

1 **CD11a regulates effector CD8 T cell differentiation and**
2 **central memory development in response to infection with *Listeria***
3 ***monocytogenes***

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11 Running Title: LFA-1 controls CD8 T cell effector development after infection

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23 **ABSTRACT**

24

25 $\beta 2$ (CD18) integrins with α chains CD11a,b,c,d are important adhesion molecules
26 necessary for leukocyte migration and cellular interactions. CD18 deficiency leads to
27 recurrent bacterial infections and poor wound healing due to reduced migration of
28 leukocytes to inflammatory sites. CD8 T cells also upregulate CD11a, CD11b, and
29 CD11c upon activation. However, the role these molecules play on CD8 T cells *in vivo*
30 is not known. To determine the function of individual $\beta 2$ integrins, we examined CD8 T
31 cell responses to *Listeria monocytogenes* (LM) infection in CD11a, CD11b and CD11c
32 deficient mice. The absence of CD11b and CD11c had no effect on the generation of
33 antigen-specific CD8 T cells. In contrast, the magnitude of the primary CD8 T cell
34 response in CD11a-deficient mice was significantly reduced. Moreover, the response in
35 CD11a-/ mice exhibited reduced differentiation of short lived effector cells (KLRG1^{hi}
36 CD127^{lo}) although cytokine and granzyme B production was unaffected. Notably,
37 CD11a-deficiency resulted in greatly enhanced generation of CD62L+ central memory
38 cells. Surprisingly, CD8 T cells lacking CD11a mounted a robust secondary response
39 to infection. Taken together, these findings demonstrated that CD11a expression
40 contributes to expansion and differentiation of primary CD8 T cells, but may be
41 dispensable for secondary responses to infection.

42

43 **INTRODUCTION**

44 Integrins are heterodimeric adhesion molecules comprised of α and β subunits that
45 participate in immune cell interactions as well as in immune cell to extracellular matrix
46 interactions. The $\beta 2$ integrin (CD18) family consists of four members based on α chain
47 pairings: CD11a (LFA-1, $\alpha L\beta 2$), CD11b (Mac-1, $\alpha M\beta 2$), CD11c (CR4, $\alpha X\beta 2$) and CD11d
48 ($\alpha D\beta 2$). Although numerous myeloid lineage cells including dendritic cells (DC),
49 neutrophils and macrophages constitutively express CD11b and CD11c lymphocytes do
50 not, but all resting leukocytes express CD11a. Upon activation CD8 T cells upregulate
51 not only CD11a, but also CD11b and CD11c (1,2). Ligands for these molecules include
52 members of the immunoglobulin superfamily (ICAM-1, ICAM-2, VCAM-1) as well as
53 fibrinogen and iC3b (3). Patients with lymphocyte adhesion deficiency type I, who lack
54 $\beta 2$ integrins, suffer from recurrent infections and impaired wound healing (4-6).
55 Similarly, CD18-deficient mice display increased rates of spontaneous infections and a
56 reduced ability to induce graft-versus-host disease (7,8). Nevertheless, the precise role
57 of each individual $\beta 2$ integrin *in vivo* in most cases is unclear.

58

59 CD11b and CD11c expressed by myeloid cells have been implicated in possible roles
60 during a number of infections and their absence may lead to an increased microbial
61 burden (9) which was attributed to their role in adhesion-mediated phagocytosis and
62 trafficking and clearance of the infectious agent by neutrophils and other innate immune
63 cells (10-13). However, the importance of CD11b and CD11c expression by activated T
64 cells during infection has not been explored in detail and little is known about the role of
65 these molecules in antigen-specific T cell activation and function. A recent report shows

that in the absence of CD11c, CD8 T cell responses to herpes simplex virus infection are enhanced, but whether this effect is a result of CD11c function on activated CD8 T cells or APC is not known (14). Conversely, CD11b or CD11d, but not CD11c deficiency results in a diminished T cell response to staphylococcus enterotoxin challenge which was hypothesized to be due to defects in T cell development mediated by integrin-expressing non-T cells (15). In contrast to the relative paucity of literature regarding CD11b and CD11c function on T cells, the immunological relevance of CD11a expression by T cells has been characterized in some detail. Studies using planar bilayers show that the adhesive interaction of CD11a to ICAM-1 leads to stabilization of the immunological synapse (16). This adhesive property may also allow CD11a to perform costimulatory functions by augmenting T cell proliferation and cytokine production *in vitro* (17,18). CD11a is also critical for lymphocyte entry into the lymph nodes (19). Interestingly, lymphocyte migration to the spleen in the absence of CD11a remains intact (20).

Establishing a role for CD11a in T cell priming *in vivo* has proven to be more complex. Prior studies illustrate an important role for CD11a in T cell activation in colitis, DTH responses and in tumor rejection (21,22). In contrast, T cell priming in viral infections is apparently unaffected in the absence of CD11a (21). CD11a and CD11b are essential for protection against *S. pneumoniae* infection. Although the T cell response was not measured, early bacterial overgrowth suggests that the defects lie with the inability of innate immune cells to clear the infection (23). In the case of pulmonary *M. tuberculosis* infection, CD11a deficiency also leads to loss of protection (24). T cell priming is

