule expression and proinflammatory cytokine production[5-10].

IL-15 is a pivotal cytokine influencing the development and function of innate immune cells[8-13]. In the present study, we investigated the role of IL-15 in CpG-induced immune activation. We show that CpG-stimulated cDCs, rather than pDCs, are major producers of both IL-15 and IL-12, but fail to produce IL-12 in pDC-depleted mice. Of note, cDC-derived IL-15 acts on cDCs to induce the expression of CD40, which interacts with CD40 ligand (CD40L) expressed on pDCs. In turn, pDCs, via CD40-CD40L interactions, stimulate cDC IL-12 production.

Results

Impaired CpG response in *Il15*$^{-/-}$ mice

CpG can stimulate DCs to secrete large quantities of IL-12 and drive T_H1-mediated immune activation[1-3]. To examine the role of IL-15 in CpG-induced immune activation, it is important to measure IL-15 protein in vivo after CpG injection. We recently generated new monoclonal antibodies (mAbs) specific for mouse IL-15 and established an ELISA system that enables us to measure IL-15 protein[11]. Using

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this system, we measured serum IL-15 and IL-12 induced at various times after injection of 50 μg CpG into WT mice. Interestingly, maximum IL-15 production was observed 24 h after CpG injection, while maximum IL-12 production was observed 48 h after CpG injection (Figure 1a). In addition, IL-12 and IFN-γ were detected in the sera of CpG-challenged WT and NK cell-depleted (anti-ASGM1 treated) WT, but not *Il15*$^{-/-}$ mice (Figure 1b). These results collectively indicate that IL-15 is essential for CpG-induced IL-12 production in vivo.

Using an established model of *Listeria monocytogenes* (LM) infection, we investigated whether the defect in CpG-induced IL-12 and IFN-γ production affects the survival of *Il15*$^{-/-}$ mice. Consistent with previous reports[12,13], CpG-treated WT mice became resistant to a lethal dose (50 LD_{50}) of LM and survived for at least 14 days after the infection, and NK cell depletion did not affect the survival of CpG-primed WT mice (Figure 1c). In contrast, CpG-treated *Il15*$^{-/-}$ mice remained sensitive and succumbed to the infection (Figure 1d). Consistent with these results, the bacterial burden in the liver of CpG-treated WT mice was substantially lower than that in the liver and spleen of CpG-treated *Il15*$^{-/-}$ mice (Figure 1e). WT and *Il15*$^{-/-}$ DCs expressed similar amounts of surface TLR9, a receptor essential for CpG-mediated activation of the immune system (Figure 1f). These results indicate that IL-15-dependent IL-12 p70 production machinery is critical for CpG-mediated immune activation in vivo.

