Distinct transcriptional programs control cross-priming in classical- and monocyte-derived dendritic cells

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Summary

Both classical DCs (cDCs) and monocyte-derived DCs (Mo-DCs) are capable of cross-priming CD8^{+} T cells in response to cell-associated antigens. We found that Ly-6C^{hi}TREML4^{−} monocytes can differentiate into *Zbtb46*^{+} Mo-DCs in response to GM-CSF and IL-4, but that Ly-6C^{hi}TREML4^{+} monocytes were committed to differentiate into Ly-6C^{lo}TREML4^{+} monocytes. Differentiation of *Zbtb46*^{+} Mo-DCs capable of efficient cross-priming required both GM-CSF and IL-4, and was accompanied by induction of *Batf3* and *Irf4*. However, monocytes require IRF4, but not BATF3, to differentiate into *Zbtb46*^{+} Mo-DCs capable of cross-priming CD8^{+} T cells. Instead, *Irf4*^{−/−} monocytes differentiate into macrophages in response to GM-CSF and IL-4. Thus, cDCs and Mo-DCs require distinct transcriptional programs of differentiation in acquiring the capacity to prime CD8^{+} T cells. These differences may be of consideration in the use of therapeutic DC vaccines based on Mo-DCs.

In Brief

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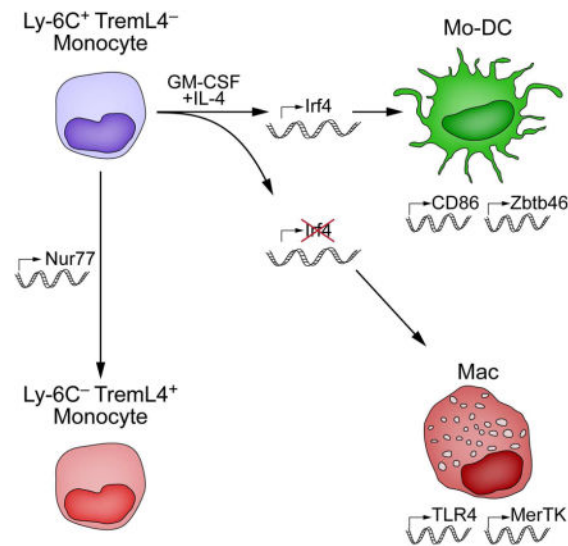The transcriptional programs required for differentiation of cross-priming APCs from various lineages are unknown. Briseño et al. show that Mo-DCs use a distinct program than cDCs, requiring IRF4 but not Batf3. These differences may impact the design of vaccines based on Mo-DCs that would require efficient cross-priming of T cells.

Introduction

Cross-presentation functions in initiating cytolytic $CD8^+$ T cell responses during viral infections (Joffre et al., 2012) and is mediated by classical dendritic cells (cDCs) derived from the common dendritic cell progenitor (Naik et al., 2007; Liu et al., 2007) and by monocyte-derived dendritic cells (Mo-DCs) (Nierkens et al., 2013). Efficient cross-presentation is carried out *in vivo* by a $CD24^+$ cDC subset requiring IRF8 and BATF3 (Briseno et al., 2014; Satpathy et al., 2012b), but the transcriptional requirements for Mo-DCs are undefined. In mice, monocytes can produce DCs under inflammatory conditions *in vivo* (Auffray et al., 2009; Cheong et al., 2010) or upon *ex vivo* treatment with granulocyte-macrophage colony stimulating factor (GM-CSF) (Inaba et al., 1992; Inaba et al., 1993; Caux et al., 1992). Human monocytes treated *ex vivo* with GM-CSF and IL-4 also acquire DC characteristics (Sallusto and Lanzavecchia, 1994; Romani et al., 1994). Mo-DCs express CD11c and MHC-II (Leon et al., 2004) and the DC-specific transcription factors *Zbtb46* and *Mycl1* (Satpathy et al., 2012a; KC et al., 2014). However, monocytes differentiated with GM-CSF alone generate a heterogeneous population of $CD11c^+$ cells (Helft et al., 2015), resembling either macrophages (GM-Macs, $CD11b^+MHC-II^{lo}$) or DCs (GM-DCs, $CD11b^+MHC-II^{hi}$). GM-DCs cross-present soluble antigen more efficiently than GM-Macs (Helft et al., 2015).

Mo-DCs can promote T_H1 and $CD8^+$ T cell responses (Leon et al., 2007; Aldridge, Jr. et al., 2009; Ji et al., 2013), but differ in the antigen processing pathways they employ (Segura et al., 2009) and the phases of infection they are involved in compared to cDCs (Ballesteros-Tato et al., 2010). Mo-DCs react distinctly from cDCs in response to adjuvant (Langlet et al.,

2012) and unlike cDCs, act independently of GM-CSF signaling *in vivo* during steady state and immunization (Greter et al., 2012). Human Mo-DCs generated *ex vivo* with GM-CSF and IL-4 can elicit CD8^{+} T cell responses against tumor antigens (Nestle et al., 1998; Holtl et al., 1999; Timmerman et al., 2002; Thurner et al., 1999) and subdominant neoantigens (Carreno et al., 2015), and have been used in cancer vaccines (Palucka and Banchereau, 2013; Carreno et al., 2015). Although CDPs have been suggested as sources of DC vaccines (Guilliams and Malissen, 2015), the abundance and practical value of monocytes motivates understanding their cross-presentation capacity for use in future vaccine design.

How IL-4 regulates Mo-DC differentiation is still unclear. In macrophages, IL-4 signaling induces M2 polarization (El Chartouni et al., 2010) by Stat6 activation and induction of Jumonji domain-containing-3 (*Jmjd3*). JMJD3 functions as a demethylase of histone 3 lysine 27 (Ishii et al., 2009) and promotes M2 polarization by regulating IRF4 expression (Satoh et al., 2010). Loss of either JMJD3 or IRF4 impairs expression of M2 macrophage genes, such as *Arg1*, *IL13*, and *Fizz1* (Satoh et al., 2010). Whether similar actions of IL-4 and IRF4 occur during Mo-DC differentiation has not been examined. In CD11b^{+} cDCs, IRF4 is required for migration (Bajana et al., 2012), survival in mucosal tissues (Schlitzer et al., 2013; Persson et al., 2013), and capacity to induce T_H17 and T_H2 responses (Gao et al., 2013; Williams et al., 2013; Schlitzer et al., 2013; Persson et al., 2013). Human Mo-DCs induce IRF4 in response to GM-CSF and IL-4 (Lehtonen et al., 2005), but its function there is undefined. In this study, we compared the transcriptional programs between cDCs and Mo-DCs for their ability to prime T cells in response to cell-associated antigens, finding that Mo-DCs do not require IRF8 and BATF3 like cDCs do, but instead require IRF4.

Results

IL-4 is required for optimal cross-priming by GM-CSF induced Mo-DCs

Splenic CD24^{+} cDCs, but not Sirp-α^{+} cDCs, efficiently cross-primed T cells with cell-associated antigen (Fig. 1A, B), as reported (den Haan et al., 2000; Becker et al., 2014). As control, both cDC subsets presented SIINFEKL peptide (Fig. 1C). Mo-DCs generated with GM-CSF and IL-4 efficiently activated T cells in response to cell-associated antigen and SIINFEKL peptide, in contrast to Ly-6C^{+} monocytes (Fig. 1A–C), as reported (Cheong et al., 2010). Unlike Mo-DCs, sorted splenic Sirp-α[+] DCs cultured in GM-CSF with or without IL-4 did not cross-prime T cells to cell-associated antigen (Fig. 1D), but presented SIINFEKL peptide (Fig. 1E). Thus, Mo-DCs, but not Sirp-α[+] cDCs, are able to cross-prime T cells to cell-associated antigens.

Monocytes cultured in GM-CSF produce a heterogeneous population of MHC-II[hi] GM-DCs and MHC-II[lo] GM-Macs in (Fig. 2A), in agreement with a recent study (Helft et al., 2015). MHC-II[hi] GM-DCs expressed *Zbtb46*[gfp] (Satpathy et al., 2012a), but MHC-II[lo] GM-Macs did not (Fig. 2A), consistent with specific *Zbtb46* expression in cDCs but not macrophages (Meredith et al., 2012; Satpathy et al., 2012a). Addition of IL-4 with GM-CSF induced uniform *Zbtb46*[gfp] expression in both MHC-II^{hi} and MHC-II^{lo} populations of CD11c^{+} cells (Fig. 2A). Both MHC-II^{hi} and MHC-II^{lo} cells that developed in GM-CSF alone were weak cross-primers of cell-associated antigen, but addition of IL-4 significantly enhanced their activity (Fig. 2B) to levels similar to CD24^{+} cDCs (Fig. 1A, B). All populations presented

SIINFEKL peptide (Fig. 2C). MHC-II^{lo} (Fig. 2D) and MHC-II^{hi} (Fig. 2E) Mo-DCs differentiated with GM-CSF alone or with IL-4 showed similar uptake of apoptotic cells. Thus, IL-4 signaling during GM-CSF-induced monocyte differentiation induces *Zbtb46* expression in MHC-II^{lo} cells and increases cross-priming in both MHC-II^{hi} and MHC-II^{lo} cell populations.

