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Id2 expression delineates differential checkpoints in the genetic program of CD8α+ and CD103+ dendritic cell lineages

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 22 March 2011

Thank you for submitting your manuscript to the EMBO Journal. Three referees have now seen your manuscript and their comments are provided below.

All three referees find the analysis interesting and suitable for publication here. They raise a number of different concerns that should not involve too much additional work to address. Regarding the 1st major concern raised by referee #2; to further functionally characterize the mature DC subtypes. If you have data on hand to address this issue you can include it in the revised manuscript, otherwise it would suffice to state why you have chosen to concentrate of the CD103+ subset. Given the comments provided I would like to ask you to submit a suitably revised manuscript for our consideration. When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

The paper by Jackson et al. reports on the construction of an Id2-GFP reporter system for analysis of dendritic cell (DC) subsets in vivo and in vitro. They found that Id2 is broadly expressed in all conventional DC (cDC) subsets but not in plasmacytoid DC (pDC). Highest Id2-GFP expression was found to CD103+ and CD8a+ subsets. Jackson et al. then went ahead to cross the Id2-GFP reporter mice with IRF-8 and Batf3 deficient mice, which lack specific DC subsets. IRF-8 deficiency caused loss of all cDC while Batf3 deficiency allowed development of Sirp-a-/CD8a equivalent DC which however showed impaired survival rates.

This is a nice and detailed study that adds very much to our understanding of DC development. The Id2-GFP reporter construct faithfully recapitulates Id2 expression in vivo and thus allows monitoring Id2 expression on the single cell level and the isolation of Id2-GFP- and Id2-GFP+ DC for further analysis.

A major finding is the abundant Id2 expression in various cDC subsets and absence (or very low expression) of Id2 in pDC. The Id2-GFP knock-in mouse system also allowed monitoring Id2 expression in hematopoietic stem/progenitor cells, including common dendritic cell progenitors (CDP) and Pre cDC in spleen and bone marrow. Following-up on these findings the authors isolated Id2-GFP- and Id2-GFP+ DC subsets employing an in vitro Flt3 ligand culture system and studied their function in cross presentation assays.

Length of text, references and number of figures are appropriate.

Major points:

It is not clear from the text on page 9, second paragraph, lines 3-5 and the respective Suppl. Fig. 3B, top panel that population 1 (pop.1) contains multipotent DC precursors. This requires rewording in text and further details in legend to Suppl. Fig. 3B.

Further, what is the message of the sentence "CD103- Id2-GFP+ CD45RA- Sirp-a+ DCs (pop. 4) only gave rise to cells of a similar phenotype ....." (page 9, second paragraph, lines 8-9 and page 10, lines 1-3)? What are these cells and what is their developmental potential?

A schematic representation of a model on DC subset specification that integrates the data shown here would be helpful (e. g. in the Supplementary Materials section). The scheme should illustrate the hierarchy of the transcription factors studied here (see also text in first paragraph of Discussion section, page 14 and text on page 15, lines 7-10 and on page 17, second paragraph, lines 2-5).

Minor points:

(1) cDC in the Abstract, line 6 should be explained.

(2) References Bachem et al., Jongbloed et al. and Poulin et al. need to be up-dated.

(3) Fig.1 represents a description of the transgenic construct and shows data confirming that the construct works as expected. This figure can be moved to the Supplementary Material section. In Fig. 1A (i), (ii) and (iii) the representation of the non-translated region of exon 1 before the arrow, which indicates start and direction of translation, is inconsistent.

(4) There is no information on the LC, dotted line in red in Fig. 2D, lower right panel in the legend to Fig. 2. Please give details.

(5) Fig. 4A, top right panels, expression Bst2 and Siglec-H is not described in the legend to Fig. 4 and in the result section. What are the CD45RAint Id2-GFP+/- cells?

In general the manuscript suffers from spelling errors, additional and missing words (e.g. page 12, line 12; page 14, lines 3 and 8). This should be corrected.

Referee #2 (Remarks to the Author):

The manuscript by Jackson et al. describes a series of experiments using a newly engineered Id2-GFP allele to delineate Id2 expression during differentiation of DC subsets in vivo. Moreover, the authors attempt to utilize their reporter allele to categorize DC subtypes in vitro in Flt3L cultures. They go on to identify non-redundant roles of transcription factors Irf8 and Batf3 for CD8+ and CD103+ DCs in their cultures, and interestingly show that GM-CSF addition can rescue formation but not function of Batf3-deficient CD103+ DCs.

Although a bit descriptive, the manuscript is very interesting and clearly written. The experiments are for the most part sound and convincingly support the conclusions made by the authors. The Id2-GFP allele appears to be a useful tool to further characterize DC heterogeneity and development in vivo. Furthermore, the Id2-GFP based in vitro DC subset analysis of Flt3L cultures may provide an instrumental foundation to work out molecular mechanisms controlling DC differentiation and diversification. However, prior to publication the authors should address the following concerns:

Major concerns:
1. The authors define 6 different DC populations by FACS using in vitro Flt3 cultures of Id2-GFP+ bone marrow progenitors (Fig. 4a). They subsequently concentrate on one of these populations, CD103+ DCs, and use an antigen cross-presentation assay to functionally characterize these cells (Fig. 4b). The authors should provide analogous functional data also for the other mature DC subtypes they have identified in their cultures to support the FACS based characterization.

2. The second part of the manuscript focuses for a large part on the in vitro generated CD103+ cells from Id2-GFP mice with or without deletion of Batf3 or Irf8 genes (Fig. 4a population 6, Fig. 5a, Fig. 6a). However, because all CD103+ DCs in the cultures appear to express uniform Id2-GFP levels the Id2 reporter did not add anything to identification or characterization of the in vitro generated CD103+ DCs. In other words, these cells could be identified solely by their CD103 expression and the Id2-GFP allele could be omitted. This is in my view a major weakness of the manuscript and the authors should convincingly explain the contribution of the Id2-GFP allele to their studies on CD103+ DCs.

Minor concerns:

1. The manuscript has many typos and consequently misplaced periods and commas.

2. The neomycin selection cassette is still present in the engineered Id2 locus. This is unfortunate because it may interfere with the spatial-temporal expression regulation of the Id2 gene at levels which may be hard to detect by the qPCR experiments shown in Fig. 1d. The authors need to mention this somewhere.

