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Host CD8 α ⁺ and CD103⁺ dendritic cells prime transplant antigen-specific CD8⁺ T cells via cross-dressing

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Abstract

The participation of dendritic cells (DCs) in CD8⁺ T cell mediated allograft rejection is a long-standing question of great clinical relevance for tissue transplantation. Here, we show that Batf3^{-/-} mice demonstrate delayed allo-skin graft rejection and are deficient in priming allo-specific CD8⁺ T cells. Batf3^{-/-} mouse priming is restored by transferring either purified CD8α⁺ or CD103⁺ DCs, demonstrating the critical role of these cells in alloreactivity. Using D^b-restricted anti-viral F5 transgenic TCR T cells, which we demonstrate are alloreactive with H-2K^d, we show that cross-dressing of CD8α⁺ and CD103⁺ primes CD8⁺ T cell or an allo-specific responses *in vitro* and *in vivo*. These findings suggest novel strategies for moderating tissue rejection based on interfering with DC cross-dressing or subsequent interaction with T cells.

INTRODUCTION

One of the major challenges faced by transplantation medicine is organ rejection caused by host T cells against donor's allogeneic (allo) MHC molecules. There are three types of alloresponses: direct alloresponse refers to host allo-specific T cells directly recognizing donor MHC-peptide complexes displayed on donor-derived antigen-presenting cells (APCs); indirect alloresponse refers to these T cells activated by host APCs that present antigenic peptides derived from the allo MHC via cross-presentation; and semi-direct alloresponse refers to the intermediate situation in which host APCs capture donor derived MHC-peptide complexes and subsequently use these molecules to activate host T cells (also referred as “cross-dressing”) ^{1,2}.

Although many virus-specific T cell clones were reported to be able to cross-react to allo HLA molecules ³, indicating donor-derived nonhemopoietic cells might be able to “initiate” an alloresponse, mouse model derived evidence demonstrated that DCs are required to prime host allo-specific T cells ². In fact, both host and donor derived DCs have been implicated in allo-priming although it is still debated which might play a bigger role. For example, using allo heart transplant mouse models, Garrod *et al.* showed that graft-derived DCs interact with host NK cells and are quickly eliminated with little role to play for allo T cell priming ⁴. Furthermore, Smyth *et al.* were not able to identify donor derived DCs in the draining lymph nodes in a skin transplantation model ⁵. Zhang *et al.* recently showed that host DCs infiltrating heart grafts are responsible for priming alloresponses ⁶. Therefore, the roles of host-derived DCs have been emphasized. However, it remains unclear whether this reflects the mechanism in other solid organ transplant scenarios. More importantly, it remains unclear which DC subtypes are able to initiate an alloresponse. In mouse models where the virus does not infect DCs directly or if the tumor antigens are not expressed by DCs endogenously, CD8⁺ T cell responses require cross-priming by CD8α⁺ and/or CD103⁺ DCs ⁷⁻⁹.

66 However, it has not been demonstrated that this is also the case during either indirect or semi-direct
67 alloresponse induction.

68 Batf3 is a transcription factor only expressed in the hematopoietic cell lineage. Batf3
69 deficiency leads to significantly reduced resident CD8 α^+ DC subset in the lymphoid organs and
70 migratory CD103 $^+$ DCs in the non-lymphoid organs¹⁰. The lack of CD8 α^+ and CD103 $^+$ DCs in
71 Batf3 deficient (Batf3 $^{-/-}$) mice has been linked to significantly reduced cellular immunity to various
72 viruses¹¹⁻¹³, bacteria¹⁴ anti-tumor immunity^{11, 15, 16}, and allo response to OVA-expressing skin
73 grafts although immunity to minor antigens seemed to be intact^{17, 18}.

74 In this study, using Batf3 $^{-/-}$ mice we demonstrate for the first time that induction of
75 alloresponses in naïve mice requires host CD8 α^+ and CD103 $^+$ DC subsets. Furthermore, we show
76 also for the first time that these DCs activate allo-specific CD8 $^+$ T cells via cross-dressing of
77 naturally derived allo MHC-peptide complexes. The implications of this observation are discussed.

78 RESULTS

79 *Batf3 KO mice lack alloresponse*

80 We previously investigated antigen cross priming by immunizing B6 mice (H-2 b)
81 intraperitoneally with irradiated allo cells (H-2 d) expressing the highly immunogenic simian virus
82 40 (SV40) large T antigen (D2.SV cells), which immortalizes the cells. Infecting cells with
83 influenza A virus to generate an innate immune signal enhanced priming of CD8 $^+$ T cell responses
84 to the T-Ag404/K b epitope¹⁹. We routinely monitored CD8 $^+$ T cell alloresponses in these
85 experiments, finding they were not modulated by innate immune activation in the SV40
86 transformed cells as an indicator of the normal cellular immunity.

87 Returning to this model system, we found Batf3 $^{-/-}$ mice fail to generate an allo response to
88 IAV infected D2.SV cells 7-d post *i.p.* immunization as measured by intracellular cytokine staining
89 (ICS) for IFN- γ in splenic and peritoneal CD8 $^+$ T cells stimulated with D2.SV cells *in vitro* (Fig 1),
90 consistent with a requirement for CD8 α^+ or CD103 $^+$ DCs in cross-priming¹¹. Notably, IAV specific
91 response were modestly reduced as measured using immunodominant viral peptides to activate
92 CD8 $^+$ T cells *in vitro* probably due to reduced cross-priming¹².