Expression of TREML4 and NUR77 identifies monocytes lacking Mo-DC potential

TREML4, a triggering receptor family member expressed on myeloid cells-like (Ford and McVicar, 2009), is induced during heme-mediated differentiation of macrophages from monocytes and BM progenitors (Haldar et al., 2014). TREML4 is expressed on CD24$^+$ cDCs, monocytes (Hemmi et al., 2012) and macrophages, where it regulates TLR7 signaling amplification (Ramirez-Ortiz et al., 2015). Ly-6C^{hi} monocytes were heterogeneous for TREML4 expression, but Ly-6C^{lo} monocytes were uniformly TREML4 positive (Fig. 3A). Only LY-6C^{hi} TREML4$^-$ monocytes were able to induce *Zbtb46*^{gfp} expression in response to GM-CSF and IL-4, whereas Ly-6C^{hi} TREML4$^+$ monocytes and Ly-6C^{lo} TREML4$^+$ monocytes could not (Fig. 3B). Thus, TREML4 may mark the commitment of monocytes to the Ly-6C^{lo} monocyte and macrophage lineages. Gene expression profiling suggested that Ly-6C^{hi} TREML4$^+$ monocytes were an intermediate stage of differentiation between Ly-6C^{hi} TREML4$^-$ and Ly-6C^{lo} monocytes (Fig. 3C). In Ly-6C^{hi} TREML4$^-$ monocytes, expression of *Ccr2* was 3-fold higher and 10-fold higher compared to Ly-6C$^+$ TREML4$^+$ and Ly-6C^{lo} TREML4$^+$ monocytes, respectively, while *Treml4* expression was about 4-fold higher in Ly-6C^{hi} TREML4$^+$ monocytes and 6-fold higher in Ly-6C^{lo} TREML4$^+$ monocytes relative to Ly-6C^{hi} TREML4$^-$ monocytes (Fig. 3C).

NUR77 (*Nr4a1*) is required for development of Ly-6C^{lo} monocytes (Martinez-Gonzalez and Badimon, 2005; Hanna et al., 2011). Analysis of NUR77-GFP reporter mice (Moran et al., 2011) shows that TREML4 expression increased along with Nur77 (Fig. 3D). NUR77-GFP was absent in Ly-6C^{hi} TREML4$^-$ monocytes, but expressed at intermediate levels in all Ly-6C^{hi} TREML4$^+$ monocytes and at high levels in all Ly-6C^{lo} TREML4$^+$ monocytes (Fig. 3D). We then tested the DC potential of monocytes expressing different levels of NUR77 (Fig. 3E). Ly-6C$^+$ NUR77-GFP$^-$ monocytes differentiated into Mo-DCs in response to GM-CSF and IL-4 (Fig. 3E). In contrast, Ly-6C$^+$ Nur77-GFP$^+$ monocytes and Ly-6C^{lo} NUR77-GFP$^+$ monocytes were unable to differentiate into CD11c$^+$MHC-II$^+$ Mo-DCs (Fig. 3E). NUR77-deficient monocytes could not develop into Ly-6C^{lo} monocytes, as reported (Moran et al., 2011), but could develop into Mo-DCs (Fig. 3F). Unsupervised analysis using SPADE (Qiu et al., 2011) reconstituted the successive steps of monocyte differentiation *in vivo* (Fig. S1, related to Figure 3). Thus, Ly-6C^{hi} TREML4$^-$ Nur77-GFP$^-$ monocytes are the last stage of monocyte differentiation that retains potential for Mo-DC development.

IL-4 induces BATF3 and IRF4 during Mo-DC differentiation

We examined gene expression microarrays of Ly-6C^{hi} TREML4$^-$ and Ly-6C^{lo} monocytes, Mo-DCs cultured with or without IL-4, and splenic CD24$^+$ and Sirp-α^+ cDCs. Several transcription factors were increased when Mo-DCs were differentiated with GM-CSF and IL-4, compared to monocytes or Mo-DCs cultured in GM-CSF alone (Fig. 4A). Specifically, *Batf3* was induced by GM-CSF and IL-4 by 10-fold and 4-fold relative to monocytes and

Mo-DCs cultured with GM-CSF alone, respectively. In addition, *Irf4* was induced more than 25-fold relative to monocytes and 2-fold relative to Mo-DCs cultured with GM-CSF (Fig. 4A, B), as reported in human Mo-DCs (Lehtonen et al., 2005). Two other factors, *Nr4a3* (DeYoung et al., 2003) and *Vdr* (Yoshizawa et al., 1997; Li et al., 1997), were induced, but have not been associated with antigen presentation. In contrast, *Batf3* is required for the development of cDCs capable of cross-presentation (Hildner et al., 2008; Torti et al., 2011) and *Irf4* was shown to be required for MHC-II expression in GM-DCs (Vander et al., 2014). Mo-DCs induced *Batf3*, but not *Batf* or *Batf2*, to levels equivalent to both splenic CD24$^+$ and Sirp-α$^+$ cDCs (Fig. 4C). Likewise, Mo-DCs expressed *Irf4* to levels similar to Sirp-α$^+$ cDCs (Fig. 4C). Also, IL-4 increased IRF4 expression in Mo-DCs (Fig. 4D). In summary, IL-4 induced both BATF3 and IRF4 during Mo-DC differentiation.

Cross-priming by Mo-DCs is independent of BATF3

To examine Mo-DC differentiation and function, we used monocytes from *Batf*, *Batf2* and *Batf3* triple knockout mice (Batf-TKO), since *Batf* and *Batf2* can compensate for *Batf3* in CD24$^+$ cDC development (Tussiwand et al., 2012). Mo-DCs developed normally from Batf-TKO monocytes (Fig, 5A), with normal expression of IRF4 and IRF8 (Fig. 5B). As reported (Tussiwand et al., 2012), Batf-TKO lacked splenic CD24$^+$ cDCs but retained Sirp-α$^+$ cDCs (Fig. 5A). We found no difference in cross-priming between WT and Batf-TKO Mo-DCs over a range of antigen concentrations or in presentation of SIINFEKL peptide (Fig. 5C, D). Splenic Batf-TKO Sirp-α$^+$ DCs did not cross-prime but could present SIINFEKL peptide (Fig. 5E, F). Thus, development and cross-priming of Mo-DCs was independent of BATF3.

IRF4 is required for development of *in vitro* derived Mo-DCs but not for Sirp-α$^+$ cDCs

IRF4 is required for migration and homeostasis of Sirp-α$^+$ cDCs (Bajana et al., 2012; Schlitzer et al., 2013; Persson et al., 2013) and promotes MHC-II expression by bone marrow-derived GM-DCs (Vander et al., 2014) but its role in priming of CD8$^+$ T cells by Mo-DCs is unknown. Mo-DCs derived from *Irf4*$^{-/-}$ Ly-6C^{hi} TREML4$^-$ monocytes were inactive for cross-priming (Fig. 6A, B). In contrast, *Irf4*$^{-/-}$ splenic CD24$^+$ cDCs were as efficient as WT CD24$^+$ DCs in cross-priming OT-I cells (Fig. 6C, D). Uptake of apoptotic cells was similar between WT and *Irf4*$^{-/-}$ Mo-DCs (Fig. 6E). Mo-DCs lacking IRF4 did not express MHC-II, as reported (Vander et al., 2014), but expressed normal MHC-I levels (Fig. 6F). However, they were unable to induce OT-I proliferation with SIINFEKL peptide (Fig. 6G).

Unlike WT Mo-DCs, *Irf4*$^{-/-}$ monocytes failed to induce *Zbtb46*-GFP, and instead acquired expression of F4/80 following treatment with GM-CSF and IL-4 (Fig. 7A). IRF4 was not required for ZBTB46 expression in CD24$^+$ or Sirp-α$^+$ splenic cDCs (Fig. 7A). By contrast, *Zbtb46*-deficient Mo-DCs expressed normal levels of MHC-II and IRF4 (Fig. 7B). Consistent with the lack of MHC-II and Zbtb46 expression, the normal dendritic cell morphology of Mo-DCs was not seen in *Irf4*$^{-/-}$ Mo-DCs, which instead had the appearance of macrophages (Fig. 7C), suggesting that IRF4 may be required for induction of a broader DC transcriptional program in Mo-DCs beyond MHC-II gene expression.