3. Fig. 2f: What is shown here, spleen or peritoneum? The legend is a bit confusing.

4. Fig. 5a: Do Irf8 and/or Batf3 control transcription of CD103? Address experimentally.

5. Fig. 5a: There are no major differences visible in the FACS plots of day 5 (upper panel) and day 8 (lower panel) after differentiation. Why are both time points shown?

6. Fig. 5a, 6a, 7a: Why do the authors show CD45RA instead of CD8a or Sirp-a? This is somewhat confusing.

7. Fig. 5d: Original FACS plots should be shown.

8. Fig. 6a: Show gates and percentages in the upper FACS panel.

Referee #3 (Remarks to the Author):

In this study, Jackson et al. develop a novel reagent to monitor transcription of the Id2 locus, which

is quite relevant as the transcriptional regulator is absolutely required for the proper development of multiple cell types within the hematopoietic compartment. They claim the resultant mouse's hematopoietic compartment is indistinguishable from wild-type controls (although only FACS plots are provided without any absolute numbers or frequencies reported). Importantly, the relative levels of GFP transcript correlate synonymously with Id2 transcript amounts, and this serves as the basis for further comparisons made about dendritic cell (DCs) subsets and Id2 levels.
The report begins by performing an extensive flow cytometric analysis of dendritic cells and their precursors in multiple lymphoid organs (Fig. 2). The authors conclude that Id2 is expressed in nearly all DCs, though the levels are quite variable. Not surprisingly, the highest levels of GFP (as measured by mean fluorescence intensity) appear in the cell types for which Id2 is absolutely required during development (e.g., CD8α+ conventional DCs, migratory CD103+ DCs and Langerhans cells). Intriguingly, in Fig. 3, Id2 expression is negligible in the immediate precursors, suggesting a role in the terminal steps of differentiation or maintenance.
Next, the investigators attempt to define six distinct cell types from Flt3 ligand cultures, which are thought to recapitulate the development of steady-state dendritic cells (Fig. 4). The major advancement to the field from this part of the study is the identification of a CD103+ DC, which presents exogenous cell-associated antigens more efficiently than the other subsets from in vitro culture. The authors argue that the high levels of Id2 combined with superior cross-presenting potential and cell-surface markers is sufficient to make the identified cell type the in vitro correlate of the migratory CD103+ DCs.

The last part of the study, in Fig. 5-7, focuses on clarifying the stage at which Batf3 or Irf8 deficiency causes the blockade of DC development. The data in Fig. 5-7 suggests that Irf8, but not Batf3, may control the expression of Id2, leading the investigators to conclude that Irf8 and Id2 are upstream of Batf3. What's more, they detect increased apoptosis from Batf3-/- bone marrow Flt3-ligand cultures and thus conclude that Batf3 is important for the maintenance of CD8α+ equivalent DCs.
Lastly, in Fig. 6-7, authors demonstrate that the a late addition of GM-CSF to Flt3-ligand cultures will enhance or induce the expression of CD103, even in the absence of Batf3 although the emergent cells still appear to be deficient at cross-presentation.

Overall, the strength of the study rests on the identification of a CD103+ DC subset in vitro that may represent a correlate of the migratory population in vivo. However, the study greatly overstates the value of the data in concluding the identification of distinct developmental checkpoints. Another weakness stems from the lack of mechanistic or molecular data on Id2, Irf8 and Batf3 function.

Specific concerns:

1. In the abstract, the authors claim that the CD103+ DCs were the only DC able to cross-present cell-associated antigens constitutively. Strictly, this is only true in vitro, and unless direct comparisons with in vivo subsets are made, such claims only lead to confusion. Therefore, the investigators need either to state that it's in vitro or perform the comparison studies with in vivo equivalents. This can be fixed by simply modifying the text at appropriate places throughout.

2. The author's claim that population 5 (at day 5) is equivalent to CD8α+ DCs is inaccurate. By their own re-plating studies, it's seems that population 5 is a heterogeneous group that ultimately gives rise to both SIRPα positive and negative DCs. Importantly, the original observation that Flt3-ligand cultures could support the development of a CD8α DC equivalent was based upon CD24 by SIRPα. The absence of CD24 as a marker in this study makes it rather hard to compare the current data with what has been reported previously in the literature. If the authors were to stain using CD24, then more direct comparisons with the literature could be made. Moreover, the use of day 5 Flt3 ligand cultures is quite different that what's been reported in the literature, where day8-10 is more commonly seen to contain fully differentiated DC subsets. The author can fix this by simply not making this claim, or by showing the plots with CD24 as a marker. I don't this the claim is too important, since the author has much novel data which substantiates publication anyway.

3. The control for cross-presentation assay shown in Figure 4B might be inappropriate. They are comparing a seemingly differentiated and homogenous population, the CD103+ DCs, to a mixed population of progenitors that ultimately gives rise to both SIRPα positive and negative cell types. Instead, the investigators should compare either to a later time point of the same terminally

differentiated CD8+ equivelents, or use in vivo CD8 cDC equivalent cells. As it is, they are comparing an in vitro-derived cross-presenting capable cells to heterogeneous and immature populations. The efficiency of the XP by the CD103 cells may appear due to its homogeneity, whereas the other cells being compared are mixed.

4. The authors attempt to clarify the order in which Id2, Irf8 and Batf3 act during dendritic cell development. This is largely based on descriptive work using knockout mice without any substantive molecular insights (Fig. 5). It is essentially interpreting epistasis from static knockout mice. Importantly, the authors have no expression data for Irf8 or Batf3 from Flt3-ligand cultures. They should perform qPCR of the 6 populations on day 5 and compare it to day 8, for example. This would greatly strengthen their correlative arguments. For example, the authors cannot claim that Id2 is upstream of Batf3, but rather only that Batf3 is not required for Id2 expression. Instead, it appears that Irf8 may be required for Id2 expression. But the suggested expression data would help clarify this, although only partially.

