Leishmania major induces differential expression of costimulatory molecules on mouse epidermal cells

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Levels of expression of costimulatory molecules have been proposed to influence the outcome of antigen-specific T cell priming. We found that *Leishmania major* selectively modulated the expression of costimulatory molecules on various populations of epidermal cells. B7.2 expression was down-regulated on Thy1.2$^+$ epidermal cells (keratinocytes) from disease-resistant C3H mice, but not from disease-susceptible BALB/c mice. In addition, epidermal cells from BALB/c mice showed a down-regulation of B7.1 expression on NLDC 145$^+$ Langerhans cells. *In vitro* T cell priming experiments, using syngeneic epidermal cells as antigen-presenting cells (APC), showed that the production of IFN-γ was inhibited when either B7.1 or B7.2 signaling pathways were blocked. Blockade of B7.2, but not B7.1, significantly inhibited the ability of epidermal cells to induce IL-4 production from CD4$^+$ T cells. In addition, C3H CD4$^+$ T cells, which were unable to secrete detectable levels of IL-4 in cultures with syngeneic APC, were now able to secrete IL-4 following presentation of *L. major* antigens by congenic BALB/K epidermal cells. Conversely, C3H epidermal cells supported the priming of BALB/K CD4$^+$ T cells for IL-4 production *in vitro*. Thus, the differential expression of B7 molecules on epidermal cells may not represent the sole factor governing the polarization of *L. major*-specific CD4$^+$ T cells *in vitro*.

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1 Introduction

The protozoan parasite *Leishmania major* induces cutaneous leishmaniasis. It exists as a flagellated promastigote in its insect vector, the sand fly. The vertebrate host becomes infected with *L. major* when the sand fly probes into the skin for a blood meal and injects parasites. Promastigotes are taken up by phagocytic cells, and within these cells they transform into amastigotes (reviewed in [1]).

In mice infected with *L. major*, selective activation of Th1 or Th2 cells occurs (reviewed in [2–5]). In resistant mice (*e.g.* C57BL/6, C3H, CBA), IFN-γ is the principal mediator of resistance to *L. major* due to its ability to activate macrophages to destroy the parasite [6–9]; treating mice with a neutralizing anti-IFN-γ Ab exacerbates the course of infection by promoting the outgrowth of Th2 cells [10, 11]. In susceptible mice (BALB/c), IL-4 can block the ability of IFN-γ to activate macrophages to destroy *Leishmania* [12, 13]. Administration of neutralizing anti-IL-4 Ab allows BALB/c mice to cure their infection by promoting the outgrowth of Th1 cells [14, 15]. Importantly, to be effective, Ab treatment must be initiated within the first week of infection, suggesting that events occurring early in the priming of CD4$^+$ T cells lead to commitment to either a Th1 or a Th2 phenotype [2–5].

Both T cell and non-T cell compartments (*e.g.* accessory cells) can be involved in determining disease outcome in mice infected with *L. major* [16]. T cell activation requires a minimum of two signaling events. The first signal is delivered by the MHC/Ag complex interacting with the TCR and is Ag specific. The second signal, also called a costimulatory signal, is crucial for complete cellular activation [17–19]. The levels of expression of costimulatory molecules, as well as the strength and affinity of the interaction between these molecules and their co-receptors on T cells have been proposed to influence the outcome of Ag-specific T cell priming [20–22]. Costimulatory molecules such as B7 and CD40 have been shown to be crucial to the outcome of *L. major* infection [23–27]. However, evidence associating differential expression of B7 and/or CD40 costimulatory molecules on professional APC with T cell cytokine profiles in disease-resistant and -susceptible mice remains to be demonstrated.