93 We next extended these experiments to additional cell lines P815 (H-2 d), LTA-5 (H-2 k),
94 SS.SV (H-2 s) as well as irradiated DC-depleted BALB/c or C3H splenocytes. Seven days after *i.p.*
95 immunization, we tested peritoneal washout cells (PWCs) and splenic cells for CD8 $^+$ T cell
96 alloreactivity using a panel of allo cell lines and ICS. While B6 mice mounted strong alloresponses
97 to all the cells tested, Batf3 $^{-/-}$ mice failed to respond to any of the allo cell lines (Figure 2a-d). As a
98 negative control neither B6 nor Batf3 $^{-/-}$ mice responded to syngeneic cell line EL-4 or B6, or Batf3 $^{-/-}$
99 splenic cells (Figure 2e). Splenic T cells gave similar results (Supplementary figure 1). Interestingly,

100 Batf3^{-/-} CD8⁺ T cells do respond to tumor antigens expressed by MC57G cells, albeit at ~20% of the
101 levels of B6 mice (Figure 2e).

102 Based on these data, we conclude that Batf3^{-/-} mice are deficient in mounting CD8⁺ alloresponses
103 after *i.p.* challenge.

104

105 ***Lack of alloresponse in Batf3^{-/-} mice is not due to altered T cell repertoire***

106 As CD8 α ⁺ DCs participate in T cell thymic education ²⁰, it was important to show that Batf3^{-/-}
107 ^{-/-} mice possess a TCR repertoire capable of mounting an alloreactive CD8⁺ T cell response. After
108 transferring FACS-sorted splenic T cells from Batf3^{-/-} mice (Ly5.2) into Ly5.1 B6 mice, we
109 immunized with irradiated DC-depleted BALB/c splenic cells, and assessed donor (CD45.2⁺) and
110 host (CD45.1⁺) CD8⁺ T cell allo-responses.

111 Although transferred Batf3^{-/-} T cells constituted only ~10% of CD8⁺ T cells, they mounted a
112 proportional alloresponse to the BALB/c splenic cells (Figure 3a). A complementary experiment
113 revealed that purified Ly5.1 B6 T cells do not mount alloresponses following their transfer into
114 Ly5.2 Batf3^{-/-} (Figure 3b).

115 These results clearly indicate the lack of CD8⁺ T cell alloresponsiveness in Batf3^{-/-} mice
116 cannot be attributed to deficiency in their T cell repertoire.

117

118 ***Batf3^{-/-} mouse deficient alloreactivity is due to lack of CD8 α ⁺ and CD103⁺ DCs***

119 We next directly addressed the ability of Batf3-dependent DCs to prime for allo-CD8⁺ T cell
120 responses. We injected Batf3^{-/-} mice *i.p.* with 5 X 10⁶ irradiated DC-depleted BALB/c splenic cells
121 alone or with 10⁶ FACS-sorted CD8 α ⁺, or CD103⁺, or both CD8 α ⁺ and CD103⁺ non-irradiated DCs
122 and measured allo responses as above. The inability of Batf3^{-/-} mice to mount an alloresponse was
123 remedied by transferring either CD8 α ⁺ or CD103⁺ DCs, with responses reaching ~50% of those of
124 B6 mice. When an equal number of CD8 α ⁺ and CD103⁺ DCs were co-transferred, Batf3^{-/-} mice
125 generated an alloresponse of similar magnitude as B6 mice (Figure 4a).

126 As we injected DCs and allo cells together in this experiment, we were concerned that the
127 close contact might have facilitated allo priming artificially. We therefore repeated the experiment
128 but waited 24 h after *i.v.* DC transfer to *i.p.* challenge with irradiated D2.SV or P815 cells. This
129 gave results very similar to results to the simultaneous transfer experiments, with mixing CD8 α ⁺
130 and CD103⁺ DCs giving an additive alloresponse (Figure 4b). Importantly, alloresponses were not
131 detected in the CD8 α ⁺CD103⁻ DC transferred group included in this experiment. Extending these
132 results to *in vitro* mixed lymphocyte responses (MLRs) we co-cultured CFSE labelled purified
133 splenic T cell populations from either B6 or Batf3^{-/-} mice with irradiated D2.SV cells with or

without DCs. Addition of either CD8 α ⁺ or CD103⁺ DC was absolutely required for T cell proliferation after 4 days of co-culture (Figure 4c).

Taken together, our findings clearly show that CD8 α ⁺ and/or CD103⁺ DCs (but not CD8 α ⁻ CD103⁻ DCs) are required to initiate an allo-specific CD8⁺ T cell response in Batf3^{-/-} mice.

Batf3^{-/-} mice show significantly delayed skin rejection

How do our findings relate to organ transplantation? First, we transplanted skin pieces from either B6 or Batf3^{-/-} mice onto both B6 and Batf3^{-/-} mice. All skin grafts were accepted with no sign of rejection, indicating that knocking out Batf3 does not affect self-tolerance. We then transplanted BALB/c skin grafts onto both B6 and Batf3^{-/-} mice. As an internal control, we transplanted self-skin on opposite flank of each mouse. Day 6 post-transplantation we began scoring graft rejection as described ²¹. BALB/c grafts were all rejected by the B6 mice by the 8th day post grafting, with the control self-grafts on the opposite flanks showing no sign of rejection. By contrast BALB/c grafts were intact on Batf3^{-/-} mice at day 8 with only slight inflammation (redness) compared to the self-graft on the opposite flank. Eventually, however, all Batf3^{-/-} mice rejected their allografts within the next 7 days (Figure 5a).

