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

Carlos G. Briseno  
*Washington University School of Medicine in St. Louis*

Malay Haldar  
*University of Pennsylvania*

Nicole M. Kretzer  
*Washington University School of Medicine in St. Louis*

Xiaodi Wu  
*Washington University School of Medicine in St. Louis*

Derek J. Theisen  
*Washington University School of Medicine in St. Louis*

*See next page for additional authors*

Follow this and additional works at: [https://digitalcommons.wustl.edu/open_access_pubs](https://digitalcommons.wustl.edu/open_access_pubs)

Please let us know how this document benefits you.

**Recommended Citation**

Briseno, Carlos G.; Haldar, Malay; Kretzer, Nicole M.; Wu, Xiaodi; Theisen, Derek J.; KC, Wumesh; Durai, Vivek; Grajales-Reyes, Gary E.; Iwata, Arifumi; Bagadia, Prachi; Murphy, Theresa L.; and Murphy, Kenneth M., "Distinct transcriptional programs control cross-priming in classical and monocyte-derived dendritic cells." Cell Reports. 15, 11. 2462-2747. (2016).  
[https://digitalcommons.wustl.edu/open_access_pubs/5091](https://digitalcommons.wustl.edu/open_access_pubs/5091)
Distinct Transcriptional Programs Control Cross-Priming in Classical and Monocyte-Derived Dendritic Cells

Graphical Abstract

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

Authors
Carlos G. Briseño, Malay Haldar, Nicole M. Kretzer, ..., Prachi Bagadia, Theresa L. Murphy, Kenneth M. Murphy

In Brief
The transcriptional programs required for differentiation of cross-priming APCs from various lineages are unknown. Briseño et al. show that Mo-DCs use a program distinct from that of 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.

Accession Numbers
GSE75015
Distinct Transcriptional Programs Control Cross-Priming in Classical and Monocyte-Derived Dendritic Cells

Carlos G. Briseño,1,4 Malay Haldar,2,4 Nicole M. Kretzer,1 Xiaodi Wu,1 Derek J. Theisen,1 Wumesh KC,1 Vivek Durai,1 Gary E. Grajales-Reyes,1 Arifumi Iwata,1 Prachi Bagadia,1 Theresa L. Murphy,1 and Kenneth M. Murphy1,3,*

1Department of Pathology and Immunology, School of Medicine, Washington University in St. Louis, St. Louis, MO 63110, USA
2Department of Pathology and Laboratory Medicine, Perelman School of Medicine and Abramson Family Cancer Research Institute, University of Pennsylvania, Philadelphia, PA 19104, USA
3Howard Hughes Medical Institute, School of Medicine, Washington University in St. Louis, St. Louis, MO 63110, USA
4Co-first author

*Correspondence: kmurphy@wustl.edu

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⁻/⁺TREML4⁻/⁺ monocytes can differentiate into Zbtb46⁺ Mo-DCs in response to granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4) but that Ly-6C⁺TREML4⁺ monocytes were committed to differentiate into Ly-6C⁻/⁺TREML4⁺ monocytes. Differentiation of Zbtb46⁺ Mo-DCs capable of efficient cross-priming required both GM-CSF and IL-4 and was accompanied by the 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.

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 (Briseño et al., 2014; Satpathy et al., 2007, 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, 1993; Caux et al., 1992). Human monocytes treated ex vivo with GM-CSF and interleukin-4 (IL-4) also acquire DC characteristics (Sallusto and Lanzavecchia, 1994; Romani et al., 1994). Mo-DCs express CD11c and major histocompatibility complex class II (MHC-II) (León et al., 2004), as well as the DC-specific transcription factors ZBTB46 and L-MYC (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⁻) or DCs (GM-DCs, CD11b⁻MHC-II⁺). GM-DCs cross-present soluble antigen more efficiently than GM-Macs do (Helft et al., 2015).