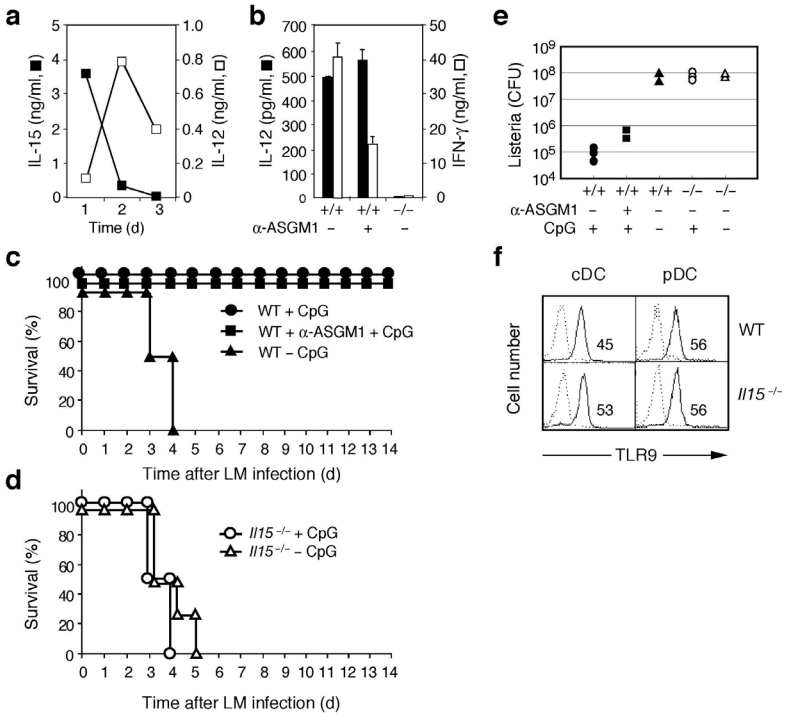

Figure 1 Impaired IL-12 and IFN-γ production in CpG-injected IL-15-deficient mice. (a) Serum IL-15 and IL-12 p70 was quantified by ELISA at indicated times after injection of 50 μg CpG. (b) Serum IL-12 p70 and IFN-γ in WT, anti-ASGM1-treated WT, and Il15$^{-/-}$ mice was determined by ELISA 2 d after injection of 50 μg CpG. (c,d) Survival of WT or anti-ASGM1-treated WT (c), and Il15$^{-/-}$ (d) mice which were or were not primed with 50 μg CpG 3 d prior to infection with a lethal dose (50 LD_{50}) of LM. Each group includes 4-5 mice. Anti-ASGM1 was administered every 4 d, starting 1 d prior to CpG injection. (e) Bacterial burden in the liver of LM-infected mice. The symbols used in this figure correspond to those in Figures. 1c and 1d. (f) TLR9 expression in cDCs and pDCs of WT and Il15$^{-/-}$ mice was determined by intracellular staining. Solid line, TLR9 staining, dashed line, isotype control staining. Numbers indicate mean fluorescence intensity (MFI).

DC-derived IL-15 in CpG response

DCs are one of the major producers of IL-15[5]. To demonstrate that DCs are a major producer of IL-15 after CpG injection in vivo, we used CD11c-DTR-GFP transgenic (DTR-GFP) mice[14]. These mice carry a transgene encoding a diphtheria toxin receptor (DTR)-GFP fusion protein under the control of the murine CD11c promoter; therefore, an injection of diphtheria toxin (DT) induces the selective depletion of DCs in vivo[14]. We constructed mice that lack DC-derived IL-15, by injecting a 1:1 mixture of *Il15*$^{-/-}$ and DTR-GFP BM cells into irradiated CD45.1^{+} congenic B6.SJL mice (*Il15*$^{-/-}$ + DTR-GFP chimeras). Control chimeras received a 1:1 mixture of WT and DTR-GFP BM cells (WT+DTR chimeras). DTR-GFP BM-derived GFP^{+} DCs constituted approximately 50% of the donor-derived DCs in the *Il15*$^{-/-}$+DTR-GFP chimeras, suggesting that *Il15*$^{-/-}$ and DTR-GFP BM contributed equally to the reconstituted DC population (Figure 2a). Upon DT injection, DCs derived from DTR-GFP BM but not those derived from *Il15*$^{-/-}$ BM were depleted (Figure 2a). In addition, DTR-GFP BM-derived monocytes and macrophages, which were potential IL-15 producers, were present in *Il15*$^{-/-}$ + DTR-GFP chimeras (data not shown), indicating that the DT-injected *Il15*$^{-/-}$ +DTR-GFP chimeras selectively lacked DC-derived IL-15. CpG injection induced substantial production of IL-15 (Figure 2b), IL-12 (Figure 2c), and IFN-γ (Figure 2d) in DT-injected control WT+DTR-GFP but not *Il15*$^{-/-}$+DTR-GFP chimeras. Upon subsequent LM infection, all DT-injected *Il15*$^{-/-}$+DTR-GFP chimeras died within 4 days, whereas the DT-injected WT+DTR-GFP chimeras survived much longer (Figure 2e). These results collectively demonstrate that DC-derived IL-15 is essential for the CpG-mediated activation of protective immune responses in vivo.