Minor Concerns:

Figure 1:
1. Frequency or absolute numbers of dendritic cells, NK cells, etc. should be included in the supplement to confirm the mutated allele faithfully recapitulates the expression pattern of the WT one.
2. Please clarify the cell type sorted in "D." Or indicate that's a mixed population that simply includes graded Id2 GFP levels

Figure 2:
1. Given the surprisingly non-selective expression profile of the internal reporter, the authors should attempt to correlate GFP levels with Id2 protein. This is especially important as Id2 acts at the protein level to titrate down E-box binding transcription factors. I would suggest that the authors perform WB for Id2 in sorted DC's and use GFP as a means to compare.
2. Please indicate the exact compartment being studied in "F" - legend indicates that it's both spleen and peritoneum. Be specific about the previous gating strategy to define monocytes and macrophages. Additionally, CD11b also marks neutrophils. Be more accurate about how these populations are being defined.

Figure 3:
1. The gating strategy for pre-cDC in "C" is inappropriate. The plot should include Flt3 by SIRPa, as pre-cDC's appear to have a smear of SIRPa.

Figure 4:
1. Histogram overlay legend doesn't make sense - what's the gray supposed to represent.
2. The markers and subsequent gating strategy to define cDC correlates in lower part of "A" is quite confusing - arrows indicate further gating but are actually the same population displayed with different markers. Instead, the authors should gate each population (clearly marked with a number) onto a plot of CD24 by SIRPa (which would allow comparison with literature) and CD45RA by SIRPa.
3. The the comparison between a terminally differentiated (and thus fully mature) cell type and a mixed population of proliferating progenitors is inappropriate for this assay. Importantly, to strength the authors claims that these in vitro cells are actually relevant correlates, the authors should be comparing the cultured cell with in vivo cDCs - splenic CD8a+ and/or CD103+ DCs from nodes.

Figure 5:
1. In A, the authors continually refer to SIRPa+ or - populations but don't display any FACS plots using this marker, which makes it hard to follow and compare against the previous figures. Worse, the reviewer is left to extrapolate almost blindly.
2. Additionally, it appears as though IRF8 may regulate Id2. The investigators could attempt to determine if this is actually true by possibly using retroviral transduction of the culture and monitoring GFP levels.
3. Please include a splenic FACS of Id2-GFP crossed to IRF8-KO. How similar is this to Batf3. This will help improve their argument that Irf8 is upstream of both Batf3 and Id2.
4. In D, please include a representative FACS plot - the survival argument critically depends on the

quality of this data.

Supplemental Figure 3:
1. The authors claim that population 2 and 5 are precursors to pDCs and CD8a+ equivalents, respectively, is greatly weakened by the sizable emergence of other cell types when these progenitors are plated. Clearly, there's mixed/uncommitted potential within these gates.

1st Revision - authors' response 02 April 2011

Response to Referees Remarks: Jackson et al.

Referee #1:
The paper by Jackson et al. reports on the construction of an Id2-GFP reporter system for analysis of dendritic cell (DC) subsets in vivo and in vitro. They found that Id2 is broadly expressed in all conventional DC (cDC) subsets but not in plasmacytoid DC (pDC). Highest Id2-GFP expression was found to CD103+ and CD8α+ subsets. Jackson et al. then went ahead to cross the Id2-GFP reporter mice with IRF-8 and Batf3 deficient mice, which lack specific DC subsets. IRF-8 deficiency caused loss of all cDC while Batf3 deficiency allowed development of Sirp-a-/CD8a equivalent DC which however showed impaired survival rates.
This is a nice and detailed study that adds very much to our understanding of DC development. The Id2-GFP reporter construct faithfully recapitulates Id2 expression in vivo and thus allows monitoring Id2 expression on the single cell level and the isolation of Id2-GFP-and Id2-GFP+ DC for further analysis. A major finding is the abundant Id2 expression in various cDC subsets and absence (or very low expression) of Id2 in pDC. The Id2-GFP knock-in mouse system also allowed monitoring Id2 expression in hematopoietic stem/progenitor cells, including common dendritic cell progenitors (CDP) and Pre cDC in spleen and bone marrow. Following-up on these findings the authors isolated Id2-GFP-and Id2-GFP+ DC subsets employing an in vitro Flt3 ligand culture system and studied their function in cross presentation assays. Length of text, references and number of figures are appropriate.

Major points:
It is not clear from the text on page 9, second paragraph, lines 3-5 and the respective Suppl. Fig. 3B, top panel that population 1 (pop.1) contains multipotent DC precursors. This requires rewording in text and further details in legend to Suppl. Fig. 3B.
We have indicated that the identity of population 1 is unclear and that we hypothesized that it contains multipotent DC precursors. We then tested this notion by *in vitro* sorting and indeed it gave rise to all the different DC subsets. This is stated on page 9, paragraph 2, lines 3-5.

Further, what is the message of the sentence "CD103-Id2-GFP+ CD45RA-Sirp-α+ DCs (pop. 4) only gave rise to cells of a similar phenotype ....." (page 9, second paragraph, lines 8-9 and page 10, lines 1-3)? What are these cells and what is their developmental potential?
This sentence has been modified to read “CD103-Id2-GFP+CD45RA-Sirp-α+ DCs (pop. 4) maintained their phenotype. These cells would be equivalent to CD4+ or DN DCs *in vivo* and as they do not adopt any alternate phenotypic markers would appear to be a terminally differentiated cell type. This is stated on page 10, end paragraph 1.
A schematic representation of a model on DC subset specification that integrates the data shown here would be helpful (e. g. in the Supplementary Materials section). The scheme should illustrate the hierarchy of the transcription factors studied here (see also text in first paragraph of Discussion section, page 14 and text on page 15, lines 7-10 and on page 17, second paragraph, lines 2-5).
We have developed a schematic model of DC subset specification that integrates our data and have provided this as Figure 8B and referenced this figure in the Discussion (p. 14, 15, 17).

Minor points:

(1) cDC in the Abstract, line 6 should be explained.
“cDC” has been modified to “conventional DCs” in the abstract. The abbreviation cDCs is outlined in the introduction.