To determine the identity of cells originating from IRF4-deficient monocytes cultured with GM-CSF and IL-4, we performed microarray analysis of WT and $Irf4^{-/-}$ Mo-DCs cells (Figs. 7D, E). Consistent with the macrophage identity observed by flow cytometry and microscopy, $Irf4^{-/-}$ monocytes cultured in GM-CSF and IL-4 induced high expression of macrophage-specific genes such as *Mertk*, *Tlr4* and *Tlr7* (Gautier et al., 2012), and unlike WT Mo-DCs, failed to induce DC-associated genes such as *Kmo*, *Traf1* and *Slamf7* (Miller et al., 2012) (Fig. 7D). Since, IRF4 has been previously implicated in the development of splenic Sirp-α⁺ cDCs (Suzuki et al., 2004) we asked if IRF4 regulated a similar genetic program in both Mo-DCs and splenic Sirp-α⁺ cDCs. Comparison of the microarrays of WT and $Irf4^{-/-}$ Mo-DCs showed 747 genes to be differentially expressed by at least 3-fold between these two populations (Fig. 7E). However, only 49 of those targets were also at least 3-fold different between WT and $Irf4^{-/-}$ Sirp-α⁺ cDCs (Fig. 7E), suggesting Mo-DCs but not splenic Sirp-α⁺ cDCs require IRF4 for their development. We identified CD86 to be specifically downregulated in Mo-DCs, but not Sirp-α⁺ cDCs, lacking *Irf4.* We confirmed this result by assaying the expression of CD86 on WT and $Irf4^{-/-}$ splenic cDCs and Mo-DCs activated with LPS. Only MoDCs, and neither CD24⁺ nor Sirp-α⁺ cDCs, required IRF4 for CD86 expression (Fig. 7F, G). Altogether, these results indicate a specific requirement for IRF4 by monocytes for their differentiation into DC-like cells.

Discussion

Vaccines based on Mo-DCs can enhance immune responses against human melanoma (Carreno et al., 2015). Mo-DCs have been generated either in culture of GM-CSF alone or with IL-4 (Linette and Carreno, 2013). We show that IL-4 augments expression of *Zbtb46* and *Irf4*, and that *Irf4* is required for monocytes to differentiate into DCs. Mo-DCs can cross-prime CD8⁺ T cells for cell-associated antigen as efficiently as CD24⁺ cDCs. We show Mo-DCs rely on a distinct transcriptional program compared with cDCs in acquiring the ability to prime CD8⁺ T cells. Cross-presenting Mo-DCs require IRF4 but not BATF3, while cross-presenting cDCs require BATF3 but not IRF4.

Circulating Ly6Chi monocytes can differentiate either into Mo-Macs, Mo-DCs, or Ly6Clo 'patrolling' monocytes. Nur77 is required for differentiation of Ly6Chi monocytes into patrolling monocytes (Hanna et al., 2011), but not into Mo-DCs (Fig. 3F). We find that Ly6Chi monocytes that express Nur77 or TREML4, lack Mo-DC potential. In CD8⁺ T cells, Nur77 may inhibit IRF4 expression (Nowyhed et al., 2015), suggesting it may act similarly in Ly6Chi TREML4⁺ monocytes to repress IRF4 and thus Mo-DC development.

The biochemical basis for cross-presentation by different cells remains incompletely understood. Several proteins implicated in cross-presentation have been analyzed only in cells generated from BM cells treated with GM-CSF alone (Joffre et al., 2012; Segura and Amigorena, 2015). In this setting, NOX2 (Savina et al., 2006; Savina et al., 2009), Rac2 (Savina et al., 2009) and VAMP8 (Matheoud et al., 2013) were shown to regulate acidification of phagosomes in GM-DCs, suggesting that they act to preserving antigens from complete degradation. While NOX2 and Rac2 also regulate phagosomal acidification in CD8⁺ cDCs (Savina et al., 2009), only NOX2, but not Rac2, deficiency reduced CD8⁺ cDC cross-presentation of soluble antigen. Rab11a (Nair-Gupta et al., 2014), Rab3b (Zou et

al., 2009) and Sec22b (Cebrian et al., 2011), which regulate vesicular trafficking, were shown to promote cross-presentation, but were studied using BM cultures treated with GM-CSF or in the DC2.4 cell line. In our studies, Mo-DCs generated with GM-CSF alone were relatively inefficient in cross-priming of cell-associated antigen compared with CD8$^+$ cDCs and Mo-DCs generated with both GM-CSF and IL-4 (Fig. 2B).

Other known proteins such as ERAP1 (Firat et al., 2007) and IRAP (Segura et al., 2009; Saveanu et al., 2009) may be also be involved in cross-presentation. ERAP1 was required *in vivo* but not in GM-CSF BM-derived cells (Firat et al., 2007), while IRAP was required for both *in vivo* and *in vitro* cross-priming of CD8$^+$ T cells to cell-associated antigen (Saveanu et al., 2009). IRAP was required for cross-presentation of soluble antigen only in inflammatory Mo-DCs generated *in vivo*, and not in CD24$^+$ DCs (Segura et al., 2009). Alternately, unknown proteins may remain unidentified that differentially act in cross-presentation.

Experimental Procedures

Mice

Zbtb46$^{gfp/+}$ mice (Satpathy et al., 2012a) were backcrossed to C57BL/6J for at least 8 generations. *Batf*$^{-/-}$*Batf2*$^{-/-}$*Batf3*$^{-/-}$(Batf-TKO), *Irf8*$^{-/-}$ and *Irf4*$^{-/-}$ mice have been described mice (Tussiwand et al., 2012; Grajales-Reyes et al., 2015). The following mice were purchased from Jackson Laboratories: *Nr4a1*$^{-/-}$ (B6;129S2-Nr4a1^{tm1Jmi}/J), OT-I (C57BL/6-Tg(TcraTcrb)^{1100Mjb}/J), CD45.1$^+$ (B6.SJL-Ptprca Pepcb/BoyJ). *Nr4a1*$^{gfp/+}$ mice were a gift from Chyi-Song Hsieh and K$^{b-/-}$D$^{b-/-}$β2m$^{-/-}$ mice (MHCI-TKO, Lybarger et al., 2003) were a gift from Herbert W. Virgin IV and Ted Hansen, Washington University in St. Louis. Mice, except Batf-TKO (129/SvEvTac), were maintained on the C57BL/6 background. All mice were housed in a specific pathogen-free animal facility following institutional guidelines with protocols approved by the Animal Studies Committee at Washington University in St. Louis. Experiments were performed with mice 8–12 weeks of age using sex-matched littermates.

Antibodies and flow cytometry

Cells were stained at 4°C in MACS buffer (PBS with 0.5% BSA and 2 mM EDTA) with CD16/32 Fc block (BD clone 2.4G2).

These antibodies were purchased from Becton Dickinson (BD): CD11b (M1/70); CD45.2 (104); CD135 (A2F10.1); MHC-II (M5/114.15.2); Ly-6C (AL-21); from eBioscience: CD4 (GK1.5); CD8α (53-6.7); CD11b (M1/70); CD45.1 (A20); CD44 (IM7); CD117 (2B8); CD115 (AFS98); CD11c (N418); CD24 (M1/69); CD172a (P84); Ly-6C (HK1.4); Ly-6A/E (D7); Ly-6G (IA8); Siglec-H (eBio440C); Ter-119 (Ter-119); CD105 (MJ7/18); Irf8 (V3GYWCH); CD45R (RA3-6B2); NK1.1 (PK136); Irf4 (3E4); 7AAD viability staining solution; from Tonbo Biosciences CD45.1 (A20); CD11c (N418); from BioLegend CD8α (53-6.7); CD45.2 (104); CD115 (ASF98); Ly-6G (IA8); TCR Vα2 (B20.1); TREML4 (16E5); from ThermoFisher Scientific: TCR Vα2 (B20.1), Live/dead Fixable Aqua Dead cell Stain kit. Carboxyfluorescein succinimidyl ester (CFSE) was purchased from Sigma.

Anti-Biotin and anti-B220 microbeads were purchased from Miltenyi. Cells were fixed and permeabilized for intracellular staining of IRF4 and IRF8 using the FoxP3/Transcription Buffer Set (eBioscience). Cells were sorted on a FACS Aria Fusion flow cytometers (BD) and with FlowJo software (Tree Star).