89 delayed and fewer antigen-specific T cells are found in the lungs after infection due to
90 additional defects in T cell migration. Care must be taken when interpreting results
91 related to situations where lymph node (LN) priming is required since CD11a plays an
92 important role in migration of naïve T cells to the LN (20,25). Thus, the paucity of T
93 cells in the CD11a-/ LN due to the migration defect could lead to ineffective T cell
94 priming, rather than indicate a direct role for CD11a in T cell activation. In the case of *L.*
95 *monocytogenes* (LM) infection, β2 integrin deficient and CD11a-/ mice exhibit
96 enhanced resistance to LM infection potentially due to neutrophilia particularly in the
97 liver as well as enhanced IL-12 and G-CSF production (26-28). In fact, while neutrophils
98 are important for LM clearance from the liver, they play much less of a role in splenic
99 LM clearance and inflammatory monocytes are essential for protection against LM
100 infection (29-31). Thus far, the role of β2 integrins in the T cell response to LM infection
101 has not been examined. Here, we assess T cell activation following LM infection of mice
102 deficient in specific β2 integrins and demonstrate a T cell intrinsic requirement for
103 CD11a in CD8 T cell activation and function independent of migration.
104

105 MATERIALS AND METHODS

106 **Mice.** C57BL/6J, CD11b-/ (B6.129S4-*Itgam*^{tm1Myd}/J) (32) and CD11a-/ (B6.129S7-
107 *Itga*^{tm1BII}/J) (15) mice were purchased from The Jackson Laboratory (Bar Harbor, ME).
108 CD11c-/ mice (15) and CD45.1+/CD45.2+ B6 mice were bred in our facility. All animal
109 protocols were carried out in accordance with NIH guidelines and approved by the
110 UCHC Animal Care Committee.

111

112 **Infection.** Mice were infected i.v. with *L. monocytogenes* expressing ovalbumin (Ova)
113 (33). A dose of 1×10^3 CFU or 5×10^4 CFU was used in primary infections and a dose of
114 1×10^4 CFU was used for secondary infections. In some imaging experiments, 1×10^6
115 CFU of the attenuated ActA- strain of LM-Ova was used in order to generate sufficient
116 endogenous Ova-specific CD8 T cells. Bacterial burdens bacterial titers within tissues
117 were determined by homogenizing the tissue in PBS containing 1% saponin and plating
118 serial dilutions of the homogenate on brain–heart infusion agar containing 5 µg/ml
119 erythromycin and incubating for 2 d at 37°C.
120
121 **Flow cytometry.** Antigen-specific CD8 T cells were identified using an H-2K^b tetramer
122 containing the Ova-derived peptide, SIINFEKL, and listeriolysin O-specific CD4 T cells
123 were identified with an LLO-I-A^b tetramer (34), generously provided by Dr. Marc
124 Jenkins (UMINN). Tetramer+ cells were characterized with monoclonal antibodies
125 reactive with the indicated antigens. All antibodies were purchased from BD,
126 Ebioscience or Biolegend. For intracellular cytokine staining, splenocytes isolated from
127 infected mice were cultured with or without 2–20 µg/ml SIINFEKL or LLO peptide for 5
128 hours in the presence of GolgiPlug (BD Biosciences) and stained as directed (BD
129 Biosciences). Viability was analyzed using LIVE/DEAD cell stain (Invitrogen). Samples
130 were collected using a LSRII (BD Biosciences) and analyzed with FlowJo software
131 (Tree Star, Ashland, OR).
132
133 **Confocal Microscopy.** Spleens were isolated from naïve or infected mice and
134 prepared as previously described (35). Antibodies reactive to the indicated antigens

135 were purchased from BD Biosciences, Invitrogen or Ebioscience. Stained samples were
136 mounted using Immumount, imaged using a LSM 780 (Zeiss) and analyzed using Imaris
137 Suite (Bitplane Inc).

138

139 **Lytic assay.** Spleens from LM-Ova-ActA- infected mice were harvested on day 7.
140 Splenocytes were enriched for CD8 T cells by negative selection using CD8 T cell
141 isolation kit from Miltenyi biotec. The CD8 enriched fraction was stained for OVA-
142 tetramer to determine the absolute number of effector T cells specific for SIINFEKL. EL4
143 cells were used as target cells and pulsed with 1 µg/mL SIINFEKL peptide for 45 min at
144 37°C. Pulsed or unpulsed EL4 were labeled with high (5 µM) or low (1 µM)
145 concentrations of CFSE, respectively. Serial dilutions of effector cells were then
146 incubated with 10⁴ pulsed and 10⁴ unpulsed EL4 for 4 H. Cells were stained for viability
147 dye and examined by flow cytometry. After gating on live EL4 cells, specific lysis was
148 calculated as follows : % specific lysis = 100 – [100 × (% CFSE^{high} infected mice/%
149 CFSE^{low} infected mice)/(% CFSE^{high} target alone /% CFSE^{low} target alone)]. E:T ratios
150 were calculated for each mouse based on the OVA-tetramer number per well.

151

152 **BrdU incorporation.** Mice were injected at days 5, 6 and 7 post infection with 1mg of
153 BrdU i.p. Eight days post infection, splenocytes were stained for surface markers as
154 described above and for BrdU incorporation according to the manufacturer's protocol
155 (BD Biosciences).