Mo-DCs can promote T₁,1 and CD8⁺ T cell responses (León et al., 2007; Aldridge et al., 2009; Ji et al., 2013) but they differ from cDCs in the antigen processing pathways they use (Segura et al., 2009) and the phases of infection in which they are involved (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; Höltl et al., 1999; Timmerman et al., 2002; Thurner et al., 1999) and subdominant neoantigens (Carreno et al., 2015) and they have been used in cancer vaccines (Palucka and Bancher-eau, 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 Chantouni 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 (Sato et al., 2010). Loss of either JMJD3 or IRF4 impairs expression of M2 macrophage genes such as Arg1, IL13, and Fizz1 (Sato et al.,...
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 (Bajanà et al., 2012), survival in GM-CSF (Bajanà et al., 2012), and capacity to induce TH17 and TH2 responses (Gao et al., 2013; Persson et al., 2013), and capacity to induce TH17 and TH2 responses (Gao et al., 2013; Persson et al., 2013), and capacity to induce TH17 and TH2 responses (Gao et al., 2013; Persson et al., 2013). Unlike Mo-DCs, sorted splenic Sirp-α+ cDCs, BM Ly-6C+ monocytes, and Mo-DCs cultured in GM-CSF purified by cell sorting. APCs were co-cultured with CFSE-labeled OT-1 cells, and the indicated number of OVA-loaded γ-irradiated K1461/2462-2474 (MHC-I TKO) splenocytes. OT-1 cells were analyzed after 3 days by flow cytometry. (A) Representative flow cytometry analysis of OT-1 proliferation after cross-presentation. (B) Summary of OT-1 proliferation after cell-associated cross-presentation assay determined as the percentage of CD44+ OT-1 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+ cDCs, and Mo-DCs. OT-1 cell proliferation was analyzed by flow cytometry 3 days after culture. n = 2 biological replicates per group. (D and E) Sorted splenic Sirp-α+ cDCs were cultured in GM-CSF with or without IL-4 for 2 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. Error bars indicate SEM.

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 (Figures 1A and 1B), as reported previously (den Haan et al., 2000; Becker 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 (Bajanà et al., 2012), survival in mucosal tissues (Schlitzer et al., 2013; Persson et al., 2013), and capacity to induce Th17 and Th2 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 they instead require IRF4.

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 (Figures 1A–1C), as reported previously (Cheong et al., 2010). Unlike Mo-DCs, sorted splenic Sirp-α+ cDCs cultured in GM-CSF with or without IL-4 did not cross-prime T cells to cell-associated antigen (Figure 1D) but they presented SIINFEKL peptide (Figure 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-IIα GM-DCs and MHC-IIα GM-Macs (Figure 2A), in agreement with a recent study (Helft et al., 2015). MHC-IIα GM-DCs expressed Zbtb46 (Satpathy et al., 2012), but MHC-IIα GM-Macs did not (Figure 2A), consistent with specific Zbtb46 expression in cDCs but not macrophages (Meredith et al., 2012; Satpathy et al., 2012). Addition of IL-4 with GM-CSF induced uniform Zbtb46 expression in both MHC-IIα and MHC-IIα populations of CD11c+ cells (Figure 2A). Both MHC-IIα and MHC-IIα cells that developed in GM-CSF alone were weak cross-primers of cell-associated antigen, but addition of IL-4 significantly enhanced their activity (Figure 2B) to levels similar to those of CD24+ cDCs (Figures 1A and 1B). All populations presented SIINFEKL peptide (Figure 2C), MHC-IIα (Figure 2D) and MHC-IIα (Figure 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<sup>lo</sup> cells and increases cross-priming in both MHC-II<sup>hi</sup> and MHC-II<sup>lo</sup> cell populations.