Isolation and culture of BM cells and splenic DCs

Femurs, pelvis and tibias were crushed using mortar and pestle in MACS buffer and filtered through a 70-μm strainer, and purified on Histopaque-119 gradient and depleted of Ly-6G and B220-expressing cells with biotinylated anti-Ly-6G and B220 antibodies and anti-biotin microbeads (Miltenyi). BM monocytes were identified as Lin$^-$SiglecH$^-$Ly-6G$^-$MHCII^{lo}CD11c$^-$CD117$^-$CD135$^-$CD115$^+$ and sorted as Ly-6C^{hi}TREML4$^-$ or Ly-6C^{lo}TREML4$^+$ for microarray analysis. Lin includes B220, CD105, NK1.1, and Ter-119. Blood monocytes were defined as Ter-119$^-$CD45.2$^+$MHC II^{lo}Ly-6G$^-$CD115$^+$ and segregated based on Ly-6C and TREML4 expression. Cells were sorted into Iscove's modified Dulbecco's medium + 10% FCS kept at 4°C. Spleens were minced and digested for 45 minutes at 37°C with stirring in 5 ml's complete media with 250 μg/ml collagenase B (Roche) and 30 U/mL DNase I (Sigma-Aldrich). Red blood cells were lysed with ACK lysis buffer and splenocytes were passed through a 70-μm strainer. CD24$^+$ cDCs were defined as B220$^-$CD11c$^+$MHC-II$^+$CD24$^+$CD172a$^-$. Sirp-α$^+$ cDCs were defined as B220$^-$CD11c$^+$MHCII$^+$CD24$^-$CD172a$^+$. For Mo-DC differentiation, sorted Ly-6C$^+$ TREML4$^-$ monocytes from BM or peripheral blood were cultured (0.25×10^5-0.5×10^5 cells/mL) at 37°C in complete media with GM-CSF and IL-4 (20ng/mL, Peprotech) for 3–4 days. Loosely adherent Mo-DCs were harvested by gentle pipetting. Sorted Sirp-α$^+$ cDCs were cultured in 20ng/mL of GM-CSF and IL-4 for 48 hours. For induction of CD86, sorted CD24$^+$ and Sirp-α$^+$ cDCs, and Mo-DCs were cultured with LPS (1ng/mL) for 16 hours.

Microscopy

Cytospins of sorted Mo-DCs generated from GM-CSF and IL-4 culture of Ly-6C$^+$TREML4$^-$ monocytes were stained with Wright-Giemsa stain using Hema 3 kit (Fisher Scientific). Images were acquired at room temperature with an Axioskop microscope (Objective: 100x, 1.25, oil) using an Axiocam ICc3 camera (Zeiss).

Gene expression microarray analysis

Total RNA was extracted from purified splenic cDCs, Mo-DCs, and monocytes from BM and peripheral blood using the RNAqueous-Micro Kit (Ambion). RNA was amplified using the Ovation Pico WTA Sytem (NuGEN) and hybridized to GeneChip Mouse Gene 1.0 ST microarrays (Affymetrix). Data was processed using robust multiarray average summarization and quartile normalization using ArrayStar software version 5 (DNASTAR). Sirp-α$^+$ cDC expression values from WT and *Irf4$^{-/-}$* mice were averaged from biological duplicates; all other expression values were from one biological sample.

Antigen presentation assays

Splenic OT-I cells were sorted as B220$^-$CD11c$^-$CD45.1$^+$TCR-Vα2$^+$CD4$^-$CD8α$^+$ to >95% purity, labeled with CFSE and plated at a density of 12.5×10^5 cells/mL. Splenocytes from

MHC-I TKO mice were processed as described above. OVA loading of MHC-I TKO splenocytes has been described before (Carbone and Bevan, 1990). Splenocytes (2.5×10^7/mL) were incubated in hypertonic medium (RPMI 1640, 0.5M sucrose, 10% w/v poly-ethylyne glycol, 10 mM HEPES pH 7.2) with 5 mg OVA (Worthington) for 10 minutes at 37°C. Cells were diluted 10-fold with hypotonic media (60% FBS, 40% Sterile water) and incubated for 2 minutes at 37°C. Cells were washed with PBS and irradiated (13.5 Gy). Sorted splenic CD24$^+$ and Sirp-α^+ cDCs, and Mo-DCs (12.5×10^5 cells/mL) were co-cultured with CFSE labeled OT-I's (12.5×10^5 cells/mL) and OVA-loaded MHC-I TKO cells (2.5×10^5-25.0×10^6 cells/mL). For peptide presentation, 2.5×10^4 APCs were cultured with SIINFEKL peptide (1.0×10^{-3}-1.0 ng/mL) for 45 minutes in complete media at 37°C, washed twice, and cultured with 2.5×10^4 CFSE labeled OT-I cells. Cells were cultured at 37°C for 3 days and analyzed by flow cytometry. OT-I proliferation was determined as the percent of CD45.1$^+$CD8α^+TCR-Vα2$^+$CD44$^+$ cells that had undergone at least one CFSE dilution.

Phagocytosis assay

To prepare target cells, CD45.1$^+$ splenocytes were harvested as described above, loaded with CFSE and γ-irradiated (13.5 Gy). Sorted CD45.2$^+$ Mo-DCs (12.5×10^5 cells/mL) were co-cultured with CFSE labeled irradiated splenocytes (2.5×10^5-25.0×10^5 cells/200uL) for 16 hours at 37° C. After culture, Mo-DCs were washed, and stained for CD45.2, CD45.1, CD11c, Ly-6C, Aqua, and MHC-II. Percent phagocytosis was determined as the percentage of live (Aqua$^-$) singlet Mo-DCs (CD45.1$^-$CD45.2$^+$Ly-6C$^-$CD11c$^+$) that were CFSE positive.

Statistical analysis

Error bars indicate standard error of mean. Statistical analyses were performed using two-way analysis of variance (ANOVA) with Sidak's multiple comparison test unless otherwise specified. All statistical analyses were performed using Prism (GraphPad Software).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- GM-CSF derived Mo-DCs require IL-4 to cross-present cell-associated antigen.

- Monocytes expressing TremL4 lose potential to differentiate into DCs.

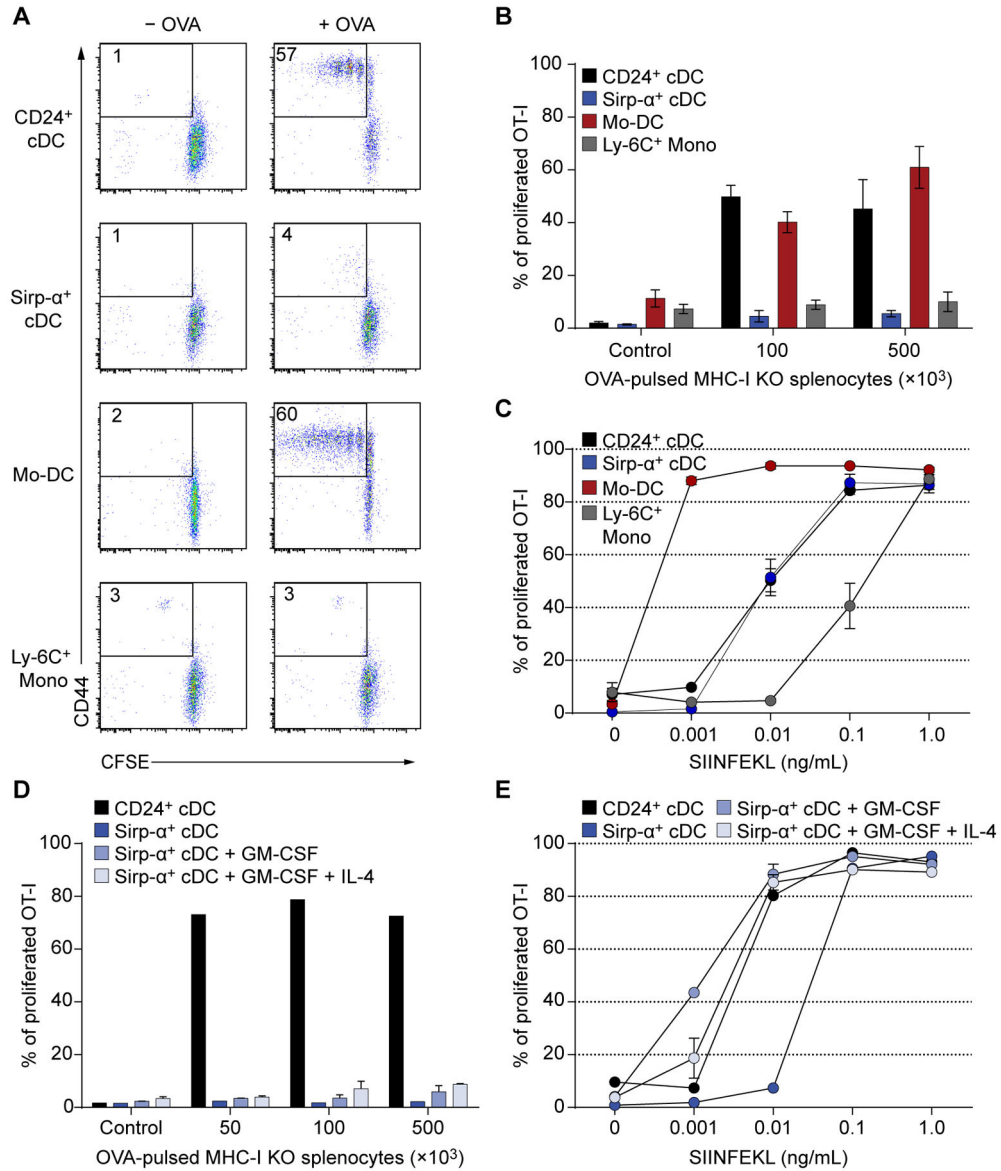- Monocytes require IRF4 but not Batf3 to become APCs that can prime CD8 T cells.