156

157 **Tetramer decay assay.** T cell avidity was determined by tetramer decay analysis as
158 described previously (36). Briefly, splenocytes of infected mice were stained using Ova-
159 K^b tetramer and subsequently Fab fragments of anti-H-2K^b mAb (clone Y-3) were added
160 to a final concentration of 5 uM. Stained aliquots of cells were removed at various time
161 points and fixed immediately in 2% paraformaldehyde/PBS solution. Tetramer mean
162 fluorescence intensity (MFI) was determined by flow cytometry and half-life was
163 calculated by loss of tetramer reactivity over time.
164

165 **Tetramer Enrichment.** Splenocytes pooled from 3 infected mice were enriched for
166 Ova-specific CD8 T cells as described (37). Briefly, splenocytes were first labeled with
167 PE- and APC- labeled Ova-K^b tetramers at room temperature for 1 hour. Samples were
168 then washed and incubated with anti-PE beads (Miltenyi Biotec) at 4°C for 30 minutes
169 before positive selection using an autoMACS (Miltenyi Biotec). The cells were then
170 stained with additional antibodies as described above and analyzed by flow cytometry.
171

172 **Bone marrow chimeras.** Bone marrow was isolated from femurs and tibias of wildtype
173 (CD45.2+/CD45.1+) and CD11a -/- (CD45.2) mice. These cells were then mixed and
174 injected i.v. into lethally irradiated (1000rads) CD45.1 hosts. After 6-8 weeks of
175 reconstitution, chimeras were immunized as described above. Donor populations were
176 separated based on CD45 expression.
177

178 **Statistical Analysis.** Statistical significance was determined by using Prism 5
179 (Graphpad Software) and the functions indicated. Error bars indicate SEM values.

180

181 **RESULTS**

182

183 **Primary CD8 T cell responses to bacterial infection are CD11b and CD11c**

184 **independent.** Previous studies have reported the induction of myeloid markers CD11b
185 and CD11c on activated antigen specific CD8 T cells during LCMV infection (38) and
186 the GVHD response (2). We tested whether a similar phenomenon occurred in
187 response to LM infection. CD8 T cell priming after intravenous LM infection occurs
188 primarily in the spleen (39) and thus circumvents any potential effects of the role of $\beta 2$
189 integrins in LN migration. At the peak of the primary CD8 T cell response, CD11a,
190 CD11b and CD11c were substantially upregulated by Ova-K^b specific CD8 T cells
191 (Figure 1A). The selective upregulation of these markers on activated antigen-specific
192 CD8 T cells suggested that engagement of these receptors may play an important role
193 in the CD8 T cell responses to infection. To test this possibility, we examined Ova-K^b
194 specific CD8 T cell responses after LM infection in mice deficient in CD11b or CD11c.
195 Neither CD11b-/ nor CD11c-/ mice exhibited any defects in generation of Ova-K^b-
196 specific CD8 T cell responses in the spleen (Figure 1B). In the lung, CD11b-/ mice
197 generated a modest but statistically significant increase in the antigen-specific CD8 T
198 cell response.

199

200 After infection, a heterogeneous pool of CD8 T cell effectors is formed that can be
201 identified based on the expression of various cell surface markers, including KLRG1
202 and CD127 (IL-7R) (40). The early effector CD8 T cells (EECs) that are KLRG1^{low}

203 CD127^{low}, are the first effector subset to emerge after LM infection (41). However, at
204 the peak of the CD8 T cell response to LM infection, the terminally differentiated short
205 lived effector cells (SLEC) constitute the majority of the responding CD8 T cells and are
206 identified by KLRG1^{hi} and CD127^{low} expression. The long-lived memory precursors
207 (MPEC) are KLRG1^{low} and CD127^{hi} and constitute a smaller fraction of responding CD8
208 T cells along with the double positive effector cells (DPECs) expressing both KLRG1
209 and CD127. An examination of effector cell heterogeneity in the absence of CD11b or
210 CD11c further indicated that these integrins did not affect the CD8 T cell response to
211 LM infection (Figure 1C). Similarly, granzyme B and cytokine production among
212 responding CD8 T cells in CD11b or CD11c deficient or wild type mice was equivalent
213 (Figure 1D-F). Thus, despite their induction on responding CD8 T cells, CD11b and
214 CD11c were not required for the generation of optimal primary antigen-specific CD8 T
215 cell responses during LM infection.

216

217 **CD11a is crucial for the optimal expansion of antigen-specific CD8 and CD4 T**
218 **cells.** Although CD11a plays a prominent role in lymphocyte migration to LN (42), the
219 role of this molecule in CD8 T cell priming during infection *in vivo* is not well understood.
220 Thus, we tested CD11a function *in vivo* by infecting CD11a-deficient mice with LM-Ova
221 and then evaluating antigen-specific CD8 T cell responses. At the peak of the CD8 T
222 cell response, CD11a-/- mice displayed a significant defect in accumulation of splenic
223 Ova-K^b-specific CD8 T cells (Figure 2A) which was also evident in liver and lungs (data
224 not shown). The reduced T cell response was not due to reduced antigen availability or
225 altered bacterial load since there were no significant differences in bacterial loads in the

226 spleens of WT and CD11a-/- mice during the peak of LM replication at 3 days post
227 infection (Figure S1). Since previous reports indicate that CD11a-/- mice are more
228 resistant to LM infection (26,27), we also challenged mice with a higher LM dose (5×10^4
229 CFU). In this case, bacterial burden in the spleen was decreased in CD11a-/- mice as
230 compared to control animals (Figure S1). At the higher dose, the CD8 T cell response in
231 the WT animals was less than that observed when a low dose was given (Figure S2).
232 Nevertheless, the CD8 T cell response was not reduced in the CD11a-/- mice
233 suggesting that increasing antigen and/or inflammation could overcome the defect.
234 Interestingly, the CD4 T cell response was not reduced in CD11a-/- mice when a low
235 LM dose was used but was decreased at the higher dose (Figure S2).