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Abbreviations: LC: Langerhans cells **Lm:** *Leishmania major* **MFI:** Mean fluorescence intensity

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Because the skin represents the primary target organ of *L. major*, epidermal APC, such as Langerhans cells (LC), are likely to play a major role in the initiation of an immune response against *L. major*. In fact, previous studies have shown that epidermal LC, but not keratinocytes, present *L. major* antigens to T cells [28–30]. Furthermore, a recent study demonstrated that ingestion of *L. major* amastigotes by LC-like immature dendritic cells derived from fetal skin resulted in an up-regulation of the expression of various accessory molecules including B7.1, B7.2, CD40, ICAM-1 and MHC class II on LC, but not on macrophages [31]. Previous studies have also demonstrated that B7 costimulation is required for the development of an early immune response in BALB/c and C3H mice infected with *L. major* [27]. Furthermore, this study showed that costimulation involving B7.1 or B7.2 did not lead to the activation of Th1 or Th2 cells, respectively; rather, both B7.1 and B7.2 could costimulate for Th1- or Th2-associated cytokines, depending on the level of expression of these costimulatory molecules [27]. Thus, levels of B7.1 and B7.2 expression on APC in the skin could represent a crucial factor to the outcome of T cell priming in *L. major*-infected mice.

In this study, we hypothesized that *L. major* can mediate differential expression of accessory molecules on epidermal cells from disease-resistant and disease-susceptible mice, and this, in turn, will influence the subsequent cytokine pattern elicited *in vitro* by Ag-specific $CD4^+$ T cells. We found that B7.1 and B7.2 costimulatory molecules differentially modulate the outcome of cytokine production by *L. major*-specific $CD4^+$ T cells. Our results also suggest that, although a differential expression of B7 molecules was observed on epidermal cells from BALB/c and C3H mice, additional factors including the $CD4^+$ T cell compartment are likely to regulate the polarization of *L. major*-specific T cells.

2 Results

2.1 *L. major* differentially influences B7.1 and B7.2 expression on BALB/c and C3H epidermal cells

We first determined whether BALB/c and C3H epidermal cells engulf *L. major* promastigotes equally. The infection rate of BALB/c and C3H epidermal cells with *L. major* promastigotes was approximately the same, with low numbers (5–11%) of LC, but not keratinocytes, harboring the parasites; this finding is in agreement with previously published reports [28–30].

Resistance and susceptibility to *L. major* infection is associated with the production of distinct cytokine profiles by $CD4^+$ T cells [2–5], and those cytokine profiles can be reversed by blocking costimulatory molecules [23, 24] *in vivo*. Because LC from the skin can take up *L. major* (shown here and in [28–30]) and present *L. major* antigen to T cells *in vitro* [30], we hypothesized that one factor leading to selective cytokine production is differential expression of costimulatory molecules (*e.g.* B7 or CD40) on *L. major*-infected epidermal APC *in vitro*. Therefore, we compared levels of expression of various costimulatory molecules on LC-enriched epidermal cells derived from disease-resistant C3H mice and disease-susceptible BALB/c mice in the presence or absence of *L. major*.

Expression of accessory molecules on epidermal cells was analyzed by flow cytometry after 3 days in culture. This time point was chosen for two reasons. First, LC migrate to the draining lymph nodes within 3 days following antigenic stimulation [32, 33]. Second, murine LC undergo maturation within 3 days in culture, a stage at which peak levels of accessory molecule expression occurs (Fig. 1 and [34–37]). Thus, this protocol allowed us to compare levels of accessory molecule expression on LC at a time when high T cell stimulatory capacity was probable.

We found that *L. major* differentially regulates B7.1 and B7.2 on epidermal cells from BALB/c and C3H mice