To better understand graft induced Batf3^{-/-} mouse alloresponses, we examined the alloreactivity of CD8⁺ T cells obtained from spleen and draining lymph nodes (DLNs) 8 days after skin transplant. B6 CD8⁺ T cells recovered from the draining lymph nodes and the spleens showed clear alloreactivity to allo APCs, while T cells recovered LN draining the self-graft side did not. T cells recovered from the Batf3^{-/-} mice showed minimal alloreactivity (Figure 5b). By 14-day post-transplant, B6 mice had very high allo-specific T cells in all LNs, including control non-draining LNs. Alloresponsive T cells were now present in draining and non-draining LN in Batf3^{-/-} mice with the percentages in non-draining nodes much lower than those detected in B6 mice (Figure 5c).

What accounts for the partial defect in Batf3^{-/-} mouse alloresponsiveness? In the original description, depletion of DC subsets varied among secondary lymph tissues in Batf3^{-/-} mice ¹¹. We confirmed Batf3^{-/-} mice exhibit a greater overall deficiency in CD103⁺ vs. CD8 α ⁺ DCs. CD8 α ⁺ DCs are detected at normal levels in the inguinal and axillary LNs, with decreases in all other LNs and tissues tested, particularly the spleen (Supplementary figure 2a). CD103⁺ DCs are decreased in the spleen, more so in the facial, axillary and kidney LNs, and reduced to residue level in mediastinal, mesenteric and inguinal LNs. lung and liver (Supplementary figure 2b). Thus, we confirm that, as reported ¹¹, the degree of CD8 α ⁺ and CD103⁺ DC deficiency is location-dependent, which is likely to account for the partial CD8⁺ T cell alloresponsive phenotype we observe.

Recipient CD8 α ⁺ and CD103⁺ DCs prime CD8⁺ alloresponse via cross-dressing

169 The most likely mechanisms for CD8 α^+ and CD103 $^+$ DC to activate allo CD8 $^+$ T cells are
170 via cross-dressing and cross-presentation. To show DC cross-dressing allo MHC, we co-cultured
171 DC with allo P815 cells (H-2 d) *in vitro* for 12 or 24hrs, followed by allo-MHC antibody-based
172 FACS or confocal readout. We were easily able to demonstrate H-2K d molecule cross-dressing as
173 these molecules were stained by FITC-conjugated allo antibody on DCs stained by PE-conjugated
174 anti- H-2K b (Supplementary figure 3). However, when we isolated DCs from either spleens or DLN
175 various times after allo cell immunization, we were not able to detect cross-dressing *ex vivo* using
176 similar FACS or confocal readout.

177 We therefore employed a different strategy that leverages our serendipitous finding that TCR
178 transgenic F5 CD8 $^+$ T cells, specific for H-2D b -complexed with an IAV peptide cross-reacts to
179 P815 cells in an H-2K d -restricted fashion (as shown by blocking with an anti-H-2K d but not anti-H-
180 2L d mAbs) (Figure 6a and b). Informed that the H-2 d alloreactivity of the H-2 b T cell clone 2C is
181 based on recognizing a single peptide complexed with H-2L d ²², we tested HPLC fractionated P815
182 derived-peptides for their ability to sensitize K d -expressing human TAP deficient cells for F5
183 activation. Indeed, F5 T cells proliferated when APCs were pulsed with HPLC fractions eluted
184 between 6.1 and 6.3 minute (Figure 6c). These data indicate that like clone 2C, F5 allorecognition is
185 based on cross-reaction of an allo-MHC molecule (K d), complexed with a specific transplant
186 derived peptide, and therefore can be used as a sensitive measure of cross-dressing.

187 To this end we primed B6 and Batf3 $^{-/-}$ mice with irradiated P815 cells *i.p* and *i.v.* transferred
188 CFSE-labeled F5-Ly5.1 T cells. Seven days later we determined the proliferation status of splenic
189 F5 cells by flow cytometry. While up to 8% of F5 cells proliferated in primed B6 mice, priming in
190 Batf3 $^{-/-}$ mice was close to background levels observed in unprimed B6 mice (Figure 7a). In this
191 experiment we also examined F5 activation in TAP $^{-/-}$ mice to exclude the unlikely possibility that
192 priming might be based on cross-presentation as almost all cross-presentations require TAP
193 function. As expected, F5 cells were activated in the absence of TAP, indicating again cross-
194 dressing as the mechanism which was known not to require TAP function ²³ (Figure 7a).

195 To directly demonstrate cross-dressed APCs in allo recipients, we incubated CFSE labelled
196 F5 cells with splenocytes recovered 2 days after allo-priming *i.p.* with P815 cells. After 4 d in
197 culture we measured F5 activation by CFSE dilution (Figure 7b). Batf3 $^{-/-}$ APCs showed limited
198 ability to activate F5 cells relative to B6 APCs, while APC from TAP $^{-/-}$ mice behaved like B6 APC.
199 We repeated this experiment but used purified splenic CD8 α^+ DCs (there are very few CD103 $^+$ DCs
200 in the spleen) as APCs. This showed that CD8 α^+ DCs from each of the mice were able to activate
201 F5 cells equally, consistent with the conclusion that the partial effect of the Batf3 knockout is due to
202 a partial effect on depleting CD8 α^+ DCs (and by inference also CD103 $^+$ DCs). Hammering this

203 point home, we found that depleting CD8 α^+ DCs from B6, Batf3 $^{-/-}$ and TAP $^{-/-}$ splenocytes
 204 completely abrogated F5 activation (Figure 7c).

205 Taken together, our data strongly support the conclusion that cross-dressing of CD8 α^+ and
 206 CD103 $^+$ DCs accounts for the CD8 $^+$ T cell alloresponse after *i.p.* challenge with allogeneic cells and
 207 likely after skin grafting as well.