**Expression of TREML4 and NUR77 Identifies Monocytes Lacking Mo-DC Potential**

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

NUR77 (encoded by Nr4a1) is required for the development of Ly-6<sup>C</sup><sup>lo</sup> monocytes (Martínez-González and Badimon, 2005; Hanna et al., 2011). Our analysis of Nr4a1<sup>-GFP</sup> reporter mice (Moran et al., 2011) shows that TREML4 expression increased along with Nr4a1 (Figure 3D). Nr4a1<sup>-GFP</sup> was absent in Ly-6<sup>C</sup><sup>lo</sup> TREML4<sup>-</sup> monocytes but was expressed at intermediate levels in all Ly-6<sup>C</sup><sup>hi</sup> TREML4<sup>+</sup> monocytes and at high levels in all Ly-6<sup>C</sup><sup>lo</sup> TREML4<sup>+</sup> monocytes (Figure 3D). Then, we tested the DC potential of monocytes expressing different levels of Nr4a1<sup>-GFP</sup> (Figure 3E). Ly-6<sup>C</sup><sup>+</sup> Nr4a1<sup>-GFP</sup> monocytes differentiated into Mo-DCs in response to GM-CSF and IL-4 (Figure 3E). In contrast, Ly-6<sup>C</sup><sup>+</sup> Nr4a1<sup>-GFP</sup> monocytes and Ly-6<sup>C</sup><sup>lo</sup> Nr4a1<sup>-GFP</sup> monocytes were unable to differentiate into CD11c<sup>+</sup> MHC-II<sup>+</sup> Mo-DCs (Figure 3E). Nr4a1-deficient monocytes could not develop into Ly-6<sup>C</sup><sup>lo</sup> monocytes, as reported (Moran et al., 2011), but could develop into Mo-DCs (Figure 3F). Unsuspected analysis using SPADE (Qiu et al., 2011) reconstituted the successive steps of monocyte differentiation in vivo (Figure S1, related to Figure 3). Thus, Ly-6<sup>C</sup><sup>hi</sup> TREML4<sup>-</sup> Nr4a1<sup>-GFP</sup> 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-6ChiTREML4+ and Ly-6Clo 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 as compared to monocytes or Mo-DCs cultured in GM-CSF alone (Figure 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 (Figures 4A and 4B), 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 these 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 Lugt et al., 2014). Mo-DCs induced Batf3, but not Batf or Batf2, to levels equivalent to those of both splenic CD24+ and Sirp-α+ cDCs (Figure 4C). Likewise, Mo-DCs expressed Irf4 to levels similar to those of Sirp-α+ cDCs (Figure 4C). Also, IL-4 increased IRF4 expression in Mo-DCs (Figure 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 (Batt 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 (Figure 5A), with normal expression of IRF4 and IRF8 (Figure 5B). As reported (Tussiwand et al., 2012), Batf-TKO lacked splenic CD24+ cDCs but retained Sirp-α+ cDCs (Figure 5A). We found no difference in cross-priming between wild-type (WT) and Batf-TKO Mo-DCs over a range of antigen concentrations or in presentation of SIINFEKL peptide (Figures 5C and 5D). Splenic Batf-TKO Sirp-α+ DCs did not cross-prime but could present SIINFEKL peptide (Figures 5E and 5F). 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 the 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 BM-derived GM-DCs (Vander Lugt et al., 2014), but its role in priming of CD8+ T cells by Mo-DCs is unknown. Mo-DCs derived from Irf4−/− Ly-6Chi TREML4+ monocytes were inactive for cross-priming (Figures 6A and 6B). In contrast, Irf4−/− splenic CD24+ cDCs were as efficient as WT CD24+ cDCs in cross-priming OT-I cells (Figures 6C and 6D). The uptake of apoptotic cells was similar between WT and Irf4−/− Mo-DCs (Figure 6E). Mo-DCs lacking IRF4 did not express MHC-II, as reported previously (Vander Lugt et al., 2014), but expressed normal MHC class I (MHC-I) levels (Figure 6F). However, they were unable to induce OT-I proliferation with SIINFEKL peptide (Figure 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 (Figure 7A). Irf4 was not required for Zbtb46 expression in CD24+ or Sirp-α+ splenic cDCs (Figure 7A). By contrast, Zbtb46-deficient Mo-DCs expressed normal levels of MHC-II and IRF4 (Figure 7B). Consistent with the lack of MHC-II and Zbtb46 expression, the normal DC morphology of Mo-DCs was not seen in Irf4−/− Mo-DCs, which, instead, had the appearance of macrophages (Figure 7C), suggesting that IRF4 may be required for the 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 (Figures 7D and 7E). 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, Thr4, and Thr7 (Gautier et al., 2012) and, unlike WT Mo-DCs, failed to induce DC-associated genes such as
requirement for IRF4 by monocytes for their differentiation into (Figures 7F and 7G). Altogether, these results indicate a specific expressed by at least 3-fold between these two populations least 3-fold different between WT and Irf4 cDCs, lacking to be specifically downregulated in Mo-DCs, but not Sirp-α cDCs, require IRF4 for their development. We identified CD86 expression of CD86 on WT and Irf4 WT and and splenic Sirp-α cDCs, lacking IRF4 regulated a similar genetic program in both Mo-DCs and not CD24+ or Sirp-α cDCs, required IRF4 for cross-presentation have been analyzed only in cells generated from BM cells treated with GM-CSF alone (Joffre et al., 2012; Se-