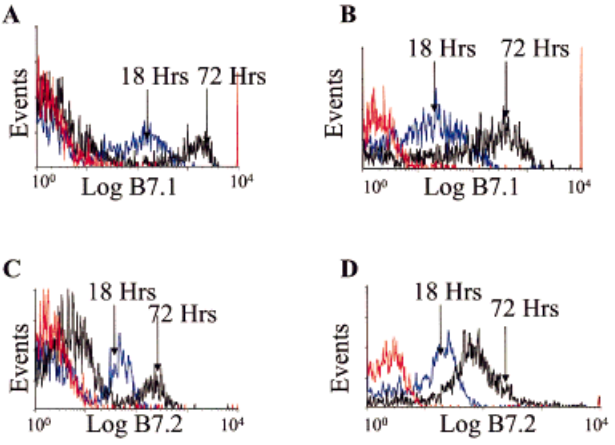

Fig. 1. Up-regulation of B7 molecules on epidermal cells cultured in the absence of *L. major*. Epidermal cells from (A, C) BALB/c and (B, D) C3H mice were analyzed for B7.1 using PE-conjugated anti-B7.1 and B7.2 expression using FITC-conjugated anti-B7.2 at 18 h (blue lines) and 72 h (black lines). Red lines represent isotype control Ab. For clarity, only isotype control Ab using cells cultured without *L. major* are shown. Similar results were obtained when epidermal cells cultured with *L. major* were stained with the same isotype control Ab. The data are representative of three experiments.

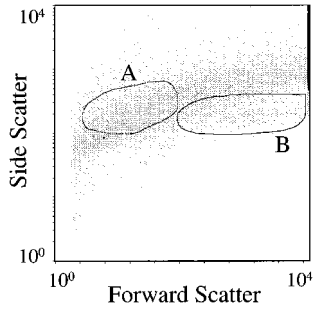

Fig. 2. Light scatter gates of NLDC 145 and Thy1.2$^+$ BALB/c epidermal cells. Epidermal cells were double-stained with NLDC-145 and anti-Thy1.2 mAb. The gates shown enclose NLDC 145$^+$ (A) and Thy1.2$^+$ (B) epidermal cells. No NLDC 145$^+$/Thy1.2 double-positive cells were identified. The light scatter gates shown here were used in subsequent experiments to enclose populations containing NLDC 145$^+$ and Thy1.2$^+$ cells.

(light scatter gates of the different epidermal cell populations are shown in Fig. 2). Both B7.1 and B7.2 were equally up-regulated on NLDC 145$^+$ epidermal cells (LC) from C3H mice in the presence or absence of *L. major* (Fig. 3A, B; *p* values in each figure were greater than 0.2). However, *L. major* induced a selective down-regulation of B7.1 expression on BALB/c NLDC 145$^+$ cells [Fig. 4A; mean fluorescence intensity (MFI) of 140±41.2 in the absence of *L. major* compared to 79±28.8 in the presence of *L. major*, p=0.05]. In contrast, B7.2 expression on BALB/c NLDC 145$^+$ was not affected in cultures containing *L. major* (Fig. 4B). Our results also showed that *L. major* differentially regulates the expression of B7.1 and B7.2 on Thy1.2$^+$ epidermal cells (*e.g.* keratinocytes and dendritic epidermal T cells). B7.2 expression on Thy-1.2$^+$ C3H epidermal cells was down-regulated in the presence of *L. major* (Fig. 3D; MFI of 36.9±2.87 in the absence of *L. major* compared to a MFI of 17.1±1.2 in the presence of *L. major*, p=0.03). *L. major* did not alter

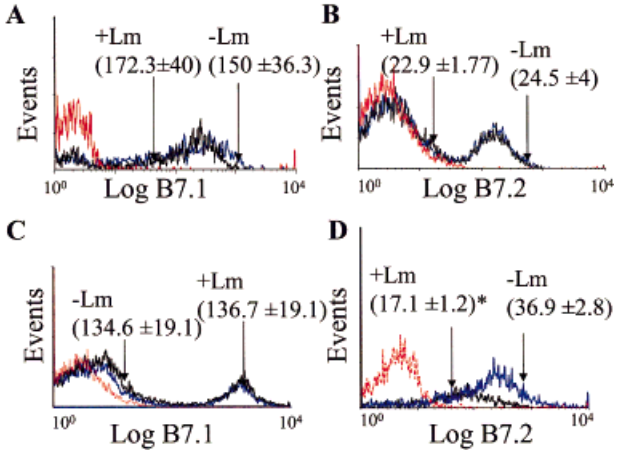

Fig. 3. L. major selectively down-regulates B7.2 expression on Thy1.2$^+$ epidermal cells from C3H mice. Epidermal cells were isolated and cultured with (black lines) or without (blue lines) *L. major* (Lm) promastigotes and analyzed by flow cytometry 3 days later by gating on (A, B) NLDC 145$^+$ or (C, D) Thy-1.2$^+$ C3H epidermal cells predetermined by light scatter. Red lines represent isotype control Ab. Numbers in parentheses indicate MFI ± SD from three experiments. The data are representative of three experiments. *p=0.03.