208 DISCUSSION

209 We have demonstrated that the reduced numbers of CD8 α ⁺ and CD103⁺ DCs in Batf3^{-/-}
210 mice greatly impacts CD8⁺ T cell alloresponses, and that this effect is partially mitigated by
211 transferring CD8 α ⁺ or CD103⁺ DCs into Batf3^{-/-} mice and fully repaired by transferring a mixture of
212 CD8 α ⁺ and CD103⁺ DCs. We recapitulated this DC requirement in an *in vitro* T cell proliferation
213 assay, showing that when co-cultured with allo cells, CD8 α ⁺ or CD103⁺ DCs activate naive allo
214 specific CD8⁺ T cells (Figure 4c). Taking advantage of our finding that F5 TCR transgenic T cells,
215 nominally specific for D^b complexed with an IAV peptide, also recognize allo H-2K^d-peptide
216 complexes, we demonstrate that cross-dressing of CD8 α ⁺ DCs, and likely CD103⁺ DCs can activate
217 allo specific CD8⁺ T cells *in vitro* and *in vivo* (Figure 7).

218 Batf3^{-/-} mice have been used to demonstrate the importance of CD8 α ⁺ and CD103⁺ DCs in
219 CD8⁺ T cell priming to viruses and tumors in the central nervous system ¹¹, lung ¹², intestine ²⁴, and
220 systemically ¹³. Interestingly, impairment of minor H responses in Batf3^{-/-} mice is still debated, with
221 varied results depending on the laboratory likely based on the model antigen studied. Based on our
222 findings, it seems likely the partial reduction of CD8 α ⁺ and CD103⁺ DCs in an organ specific
223 manner is a major contributor to this variability.

224 Our studies were prompted by the limited data available regarding the mechanism of
225 priming of allospecific CD8⁺ T cells. In the original description of Batf3^{-/-} mice, normal allo
226 reactivity was reported based on *in vitro* MLRs ¹¹. Atif *et al.* reported that Batf3^{-/-} mice rejected
227 allogeneic CD8⁺ T cells based on the loss of transferred cells ¹⁷. Smyth *et al.* showed that recipient
228 DCs are cross-dressed with minor H or allo graft derived MHC-peptide complexes but did not link
229 this finding to *in vivo* alloresponses ⁵.

230 By using various allo cell lines and DC-depleted allo splenic cells with Batf3^{-/-} mice we
231 demonstrate that CD8 α ⁺ and CD103⁺ DCs are required for priming allo-specific CD8⁺ T cells,
232 providing the first direct demonstration of a central role for CD8 α ⁺ and CD103⁺ DCs, in allo-
233 specific CD8⁺ T cell activation. While our data demonstrate that CD103⁺ DCs are capable of
234 priming allo CD8⁺ T cells, we have not established where such priming occurs, an important issue
235 given the paucity of CD103⁺ cells in LNs. Intriguingly, CD103⁺ DCs were recently reported to
236 prime virus-specific CD8⁺ T cell response in the liver ²⁵. Furthermore, we could not formally
237 exclude a possible migration defect in the “migratory” CD103⁺ DCs by purifying now very rare
238 CD103⁺ DCs from the Batf3^{-/-} mice and transfer them back into the naïve knockout mice to see
239 whether the allo responses are restored to the same degree as those in the knockout mice receiving
240 CD103⁺ DCs from B6 mice.

241 Cross-dressing of APC by MHC-peptide complexes derived from other cells has gained
242 significant attention in the recent years. The contribution of transferred preformed MHC class II or

class I complexes to cross-priming between cells was first proposed by Simth and Fazekas de St Groth²⁶ and subsequently dubbed “cross-dressing” by Yewdell and Haeryfar²⁷. The *in vivo* relevance of cross-dressing was initially demonstrated by Dolan *et al.* for class II-tumor peptides and then for K^b-SIINFEKL complexes^{28, 29}, and later shown to contribute to virus-²³ and allo⁵-specific T cell priming. Li *et al.* compare cleverly constructed single-chain H-2K^b-SIINYEKL and OVA-SIINYEKL DNA vaccines. As SIINYEKL has low affinity for H-2K^b, OVA-SIINYEKL failed to prime T cell via traditional cross-presentation; but as part of the single chain construct, it was able to prime T cells via cross-dressing. Most relevant to our findings, Li *et al.* were the first to link CD8 α ⁺ and CD103⁺ DCs to cross-dressing capable host DCs, as this activity was significantly reduced in Batf3^{-/-} mice³⁰. Our finding now demonstrate that these two DC subsets mediate allo-specific CD8⁺ T cell priming largely via cross-dressing.

Consistent with prior reports¹¹, we found that Batf3^{-/-} mice contain substantial numbers of CD8 α ⁺ and CD103⁺ DCs (Supplementary figure 2). How can this partial defect result in such a profound defect in T cell priming to *i.p.* introduced allo cells? First, by happy coincidence, CD8 α ⁺ and CD103⁺ DC numbers are lowest in Batf3^{-/-} mesenteric LN, which drain the peritoneum. Second, it is possible that it is relatively difficult for DCs to encounter allo cells for cross-dressing since direct contact is required, and that Batf3^{-/-} mice have a sufficient decrease in DC numbers to compromise priming. Third, residual CD8 α ⁺ DCs in Batf3^{-/-} mice have been recently reported to be functionally impaired, particularly in endocytic/phagocytic function¹⁸. We can probably rule out this last possibility, however, as we show (Figure 7c) that on a per cell basis, B6 and Batf3^{-/-} CD8 α ⁺ DCs function equivalently in cross-dressing for allo presentation.