(2) References Bachem et al., Jongbloed et al. and Poulin et al. need to be up-dated.
These 3 references have now been updated to include the full print page numbers as follows:

p. 34:Bachem A, Guttler S, Hartung E, Ebstein F, Schaefer M, Tannert A, Salama A, Movassaghi K, Opitz C, Mages HW, Henn V, Kloetzel PM, Gurka S, Kroczek RA(2010) Superior antigen cross-presentation and XCR1 expression define human CD11c+CD141+ cells as homologues of mouse CD8+ dendritic cells. *J Exp Med* **207**:1273-1281
p. 28: Jongbloed SL, Kassianos AJ, McDonald KJ, Clark GJ, Ju X, Angel CE, Chen CJ, Dunbar PR, Wadley RB, Jeet V, Vulink AJ, Hart DN, Radford KJ (2010) Human CD141+ (BDCA-3)+ dendritic cells (DCs) represent a unique myeloid DC subset thatcross-presents necrotic cell antigens. *J Exp Med* **207**:1247-1260
p.30: Poulin LF, Salio M, Griessinger E, Anjos-Afonso F, Craciun L, Chen JL, Keller AM, Joffre O, Zelenay S, Nye E, Le Moine A, Faure F, Donckier V, Sancho D, Cerundolo V, Bonnet D, Reis ESC (2010) Characterization of human DNGR-1+ BDCA3+ leukocytes as putative equivalents of mouse CD8{alpha}+ dendritic cells. *J Exp Med* **207**:1261-1271

(3) Fig.1 represents a description of the transgenic construct and shows data confirming that the construct works as expected. This figure can be moved to the Supplementary Material section. In Fig. 1A (i), (ii) and (iii) the representation of the non-translated region of exon 1 before the arrow, which indicates start and direction of translation, is inconsistent.
We would prefer that the description of the mouse remain in the main part of the figure if possible as we feel it is important for the reader to have direct access to this information given the mouse is likely to be a valuable tool for future immunological analyses. We have now amended the figure as suggested by the reviewer.

(4) There is no information on the LC, dotted line in red in Fig. 2D, lower right panel in the legend to Fig. 2. Please give details.
We have clarified the meaning of the red line by adding "In D, the fluorescenceintensity of Langerhans cells has been shown in red for comparison" to the legend (p. 32).

(5) Fig. 4A, top right panels, expression Bst2 and Siglec-H is not described in the legend to Fig. 4 and in the result section. What are the CD45RA^{int} Id2-GFP+/- cells?
CD45RAint Id2-GFP+/- cells refers to analyses of all cells that stain for CD45RA. This demonstrates that in the absence of Id2-GFP is is not possible to distinguish cDCs and immature pDCs. However, using Id2-GFP we were able to show that CD45RA^{int} Id2-GFP-DCs are immature pDCs expressing the pDC markers Bst-2 and Siglec-H.
We have added a description of the Bst-2 and Siglec-H on page 9 of results as follows: "(1) CD45RA- DCs; (2) CD45RA^{int} DCs that expressed the pDC markers Bst-2 and Siglec-H (Figure 4A);".
To clarify this we have added the following to the figure 4 legend: *Right panels*. CD11c+CD45RA+Id2-GFP-cells expressed markers of pDCs. Histograms show expression of Bst2 (upper right panel) and Siglec-H (lower right panel) of total CD45RA^{int} cells (grey shading), CD45RA^{int} Id2-GFP-immature pDCs (black line). The expression of markers for CD103-CD45RA ^{high} (mature) pDC are indicated in red.

In general the manuscript suffers from spelling errors, additional and missing words (e.g. page 12, line 12; page 14, lines 3 and 8). This should be corrected.
We have corrected these problems throughout the text.

***Referee #2**:*
The manuscript by Jackson et al. describes a series of experiments using a newly engineered Id2-GFP allele to delineate Id2 expression during differentiation of DC subsets in vivo. Moreover, the authors attempt to utilize their reporter allele to categorize DC subtypes in vitro in Flt3L cultures. They go on to identify non-redundant roles of transcription factors Irf8 and Batf3 for CD8+ and CD103+ DCs in their cultures, and interestingly show that GMCSF addition can rescue formation but not function of Batf3-deficient CD103+ DCs. Although a bit descriptive, the manuscript is very interesting and clearly written. The experiments are for the most part sound and convincingly support the conclusions made by the authors. The Id2-GFP allele appears to be a useful tool to further characterize DC heterogeneity and development in vivo. Furthermore, the Id2-GFP based in vitro DC subset analysis of Flt3L cultures may provide an instrumental foundation to work out molecular mechanisms controlling DC differentiation and diversification. However, prior to publication the authors should address the following concerns:

Major concerns:

1. The authors define 6 different DC populations by FACS using in vitro Flt3 cultures of Id2-GFP+ bone marrow progenitors (Fig. 4a). They subsequently concentrate on one of these populations, CD103+ DCs, and use an antigen cross-presentation assay to functionally characterize these cells (Fig. 4b). The authors should provide analogous functional data also for the other mature DC subtypes they have identified in their cultures to support the FACS based characterization.
We have quite extensively analysed the 6 different populations for a variety of functions including cross-presentation (in part presented in Figure 4), maturation (Supplementary Figure 4), cytokine production and gene array (not presented). This represents a large body of data in which more detailed analysis is currently being undertaken investigating the various aspects of the findings. It would be beyond the scope of the current manuscript and thus we have focused on the value of the Id2-GFP mouse and the identification of the CD103+ DCs.