DISCUSSION

Vaccines based on Mo-DCs can enhance immune responses against human melano-

toma (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 aug-

ments 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 that 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 Ly-6C hi monocytes can differentiate into either Mo-Macs, Mo-DCs, or Ly-6C lo “patrolling” monocytes. NUR77 is required for differentiation of Ly-6C hi monocytes into patrolling monocytes (Hanna et al., 2011) but not into Mo-DCs (Figure 3F). We find that Ly-6C hi monocytes that express NUR77 or TREM4 lack Mo-DC potential. In CD8+ T cells, NUR77 may inhibit IRF4 expression (Nowyhed et al., 2015), suggesting that it may act similarly in Ly-6C hi TREM4+ 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, 2009), Rac2 (Savina et al., 2009) and VAMP8 (Matheoud

Kmo, Traf1, and Slamt7 (Miller et al., 2012) (Figure 7D). Since IRF4 has been previously implicated in the development of splenic Sirp-α cDCs (Suzuki et al., 2004), we asked whether 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 (Figure 7E). However, only 49 of those targets were also at least 3-fold different between WT and Irf4 −/− Sirp-α cDCs (Figure 7E), suggesting that 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 lipopolysaccharide (LPS). Only Mo-DCs, not CD24+ or Sirp-α cDCs, required IRF4 for CD86 expression (Figures 7F and 7G). Altogether, these results indicate a specific requirement for IRF4 by monocytes for their differentiation into DC-like cells.
et al., 2013) were shown to regulate acidification of phagosomes in GM-DCs, suggesting that they act to preserve 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., 2013) were shown to regulate acidification of phagosomes and in vivo but not in GM-CSF BM-derived cells (Firat et al., 2007), 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 the cross-priming of cell-associated antigen, compared with CD8+ cDCs and Mo-DCs generated with both GM-CSF and IL-4 (Figure 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<sup>+/–</sup> mice (Satpathy et al., 2012a) were backcrossed to C57BL/6J for at least eight generations. Batf<sup>–/–</sup> Batf2<sup>–/–</sup> Batf3<sup>–/–</sup> (Batf-TKO), Ifnγ<sup>/–</sup>, and Irf4<sup>/–</sup> mice have been described previously (Tussiwand et al., 2012; Grajales-Reyes et al., 2015). The following mice were purchased from Jackson Laboratory: Nrd4<sup>1–/–</sup> (B6;129S2-Nrd4<sup>1tm1Jmi</sup>/J), OT-I (C57BL/6-Tg(TcraTcrb)[129OBy])<sup>100Mth/J</sup>, and CD45.1<sup>+</sup> (B6.SJL-Ptprca Pepcb/BoyJ). Nrd4<sup>1</sup><sup>/</sup><sup>–</sup><sup>/</sup><sup>–</sup> mice were a gift from Chyi-Song Hsieh, and K<sup>b</sup> mice were a gift from Herbert W. Virgin IV and Graciela Sánchez, 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 use 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 (Becton Dickinson [BD] clone 2.4G2).

The following antibodies were purchased from BD: CD11b (M1/70); CD45.2 (104); CD135 (A2F10.1); MHC-II (M5/114.15.2); and Ly-6C (AL-21). The

**Figure 5. Mo-DCs Do Not Require BATF3 for Differentiation into APCs Capable of Cross-Presentation**

(A) Flow cytometry analysis of splenocytes and Mo-DCs generated with GM-CSF and IL-4 from WT and Batf<sup>–/–</sup> Batf3<sup>–/–</sup> (Batf-TKO) mice. Splenic CD24+ cDCs were pre-gated as B220<sup>+</sup> CD11c<sup>+</sup>MHC-II<sup>+</sup> cells. Mo-DCs are gated as Ly-6C<sup>+</sup> 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 are 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<sup>+</sup> OT-I cells that had undergone at least one CFSE dilution. n = 3 biological replicates per group; control: 1 x 10<sup>6</sup> 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 3 days of culture. n = 2 biological replicates per group.

(E) Cell-associated cross-presentation assay by Batf-TKO Sirp-α<sup>–</sup> cDCs, as in (C). Splenic WT CD24<sup>+</sup> and Sirp-α<sup>–</sup> cDCs were used as controls.

(F) SIINFEKL peptide presentation by WT CD24<sup>+</sup> and Sirp-α<sup>–</sup> cDCs and Batf-TKO Sirp-α<sup>–</sup> cDCs, as in (D), n = 2 biological replicates per group. Error bars indicate SEM.
Figure 6. Mo-DCs Require IRF4 for Cross-Priming CD8+ T Cells to Cell-Associated Antigen

(A and B) Cross-presentation of cell-associated antigen by WT and Irf4−/− Mo-DCs. OT-I cell proliferation was analyzed by flow cytometry 3 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 six 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.