Batf3^{-/-} mice have been recently reported to have significantly reduced cross-dressing capacity in the context of class I- β_2 -peptide single chain-based DNA vaccination³⁰, consistent with our findings. The mechanistic details of how cross-dressed APCs activate naïve CD8⁺ T cells *in vivo* are an important area of future research. A number of scenarios are possible in skin graft rejection. CD103⁺ DCs might enter grafts to cross-dress and then migrate to LN to activate CD8⁺ T cells directly or transfer allo class I molecules to CD8 α ⁺ DCs (cross-dressing) which then prime CD8⁺ T cells. Alternatively, graft Langerhans cells might migrate to DLN and cross-dress CD8 α ⁺ DCs. LCs are reported to transfer antigens to the resident CD8 α ⁺ DCs³¹. In the case of peritoneal allo-priming, an unusual cross-presenting CD8 α and CD103 double positive DC (CD8 α ⁺CD103⁺ DC) subset reported to reside in the intestinal lamina propria and mesenteric DLN may be relevant^{32, 33}. In our *in vivo* cross-dressing experiments, although we failed to identify CD8 α ⁺CD103⁺ DC *ex vivo*, we detected many CD103⁺ DCs in the peritoneum, suggesting an important role in priming.

A very recent preclinical study has demonstrated cross-dressing directly in organ transplantation models using the famous model antigen and TCR transgenic T cell system, i.e.

OVA₂₅₇₋₂₆₄ (SIINFEKL) and OT-I TCR T cells. The study showed convincingly that CD11c⁺ DCs in the grafts cross-dressed H-2K^b/OVA₂₅₇₋₂₆₄ complexes using both advanced imaging technique as well as functional OT-I T cell activation ³⁴. However, in our study, we have demonstrated such cross-dressing capacity mainly lies with the Batf3-dependent CD8 α ⁺ and CD103⁺ DC subsets. Further, we are the first to demonstrate naturally occurring allo MHC-peptide complexes being cross-dressed *in vivo* using F5 T cells that happen to cross-react with probably a single H-2K^d/peptide complex.

In summary, we demonstrate a clear dependence of allo CD8⁺ T cell priming on CD8 α ⁺ and CD103⁺ DC subsets. In the absence of donor derived DCs, the CD8 α ⁺ and CD103⁺ DC subsets prime allo CD8⁺ T cells mainly via an MHC-peptide complex cross-dressing mechanism. It might therefore be possible to reduce allo transplant rejection through manipulating the cross-dressing DC subsets, for example target them to express immune check-point molecules, such as PD-L1 to enable immunotolerance induction ³⁵. Alternatively, as the molecular mechanisms for cross-dressing are revealed in the coming years, it might be possible to specifically modulate cross-dressing to rationally control T cell priming.

METHODS

Mice

SPF female BALB/c, C3H, C57BL/6 (B6) (Ly5.2) and B6-Ly5.1 mice (~6-week-old) were purchased from the Walter Eliza Hall Institute of Medical Research (Melbourne, Australia). B6 Batf3^{-/-} mice were kindly provided by Dr Kenneth M. Murphy (Washington University, St. Louis, USA). B6 TAP^{-/-} mice ³⁶ were kindly provided by Dr Luc Van Kaer (Vanderbilt University, Nashville, USA). B6 F5 TCR Tg mice was provided by Dr Gunasegaran Karupiah (University of Tasmania) and originally generated by Dr Mamalaki *et al.* ³⁷. We generally use the MHC-II-depleted CFSE-labelled F5 T cells either in *in vivo* or *in vitro* proliferative experiments.

All animal experiments were approved by the La Trobe University Animal Ethics Committee in accordance with the National Health and Medical Research Council Australia code of practice for the care and use of animals for scientific purposes.

Cell culture and virus infection

The following cell lines were used in this study: D2.SV (H-2^d) and SS.SV (H-2^s) are SV40 transformed mouse fibroblast lines ³⁸; P815 is a mouse mastocytoma (H-2^d, ATCC[®] TIB-64[™]); MC57G, is a carcinogen methylcholanthrene-induced fibrosarcoma cell line (H-2^b), EL-4 is a thymoma cell line (H-2^b), LTA-5 is a mouse fibroblast cell line (H-2^k, ATCC[®] CRL-2648[™]) and T2-K^d, a TAP-deficient lymphoid cell line ³⁹. All cell lines were cultured in RF-10 (RPMI-1640 supplemented with 10% FCS, 2mM L-glutamine, 100U mL⁻¹ penicillin and 100 U mL⁻¹

streptomycin) in 5% CO₂ incubator. Influenza A virus (IAV) Puerto Rico/8/34 (H1N1, PR8) was propagated in 10-day embryonic chicken eggs. The infectious allantoic fluid was harvested, titered and stored at -80°C before use. IAV was used at a multiplicity of infection of 50 (MOI=50) for D2.SV in FCS-free, acidified RPMI-1640 for 1h at 37°C. Then, 2 mL of RF-10 was added for a further 4-hour infection. Infected D2.SV-PR8 were washed three times before use.

Allo immunization

Cell lines P815 (H-2^d), D2.SV (H-2^d), D2.SV-PR8 (H-2^d), LTA-5 (H-2^k), SS.SV (H-2^s), EL-4 (H-2^b), MC57G (H-2^b) and DC-depleted splenic cells purified by MACS[®] Microbeads (Miltenyi Biotech Australia, Macquarie Park NSW, Australia) isolated from BALB/c, C3H, B6 and Batf3^{-/-} mice were prepared in PBS and irradiated (100Gy). B6 or Batf3^{-/-} mice were immunized with these irradiated cells at 5×10⁶ cells per mouse via *i.p.*, unless otherwise stated.