236

237 To examine the reason for the reduced response magnitude of the CD8 T cell response,
238 we utilized tetramer enrichment to track small populations of antigen-specific CD8 T
239 cells early after infection (day 4). At this time, markedly reduced numbers of Ova-K^b
240 specific CD8 T cells were present in CD11a -/- mice (Figure 2B). Next, we measured
241 the proliferation of CD11a-/- CD8 T cells by BrdU uptake on days 5-7 PI. Fewer antigen-
242 specific CD8 T cells in CD11a -/- mice incorporated BrdU (Figure 2C) and the amount of
243 BrdU incorporated into the CD11a-/- CD8 T cells was also significantly less than that of
244 control cells (Figure 2C). Thus, optimal early expansion and continued proliferation both
245 required CD11a.

246

247 **Differential requirement for CD11a in cytokine production and lytic activity of**
248 **antigen-specific CD8 T cells.** Early data implied that CD11a plays a compulsory role in

249 cytotoxic functions of CD8 T cells *in vitro* (43). We investigated whether the
250 engagement of CD11a impacted the function of responding CD8 T cells after LM
251 infection *in vivo*. Surprisingly, Ova-K^b specific CD8 T cells in WT and CD11a -/- mice at
252 day 8 post infection exhibited similar levels of granzyme B *ex vivo*. Moreover, after
253 antigen stimulation *in vitro* CD11a-/- and CD8 T cells expressed similar levels of LAMP-
254 1 (CD107a), a marker of degranulation (44) (Figure 3A). Despite these results, direct ex
255 vivo lytic activity of CD11a-/- CD8 T cells was ~10-fold less than that of WT CD8 T cells
256 (Figure 3B). We next determined whether *in vivo* generated CD11a-/- Ova-K^b-specific
257 CD8 T cells produced normal levels of IFN γ and TNF α at day 8 PI. Concomitant with
258 the reduced frequency of tetramer+ cells in CD11a-/- mice (Figure 2A), we observed a
259 reduced frequency of IFN γ producing CD8 T cells (Figure 3C, upper panel), although
260 IFN γ levels between WT and CD11a-/- cells were similar. In addition, the frequency of
261 TNF α producers among the IFN γ positive cells was similar in WT and CD11a-/- mice
262 (Figure 3C, lower panel). Thus, the absence of CD11a led to an overall reduction in the
263 magnitude of the CD8 T cell response and reduced lytic activity but did not impact the
264 cytokine-producing capacity of the responding CD8 T cells.

265

266 It is possible that the absence of CD11a could alter the selection of CD8 T cells that
267 contribute to the response whereby only those CD8 T cell clones that posses a higher
268 avidity for MHC-peptide complexes are equipped to aptly respond to infection. Although
269 the fluorescence intensity of tetramer staining was similar between CD11a -/- and WT
270 CD8 T cells (Figure 2A), we wanted to directly test the avidity of WT and CD11a-/- CD8
271 T cells using a tetramer decay assay that compares the dissociation kinetics of

272 peptide/MHC and TCR interaction (36). In this assay, the half life is defined by the time
273 required to lose 50% of the maximal tetramer binding for each group. Combined results
274 from two individual experiments indicated that the half life of tetramer binding of WT and
275 CD11a -/- T cells was similar; WT averaged 9.04 minutes while CD11a -/- T cells
276 averaged 10.2 minutes (Figure 3D). These data indicated that although the magnitude
277 of the CD8 T cell response was blunted in the absence of CD11a, there was no
278 evidence that the responding cells represented an atypical population made up of a
279 subset of clones with distinct avidities.

280

281 **CD11a regulates effector subset development of CD8 T cells.** Several studies show
282 that numerous cell extrinsic factors such as antigen availability, precursor frequency and
283 the inflammatory milieu are integrated to subsequently determine the differentiation
284 pattern of effector and memory CD8 T cells after infection (37,45-47). We
285 hypothesized that the absence of CD11a may lead to reduced interaction of antigen
286 presenting cell (APC) and CD8 T cells and that this could result in a reduced “strength”
287 of signal downstream of TCR engagement. To determine if CD11a deficiency affected
288 differentiation of effector CD8 T cells, we examined the SLEC-MPEC differentiation
289 profile of Ova-K^b specific CD8 T cells. Interestingly, CD11a -/- mice had a reduced
290 frequency of SLECs concomitant with a significant increase in the frequency of CD127
291 and KLRG1 double positive cells (DPEC) compared to WT mice. We also observed a
292 trend toward increased accumulation of MPEC, although this difference failed to reach
293 statistical significance (Figure 4A).

294

295 We previously demonstrated that programmed death-1 (PD-1) expression inversely
296 correlates with CD62L expression on antigen specific CD8 T cells after infection (47).
297 Moreover, limiting antigen availability during infection resulted in increased expression
298 of CD62L with a decrease in PD-1 expression. CD11a -/- antigen-specific CD8 T cells
299 displayed reduced expression of PD-1 with a concurrent major increase in expression of
300 CD62L (Figure 4B). Our previous results show that CD62L expression is restricted to
301 the MPEC subset (48) leading to development of central memory CD8 T cells (T_{CM} ,
302 Figure 4C, WT). T_{CM} express the homing molecules CD62L and CCR7 while effector
303 memory cells (T_{EM}) lack their expression. However, although CD62L expression was
304 greatly increased in the CD11a-/ MPEC, CD62L was also expressed by significant
305 populations of CD11a-/ EEC and SLEC, which we have never observed in wild type
306 mice, even when TCR triggering is limited (48,49). Because EEC are formed early
307 after initial CD8 T cell activation, our findings suggested that CD11a may be important
308 for the initial downregulation of CD62L.