2. The second part of the manuscript focuses for a large part on the in vitro generated CD103+ cells from Id2-GFP mice with or without deletion of Batf3 or Irf8 genes (Fig. 4a population 6, Fig. 5a, Fig. 6a). However, because all CD103+ DCs in the cultures appear to express uniform Id2-GFP levels the Id2 reporter did not add anything to identification or characterization of the in vitro generated CD103+ DCs. In other words, these cells could be identified solely by their CD103 expression and the Id2-GFP allele could be omitted. This is in my view a major weakness of the manuscript and the authors should convincingly explain the contribution of the Id2GFP allele to their studies on CD103+ DCs.
The Id2-GFP allele extends our understanding of DC in at least two significant ways. Firstly, it has not been previously possible to trace the expression of Id2 during differentiation of DCs and to be able to subsequently use these cells to perform functional studies. In the case of characterization of the *in vitro* CD103+ DC, the Id2GFP high expression that we observed in our cultures hinted that we had multiple Sirp-α -DC populations rather than the single subset that has previously been referred to and thus prompted an extensive search for markers of such subsets. Without such an allele, this subset would have remained undiscovered for a much greater period of time. A beauty of the finding subsequent to this, is that by back tracking and using a variety of markers we have verified molecularly it is possible to now extend the analyses of these cells in mice not expressing the $Id2^{gfp}$ reporter although the purity of cells is greatly enhanced by use of these mice. The second important feature is that it allows us to track CD103 cells in the absence of this surface marker. This is a very important feature given that our data demonstrates that CD103 is not necessarily a definitive marker of this DC subset. This is achieved as genetic deletion of CD103 (Id2 × $CD103^{-/-}$ mice) does not lead to loss of Id2-GFP^{high} cells (ie. the CD103 DCs) but the cross to Batf3 does result in the loss of these cells (Figure 6) allowing us to distinguish the presence or absence of particular DC subsets that cannot otherwise be discriminated.
To clarify this point we have modified the first sentence of the last paragraph to read: “In conclusion, we describe an approach that allowed the prospective identification ofindividual DC populations using the Id2-GFP reporter mouse but which can now bemore broadly applied to other mouse strains.”

Minor concerns:

1. The manuscript has many typos and consequently misplaced periods and commas.
We have corrected these problems throughout the text.

2. The neomycin selection cassette is still present in the engineered Id2 locus. This is unfortunate because it may interfere with the spatial-temporal expression regulation of the Id2 gene at levels which may be hard to detect by the qPCR experiments shown in Fig. 1d. The authors need to mention this somewhere.
The Neo cassette is still present down-stream of the Ires-GFP. We are aware that in a few occasions the Neo cassette has been shown to influence gene expression. However in this case the Neo resistance gene is at the opposite end of the ID2 gene to the endogenous promoter. More importantly we have demonstrated that the homozygous Id2-GFP mice are identical to C57BL/6 wild-type mice. This applies to cell numbers, cell distribution, normal phenotype of $Id2^{gfp/gfp}$ and $Id2^{gfp/+}$ mice (Results p. 7), and qPCR analysis to correlate GFP and ID2 expression (Figure 1). Furthermore, in data not shown we have performed extensive gene array analysis and quantitative PCR analysis of cells isolated from these mice and they do not have impaired expression of either Batf3 or IRF-8.

3. Fig. 2f: What is shown here, spleen or peritoneum? The legend is a bit confusing.
These cells are derived from peritoneal lavage. We have now modified the legend to clarify this point to read: (**F**) monocyte and macrophage (from peritoneal lavage) lineages.

4. Fig. 5a: Do Irf8 and/or Batf3 control transcription of CD103? Address experimentally.

This is an important and interesting question, but non-trivial as little is known about CD103 regulation with only a single study (Robinson et al., Immunology, 2001, 103:146-154) that showed that the *Itgae* promotor lacks lineage specific information. We are currently addressing this question in a separate study to understand in much greater detail the precise molecular regulation of CD103. This work, however, is beyond the scope of the current study. We can however, conclude that *Bat3* is not essential for CD103 expression as treatment with GM-CSF induces its expression even in the absence of *Batf3* (see Figure 7A).

5. Fig. 5a: There are no major differences visible in the FACS plots of day 5 (upper panel) and day 8 (lower panel) after differentiation. Why are both time points shown?
We agree with the referee that there appear to be no major differences in frequency of the different subsets between the days. It was important to show that the CD45RA+ Id2-GFP+ Sirp-α-precursor subset was still present in our Batf3$^{-/-}$cultures and not deleted as might be indicated by the original publication. Instead, the loss is selectively within the CD103+ DC subset.

6. Fig. 5a, 6a, 7a: Why do the authors show CD45RA instead of CD8a or Sirp-α ? This is somewhat confusing. CD8α can not be used in *in vitro* cultures as it is not expressed. The surrogate marker for CD8α DCs is Sirp-α. In our analyses of *in vitro* cultures, we have found that expression of Id2-GFP combined with CD45RA separates the CD103+ DCs from the CD8α-equivalent DCs. The use of Sirp-α alone does not distinguish these two subpopulations of DCs.

7. Fig. 5d: Original FACS plots should be shown.
We have now added representative profiles to Figure 6E (Figure 5 has now been renumbered and is Figure 6) and referenced this figure on p.12 in the text. The additional description has been added to the legend of Figure 5 as follows: (**E**) Histograms show representative profiles of Annexin V staining on day 8 in total CD11c+ DCs, Id2-GFP+ CD45RA ^{int} and Id2-GFP+ CD45RA- DC subsets derived from *Id2* ^{gfp} or *Id2*$^{gfp/gfp}$ *Batf3*$^{-/-}$ bone marrow.

8. Fig. 6a: Show gates and percentages in the upper FACS panel.
We have now shown the percentages in the upper FACS panels. We have clarified the gate for analyses in the legend to Figure 6a as "Profiles are gated on CD11c+ CD19NK1.1-CD3- cells."

***Referee #3**:*
In this study, Jackson et al. develop a novel reagent to monitor transcription of the Id2 locus, which is quite relevant as the transcriptional regulator is absolutely required for the proper development of multiple cell types within the hematopoietic compartment. They claim the resultant mouse's hematopoietic compartment is indistinguishable from wild-type controls (although only FACS plots are provided without any absolute numbers or frequencies reported). Importantly, the relative levels of GFP transcript correlate synonymously with Id2 transcript amounts, and this serves as the basis for further comparisons made about dendritic cell (DCs) subsets and Id2 levels.
The report begins by performing an extensive flow cytometric analysis of dendritic cells and their precursors in multiple lymphoid organs (Fig. 2). The authors conclude that Id2 is expressed in nearly all DCs, though the levels are quite variable. Not surprisingly, the highest levels of GFP (as measured by mean fluorescence intensity) appear in the cell types for which Id2 is absolutely required during development (e.g., CD8α+ conventional DCs, migratory CD103+ DCs and Langerhans cells). Intriguingly, in Fig. 3, Id2 expression is negligible in the immediate precursors, suggesting a role in the terminal steps of differentiation or maintenance. Next, the investigators attempt to define six distinct cell types from Flt3 ligand cultures, which are thought to recapitulate the development of steady-state dendritic cells (Fig. 4). The major advancement to the field from this part of the study is the identification of a CD103+ DC, which presents exogenous cell-associated antigens more efficiently than the other subsets from in vitro culture. The authors argue that the high levels of Id2 combined with superior cross-presenting potential and cell-surface markers is sufficient to make the identified cell type the in vitro correlate of the migratory CD103+ DCs.
The last part of the study, in Fig. 5-7, focuses on clarifying the stage at which Batf3 or Irf8 deficiency causes the blockade of DC development. The data in Fig. 5-7 suggests that Irf8, but not Batf3, may control the expression of Id2, leading the investigators to conclude that Irf8 and Id2 are upstream of Batf3. What's more, they detect increased apoptosis from Batf3-/-bone marrow Flt3-ligand cultures and thus conclude that Batf3 is important for the maintenance of CD8α+ equivalent DCs. Lastly, in Fig. 6-7, authors demonstrate that the a late addition of GM-CSF to Flt3-ligand cultures will enhance or induce the