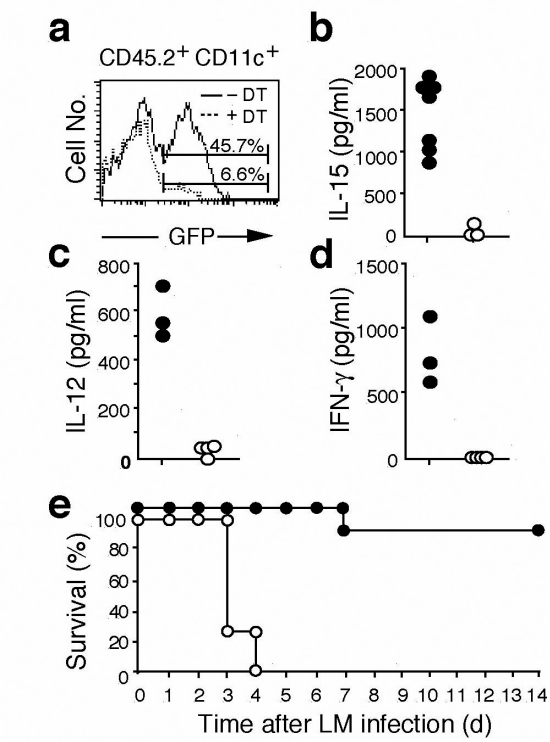

Figure 2 DC-derived IL-15 is critical for CpG-induced immune activation. (a) DC chimerism was evaluated based on GFP expression among CD45.2^{+}CD11c^{+} cells. (b-d) Serum was collected from DT-injected WT+DTR-GFP control (filled circles) and *Il15*$^{-/-}$+DTR-GFP chimeras (empty circles) 1 d after CpG injection for IL-15 (b), and 2 d after CpG injection for IL-12 p70 (c) and IFN-γ (d) measurement. (e) Survival of DT-injected and CpG-primed WT + DTR-GFP control (filled circles) and *Il15*$^{-/-}$+DTR-GFP chimeras (empty circles) was monitored after LM infection.

Specific DC subsets in CpG response

DCs are divided into cDCs and pDCs[15,16]. To examine whether cDCs and pDCs have distinct roles in CpG-induced IL-15 production and subsequent immune responses, we selectively depleted pDCs in vivo by injection of anti-mPDCA-1[17-19]. CpG-induced IL-15 production was unaffected by pDC depletion (Figure 3a). In contrast, neither IL-12 nor IFN-γ was produced in pDC-depleted WT mice upon CpG injection (Figure 3b,c). Consistent with these results, intracellular staining showed that the majority of IL-15 was

produced by cDCs (12%) but not pDCs (<1%) in CpG-injected WT mice (Figure 3d). These results together with Figure 3c demonstrate that cDCs rather than pDCs are the major source of IL-15 upon CpG-injection in vivo, and suggest two possibilities: (1) pDCs themselves are the major IL-12 producer or (2) cDCs produce IL-12 only in the presence of pDCs. To address this question, DCs were isolated from CpG-injected WT mice, and intracellular staining was performed to determine whether cDCs or pDCs produce IL-12. The results clearly show that cDCs rather than pDCs produced IL-12 upon CpG-stimulation (Figure 3e). As expected from the lack of IL-12 and IFN-γ production, pDC-depleted mice were unable to mount a CpG-induced protective immune response and died within 6 days of LM infection (Figure 3f,g). These results indicate that cDCs are the major source of both IL-15 and IL-12, and that cDCs fail to produce IL-12 in the absence of pDCs, which suggest the existence of a cross-talk between cDCs and pDCs.