309

310 Our earlier studies demonstrated that CD25 (IL-2R) expression by antigen-specific CD8
311 T cells correlates with antigen availability and is maximal at four days post LM infection
312 (47,49). The frequency of CD25⁺ cells as well as the level of CD25 expression was
313 significantly lower in CD11a-/ Ova-K^b-specific CD8 T cells (Figure 4D). The reduced
314 CD25 expression is likely a result of reduced TCR triggering which we have linked to
315 enhanced CD62L expression and increased MPEC generation (47,49).

316

317 **Effect of CD11a on splenic anatomy and CD8 T cell localization.** Recent data show
318 that the absence of chemokine receptors alters localization of CD8 T cells to
319 inflammatory sites resulting in changes in effector subset differentiation and memory
320 generation (50). Using confocal microscopy we tested whether CD11a was involved in
321 anatomical organization of the spleen in normal or infected mice. A comparison of
322 spleen sections from WT and CD11a-/ mice did not reveal any gross differences in the
323 localization of T, B, CD11c+, CD11b+ or Moma+ marginal zone macrophages (Figure 5
324 and data not shown). After infection we examined the localization of antigen-specific
325 CD8 T cells using *in situ* MHC class I tetramer staining and confocal microscopy (35)
326 (Figure 5). In order to enhance detection of Ova-K^b-specific CD8 T cells at early time
327 points, we infected mice with a higher dose of an attenuated ActA deficient strain of LM-
328 Ova (35), which generates a larger response. Both the CD4 and CD8 T cell responses
329 were significantly reduced after ActA LM infection (Figure S3). At D5 p.i., in both the WT
330 and CD11a-/ spleens, a readily detectable population of tetramer+ CD8 T cells were
331 located in T cell zones (PALS) as well as in the red pulp (RP) (Figure 5). We also
332 examined the spleen at 8 days after infection with WT LM-Ova. Consistent with the flow
333 cytometry data, the CD11a-/ spleen contained fewer Ova-specific CD8 T cells than did
334 the WT spleen (Figure 5). Moreover, in WT and CD11a-/ spleens, the majority of
335 antigen-specific CD8 T cells were located in the RP, with a smaller percentage present
336 in the B and T cell zones. Overall, these data indicated that CD11a expression was not
337 required for localization or movement of CD8 T cells during priming in the spleen.
338

339 **CD11a requirement for CD8 T cell priming is cell intrinsic.** In CD11a -/- mice, all
340 cells lack CD11a expression. In order to test whether T cells intrinsically require CD11a
341 to mount a normal response, we generated mixed chimeras by reconstituting lethally-
342 irradiated WT mice with equal numbers of bone marrow cells from congenically distinct
343 WT and CD11a -/- mice. This system also alleviates any concerns of potential
344 differences in bacterial burdens or antigen loads since WT and CD11a-/ cells are
345 responding in the same host. Interestingly, 8 weeks after reconstitution we observed an
346 incomplete reconstitution of the T cell compartment from CD11a-/ donor cells that
347 appears to be due to a partial block in T cell development (Bose et al, in preparation).
348 To compensate for differences in reconstitution we generated chimeras using a 1:3 ratio
349 (WT:CD11a-) of bone marrow cells. Although reconstitution remained unequal (~3:1,
350 WT:CD11a-), it was nevertheless possible to accurately assess the CD8 T cell
351 response after infection. The defect in CD8 T cell priming observed in CD11a-/ mice
352 was recapitulated in CD11a-/ CD8 T cells in chimeras (Figure 6A). Moreover, CD11a-/
353 CD8 T cells in chimeras also displayed altered effector subset development
354 characterized by fewer SLEC and an increase in EEC and MPEC compared to their WT
355 counterparts (Figure 6B). Cytokine production was also unaffected in the chimeras
356 (Figure 6C). Thus, CD11a expression by CD8 T cells was required for optimal CD8 T
357 cell expansion and normal effector subset development in response to LM infection.
358

359 **Generation and reactivation of memory CD8 T cells is CD11a independent.** A
360 previous report demonstrated that in the absence of ICAM-1, the presumed ligand for
361 CD11a, memory CD8 T cells did not develop due to a drastic contraction of effector OT-

362 I T cells (51). We asked whether a similar phenomenon occurred in the absence of
363 CD11a, and examined the kinetics of the antigen-specific CD8 T cell response to
364 infection in the peripheral blood of WT and CD11a -/- mice. Although CD11a-/- mice
365 had lower frequencies of Ova-K^b-specific CD8 T cells at all time points tested, the rate
366 of contraction did not appear to be greater in CD11a -/- vs. WT mice (Figure 7A).
367 Additionally, even at day 70 p.i. memory cells were present in the blood of CD11a-/-
368 mice, albeit at a reduced frequency, concomitant with the reduced magnitude of the
369 primary response. Moreover, memory CD8 T cells remained phenotypically distinct
370 between CD11a-/- and WT mice with regard to KLRG1, CD127 and CD62L expression,
371 which was again reflective of the primary response (Figure 7C,D). Similar results were
372 obtained from spleen (data not shown). Thus, CD11a was not required for memory
373 development *per se*. To determine whether CD11a played a role in memory CD8 T cell
374 reactivation, WT and CD11a -/- mice were first infected with 1x10³ CFU of LM-Ova and
375 70 days later were re-challenged with 1x10⁴ CFU of LM-Ova. Surprisingly, Ova-K^b-
376 specific CD8 T cells in the spleens of both groups of mice underwent a massive
377 expansion in the blood (Figure 7E) and spleen (Figure 7F) by day 6 after recall. CD8 T
378 cells in WT mice underwent a ~102-fold increase while CD11a -/- CD8 T cells increased
379 ~184-fold in comparison to pre-challenge memory cell levels (Figure 7E). Effector
380 subset composition in WT and CD11a -/- mice after recall was essentially identical
381 (Figure 7G). However, an increased proportion of CD11a-/- recalled CD8 T cells
382 expressed CD62L, which may be reflective of the heightened levels of CD62L
383 expression prior to challenge (Figure 7H). Analysis of cytokine production indicated
384 similar frequencies of TNF α ⁺ CD8 T cells in WT and CD11a -/- mice (Figure 7I).