(legend continued on next page)
following were purchased from eBioscience: CD4 (GK1.5); CD8 (53-6.7); CD11b (M1/70); CD45.1 (A20); CD44 (IM7); CD117 (2B8); CD115 (AFS98); CD11c (N418); CD24 (M1/69); CD172a (P84); Ly-6C (HK.1.4); Ly-6A/E (D7); Ly-6G (IA8); Sglec-H (eBio440C); Ter-119 (Ter-119); CD105 (MJ7/18); Ir8 (VG3YWH); CD45R (RA3-6B2); NK1.1 (PK136); Irf3 (SE4); and 7-AAD viability staining solution. The following were purchased from Tonbo Biotix: CD45.1 (A20); and CD11c (N418). The following were purchased from BioLegend: CD83 (S3-6.7); CD45.2 (104); CD115 (ASFP8); Ly-6G (A68); TCR Vα2 (B20.1); and TREML4 (16E5). The following were purchased from ThermoFisher Scientific: TCR Vα2 (B20.1) and the 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 Milteny Biotec. Cells were fixed and permeabilized for intracellular staining of IFNγ and IRF8 using the FoxP3/Transcription Buffer Set (eBioscience). Cells were sorted on a BD FACSAria Fusion flow cytometer (BD) and with FlowJo software (TreeStar).

Isolation and Culture of BM Cells and Splenic DCs
Femurs, pelvises, and tibias were cultured in a mortase and pestle in MACS buffer, filtered through a 70-μm strainer, purified on Histopaque-1119 gradient, and depleted of Ly-6G- and B220-expressing cells with biotinylated anti-Ly-6G and B220 antibodies and anti-biotin microbeads (Milteny Biotec). BM monocytes were identified as Lin−Siglec-H+Ly-6G−MHC-IIloCD11c+CD117−CD135−CD115− and sorted as Ly-6C−TREM4+ or Ly-6C+TREM4+ for microarray analysis. Lin includes B220, CD105, NK1.1, and Ter-119. Blood monocytes were defined as Ter-119+CD45.2−MHC-II−Ly-6G−CD115+ and segregated based on Ly-6C and TREM4 expression. Cells were sorted into isovisc’s modified Dulbecco’s medium + 10% fetal calf serum (FCS) kept at 4°C. Splenets were minced and digested for 45 min at 37°C with stirring in 5 ml of complete media with 250 μg/mL collagenase B (Roche) and 30 U/mL DNase I (Sigma-Aldrich). Red blood cells were lysed by washing with ACK (ammonium-chloride-potassium) lysis buffer, and splenocytes were washed through a 70-μm strainer. CD24+ cDCs were defined as B220+CD11c−MHC-II−CD24+CD172a+. Sirpα+ cDCs were defined as B220+CD11c−MHCII+CD24−CD172a+. For Mo-DC differentiation, sorted Ly-6C+ TREM4− monocytes from BM or peripheral blood were cultured to levels (0.25 × 105–5.5 × 105 cells/mL) at 37°C in complete media with GM-CSF and IL-4 for 3–4 days. Loosely adherent Mo-DCs were harvested by gentle pipetting. Sorted Sirpα− cDCs were cultured in 20 ng/mL of GM-CSF and IL-4 for 48 hr. For induction of CD86, sorted CD24+ and Sirpα+ cDCs, as well as Mo-DCs, were cultured with LPS (1 ng/mL) for 16 hr.

Microscopy
Cytopsins of sorted Mo-DCs generated from GM-CSF and IL-4 culture of Ly-6C−TREM4+ monocytes were stained with Wright-Giemsa stain using a Neubauer hemocytometer (Fisher Scientific). Images were acquired at room temperature with an Axioskop microscope (objective: 100x, 1.25 oil) and an Axioscam 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 RNeasy Micro Kit (Ambion). RNA was amplified using the Ovation Pico WTA System (NuGEN) and hybridized to GeneChip Mouse Gene 1.0 ST microarrays (Affymetrix). Data were processed using robust multivariate average summarization and quartile normalization using ArrayStar software, version 5 (DNASTAR). Expression values for cell lineages were averaged from biological duplicates, except for WT CD24+ cDCs and peripheral blood monocyte subsets, which were from one biological sample.