Cross-talk between cDCs and pDCs

To investigate whether cDC-derived IL-15 acts on cDCs or pDCs, both DC subsets were isolated from WT and *Il15ra*$^{-/-}$ mice[20] 24 h after CpG injection, and were co-cultured in vitro in the presence of 2μM CpG. WT cDCs co-cultured with WT or *Il15ra*$^{-/-}$ pDCs produced substantial amounts of IL-12, whereas *Il15ra*$^{-/-}$ cDCs co-cultured with WT or *Il15ra*$^{-/-}$ pDCs produced little, if any, IL-12 (Figure 4a). These results

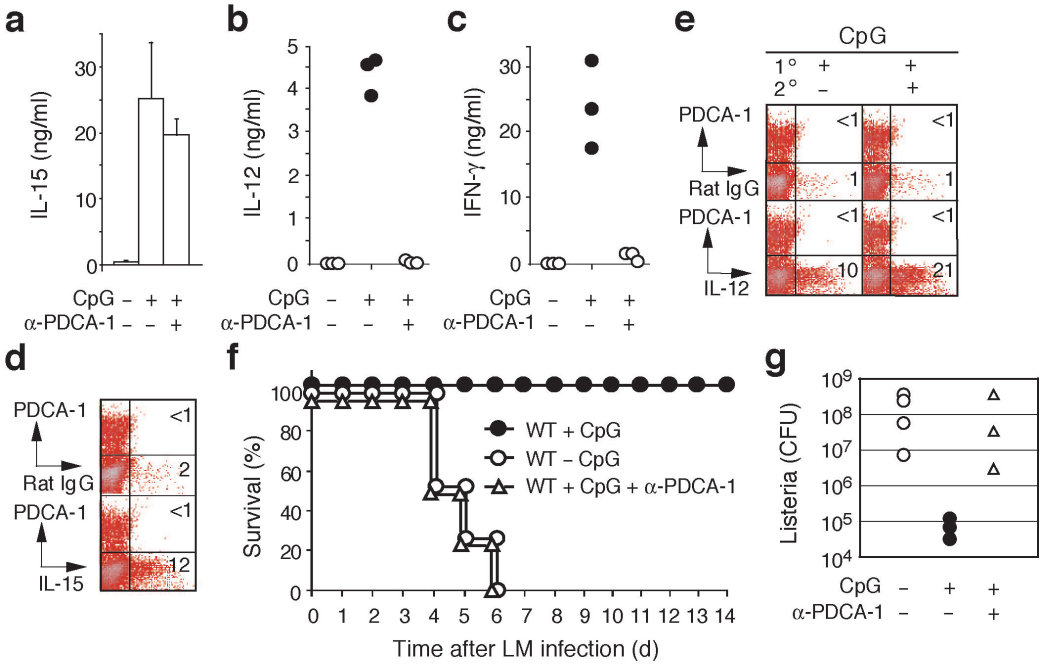

Figure 3 pDCs are required for CpG-induced immune activation. (a-c) Serum IL-15 (a), IL-12 p70 (b), and IFN-γ (c) in pDC-depleted mice was assessed as described in Figure 3. (d) IL-15 production by CpG-stimulated DC subsets. CD11c^{+} DCs were isolated from WT mice 12 h after CpG injection and were stained for mPDCA-1 and intracellular IL-15. (e) IL-12 production by CpG-stimulated DC subsets. CD11c^{+} DCs were isolated from WT mice 24 h after CpG-injection and were stained for mPDCA-1 and intracellular IL-12 p40 with or without a secondary stimulation with 2μM CpG in vitro. (f,g) WT and pDC-depleted WT mice were primed with 50μg CpG 3 d prior to infection with 50 LD_{50} LM. Survival was monitored (f) and bacterial burden in the spleen was estimated 2 d after LM infection (g).