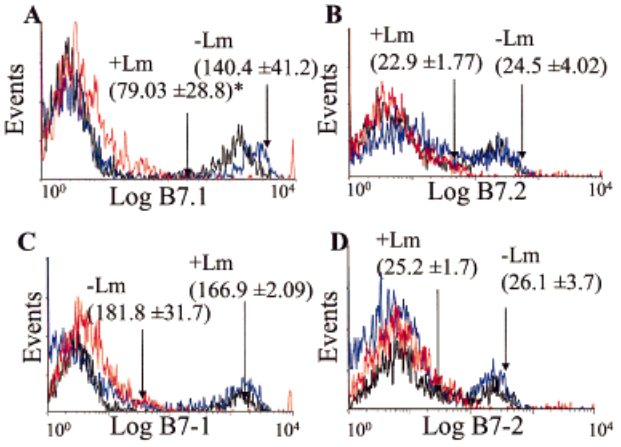

Fig. 4. L. major selectively down-regulates B7–1 expression on BALB/c NLDC 145$^+$ LC. Epidermal cells were isolated and cultured with (black lines) or without (blue lines) *L. major* (Lm) promastigotes and analyzed by flow cytometry 3 days later by gating on (A, B) NLDC 145$^+$ or (C, D) Thy-1.2$^+$ BALB/c epidermal cells predetermined by light scatter. Red lines represent isotype control Ab. Numbers in parentheses indicate MFI ± SD from three experiments. The data are representative of three experiments. *p=0.05.

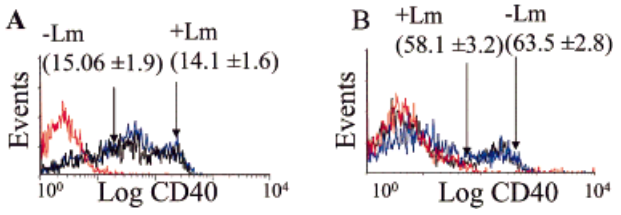

Fig. 5. Equal up-regulation of CD40 expression on NLDC 145$^+$ epidermal cells derived from BALB/c and C3H mice in the presence or absence of *L. major*. Epidermal cells derived from (A) C3H and (B) BALB/c mice were cultured in the presence (black lines) or absence (blue lines) of *L. major* (Lm) and CD40 expression was analyzed 3 days later. Light scatter gates were predetermined to enclose NLDC 145$^+$ cells. Red lines represent isotype control Ab. Numbers in parentheses indicate intensity MFI ± SD from three experiments. The data are representative of three experiments.

the expression of B7.1 and B7.2 molecules on Thy1.2$^+$ BALB/c epidermal cells (Fig. 4C, D), as differences in the MFI values were not statistically significant ($p>0.2$). These results demonstrate that *L. major* differentially regulates the expression of B7.1 and B7.2 on disease-resistant and disease-susceptible mice, and that the target populations of epidermal cells were not limited to LC.

2.2 CD40 expression is equally up-regulated on C3H and BALB/c epidermal cells in the presence or absence of *L. major*

In the mouse, CD40-CD40L-mediated IL-12 production [38] was shown to play a crucial role in the elicitation of a protective Th1 response during infection with *L. major* [25, 26]. Therefore, we analyzed levels of CD40 expression on C3H and BALB/c epidermal cells. MFI values of CD40 expression on NLDC 145$^+$ epidermal cells were not statistically significant in both strains of mice after 3 days of culture, regardless of the presence or absence of *L. major* (Fig. 5A, B; $p>0.2$).