Skin transplantation

Naïve BALB/c mice were sacrificed as skin donors. The tail skin was disinfected and harvested. Recipient mice (B6 and Batf3^{-/-}) were anesthetized, dorsal flank shaved and disinfected; a circular incision of ~ 0.6cm × 0.6cm was made using scissors on the right flank. BALB/c tail-skins were trimmed to size and placed in the graft bed. Tissue adhesive (3M[™] Vetbond[™] Tissue Adhesive, 3M, St. Paul, MN, USA) was applied on the contact zone exclusively. Self-grafts from naïve B6 or Batf3^{-/-} mice were placed on the opposite flank using the same procedure. Then, bandages were applied around the mouse waist. On day five after the transplantation, bandages were removed and transplanted mice were monitored and scored according to the graft score system²¹.

Assessing antigen-specific CD8⁺ T cell functions

Seven days after immunization or on the day specified in the text post transplantation, an intracellular cytokine staining (ICS) was performed to assess allo antigen-specific CD8⁺ T cells. Spleens, peritoneal washout cells (PWCs) or draining lymph nodes (LNs) were harvested. 5×10⁵ cells were plated into U-bottom 96-well plates with 10⁵ allogeneic or syngeneic APC added. Then, the cells were incubated for 5h in the presence of Brefeldin A (BFA) before ICS⁴⁰. Fluorescent anti-CD8 and anti-IFN-γ antibodies were used to identify the allo-specific CD8⁺ T cells. When IAV infection was involved, 1μM of IAV immunodominant peptides (NP₃₆₆₋₃₇₄ or PA₂₂₄₋₂₃₃) was also included in the assay.

Adoptive transfer of purified T cells or DCs

For assessing T cell repertoire, naïve B6-Ly5.1 and Batf3^{-/-} (Ly5.2) mice were sacrificed and splenic T cells were FACS-sorted. Then, 2×10⁶ sorted splenic T cells from Ly5.1 mice were transferred into Batf3^{-/-} mice or Batf3^{-/-} T cells transferred into Ly5.1 mice *i.v.* The next day, these mice were immunized *i.p.* with 5×10⁶ irradiated DC-depleted splenic cells per mouse from BALB/c

348 mice. Seven days later, peritoneal washout cells from immunized mice were assessed by ICS for
349 allo-specific CD8⁺ T cells.

350 DC purification was previously published ¹⁹. Briefly, spleens, lymph nodes of naïve B6 mice
351 were digested into single cell suspension using RPMI-1640 containing 2% FCS, 1mg mL⁻¹ Type 3
352 collagenase and 0.02mg mL⁻¹ DNase I at room temperature for 20min. Subsequently, cells were
353 treated with 10mM EDTA for 5min followed with DAPI, anti-CD45, anti-MHC-II, anti-CD11c,
354 anti-CD8 α and anti-CD103 antibody staining. Then, CD8 α ⁺ DCs and CD103⁺ DCs were FACS-
355 sorted. The sorted CD8 α ⁺ DCs, or CD103⁺ DCs or both CD8 α ⁺ DCs and CD103⁺ DCs were mixed
356 with irradiated DC-depleted splenic cells from BALB/c mice and injected into Batf3^{-/-} mice *i.p* at
357 5 $\times$ 10⁶ allogeneic cells and 10⁶ DCs per mouse. In some cases, the purified DCs were transferred *i.v*
358 although irradiated allo cells were injected via *i.p* route.

359 ***In vitro* mix lymphocytes reaction**

360 FACS-sorted splenic CD8⁺ T cells from naïve B6 and Batf3^{-/-} mice were labelled with 20 μ M
361 CFSE in PBS containing 5% FCS for 5min at room temperature. CFSE labelled T cells were
362 suspended in RF-10 and set up in 96-well plate (U-bottom) at 5 $\times$ 10⁴ cells/well. FACS-sorted
363 CD8 α ⁺ DCs or CD103⁺ DCs and irradiated D2.SV cells (100Gy) were added at 10⁴ DCs and 10³
364 D2.SV cells/well. The proliferation of CFSE labelled CD8⁺ T cells in these co-cultures was
365 assessed using flow cytometry from day 3 to day 7 ⁴¹.

366 **Cross-dressing experiments using F5 T cell proliferation**

367 For *in vivo* F5 proliferation, mice were *i.p.* primed with irradiated P815 cells (10⁷/mouse);
368 meanwhile, MHC-II-depleted F5-Ly5.1 splenocytes (CFSE-labelled, 2.5 $\times$ 10⁵/mouse) were *i.v.*
369 injected into P815-primed or unprimed control mice. Seven days later, injected F5-Ly5.1 T cells
370 were recovered from the host spleens and their proliferation were analyzed by flow cytometry. For
371 *in vitro* F5 proliferation, mice were *i.p.* primed with irradiated P815 cells (10⁷/mouse) or *i.p.*
372 injected with PBS; two days later, splenocytes of primed and unprimed mice were harvested,
373 followed by T cell depletion using anti-CD90.2 antibody; such T cell depleted splenocytes were
374 then co-cultured with F5-Ly5.1 T cells (CFSE-labelled, 10⁴/well) at a ratio of 50:1 (splenocytes: T).
375 In some experiments, CD8 α ⁺ DCs, or CD8 α ⁺ DC-depleted splenic cells were FACS-sorted from
376 primed B6 or Batf3^{-/-} mice and used *ex vivo* to stimulate F5 T cell proliferation. In some
377 experiments, a set of controls (T cell only, T + EL-4 (1:1), T + P815 (1:1) in the presence or
378 absence of H-2K^d (HB215 ⁴²) or H-2L^d (HB-27 also called 28-14-8.S ⁴³) blocking antibody and T +
379 NT60-NP₃₆₆₋₃₇₄-pulsed EL-4 (1:1)) were included. Four days post co-culture, F5 T cell proliferation
380 were assessed by flow cytometry.