indicate that cDC-derived IL-15 likely acts on cDCs themselves and not on pDCs. In addition, neither the cDCs nor pDCs alone produced IL-12 at a detectable level (Figure 4a), which was consistent with our in vivo observations in pDC-depleted mice (Figure 3b). As IL-15Rα on DCs is capable of mediating trans-presentation of IL-15 to the cells expressing IL-2Rβ and common γ chains[21], the results shown in Figure 4a might indicate that the trans-presentation of cDC-derived IL-15 to pDCs is critical for cDC-derived IL-12 p70 production. To exclude this possibility, *Il2rb*$^{-/-}$ DC subsets[22], which have a defect in IL-15 responsiveness, were co-cultured as shown in Figure 4a (Figure 4b). WT cDCs co-cultured with WT and *Il2rb*$^{-/-}$ pDCs produced IL-12, whereas *Il2rb*$^{-/-}$ cDCs co-cultured with WT and *Il2rb*$^{-/-}$ pDCs exhibited impaired IL-12 production (Figure 4b). Given that *Il2rb*$^{-/-}$ pDCs were unable to respond to IL-15 trans-presented by cDCs, these results collectively indicate that cDC-derived IL-15 acts on cDCs themselves and not on pDCs, and imply that cDC-derived IL-15 induces the expression of critical molecule (s) on cDCs that interact with pDCs. In this context, we noted that CpG induced a substantial upregulation of CD40 on WT but not *Il15*$^{-/-}$ cDCs (Figure 4c), indicating that cDC-derived IL-15 induces CD40 on cDCs. In contrast, CpG-induced CD40L expression was observed on a subset of pDCs in both WT and *Il15*$^{-/-}$ mice (Figure 4d).

To further examine whether cell-to-cell contact is required for the cDC-pDC cross-talk, cDCs and pDCs isolated from CpG-injected WT mice were co-cultured in the presence of CpG in a transwell system in which the two DC subsets were unable to contact each other, but DC-derived cytokines diffused freely between both populations. Importantly, cDCs did not produce IL-12 in the transwell system, indicating that cell-to-cell contact between cDC and pDC is required for the cDC-derived IL-12 production (Figure 4e). Supporting this concept, the cDC-derived IL-12 production was impaired when a CD40L blocking mAb was added to co-cultured cDCs and pDCs (Figure 4e), and this impairment was due to the blockage of CD40L expressed on pDCs but not cDCs (Figure 4f). Collectively, these results demonstrate that the IL-15-dependent cross-talk between cDCs and pDCs is mediated, at least in part, by CD40-CD40L interactions, which are essential for production of cDC-derived IL-12.