385 Overall, these findings indicated a more stringent CD11a requirement for primary versus
386 memory CD8 T cell activation.

387

388 **DISCUSSION**

389 Precursor frequency, inflammatory milieu, affinity and duration of T cell-APC
390 interactions are important determinants of T cell response kinetics and function (45,52-
391 54). Recently, two-photon microscopy revealed that interaction of DC with naïve T cells
392 is complex. Initial transient DC/T cell interactions give way to more sustained synapse
393 formation that can be hours long (55). Perturbation of DC-T cell interactions can lead
394 to altered T cell responses (51). Thus, regulation of these interactions is crucial for the
395 formation of efficient immune responses. In the current study, we highlighted the
396 important role of CD11a in CD8 T cell priming and function.

397

398 CD11a reorganization is fundamental to the ‘bulls-eye’ like architecture of the
399 immunological synapse (56). Several *in vitro* studies provide evidence that the
400 interaction of CD11a on T cells with ICAM-1 on APC facilitates stabilization of the
401 immune synapse that promotes costimulatory function leading to increased T cell
402 proliferation and cytotoxicity (18,57). Confirming a similar role for CD11a *in vivo* has
403 proven to be more complicated though studies have illustrated the importance of CD11a
404 during induction of colitis, DTH responses and tumor rejection (21,22). Based on *in*
405 *vitro* killing assays, the CD8 T cell response to VSV or LCMV infection in CD11a-/- mice
406 is equivalent to that of WT mice (21), but quantitation of antigen-specific CD8 T cell
407 numbers was not performed. In our studies, taking into account the magnitude of the

408 CD8 T cell response revealed defective lytic activity but normal cytokine production by
409 CD11a-/ CD8 T cells. The role of CD11a in migration should also be taken into account
410 in studies using CD11a-/ or β 2-integrin-deficient mice. Not only are β 2 integrins
411 involved in migration of T cells to LN, they are also involved in establishing the normal
412 complement of immune components in certain tissues such as the intestine (58). To
413 circumvent LN involvement we took advantage of the i.v. LM infection system, in which
414 T cell priming occurs primarily in the spleen (39).

415

416 Our results indicated that CD11a deficiency significantly impacted the differentiation of
417 the responding CD8 T cells. The development of effector subsets was skewed away
418 from SLEC generation and favored EEC and MPEC formation. This result mirrored our
419 previous findings where similar patterns of effector subsets developed in the absence of
420 CD4 T cell help or CD25 signaling (49). CD4 T cells play an important role in CD25
421 upregulation by CD8 T cells which bestows IL-2 responsiveness, an important
422 component of the 'help' provided by CD4 T cells during LM infection (49). Moreover,
423 sustained IL-2 signaling drives SLEC differentiation after infection (49). Yet, in the case
424 of CD11a deficiency the CD4 T cell response to LM infection was normal (data not
425 shown). In addition, the mixed chimera studies showed that cell intrinsic CD11a
426 expression by CD8 T cells was needed for an optimal response. Thus, CD11a appeared
427 to play a direct role in CD25 upregulation and effector CD8 T cell subset development
428 presumably through augmentation of T cell-APC interactions. The precise signaling
429 mechanism by which CD11a regulates CD8 T cell responses remains unclear. β 2
430 integrins lack immunoreceptor tyrosine-based activation motifs (ITAM), but activation of

431 protein tyrosine kinases via CD11a may initiate outside-in signaling which could then
432 control activation and programming of CD8 T cells (6,59). In addition, ligand-
433 independent signaling via CD11a coupled to TCR triggering may also play a role in T
434 cell activation (60). Thus, the absence of CD11a could lead to altered downstream
435 signaling events that yield the observed results. In support of this possibility, a recent
436 study showed that the proline-rich tyrosine kinase-2 (PYK2), which is important in
437 CD11a mediated adhesion and costimulation, regulates SLEC differentiation during
438 LCMV infection (61). These findings along with ours point to CD11a as an important
439 determinant of effector CD8 T cell differentiation.