381 **Peptide extraction, RP-HPLC fractionation and fraction assessment.**

382 The method was published previously ⁴⁴. Briefly, P815 cells were cultured in 1.5 liter roller
383 bottles (Corning, NY). 10⁹ P815 cells were pelleted, lysed by 1% TFA, and dounced with a tissue
384 homogenizer; peptide-containing supernatant were then collected after ultra-centrifugation at
385 23,000rpm and passed through a 3K cut-off filter (Filtron, Northeborough, MA). The filtrate was
386 then concentrated to < 400 µL on a SpeedVac (Thermo Scientific™ SPD121P P1-230 SpeedVac™,
387 Waltham, USA). The concentrated sample was then fractionated on an Agilent 1100 RP-HPLC
388 (Santa Clara, CA) using a C18 column (Waters, Milford, MA) under a continuous acetonitrile
389 gradient and collected between 5 and 35 minutes ⁴⁴.

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393

394 **AUTHOR CONTRIBUTION**

395 BL, CL, SO and JS designed and performed experiments; KX, MD and DZ performed experiments;
396 HX, JC, QZ and CW provided funding, critical reagents and contributed to manuscript preparation;
397 WC conceived the project; BL, JWY and WC wrote the manuscript.

398

399 **CONFLICT OF INTEREST**

400 We declare that none of the authors have a competing interest.

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FIGURE LEGENDS

Figure 1. Responses in Batf3^{-/-} and B6 mice immunized with allogeneic cell line D2.SV and PR8 infected D2.SV. Batf3^{-/-} and B6 mice were immunized with D2.SV or D2.SV/PR8 *i.p.* Seven days

later, mice were sacrificed, spleens and peritoneal washout cells harvested for detection of CD8⁺ T cell responses to IAV peptides (H-2D^b-NP₃₆₆₋₃₇₄ and H-2D^b-PA₂₂₄₋₂₃₃) and allogeneic cell D2.SV. **(a)** Splenic CD8⁺ T cells secreting IFN- γ in immunized mice were assessed using ICS assays. **(b)** Responses of CD8⁺ T cells from peritoneal cavity of immunized mice were determined. All experiments were performed 5 times. Data are from one representative experiment with five individual mice for each group and expressed as means $\pm$ S. D.

Figure 2. Batf3^{-/-} mice lack alloresponses when immunized with allo cell lines or allo DC-depleted splenic cells. Irradiated allo cell lines (5x10⁶) or allo DC-depleted splenic cells (5x10⁶) were used to prime B6 and Batf3^{-/-} mice *i.p* respectively. Seven days later, peritoneal washout cells were harvested, and the CD8⁺ T cell responses to allogeneic cells were analyzed using ICS assays with a panel of splenic cells and the priming and control cell lines. **(a)** H-2^d cell line (P815 and D2.SV) primed B6 and Batf3^{-/-} mice, respectively. **(b)** and **(c)** LTA-5 (H-2^K) and SS.SV (H-2^s) primed B6 and Batf3^{-/-} mice. **(d)** Allo BALB/c and C3H DC-depleted splenic cells primed B6 and Batf3^{-/-} mice. **(e)** Syngeneic cell lines (EL4 and MC57G) or B6 and Batf3^{-/-} DC-depleted splenic cells immunized B6 and Batf3^{-/-} mice. The results are a representation of 5 similar assessments.

Figure3. Batf3^{-/-} mice have similar T cell repertoire. **(a)** T cells from Batf3^{-/-} mice (Ly5.2) were FACS sorted and 2x10⁶ were transferred into Ly5.1 B6 mice *i.v*. These mice were then immunized with irradiated BALB/c DC-depleted splenic cells *i.p* the next day after T cell transfer. Seven days later, peritoneal washout cells were harvested, and alloresponses of the donor cells (CD45.2⁺) and the host cells (CD45.1⁺) were assessed. Alloresponses of donor cells are shown, and the percentage of allospecific T cells in the donor cells and host cells from five mice were compared. Data are expressed as means $\pm$ S. D. **(b)** FACS-sorted T cells from Ly5.1 B6 mice were transferred into Ly5.2 Batf3^{-/-} mice *i.v* as described above. These mice were then also primed with allo BALB/c DC-depleted splenic cells *i.p*. Seven days later, alloresponses of the donor cells (CD45.1⁺) and the host cells (CD45.2⁺) were determined by ICS. The results of **a** and **b** are a representation of three separate experiments.

Figure 4. Restore alloresponses in Batf3^{-/-} mice by transferring purified CD8 α ⁺ and/or CD103⁺ DCs. **(a)** CD8 α ⁺ DCs and CD103⁺ DCs from B6 mice were FACS sorted. Then, Batf3^{-/-} mice were primed *i.p* with irradiated BALB/c DC-depleted splenic cells together with sorted CD8 α ⁺ DCs, or CD103⁺ DCs or both CD8 α ⁺ DCs and CD103⁺ DCs, respectively. B6 mice immunized with such irradiated DC-depleted splenic cells alone were used as positive control. Batf3^{-/-} mice immunized with the same DC-depleted splenic cells without DC transfer were used as negative control. Seven