2.3 Roles of B7 molecules expressed on epidermal cells in the production of *L. major*-specific cytokines

When naive BALB/c CD4$^+$ T cells were primed *in vitro* with LC-enriched epidermal cells in the presence of *L. major*, they produced both IL-4 and IFN-γ (Fig. 6, 7). In contrast, CD4$^+$ T cells from C3H mice co-cultured with syngeneic LC-enriched epidermal cells and *L. major* secreted IFN-γ, but not IL-4 (Fig. 7). This CD4$^+$ T cell response was antigen specific, in that no or low levels of IL-4 or IFN-γ were observed when APC alone, or *L. major* and T cells alone, were co-cultured (Fig. 6, 7). Moreover, this primary CD4$^+$ T cell response occurred in the absence of macrophages because all MHC class II$^+$ epidermal cells co-stained for NLDC 145 as assessed by flow cytometry (data not shown). Thus, these observations argue against an absolute requirement of macrophages to release amastigotes for LC to prime *L. major*-specific T cells.

Addition of neutralizing anti-B7.2, but not anti-B7.1, mAb significantly inhibited IL-4 production by BALB/c CD4$^+$ T cells (Fig. 6). In contrast, significant inhibition of IFN-γ production by BALB/c or C3H CD4$^+$ T cells was achieved when either anti-B7.1 or anti-B7.2 mAb were present in the cultures; this reduction was enhanced in the presence of both mAb (Fig. 7). These results suggest that the differential expression of B7 molecules on epidermal cells may represent one factor involved in outcome of CD4$^+$ T cell priming by LC.

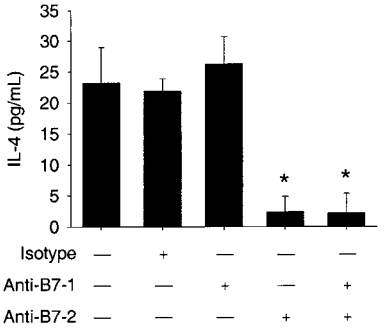

Fig. 6. B7.2, but not B7.1, on BALB/c epidermal cells costimulates for IL-4 production by *L. major*-specific CD4$^+$ T cells *in vitro*. Epidermal cells were cultured with *L. major*. Purified CD4$^+$ T cells were added to cultures (ratio APC:CD4$^+$ T cells: 1:10), and culture supernatant taken 5 days later. Neutralizing anti-B7.1 (clone 1G10) and/or anti-B7.2 (clone GL1) were added at 50 μg/ml. Equal amounts of irrelevant rat IgG antibodies were added to the cultures as negative control. Levels of IL-4 in the culture supernatants were determined by ELISA. No IL-4 was detected in supernatants collected from cultures containing epidermal cells and CD4$^+$ T cells without *L. major*, or from cultures containing CD4$^+$ T cells and *L. major* without epidermal cells. The data represent means ± SD from four experiments. *$p<0.05$. Note that no IL-4 was detected using C3H cells.

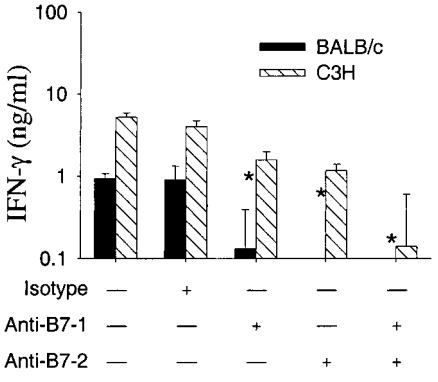

Fig. 7. B7.1 and B7.2 on C3H and BALB/c epidermal cells costimulate for IFN-γ production by *L. major*-specific CD4$^+$ T cells *in vitro*. Primary *in vitro* responses were carried out as described in Fig. 6. IFN-γ levels in culture supernatants collected 5 days after addition of CD4$^+$ T cells were measured by ELISA. Black bars represent BALB/c cells and hatched bars represent C3H cells. IFN-γ background levels in culture supernatants collected from cultures containing epidermal cells and CD4$^+$ without *L. major* (ranging from 0.44 to 0.9 ng/ml) were subtracted from the indicated IFN-γ levels. IFN-γ levels in culture supernatants containing CD4$^+$ T cells and *L. major* without epidermal cells were below background levels (ranging from 0.15 to 0.22 ng/ml). The data represent means ± SD from four experiments. *$p<0.05$.