Figure 1. Mo-DCs, but not Sirp-α^{+} cDCs, cross-present cell-associated antigen as efficiently as CD24^{+} cDCs

(A, B) Splenic CD24^{+} and Sirp-α^{+} cDCs, BM Ly-6C^{hi} monocytes and Mo-DCs cultured in GM-CSF + IL-4 were purified by cell sorting. APCs were co-cultured with CFSE-labeled OT-1 cells and the indicated number of OVA-loaded γ-irradiated K[b–/–]D[b–/–]β2m^{–/–} (MHC-I TKO) splenocytes. OT-I cells were analyzed after three days by flow cytometry. (A) Representative flow cytometry analysis of OT-I proliferation after cross-presentation assay. (B) Summary of OT-I proliferation after cell-associated cross-presentation assay determined as the percentage of CD44^{+} OT-I cells that had at least one CFSE dilution. n=3 biological replicates per group; control: 1×10^{5} γ-irradiated MHC-I TKO splenocytes without OVA. (C) SIINFEKL peptide presentation by sorted splenic CD24^{+} and Sirp-α^{+} cDCs, BM Ly-6C^{hi} monocytes and Mo-DCs. OT-I cell proliferation was analyzed by flow cytometry three days after culture. n=2 biological replicates per group. (D, E) Sorted splenic Sirp-α[+] cDCs were

cultured in GM-CSF with or without IL-4 for two days and tested for cross-presentation (D) as in A and for SIINFEKL peptide presentation (E) as in C. Sorted splenic CD24$^+$ and Sirp-α$^+$ cDCs without treatment were used as positive and negative controls respectively; n=2 biological replicates per group.
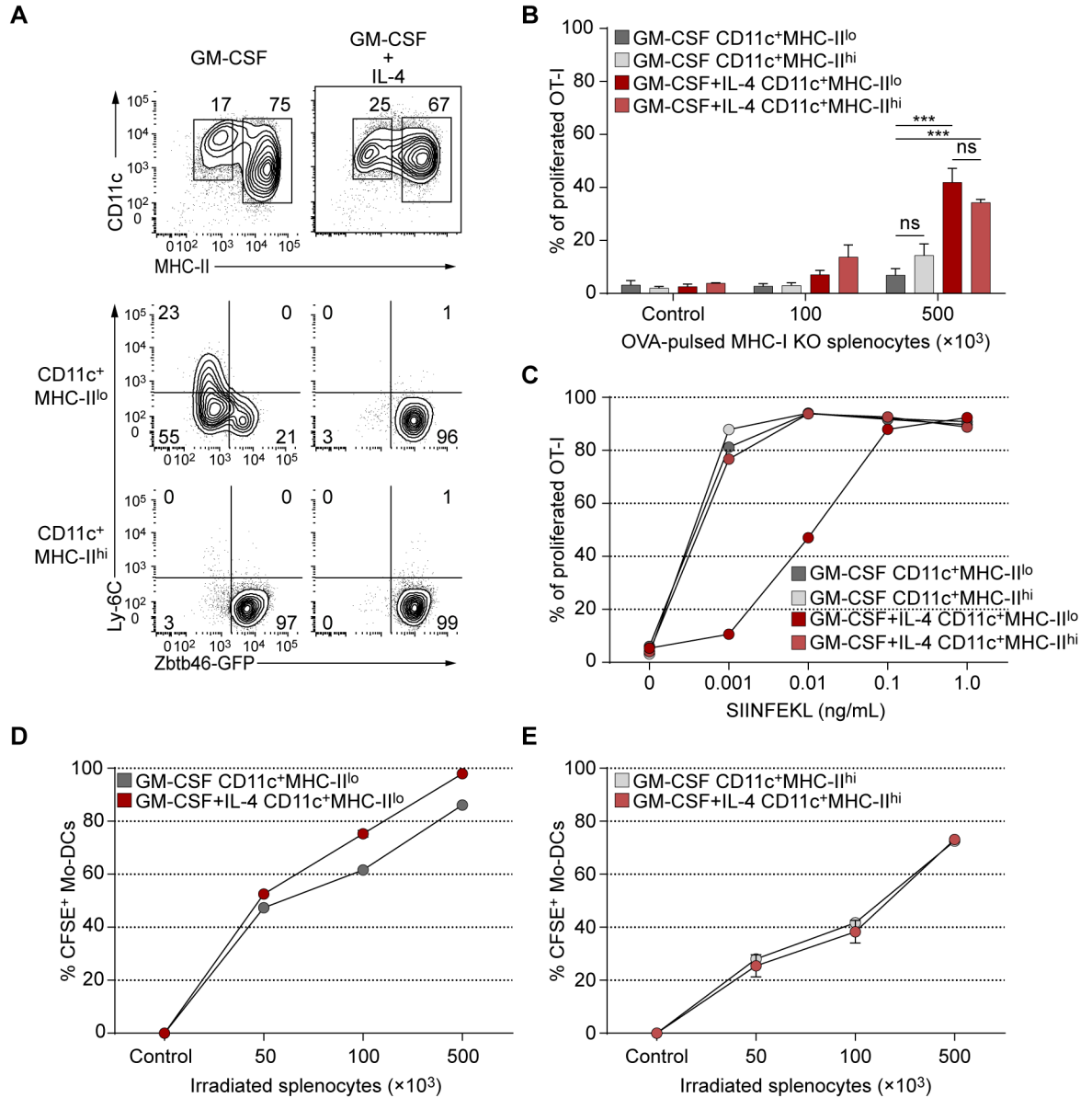
Figure 2. Mo-DCs require IL-4 treatment during differentiation for optimal cross-priming
(A) Ly-6C^{hi} BM monocytes from *Zbtb46*$^{gfp/+}$ mice were sorted and cultured in GM-CSF
with or without IL-4 for 4 days, and analyzed by flow cytometry for expression of Zbtb46-
GFP. Data are representative of three independent experiments. (B) WT Mo-DCs were
generated as in A. CD11c$^+$ Mo-DCs were then sorted as MHC-II$^-$ or MHC-II$^+$ and co-
cultured with CFSE-labeled OT-I cells and OVA-loaded γ-irradiated MHC-I TKO
splenocytes. OT-I proliferation was analyzed by flow cytometry after three days. Data are
pooled from three independent experiments, with at least 4 biological replicates per group.
Two-way analysis of variance (ANOVA) with Sidak's multiple comparison test; n.s. not
significant; ***P<0.001. (C) SIINFEKL peptide presentation by Mo-DCs to CFSE labelled
OT-I cells. OT-I proliferation was analyzed on the third day as in B. n=2 biological replicates
per group. (D, E) Mo-DCs were sorted as in B, and co-cultured with γ-irradiated CFSE