555 days later, peritoneal cells were harvested, and the allo responses to BALB/c splenic cells were
556 assessed. **(b)** FACS-sorted CD8 α^+ , or CD103 $^+$, or CD8 α^+ CD103 $^-$, or CD8 α^+ and CD103 $^+$ DCs from
557 B6 mice were transferred into Batf3 $^{-/-}$ mice *i.v.* The next day, these mice were primed with
558 irradiated allo cell lines D2.SV or P815. Batf3 $^{-/-}$ mice without DCs transfer and B6 mice immunized
559 with irradiated cells were used as negative and positive controls. Seven days later, peritoneal
560 washout cells were harvested, and alloresponses to the priming cell lines were assessed. Data are
561 expressed as means $\pm$ S. D. (3 mice per group). * $P < 0.05$. **(c)** CD8 $^+$ T cells were purified from
562 spleens of naïve B6 or Batf3 $^{-/-}$ mice and labeled with CFSE. These T cells were then co-cultured *in*
563 *vitro* with irradiated allogeneic cell line D2.SV with or without FACS-sorted CD8 α^+ or CD103 $^+$
564 DCs from B6 mice. Purified T cells without D2.SV or sorted DCs were used as controls. The CD8 $^+$
565 T cell proliferation was determined used Flow cytometry and the day 4 data are shown. Data in **a**
566 are a representative of five similar experiments. Data in **b** are a representative of three similar
567 experiments. Data in **c** are a representative of six similar experiments, and the others are shown and
568 analysed in Supplementary figure 4.

570 **Figure 5.** Batf3 $^{-/-}$ mice show delayed skin graft rejection. **(a)** Allo skin grafts from BALB/c tail
571 were transplanted onto B6 and Batf3 $^{-/-}$ mice. Self-skin grafts were placed on the opposite flanks on
572 the same mice as a control. Five days after grafting, the bandages were removed and transplants
573 were monitored and scored. Allo and elf-grafts on B6 and Batf3 $^{-/-}$ mice on day 8 and Day 14 after
574 the transplantation were shown. Allograft scores (mean $\pm$ SEM) and survival of allo skin grafts on
575 five mice per group are shown. **(b)** On day 8 post grafting, groups of transplanted B6 and Batf3 $^{-/-}$
576 mice were sacrificed. Draining (allo-graft side) and non-draining lymph nodes (self-graft side) and
577 spleens were harvested. The responses of CD8 $^+$ T cells to syngeneic and allogeneic APCs were
578 assessed using ICS assays. **(c)** On day 14 post grafting, draining and non-draining lymph nodes and
579 spleens of groups of transplanted B6 and Batf3 $^{-/-}$ mice were harvested. The Allo CD8 $^+$ T cell
580 responses and numbers were determined. These data are a representation of 5 separate experiments.

582 **Figure 6.** F5 CD8 $^+$ T cells cross-react to H-2K d -peptide complex. **(a)** CFSE-labelled naïve F5-
583 Ly5.1 T cells (10^4 /well) were co-cultured with indicated cell lines as APC at a ratio of 1:1 (F5-
584 T:APC), including F5 T cell only, F5 T + EL-4, F5 T + NT60 NP₃₆₆₋₃₇₄ (ASNENMDAM)-pulsed
585 EL-4, F5 T + P815 in the presence or absence of H-2K d or H-2L d specific antibodies. Four days
586 later, F5 T cell proliferation was analyzed. Data are shown as FACS profiles (left) and bar-graphs
587 (right) **(b).** **(c)** 10^9 P815 cells were pelleted, surface peptides were acid extracted and the peptide-
588 containing supernatant was then ultra-centrifuged and passed through a 3-kDa cut-off filter before
589 being fractionated on a RP-HPLC. The fractions were then tested using low-temperature-induced

590 T2-K^d as APC in a co-culture with CFSE-labelled F5 T cells. Four days later, F5 T cell proliferation
591 was FACS-analyzed. Fractions collected between elution time 5min to 35min were tested. Similar
592 experiments were performed twice with similar results.

593

594 **Figure 7.** Host CD8 α^+ and CD103 $^+$ DCs prime CD8 $^+$ alloresponse via cross-dressing. **(a)** B6, Batf3 $^{-/-}$
595 $^{-/-}$ and TAP $^{-/-}$ mice were *i.p.* primed with irradiated P815 cells (10^7 /mouse) or *i.p.* injected with PBS;
596 these mice were then *i.v* transferred CFSE-labelled, MHC-II-depleted F5 splenic cells
597 (2.5×10^5 /mouse). Seven days later, splenocytes of primed and unprimed mice were harvested and
598 F5 T cell proliferation was analyzed by FACS. **(b)** B6, Batf3 $^{-/-}$ and TAP $^{-/-}$ mice were *i.p.* primed
599 with irradiated P815 cells (10^7 /mouse) or *i.p.* injected with PBS; two days later, splenocytes were
600 harvested, T cells were depleted by anti-CD90.2 (Thy1.2) before being co-cultured with CFSE-
601 labelled F5-Ly5.1 T cells (10^4 /well) at a ratio of 30:1 (APC:T). T cell proliferation was FACS-
602 analyzed on day 4. **(c)** B6, Batf3 $^{-/-}$ and TAP $^{-/-}$ mice were primed as in **b**. On day 2, splenocytes were
603 harvested and their CD8 α^+ DC, CD8 α^+ DC-depleted splenocytes were isolated by cell sorting;
604 splenocytes, CD8 α^+ DC and CD8 α^+ DC-depleted splenocytes were then co-cultured with CFSE-
605 labelled F5-Ly5.1 T cells (10^4 /well) at a ratio of 30:1 (APC:T); T cell proliferation was FACS-
606 analyzed on day 4. Similar experiments were performed twice with similar results (4 mice per
607 group). Bars represent mean $\pm$ SEM. ns means not significant, $**P < 0.01$, $***P < 0.001$.

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(a) Ly5.1 PWC

(b) Batf3-/- PWC

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