Table 1. *In vitro* production of IL-4 using a congenic C3H-BALB/K mouse model[a)]

APC-T cells	IL-4 (pg/ml)				
	No Ab	Isotype	anti-B7.1	anti-B7.2	anti-B7.1 + anti-B7.2
C3H-C3H	0	0	0	0	0
C3H-BALB/K	42.6 ± 5.1	35.2 ± 3.4	45.8 ± 6.2	10.4 ± 2.2*	3.1 ± 0.75*
BALB/K-BALB/K	52.1 ± 3.8	55.7 ± 4.3	59 ± 6.8	8.6 ± 3.5*	2.4 ± 0.65*
BALB/K-C3H	69 ± 4.3	55 ± 7.4	58.8 ± 7.1	6.7 ± 1.2*	2.9 ± 0.32*

a) The cultures were set up as described in Fig. 6 and Sect. 4. The data represent means ± SD from three experiments. * $p < 0.05$.

Table 2. *In vitro* production of IFN-γ using a congenic C3H-BALB/K mouse model[a)]

APC-T cells	IFN-γ (ng/ml)				
	No Ab	Isotype	anti-B7.1	anti-B7.2	anti-B7.1 + anti-B7.2
C3H-C3H	5.8 ± 1.3	4.1 ± 1.8	2.8 ± 0.5*	1.9 ± 0.78*	0.29 ± 0.1*
C3H-BALB/K	4.6 ± 2.3	5.3 ± 1.7	3.2 ± 1.1*	2.5 ± 1.05*	0.35 ± 0.2*
BALB/K-BALB/K	6.9 ± 1.7	6.3 ± 2.2	3.4 ± 1.4*	2.9 ± 1.5*	0.28 ± 0.17*
BALB/K-C3H	5.7 ± 2.3	6.1 ± 1.8	2.8 ± 0.8*	3.3 ± 1.9*	0.4 ± 0.18*

a) The cultures were set up as described in Fig. 7 and Sect. 4. The data represent means ± SD from three experiments. * $p < 0.05$.

2.4 *In vitro* cytokine production using a BALB/K-C3H congenic mouse model

To determine whether factors other than B7 expression on epidermal cells influence cytokine production by $CD4^+$ T cells, we used a congenic BALB/K (disease-susceptible)-C3H (disease-resistant) model to analyze IL-4 and IFN-γ production *in vitro*. As shown in Table 1, epidermal cells from C3H mice were able to prime BALB/K $CD4^+$ T cells for IL-4 production. Conversely, BALB/K epidermal cells supported the production of IL-4 by $CD4^+$ T cells from C3H cells. In all cases, anti-B7.2, but not anti-B7.1 mAb, significantly inhibited the generation of IL-4 *in vitro*. These results suggest that costimulation for IL-4 production by $CD4^+$ T cells *in vitro* may be regulated by more than costimulatory molecules on epidermal cells since BALB/K T cells produced IL-4 when activated by either BALB/K T cells or C3H epidermal cells.

The production of IFN-γ was also assessed in the congenic model described above. Both C3H and BALB/K epidermal cells were able to prime $CD4^+$ T cells from BALB/K and C3H mice, respectively (Table 2). Consistent with the data reported in the syngeneic model (Fig. 7), the generation of IFN-γ was dependent on both B7.1 and B7.2 molecules, because addition of anti-B7.1 or anti-B7.2 to the cultures significantly inhibited the secretion of IFN-γ (Table 2).

3 Discussion

The T cell cytokine profile elicited in the host represents a crucial factor in determining disease outcome in mice infected with *L. major* [2–5]. During primary T cell responses, the pattern of cytokines elicited by Ag-specific T cells may be regulated, at least in part, by the strength and affinity of the interaction between accessory molecules and their coreceptors on T cells [19, 20, 22]. Therefore, the levels of expression of accessory molecules on APC able to initiate a primary immune response could represent a crucial factor in determining the outcome of T cell priming. Because the skin represents the first target organ during infection with *L. major*, APC in the skin are likely to play important roles in the initiation of an immune response against *L. major* antigens.