expression of CD103, even in the absence of Batf3 although the emergent cells still appear to be deficient at cross-presentation.
Overall, the strength of the study rests on the identification of a CD103+ DC subset in vitro that may represent a correlate of the migratory population in vivo. However, the study greatly overstates the value of the data in concluding the identification of distinct developmental checkpoints. Another weakness stems from the lack of mechanistic or molecular data on Id2, Irf8 and Batf3 function.

Specific concerns:

1. In the abstract, the authors claim that the CD103+ DCs were the only DC able to cross-present cell-associated antigens constitutively. Strictly, this is only true in vitro, and unless direct comparisons with in vivo subsets are made, such claims only lead to confusion. Therefore, the investigators need either to state that it's in vitro or perform the comparison studies with in vivo equivalents. This can be fixed by simply modifying the text at appropriate places throughout.
We have modified the statement in the abstract to reflect that the constitutive cross-presentation was limited to CD103+ DCs *in vitro.* The abstract now reads "Notably, CD103+ DCs were the only DC able to constitutively cross-present cell-associated antigens *in vitro*."

2. The author's claim that population 5 (at day 5) is equivalent to CD8α+ DCs is inaccurate. By their own re-plating studies, it's seems that population 5 is a heterogeneous group that ultimately gives rise to both SIRPα positive and negative DCs.
We acknowledge this point and have modified the text to reflect this point. The original sentence read: "CD103-Id2-GFP+CD45RA-Sirp-α+ DCs (pop. 4) maintained their phenotype while CD103-Id2-GFP+CD45RA ^{int}Sirp-α-DCs (pop. 5) generated predominantly Sirp-α -DCs that expressed varying levels of CD103, and a smaller population of CD103-Sirp-α+ DCs." The words and punctuation underlined have been deleted from the sentence.

Importantly, the original observation that Flt3-ligand cultures could support the development of a CD8α DC equivalent was based upon CD24 by SIRPα. The absence of CD24 as a marker in this study makes it rather hard to compare the current data with what has been reported previously in the literature. If the authors were to stain using CD24, then more direct comparisons with the literature could be made. Moreover, the use of day 5 Flt3 ligand cultures is quite different that what's been reported in the literature, where day8-10 is more commonly seen to contain fully differentiated DC subsets. The author can fix this by simply not making this claim, or by showing the plots with CD24 as a marker. I don't this the claim is too important, since the author has much novel data which substantiates publication anyway.
We now provide data showing the expression of CD24 as Figure 4B to enable cross-correlation of marker expression within different subsets. The antibody clone has been added to the Methods: Flow cytometric staining (p. 21).
The wording has been modified on page 9 to read: "This analysis also showed that the Id2-GFP+CD45RA-population (pop. 4) DCs was Sirp-α+ while the Id2GFP+CD45RA ^{int} population (pop. 5) was predominantly Sirp-α -and these populations expressed distinct levels of CD24 consistent with the previously described *in vitro* phenotype of CD8α -DCs and CD8α+ DCs (Figure 4C) (Naik et al, 2005)."

3. The control for cross-presentation assay shown in Figure 4B might be inappropriate. They are comparing a seemingly differentiated and homogenous population, the CD103+ DCs, to a mixed population of progenitors that ultimately gives rise to both SIRPα positive and negative cell types. Instead, the investigators should compare either to a later time point of the same terminally differentiated CD8+ equivelents, or use in vivo CD8 cDC equivalent cells. As it is, they are comparing an in vitro-derived cross-presenting capable cells to heterogeneous and immature populations. The efficiency of the XP by the CD103 cells may appear due to its homogeneity, whereas the other cells being compared are mixed.
This is a good point raised by the referee. In our experiments we compared cross-presenting capacity at both day 5 (shown in Figure 4) and day 8 (not shown). We observed that the ability of the different populations to cross-present antigens was not different although the overall magnitude of this response was lower at day 8, perhaps because late cultures contain more mature cells that may limit their ability to take up antigens. We now provide the day 8 data as Supplementary Figure 5. The text now reads (p. 11) "In contrast, all DC populations were able to present exogenous OVAantigen to CD4+ T cells. Similar analysis of DC populations at day 8 of culture showed that CD103+Id2-GFP+CD45RA-DCs remained the only *in vitro* DC subset capable of cross-presenting cell-associated antigen (Figure S5)." This has necessitated renumbering of the supplementary figures.