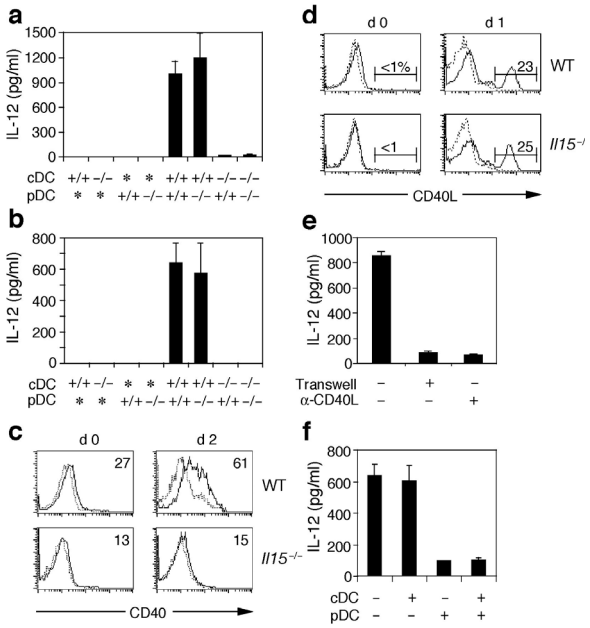

Figure 4 Mechanism of cross-talk between cDCs and pDCs. (a,b) cDCs and pDCs from WT (+/+) and *Il15ra*$^{-/-}$ (-/-) mice (a) , and *Rag2*$^{-/-}$ (+/+) and *Il2rb*$^{-/-}$*Rag2*$^{-/-}$ (-/-) mice (b) isolated 1 d after CpG injection were co-cultured for 24 h in the presence of 2μM CpG, after which supernatant IL-12 p70 was estimated by ELISA. *no addition of the DC subset. (c,d) Expression of CD40 on cDCs (c) and CD40L on pDCs (d) of WT and *Il15*$^{-/-}$ mice at indicated time after CpG injection. Solid line, CD40 or CD40L staining, dashed line, isotype control staining. Numbers indicate MFI (c) and percentage (d). (e) WT cDCs and pDCs were co-cultured in a transwell system in the presence of 2 μM CpG for 24 h after which supernatant IL-12 p70 was measured by ELISA. Anti-CD40L (10 μg/ml) was included in the culture where indicated. (f) Untreated (-) or anti-CD40L (10 μg/ml) -treated (+) WT DC subsets were co-cultured in the presence of 2 μM CpG for 24 h after which IL-12 p70 was measured by ELISA.

Discussion

It is well established that CpG stimulates host production of IL-12 and drives T_H1-skewed immune responses[1-3]. Therefore, CpG has potential therapeutic value in treating infectious diseases, cancer, and allergy. To test whether DC-derived IL-15 is important in the activation of CpG-mediated immune responses in vivo, we injected CpG into *Il15*$^{-/-}$ mice, DC-depleted mice, and mixed BM chimeric mice specifically lacking DC-derived IL-15; collectively these experiments demonstrated that DC-derived IL-15 is essential for CpG-induced immune activation in vivo. To our surprise, we found that cDCs rather than pDCs are the major producers of both IL-15 and IL-12 upon CpG injection, and that cDC-derived IL-12 production is regulated by IL-15-dependent cross-talk between cDCs and pDCs. cDC-derived IL-15 induces CD40 on cDCs, which enable cDCs to interact with CD40L-expressing pDCs and to receive signals necessary for IL-12 production. Consistent with these results, CD40 expression on DCs is upregulated by microbial stimuli including LPS and CpG, and, in combination with CD40 ligation, CpG effectively primes DCs to enhance antigen presentation, costimulation, and immunomodulatory cytokine production[23,24].

Interestingly, a recent study showed that, in herpes simplex virus type-1 (HSV-1) infection, pDCs express CD40L and play a critical role in licensing of cDCs via CD40 cross-linking, which is essential for the induction of anti-HSV-1 CTL in vivo[19]. These studies and our results indicate that pDCs are probably required, as an initial source of CD40L, for the induction of licensed cDC in response to HSV-1 and CpG in vivo. CD40L can be expressed on multiple cell-types, including activated T cells, NK cells, and pDCs. Involvement of CD40L on NK cells can be excluded, because CpG-induced IL-12 production was unaffected by NK cell-depletion in vivo. IL-12 production was severely impaired in mice depleted pDCs, which express CD40L, strongly suggesting that CD40L on pDCs is critical for IL-12 production in vivo, although we cannot completely exclude the possibility that other cell types expressing CD40L may also be involved in the process. In this context, upon CpG injection, not only WT mice but also T-cell-deficient *Rag1*$^{-/-}$, nude and SCID mice produce IL-12 and become resistant to a challenge with a lethal dose of LM, whereas CpG-primed *Il12*$^{-/-}$ mice remain susceptible[25]. Consistent with these results, serum IL-12 production was intact in WT, *Rag2*$^{-/-}$, and anti-ASGM1-treated *Rag2*$^{-/-}$ mice after CpG injection (data not shown). These studies and our results also suggest that CpG-induced licensing of cDCs can be mediated, at least in part, by pDCs, even in the absence of $CD4^+$ T cells.

Of interest, HSV-1 DNA contains abundant CpG motifs, and HSV-1-induced immune cell responses are mediated in part through a TLR9-dependent pathway[26,27]. An essential role for the pDC-dependent licensing of cDCs, which is mediated via CD40-CD40L interactions, is a common feature of both synthetic CpG- and HSV-1-induced immune responses in vivo[19]. Given that not only HSV-1 but also HSV-2[28] stimulate the immune system in a TLR9-dependent fashion, it will be intriguing to examine whether IL-15 plays critical regulatory roles in the cross-talk between cDCs and pDCs in these viral infection models.

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