labelled CD45.1$^+$ splenocytes for 16 hours. Uptake of apoptotic cells was determined as the percentage of CD45.2$^+$CD45.1$^-$CD11c$^+$ Mo-DCs that were CFSE$^+$. n=2 biological replicates per group.
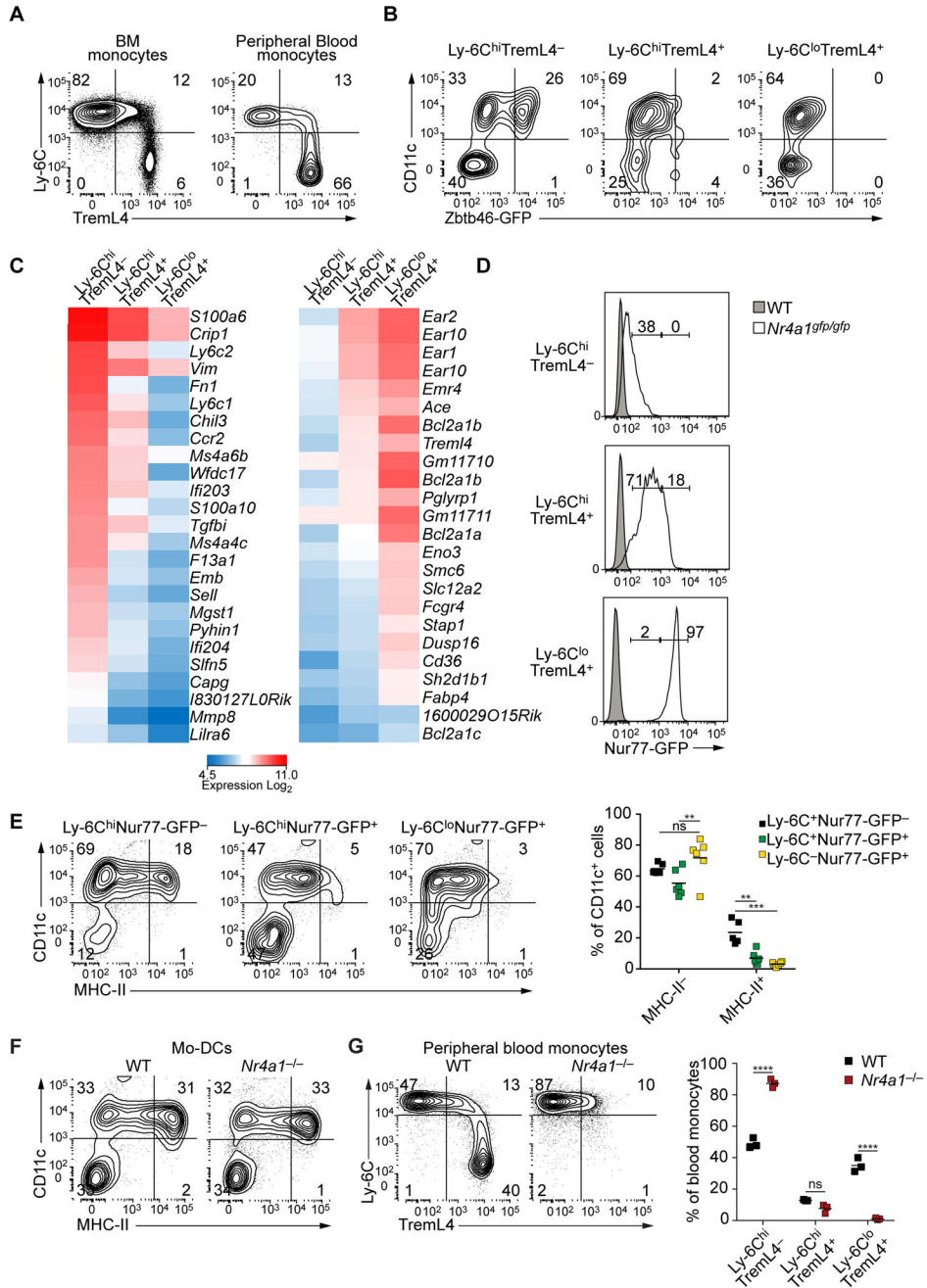
Figure 3. TREML4 identifies a subset of Ly-6C^hi monocytes committed to macrophage-lineage differentiation

(A) Flow cytometry of bone marrow (BM) and peripheral blood (PB) cells from WT mice. BM monocytes were gated as Ter-119⁻B220⁻Ly-6G⁻CD117⁻CD135⁻CD11c⁻MHC-II⁻CD115⁺CD11b⁺ live cells. PB monocytes were gated as CD45.2⁺B220⁻Ter-119⁻MHC-II⁻Ly-6G⁻CD115⁺CD11b⁺ live cells. Data is representative of three independent experiments. (B) Ly-6C^hiTREML4⁻, Ly-6C^hiTREML4⁺ and Ly-6C^loTREML4⁺ monocytes were sorted from PB of *Zbtb46^gfp/+* mice, cultured in GM-CSF and IL-4 for 3 days and analyzed by flow cytometry for Zbtb46-GFP expression. Data are representative of three

independent experiments. (C) Gene expression microarray analysis of sorted Ly-6C^{hi}TREML4$^-$, Ly-6C^{hi}TREML4$^+$, Ly-6C^{lo}TREML4$^+$ PB monocytes. Shown are genes that were at least 3-fold different between Ly-6C^{hi}TREML4$^-$ and Ly-6C^{lo}TREML4$^+$ monocytes. (D) Flow cytometry analysis of Nur77-GFP expression in the indicated monocyte populations from peripheral blood of *Nr4a1$^{gfp/+}$* mice. Monocytes were pre-gated as in A. (E) Sorted Ly-6C^{hi}Nur77-GFP$^-$, Ly-6C^{hi}Nur-77GFP$^+$, Ly-6C^{lo}Nur-77-GFP$^+$ peripheral blood monocytes from *Nr4a1$^{gfp/gfp}$* mice were cultured as in B. Left panels show representative two-color histograms for CD11c and MHC-II expression. Right panel shows summarized data; each dot represents a biological replicate. n=6 biological replicates from two independent experiments. (F) Sorted Ly-6C^{hi}TREML4$^-$ PB monocytes from *Nr4a1$^{-/-}$* and WT littermate controls were cultured as in B and analyzed by flow cytometry. Data are representative of three independent experiments. (G) Flow cytometry analysis of PB from WT and *Nr4a1$^{-/-}$* mice. Cells were gated as in A. Right panel shows summarized data; each dot represents a biological replicate; two-way analysis of variance (ANOVA) with Sidak's multiple comparison test; n.s. not significant, **P<0.01; ***P<0.001; ****P<0.0001. See also Figure S1.
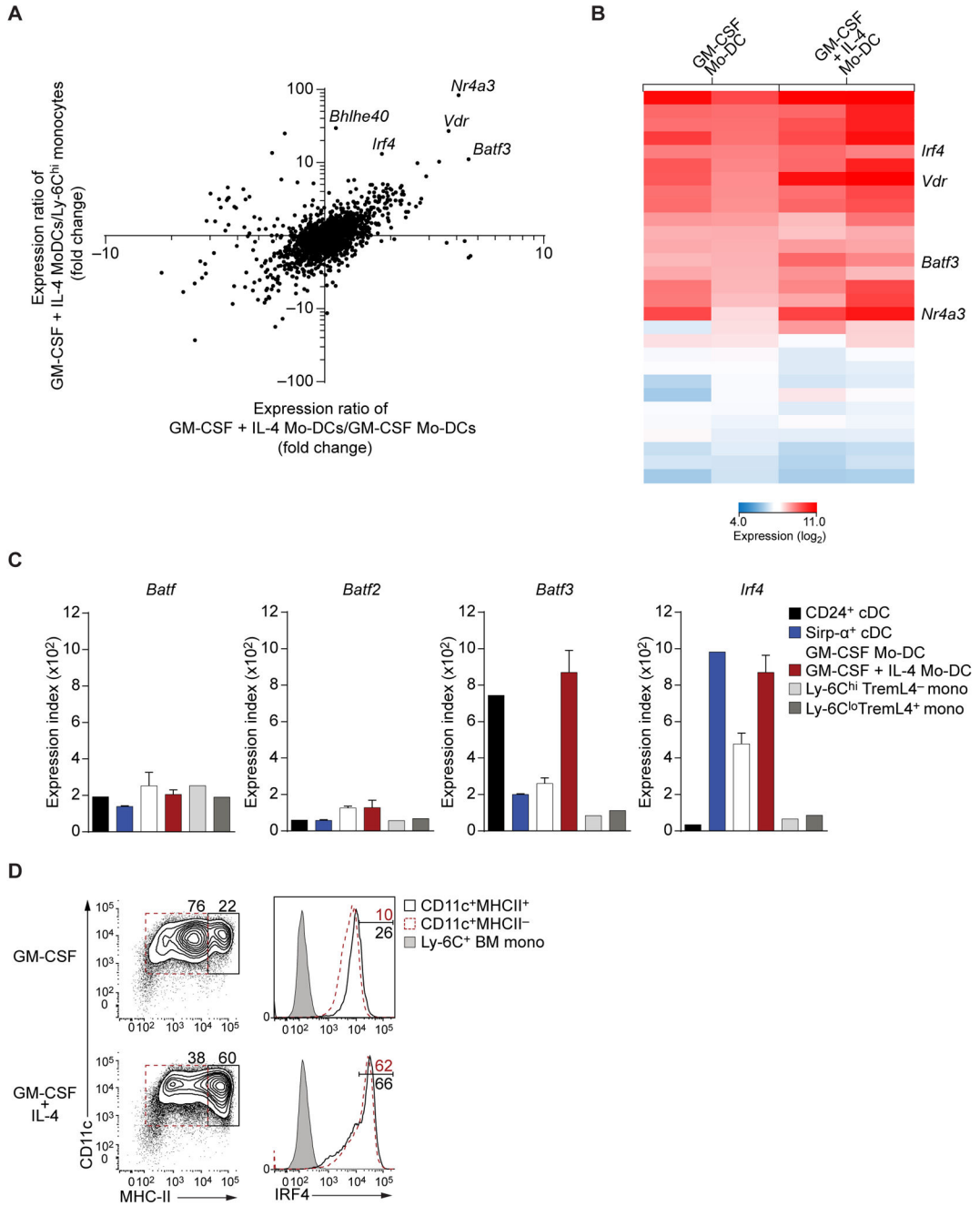
Figure 4. Mo-DCs induce expression of *Batf3* and *Irf4* in response to IL-4

(A) Gene expression microarray analysis of Ly-6C^{hi} monocytes and Mo-DCs differentiated with GM-CSF alone or GM-CSF and IL-4. Shown is the ratio of expression in Mo-DCs generated with GM-CSF and IL-4 versus that of Mo-DCs generated with GM-CSF alone (horizontal axis) plotted against the ratio of expression in Mo-DCs generated with GM-CSF and IL-4 versus that in monocytes (vertical axis) for all transcription factor-encoding genes.