4. The authors attempt to clarify the order in which Id2, Irf8 and Batf3 act during dendritic cell development. This is largely based on descriptive work using knockout mice without any substantive molecular insights (Fig. 5). It is essentially interpreting epistasis from static knockout mice. Importantly, the authors have no expression data for Irf8 or Batf3 from Flt3-ligand cultures. They should perform qPCR of the 6 populations on day 5 and compare it to day 8, for example. This would greatly strengthen their correlative arguments. For example, the authors cannot claim that Id2 is upstream of Batf3, but rather only that Batf3 is not required for Id2 expression. Instead, it appears that Irf8 may be required for Id2 expression. But the suggested expression data would help clarify this, although only partially.
We have now provided gene array and qPCR analysis of the different dendritic cell populations in new Figure 5. We agree that Batf3 is not required for Id2 expression as the reviewer suggests. This demonstrates that although Irf-8 looks like it could be necessary for aspects of Id2 expression, the restoration of Id2-GFP seen after the addition of GM-CSF to the culture (Figure 8) indicates that Irf-8 is not essential for Id2 expression and other stimuli can induce this transcription factor.
We have referenced Figure 5 as follows on p. 12 of the results: "First we analysed the expression pattern of the transcription factors Id2, Irf-8 and Batf3 in the different DC populations *in vitro* and *in vivo* (Figure 5). While this demonstrated differential expression of the transcription factors amongst different DC subsets, the *in vitro* (Id2GFP+CD45RA+) and *in vivo* CD8α+ and CD103+ DCs concordantly expressed high levels of *Id2*, *Irf-8* and *Batf3*."
Analysis of gene expression data was provided by Yifang Hu, Cynthia Liu, and Gordon K. Smyth (Bioinformatics Division, WEHI), thus these collaborators have now been added to the author list. Their attributions have also been added to the Authorship section "J.T.J., J.H., C.L. and G.K.S. analysed gene expression data;".

<u>Minor Concerns</u>: Figure 1: 1. Frequency or absolute numbers of dendritic cells, NK cells, etc. should be included in the supplement to confirm the mutated allele faithfully recapitulates the expression pattern of the WT one.
We have now added cell numbers for NK cells and DCs as follows (p. 7): (NK cells: C57BL/6, $2.3 \times 10^5 \pm 6.7 \times 10^4$/spleen; *Id2* ^{gfp}/+, $1.94 \times 10^5 \pm 4.2 \times 10^4$/spleen; *Id2* $^{gfp/gfp}$, $1.94 \times 10^5 \pm 1.9 \times 10^4$/spleen; total DCs: C57BL/6, $2.1\times 10^6 \pm 6.4\times 10^4$/spleen; *Id2* $^{gfp/gfp}$, $1.9 \times 10^6 \pm 8.3 \times 10^4$/spleen; *Id2* $^{gfp/gfp}$, $2.1 \times 10^6 \pm 5.8 \times 10^4$/spleen; and data not shown).

2. Please clarify the cell type sorted in "D." Or indicate that's a mixed population that simply includes graded Id2 GFP levels
We have modified the figure legend to clarify the cells sorted. The legend now reads
(p. 33, Figure 1) "(**D**) Quantitative PCR analysis for the indicated transcripts of live (PI-) mixed populations of cells from spleen, thymus and bone marrow purified on thebasis of their expression of Id2-GFP."

Figure 2:
1. Given the surprisingly non-selective expression profile of the internal reporter, the authors should attempt to correlate GFP levels with Id2 protein. This is especially important as Id2 acts at the protein level to titrate down E-box binding transcription factors. I would suggest that the authors perform WB for Id2 in sorted DC's and use GFP as a means to compare.
We agree that such an experiment would be ideal. A central motivation for generating the Id2-GFP reporter mouse is that no reagents are available that are able to detect or report on endogenous levels of Id2. Although 5 antibodies are commercially available, and we have generated 2 ourselves, none of these detect Id2 except when it is highly overexpressed in cells. Thus, it is unfortunately not possible to performs the experiment proposed by the referee.

2. Please indicate the exact compartment being studied in "F" -legend indicates that it's both spleen and peritoneum. Be specific about the previous gating strategy to define monocytes and macrophages. Additionally, CD11b also marks neutrophils. Be more accurate about how these populations are being defined.
We have amended this. Please see comment Referee 2, point 3.

Figure 3: 1. The gating strategy for pre-cDC in "C" is inappropriate. The plot should include Flt3 by SIRPa, as pre-cDC's appear to have a smear of SIRPa.
The Figure legend has been modified to expand the full staining regime which was exactly as described in Liu & Nussenzweig, 2010. We have also stained as described by Naik & Shortman using CD43 with similar results although this approach appears a little less clean.

The legend now reads "(**C**) Bone marrow and splenic DC progenitors were analysed by depletion of lineage expressing cells (CD19, NK1.1, CD3, Ter119) then stained for CD11c, Flt3, MHC II and Sirp-α and analyzed for the pre-cDC population by flow cytometry (Liu & Nussenzweig, 2010). Profiles show the gating strategy in which CD11c+MHCII-cells (region 1, R1) were then selected for expression of Flt3 and Sirp-α (region 2, R2)." We have also modified the figure to show the Flt3 gating.

Figure 4:
1. Histogram overlay legend doesn't make sense -what's the gray supposed to represent.
The legend to this part of the figure has been amended. Please see comment to Referee 1, point 5.
2. The markers and subsequent gating strategy to define cDC correlates in lower part of "A" is quite confusing -arrows indicate further gating but are actually the same population displayed with different markers. Instead, the authors should gate each population (clearly marked with a number) onto a plot of CD24 by SIRPa (which would allow comparison with literature) and CD45RA by SIRPa. We have modified Figure 4 to improve the clarity. We now provide data showing the relating CD24 expression to that of Sirp-α as Figure 4B to enable cross-correlation of marker expression within different subsets. The antibody clone has been added to the Methods: Flow cytometric staining (p. 21).
The wording has been modified on page 9 to read: "This analysis also showed that the Id2-GFP+ CD45RA-population (pop. 4) DCs was Sirp-α+ while the Id2GFP+CD45RA^{int} population (pop. 5) was predominantly Sirp-α -and these populations expressed distinct levels of CD24 consistent with the previously described *in vitro* phenotype of CD8α -DCs and CD8α+ DCs (Figure 4C) (Naik et al, 2005)."

3. The comparison between a terminally differentiated (and thus fully mature) cell type and a mixed population of proliferating progenitors is inappropriate for this assay. Importantly, to strength the authors claims that these in vitro cells are actually relevant correlates, the authors should be comparing the cultured cell with in vivo cDCs - splenic CD8a+ and/or CD103+ DCs from nodes.
Figure 5: 1. In A, the authors continually refer to SIRPa+ or -populations but don't display any FACS plots using this marker, which makes it hard to follow and compare against the previous figures. Worse, the reviewer is left to extrapolate almost blindly.
We agree that the terminology was not particularly clear and consistent and have modified the text to Id2-GFP+CD45RA^{int}Sirp-α - (two places on p. 12) to more accurately reflect the population referred to. The cross-correlation of Sirp-α expression in the different populations is also provided in Figure 4a and have modified this figure to improve the flow of gating.