440

441 The ligands for CD11a *in vivo* that drive CD8 T cell responses are not well-defined.
442 ICAM-1 is thought to be a major counterreceptor for CD11a (17). The role of ICAM-1 in
443 CD8 T cell priming after immunization with DC-targeted antigen and CD40 costimulation
444 (51) was examined by intravital microscopy. ICAM-1 expression by DC enhanced long-
445 lived stable interactions with responding OT-I TCR transgenic CD8 T cells. However,
446 immunization of ICAM-1-/- hosts results in a numerically and functionally normal primary
447 response of adoptively transferred OT-I cells, suggesting that, at least in this
448 immunization scheme, ICAM-1 is not a major contributor to initial CD8 T cell activation
449 *in vivo*. Our results indicated that CD11a was essential for optimal CD8 T cell expansion
450 in response to LM infection implying that ICAM-1 may not always play a dominant role
451 in CD11a-mediated T cell activation or that initiation/amplification of different immune
452 responses may involve distinct integrin-ligand combinations.

453

454 Our data also demonstrated an important function for CD11a in regulating the T_{CM} / T_{EM}
455 ratio through control of CD62L expression. In the absence of CD11a, the number of
456 CD62L+ MPEC was greatly increased and unusual expression of CD62L by EEC and
457 SLEC was noted. This correlated with decreased PD-1 expression which was similar to
458 our previous results where specifically limiting antigen availability results in a decrease
459 in CD25 levels, and an increase in CD62L by MPEC (47,48). Coupled with these
460 results, our findings with CD11a-deficient CD8 T cells suggested a differential regulation
461 of effector subset development and the T_{CM} / T_{EM} ratio. Thus, CD62L expression is more
462 sensitive to reductions in signal strength, while altering MPEC and SLEC development
463 occurs only when signal transduction is more severely limited. Furthermore, the
464 induction of aberrant CD62L expression by CD11a-deficient EEC and SLEC indicated
465 an additional level of CD62L regulation related to signal strength, perhaps at an early
466 activation stage when CD62L is enzymatically cleaved (62,63).

467

468 Of particular note was the role of CD11a in CD8 memory T cell responses. Although
469 the primary response of CD11a-/ CD8 T cells was diminished, development of memory
470 was not overtly affected. Indeed, based on the maximum peak of each response,
471 CD11a-/ mice tended to generate a larger proportion of memory cells as compared to
472 WT mice, perhaps as a result of enhanced T_{CM} generation, since these cells undergo
473 greater proliferation than do T_{EM} (64,65). In contrast, the aforementioned ICAM-1 study
474 shows that memory is not generated in the absence of ICAM-1 despite a normal primary
475 response (51). This is difficult to reconcile with our findings and other available
476 literature and possibly suggests that ICAM-1 is involved in survival of cells during or

477 after the contraction phase. Another possibility is that the adoptively transferred OT-I
478 CD8 T cells, upregulate ICAM-1 after activation and are rejected by the ICAM-1-/ host.
479 Further studies will be needed to resolve these issues. Our results also showed that
480 reactivation of memory CD8 T cells was largely CD11a independent. One interpretation
481 of these data is that long-lived stable interactions were not compulsory for memory CD8
482 T cell activation. This is in agreement with our previous findings that memory CD8 T cell
483 reactivation requires a significantly shorter window of antigen availability than do naïve
484 T cells (54).

485

486 Collectively, our results delineated several key features of CD11a-mediated
487 costimulation that regulated the CD8 T cell response to infection. First, optimal initial
488 priming for proliferation and induction of lytic activity, but not effector cytokine
489 production, required CD11a. Second, effector subset ratios were controlled by CD11a.
490 Third, CD11a played a major role in controlling CD62L expression and therefore the
491 T_{CM}/T_{EM} ratio. Finally, memory CD8 T cell reactivation was CD11a independent. These
492 data provide a comprehensive view of the important role of CD11a in the CD8 T cell
493 response to infection.

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708 **FIGURE LEGENDS**

709

710 **FIGURE 1. Absence of CD11b or CD11c does not affect priming of CD8 T cells**
711 **during *Listeria* infection.** (A) Representative plots show gated CD8 T cells from
712 spleens of WT C57BL/6 mice that were infected 8 days prior with LM-Ova. Histograms
713 show expression of CD11a, CD11b and CD11c by tetramer+ CD8 T cells in open
714 histogram and endogenous naïve CD8 T cells (tetramer- CD44^{low}) in filled gray
715 histogram. (B) Graphs show frequency of Ova-K^b specific CD8 T cell in WT versus
716 CD11b-/- and CD11c -/- mice in spleen and lung D8 p.i. Graphical representation of
717 various effector subsets (C) and granzyme B production (D) within Ova-K^b specific CD8
718 T cells. Eight days p.i. splenocytes were re-stimulated *in vitro* with peptide to assess
719 cytokine production from CD8 (E) and CD4 T cells (F). These data are representative
720 of two individual experiments with four to five mice per group. Statistical significance
721 was determined using one-way ANOVA and Bonferroni post test. *p< 0.05.
722

723 **FIGURE 2. CD11a drives optimal proliferation of responding CD8 T cells.** (A) Dot
724 plot and graph shows magnitude of the Ova-K^b specific CD8 T cell response in WT and
725 CD11a -/- mice in the spleen at D8 p.i.. (B) Data plot and graph show the magnitude of
726 Ova-K^b specific response in tetramer enriched splenocytes from at D4 p.i.. Each
727 representative dot plot and data point on the graph is representative of 3 pooled mice
728 from two individual experiments. (C) Histogram analysis and graphs of BrdU
729 incorporation among tetramer+ CD8 T cells (open histogram) or naïve (CD44^{low}) CD8 T
730 cells in individual spleens at D8 p.i.. Indicated numbers on dot plots and histograms

731 signify percentage of cells that are within each gated region of representative samples.
732 Data is representative of two or more individual experiments which included 4-5
733 individual mice per group (A, C). Student's t test was used to determine statistical
734 significance. *p<0.05.