(B) Gene expression of transcription factors induced at least 2-fold in Mo-DCs cultured with IL-4 relative to Mo-DCs cultured with GM-CSF alone. Shown are biological replicates for

each cell lineage. (C) Relative expression of *Batf*, *Batf2*, *Batf3* and *Irf4* from microarrays of the indicated cell type. (D) Representative intracellular flow cytometry analysis of sorted Ly-6C^hiTREML4^− BM monocytes cultured in GM-CSF with or without IL-4. Ly-6C^+ BM monocytes are shown as control. Data is representative of three independent experiments.
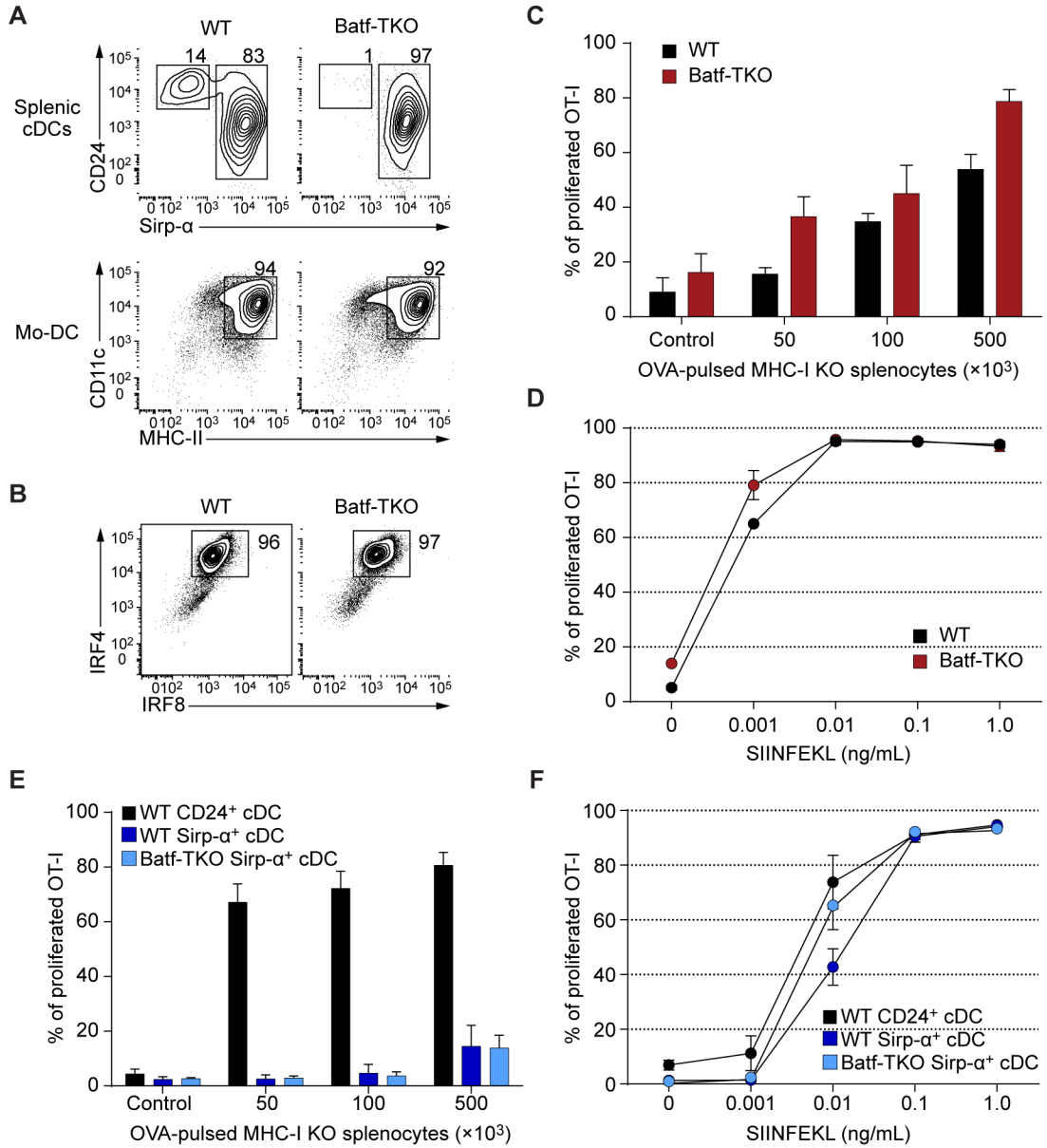
Figure 5. Mo-DCs do not require BATF3 for differentation into APCs capable of cross-priming
(A) Flow cytometry analysis of splenocytes and Mo-DCs generated with GM-CSF and IL-4
from WT and *Batf−/−Batf2−/−Batf3−/−* (Batf-TKO) mice. Splenic cDCs are pre-gated as
B220−CD11c+MHC-II+ cells. Mo-DCs are gated as Ly-6C− cells. Data are representative of
two independent experiments. (B) Intracellular flow cytometry analysis for IRF4 and IRF8
in WT and Batf-TKO Mo-DCs. Data is representative of three independent experiments. (C)
Cross-presentation of cell-associated antigen by WT and Batf-TKO Mo-DCs. Percent
proliferation was determined as the percentage of CD44+ OT-I cells that had undergone at
least one CFSE dilution. n=3 biological replicates per group; control: 1×10^5 γ-irradiated
MHC-I TKO splenocytes without OVA. (D) SIINFEKL peptide presentation by WT and
Batf-TKO Mo-DCs. OT-I proliferation was analyzed by flow cytometry as in C after three
days of culture. n=2 biological replicates per group. (E) Cell-associated cross-presentation

assay by Batf-TKO Sirp-α^+ cDCs as in C.Splenic WT CD24$^+$ and Sirp-α^+ cDCs were used as controls. (F) SIINFEKL peptide presentation by WT CD24$^+$ and Sirp-α^+ cDCs and Batf-TKO Sirp-α^+ cDCs as in D, n=2 biological replicates per group.
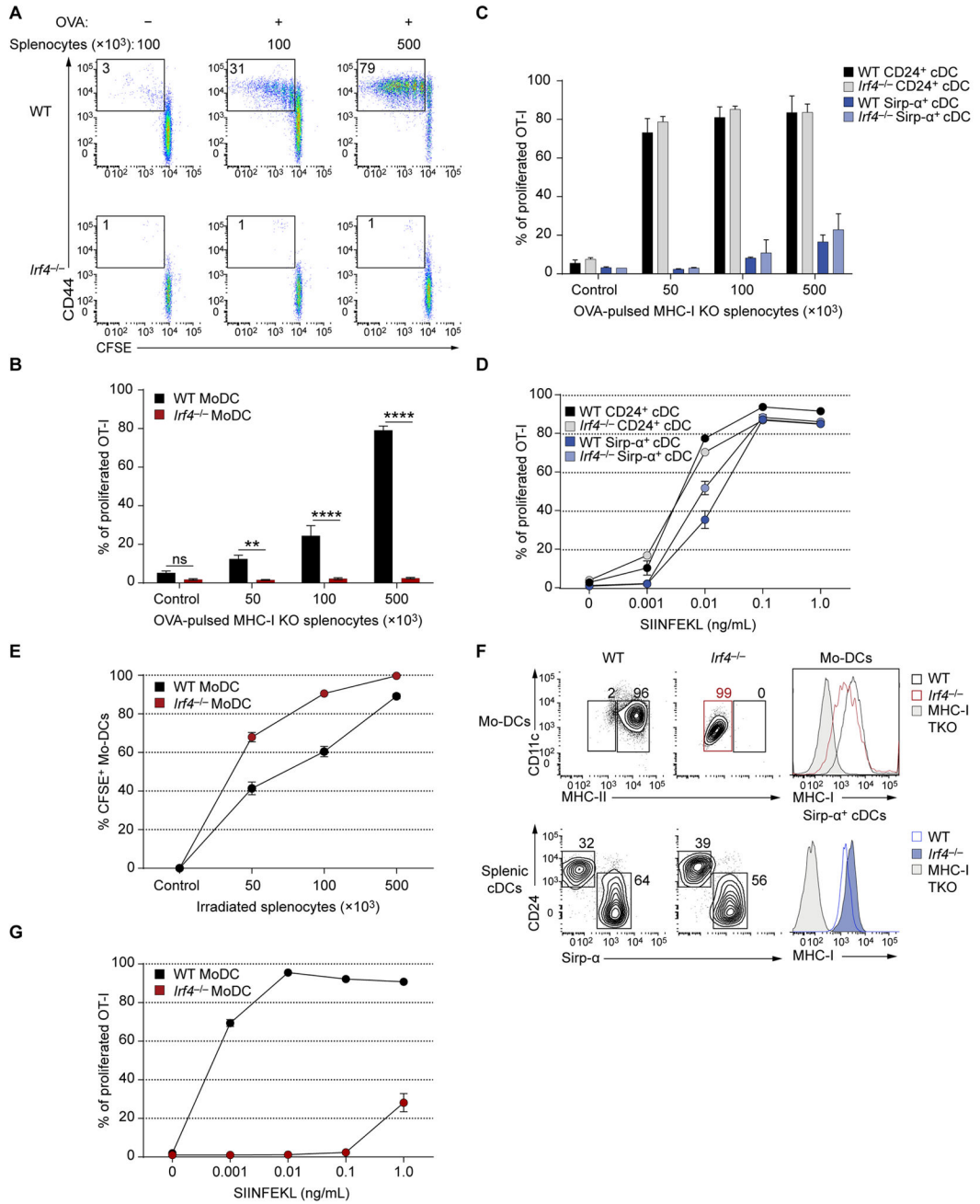
Figure 6. Mo-DCs require IRF4 for cross-priming CD8$^+$ T cells to cell-associated antigen
(A, B) Cross-presentation of cell-associated antigen by WT and *Irf4$^{-/-}$* Mo-DCs. OT-I cell
proliferation was analyzed by flow cytometry three days after culture. (A) Representative
two color histograms of OT-I cell proliferation after cross-presentation assay. (B) Summary
of cell-associated cross-presentation by WT and *Irf4$^{-/-}$* Mo-DCs. Percent proliferation of
OT-I cells was determined as CD44$^+$ OT-I cells that had undergone at least one CFSE
dilution. Data are pooled from 6 biological replicates per group; control: 1×10^5 γ-irradiated
MHC-I TKO splenocytes without OVA. (C) Cross-presentation of cell-associated antigen by
sorted CD24$^+$ and Sirp-α^+ DCs from spleens of WT and *Irf4$^{-/-}$* mice as in A; n=2 biological