Antigen Presentation Assays
Splenic OT-I cells were sorted as B220−CD11c+CD45.1+CD8−CD4+ CD8a+ to > 95% purity, labeled with CFSE, and plated at a density of 12.5 × 107 cells/mL. Splenocytes from MHC-I−TKO mice were processed as described earlier. OVA loading of MHC-I−TKO splenocytes has been described previously (Carbone and Bevan, 1990). Splenocytes (2.5 × 107/mL) were incubated in hypertonic medium (RPMI 1640, 0.5 M sucrose, 10% w/v polyethylyene glycol, 10 mM HEPES [pH 7.2]) with 5 mg OVA (Worthington) for 10 min at 37°C. Cells were diluted 10-fold with hypotonic medium (5% FBS, 0.5% sterile water) and incubated for 2 min at 37°C. Cells were washed with PBS and irradiated (15.3 Gy). Sorted splenic CD24+ and Sirpα+ cDCs, as well as Mo-DCs (12.5 × 106 cells/mL), were co-cultured with CFSE-labeled OT-I cells (12.5 × 106 cells/mL) and OVA-loaded MHC-I−TKO cells (2.5 × 107–25.0 × 106 cells/mL). For peptide presentation, 2.5 × 105 antigen-presenting cells (APCs) were cultured with SINIFKEL peptide (1.0 × 10−6M) for 45 min in complete media at 37°C, washed twice, and cultured with 2.5 × 104 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 percentage 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 and stained with CFSE and stained with γ-irradiated (13.5 Gy). Sorted CD45.2+ Mo-DCs (12.5 × 106 cells/mL) were co-cultured with CFSE-labeled irradiated splenocytes (2.5 × 107–25.0 × 106 cells/200 μL) for 16 hr at 37°C. After culture, Mo-DCs were washed and stained for CD45.2, CD11c, Ly-6C, 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
Statistical analyses were performed using a two-way ANOVA with Sidak’s multiple comparison test unless otherwise specified. All statistical analyses were performed using Prism (GraphPad Software).

ACCESSION NUMBERS
The accession number for the microarray data reported in this paper is GEO: GSE75015.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures and one figure and can be found with this article online at http://dx.doi.org/10.1016/j.cellrep.2016.05.025.

AUTHOR CONTRIBUTIONS
C.G.B., M.H., T.L.M. and K.M.M. designed the study; C.G.B., X.W., and W.K. performed microarray experiments, with advice from A.I.; C.G.B., N.M.K. and D.J.T. performed cross-presentation assays; C.G.B. and M.H. performed experiments related to cell sorting, culture, and flow cytometry, with advice from
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*+/+ and *Zbtb46*+/+ *Irf4*−/− mice. Splenic cDCs were gated as B220−CD11c+MHC-II+, and pDCs are shown as negative control. Data are representative of three independent experiments.

(B) Flow cytometry analysis of WT, *Zbtb46*+/+, 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, 10 μm.

(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 3-fold higher (red) or lower (blue) in WT Mo-DCs than in *Irf4*−/− Mo-DCs. Welch’s t test, p value (vertical axis).

(F and 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 hr. Data are representative of two independent experiments.
V.D., G.E.G.-R., D.J.T. and P.B.; C.G.B., M.H. and K.M.M. wrote the manuscript with contributions from all authors.

ACKNOWLEDGMENTS

We thank C.S. Hsieh for Nr4a1-GFP mice, the Alvin J. Siteman Cancer Center at Washington University School of Medicine for use of the Center for Biomedical Informatics and Multiplex Gene Analysis Genechip Core Facility, and Ansuman T. Satpathy for helpful discussions. This work was supported by the Howard Hughes Medical Institute (to K.M.M.), the U.S. NIH (F30DK108498 to V.D., 1F31CA189491-01 to G.E.G.-R., and 1K08AI106953 to M.H.), the American Heart Association (12PRE12050419 to W.K.), and the Burroughs Wellcome Fund Career Award for Medical Scientists (to M.H.).

Received: November 19, 2015
Revised: March 11, 2016
Accepted: May 4, 2016
Published: June 2, 2016

REFERENCES


dendritic cells arise from a common major histocompatibility complex class II-negative progenitor in mouse bone marrow. Proc. Natl. Acad. Sci. USA 90, 3038–3042.


alkalinization and antigen crosspresentation selectively in CD8(+) dendritic cells. Immunity 30, 544–555.