735

736 **FIGURE 3. Normal effector functions of responding CD8 T cells in CD11a
737 deficient mice.** (A) Splenocytes from D8 infected mice were isolated and directly
738 stained for granzyme B expression or restimulated *in vitro* for 5 hours with SIINFEKL
739 before analysis of LAMP-1 (CD107a). Bar graphs show granzyme expression among
740 Ova-K^b specific CD8 T cell and LAMP expression among IFN γ producing CD8 T cells.
741 (B) In vitro killing activity of CD11a-/ and WT CD8 T cells. Each line represents one
742 mouse. (C) Representative dot plots of TNF α producers (bottom panel and bar graph)
743 gated on CD8 T cells producing IFN γ (top panel) after *in vitro* restimulation with peptide.
744 (D) Tetramer binding decay assay of Ova-K^b specific CD8 T cells of D8 p.i. splenocytes.
745 Data is representative of combined results of two individual experiments with a total of
746 9-10 mice per group. Half life of Ova-K^b tetramer binding ($T_{1/2}$) is defined as the amount
747 of time required to lose 50% of the maximal tetramer binding. Mean $T_{1/2}$ is indicated for
748 each group on the graph. Indicated numbers on dot and zebra plots signify percentage
749 of cells that are within each gated region of representative samples.

750

751 **FIGURE 4. CD11a regulates effector CD8 T cell differentiation.** (A) KLRG1 and
752 CD127 expression was used to subdivide Ova-K^b specific CD8 T cells into EEC
753 (KLRG1^{low}CD127^{low}), MPEC (KLRG1^{low}CD127^{hi}), DPEC (KLRG1^{hi}CD127^{low}) and SLEC

754 (KLRG1^{hi}CD127^{low}). Representative zebra plots and bar graphs show CD62L versus
755 PD-1 expression either among total Ova-K^b specific CD8 T cells (B) or within specific
756 effector subsets (C) in splenocytes of D8 infected mice. (D) Histogram displays CD25
757 expression of antigen-specific CD8 T cells in tetramer enriched splenocytes of D4
758 infected mice. Splenocytes from 3 mice were pooled per sample with a total of 12 mice
759 per group. Bar graphs show frequency and mean fluorescence intensity (MFI) of CD25
760 expression in total tetramer enriched CD8 T cells. Indicated numbers on zebra plots
761 and histograms signify percentage of cells that are within each gated region of
762 representative samples. Data is representative of two or more experiments. Student's t
763 test was used for statistical analysis. *p<0.05.

764

765 **FIGURE 5.** Normal localization of Ova-K^b specific CD8 T cells in CD11a-/ mice after
766 LM infection. Thick sections of spleen were stained with Ova-K^b tetramer and
767 antibodies to other other surface markers to indicate B (B220) and T cell zones (CD8)
768 and CD31 to identify blood vessels. Images of uninfected spleens from WT mice (A)
769 were acquired with a 20X 0.75 numerical aperture (NA) objective. Infection with 1x10⁶
770 CFU ActA-/ LM-Ova was used to assess the early T cell response (D5 p.i.) in WT (B)
771 and CD11a-/ (C) mice. Sections from spleens of WT (D) and CD11a KO (E) mice that
772 had been infected with 1x10³ CFU LM-Ova 8 days prior. B=B cell zone; PALS=peri-
773 arteriolar lymphoid sheath (T cell zone); RP=red pulp; CA=central arteriole.

774

775 **FIGURE 6. CD11a requirement for CD8 T cell activation is cell intrinsic.** Chimeric
776 mice reconstituted with a mixture of bone marrow from WT and CD11a-/ mice were

777 infected with 1×10^3 CFU LM-Ova and assessed for T cell responses in the spleen 8
778 days post infection. Bar graphs display overall frequency of antigen specific T cell
779 responses (A) and differentiation among responding cells (B). Splenocytes were used
780 for *in vitro* peptide restimulation to determine the frequency of cells producing TNF α
781 among IFN γ CD8 T cells. Data is representative of two individual experiments with 4
782 chimeric mice per experiment.

783

784 **FIGURE 7. Memory CD8 T cell reactivation is CD11a independent.** (A) Graph
785 shows the kinetics of Ova-K b specific CD8 T cells in the peripheral blood at various time
786 points after infection with 1×10^3 CFU of LM-Ova. Representative dot plots show
787 staining of Ova-K b specific CD8 T cells (B) and KLRG1/CD127 expression (C) among
788 Ova-K b specific CD8 T cells in peripheral blood 70 days post infection. (D) CD62L
789 expression among Ova-K b specific MPEC (KLRG1 low CD127 hi) at D70 in the peripheral
790 blood. Memory mice were challenged with 1×10^4 CFU of LM-Ova and recall responses
791 were assessed in the blood before and after recall (E). (F-I) Splenocytes were
792 harvested six days post recall to determine the frequency of Ova specific CD8 T cells
793 (F) or SLEC/ MPEC differentiation of antigen-specific CD8 T cells (G). (H) Bar graphs
794 represent CD62L expression in total Ova-K b specific CD8 T cells or among the MPEC
795 population. (I) *In vitro* restimulation of recalled splenocytes was used to assess the
796 frequency of TNF α producers (gated first on CD8 T cells producing IFN γ). Indicated
797 numbers on dot plots and histograms signify percentage of cells that are within each
798 gated region of representative samples. These data are representative of two individual
799 experiments with a total of 5 mice per group.