replicates per group. (D) SIINFEKL peptide presentation by splenic CD24$^+$ and Sirp-α$^+$ DCs from WT and *Irf4*$^{-/-}$ mice; n=2 biological replicates per group. (E) Apoptotic cell uptake after by WT and *Irf4*$^{-/-}$ Mo-DCs after 16 hours of culture. Cells were analyzed by flow cytometry, pre-gated as CD45.2$^+$CD45.1$^-$CD11c$^+$; n=2 biological replicates per group. (F) Flow cytometry analysis of WT and *Irf4*$^{-/-}$ *ex vivo* derived Mo-DCs and splenic cDCs. Splenic cDCs were gated as B220$^-$SiglecH$^-$CD11c$^+$MHC-II$^+$. One-color histograms show MHC-I expression for the indicated populations. Cells from MHC-I TKO mice are shown as control. Data are representative of three biological replicates. (G) SIINFEKL peptide presentation assay by WT and *Irf4*$^{-/-}$ Mo-DCs. OT-I proliferation was measured as in A; n=3 biological replicates per group. Two-way analysis of variance (ANOVA) with Sidak's multiple comparison test; n.s. not significant, **P<0.01, ****P<0.0001.
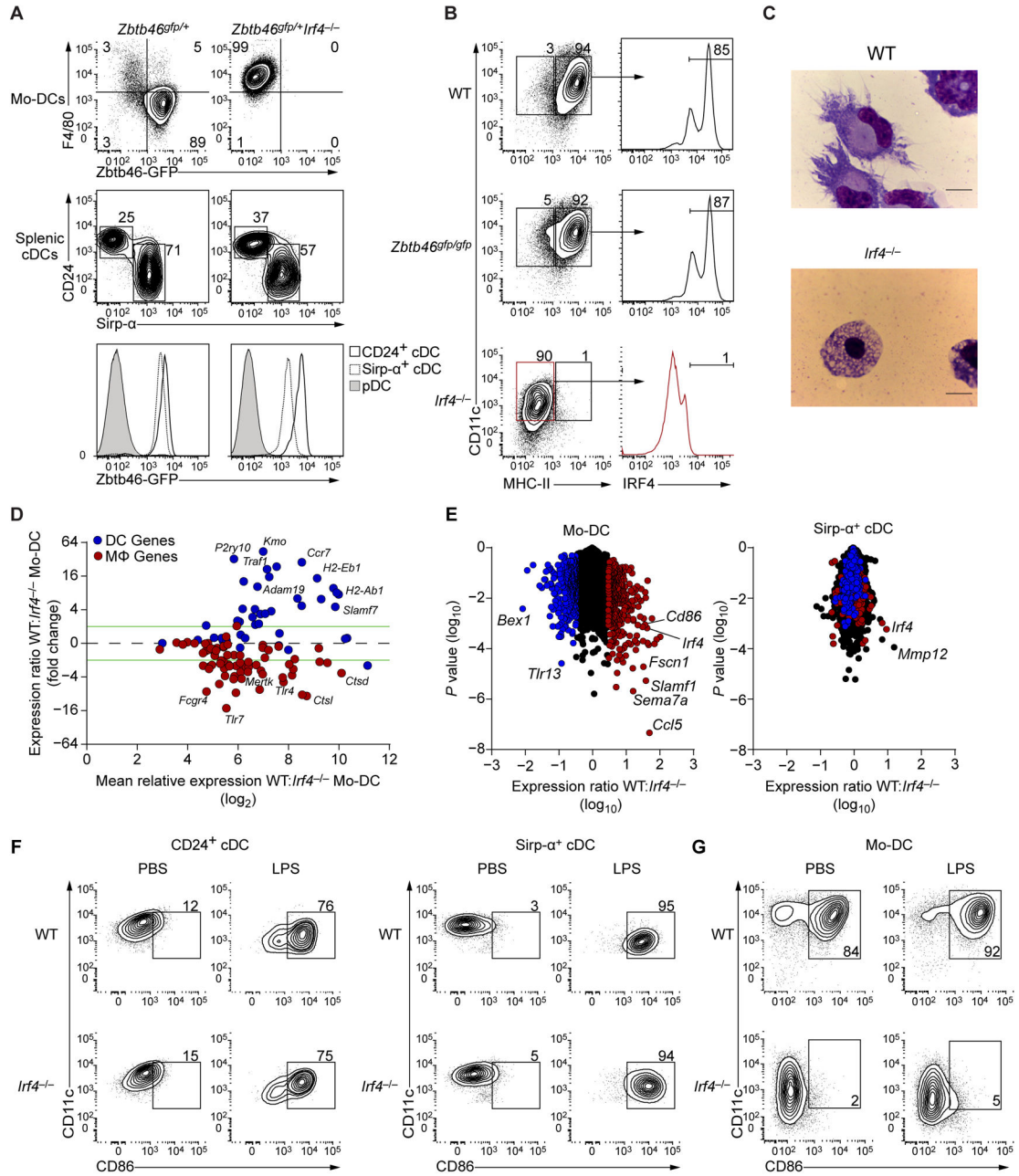
Figure 7. *Irf4*^{−/−} monocytes divert to macrophages upon GM-CSF and IL-4 signaling

(A) Flow cytometry analysis of Zbtb46 expression in Mo-DCs generated with GM-CSF and IL-4 and splenic cDCs from *Zbtb46*^{gfp/+} and *Zbtb46*^{gfp/+}*Irf4*^{−/−} mice. Splenic cDCs were gated as B220^{−}CD11c⁺MHC-II⁺, pDCs are shown as negative control. Data are representative of three independent experiments. (B) Flow cytometry analysis of WT, *Zbtb46*^{gfp/gfp} and *Irf4*^{−/−} Mo-DCs generated as in A. Expression of IRF4 in the indicated gates is shown in right panels. Data are representative of three biological replicates per group. (C) Microscopy of WT and *Irf4*^{−/−} Mo-DCs stained with Wright-Giemsa stain. Scale bars: 10μm. (D) MA plot of the expression ratio of DC and macrophage (MΦ) specific genes

from (Miller et al., 2012; Gautier et al., 2012) in WT and $Irf4^{-/-}$ Mo-DCs. (E) Gene expression analysis of Mo-DCs and splenic Sirp-α^+ cDCs from WT and $Irf4^{-/-}$ mice. Colors indicate expression three fold higher (red) or lower (blue) in WT MoDCs than in $Irf4^{-/-}$ Mo-DCs. Welch's t test, P value (vertical axis). (F, G) Flow cytometry analysis of sorted CD24$^+$ and Sirp-α^+ splenic DCs (F) and Mo-DCs (G) from WT or $Irf4^{-/-}$ mice treated with LPS for 16 hours. Data is representative of two independent experiments.