2. Additionally, it appears as though IRF8 may regulate Id2. The investigators could attempt to determine if this is actually true by possibly using retroviral transduction of the culture and monitoring GFP levels.
This is an interesting point raised by the referee. We agree that there may be interactions between Irf-8 and Id2 and intend to pursue this in further studies as the experiments are non-trivial and beyond the scope of the current work. This is in part due to the fact that in the absence of Irf-8 the target cells do not appear to develop. However, Figure 6a shows that some Id2 expression occurs even in the absence of Irf8 and that this is amplified significantly in the presence of GM-CSF. Thus, although expression of Id2 may be regulated by Irf-8 to some extent in the presence of Flt3L, Irf-8 does not appear to be an absolute requirement for the induction of Id2.

3. Please include a splenic FACS of Id2-GFP crossed to IRF8-KO. How similar is this to Batf3. This will help improve their argument that Irf8 is upstream of both Batf3 and Id2.
We have now included this data in Supplementary Figure 6. This data shows nicely that Irf-8 is required for CD8+ DC and CD103+ DC, while in the absence of Batf3 these populations are formed but appear to be blocked in further maturation.

4. In D, please include a representative FACS plot -the survival argument critically depends on the quality of this data.
We have now included this data in Figure 6.

Supplemental Figure 3: 1. The authors claim that population 2 and 5 are precursors to pDCs and CD8a+ equivalents, respectively, is greatly weakened by the sizable emergence of other cell types when these progenitors are plated. Clearly, there's mixed/uncommitted potential within these gates.
We have modified the text to reflect this point.

2nd Editorial Decision 26 April 2011

Thank you for submitting your revised manuscript to the EMBO Journal. It has now been re-reviewed by the original referees and their comments are provided below. As you can see the referees appreciate the revisions and support publication here. Referee #2 has a few remaining issues. After further discussions with one of the other referees, I have come to the conclusion that only the last two issues need to be addressed in a revised version: 1) to better explain why you used CD45RA instead of CD8a (either in the point -by-response or in the text) and 2) to add standard deviations for the new figure 5. Once these issues have been resolved we will accept the paper for publication here.

Thank you for submitting your interesting study to the EMBO Journal!

Best wishes

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1:

The authors addressed the points made.

Referee #2:

The revised version of the manuscript by Jackson et al. is much improved. However, the authors have not addressed my following critique points adequately:

"The second part of the manuscript focuses for a large part on the in vitro generated CD103+ cells from Id2-GFP mice with or without deletion of Batf3 or Irf8 genes (Fig. 4a population 6, Fig. 5a, Fig. 6a). However, because all CD103+ DCs in the cultures appear to express uniform Id2-GFP levels the Id2 reporter did not add anything to identification or characterization of the in vitro generated CD103+ DCs. In other words, these cells could be identified solely by their CD103 expression and the Id2-GFP allele could be omitted. This is in my view a major weakness of the manuscript and the authors should convincingly explain the contribution of the Id2-GFP allele to their studies on CD103+ DCs."
I understand that the Id2-GFP allele is nice to delineate the in vivo expression of Id2 and helps to define DC populations. But I still do not see the benefit of the reporter for the CD103 population at least in the wildtype background (in figures 4b (bottom left), 7a (bottom panel) and 8a (bottom panel) all CD103+ cells are uniformly positive for GFP).

"Fig. 5a: Do Irf8 and/or Batf3 control transcription of CD103? Address experimentally."
This point has still not been adequately addressed. The authors should show experimental data because it is important for their conclusions (Figures 6-8) that CD103 expression is not transcriptionally dependent on IRF8 or BATF3. I have two experimental suggestions:
a. The authors should transduce cells with an Irf8 and Batf3 expressing retrovirus and check CD103 expression afterwards.
b. Are there consensus binding motifs for these factors in the upstream region of the CD103 gene? If so, the authors should perform ChIP experiments to evaluate whether IRF8 or BATF3 indeed occupy these sites.
I am aware that both experiments are not fully conclusive but at least are attempts to evaluate whether CD103 may be a transcriptional target of IRF8 and/or BATF3 or not.

"Fig. 5a: There are no major differences visible in the FACS plots of day 5 (upper panel) and day 8 (lower panel) after differentiation. Why are both time points shown?"
The authors should put the day 8 FACS plots in the suppl. material.

"Fig. 5a, 6a, 7a: Why do the authors show CD45RA instead of CD8a or Sirp-a? This is somewhat

confusing."
The authors should modify the text to better explain this point.

Additional critique point:

New figure 5: Standard deviations should be shown.

Referee #3:

I am satisfied with the revisions made by the authors to my comments in the first review. I am fine with the EMBO to now publish the paper.

2nd Revision - authors' response 27 April 2011

Response to Referees Remarks: Jackson et al.

Points to be addressed:

(1) To better explain why you used CD45RA instead of CD8a (either in the point -by-response or in the text).

We appreciated that the markers used for subsetting DCs can be quite confusing. In order to clarify not using CD8α universally to analyse the *in vitro* and *in vivo* DCs we have added the following sentences in the text (p. 9) "It should be noted that although the surface molecule CD8α is expressed on DCs isolated directly *ex vivo*, this marker is not expressed on *in vitro*-derived CD8α-equivalent DCs. These cells have been previously identified by their lack of expression of Sirp-α, and as shown below, intermediate expression of CD45RA."

We have presented our profiles displaying CD45RA to allow us to show the expression differences between the conventional DCs (Id2-GFP$^+$) and pDCs (Id2-GFP$^-$) cells. Our data also show the novel finding that precursor CD8α DCs express CD45RA at an intermediate level allowing them to be delineated from CD103$^+$ DCs that do not express this marker. All our DC analyses have been stained for Sirp-α and both CD8α-equivalent DCs and CD103 DCs lack expression of this surface molecule. Thus, it has not proven useful in delineating between these two different DC subsets.

(2) To add standard deviations for the new figure 5.

Standard deviations have now been added to the revised version of new figure 5. This has been added to the legend of Figure 5 (p. 36) "Data show the mean and s.d. relative to HPRT of two biological replicates each assayed in triplicate for each DC population."