We found that *L. major* selectively and differentially altered the expression of B7.1 and B7.2 on various epidermal cell populations derived from BALB/c and C3H mice (a summary of these findings is presented in

Table 3). On BALB/c epidermal cells, B7.2 expression was equally up-regulated on Thy-1.2$^+$ and NLDC-145$^+$ cells in the presence or absence of *L. major*, whereas B7.1 expression was down-regulated on NLDC 145$^+$ cells (Fig. 4A). Furthermore, the B7.2 signaling pathway on BALB/c epidermal cells appeared to be involved in IL-4 production by *L. major*-specific CD4$^+$ T cells, because addition of neutralizing anti-B7.2, but not anti-B7.1, mAb significantly reduced the levels of IL-4 produced *in vitro* (Fig. 6). These results suggest that costimulation via B7.2 on LC could promote the production of IL-4 in susceptible mice infected with *L. major*. This hypothesis is supported by previous findings showing that treatment of BALB/c mice with neutralizing anti-B7.2 mAb dramatically reduced the levels of IL-4 produced in the lymph nodes draining leishmanial lesions, and enhanced resistance to *L. major* infection [24]. Likewise, Corry et al. [23] reported that treatment of BALB/c mice with CTLA4Ig within the first week of infection completely abrogated progressive disease, suggesting that the priming of Th2 cells is more dependent upon CD28-B7 pathway than the priming of Th1 cells. However, other studies have demonstrated that CD28$^{-/-}$ BALB/c mice exhibit normal susceptibility to *L. major* infection and generated high levels of IL-4 production [27, 39]. These observations suggest that either compensatory pathways may have driven the production of Th2 cytokine in these mice, or that alternative B7 ligands could be involved in the costimulation for cytokine production.

Disease-resistant C3H mice showed an equal up-regulation of B7.1 and B7.2 on epidermal LC in the presence or absence of *L. major* (Fig. 3A, B). When C3H CD4$^+$ T cells were primed *in vitro* with epidermal cells pulsed with *L. major*, they produced detectable amounts of IFN-γ but no IL-4. Optimal production of IFN-γ required both B7.1 and B7.2 pathways, since a significant reduction in the levels of IFN-γ production was observed when neutralizing anti-B7.1 or anti-B7.2 mAb were present in cultures (Fig. 7).

Table 3. Differential expression of B7 molecules on epidermal cells induced by *L. major in vitro*

	BALB/c		C3H	
	NLDC 145	Thy1.2[a)]	NLDC 145	Thy1.2
B7.1	* ↓	↔	↔	↔
B7.2	↔	↔	↔	** ↓
CD40	↔	ND	↔	ND

a) Freshly isolated epidermal cells were co-cultured with or without *L. major* promastigotes for 3 days as described in Materials and Methods. Expression of B7.1, B7.2 or CD40 on epidermal cells in culture with or without *L. major* was compared by flow cytometry by gating on light scatter gates predetermined to contain NLDC 145+ or Thy1.2+ cells. Downward arrows indicate down-regulation of the expression of the indicated molecules (* $P = 0.05$; ** $P = 0.03$); horizontal arrows indicate no *change.*

Overall, these observations point to a complex regulation of Th1 and Th2 cytokines by CD4$^+$ T cells primed with *L. major*-pulsed LC. Although the B7.2 signaling pathway appears to promote the production of IL-4 by BALB/c T cells, the observation that BALB/c LC down-regulated the expression of B7.1 in the presence of *L. major* raises a possible involvement of B7.1 in the overall promotion of a Th2 response. This is likely to occur indirectly, as a result of a decreased production of IFN-γ, thus allowing the expansion of Th2 secreting T cells [2–5]. This hypothesis would be consistent with our findings showing that (1) IFN-γ production required both B7.1 and B7.2 pathways, and (2) BALB/c LC, but not C3H LC, showed a decreased level of B7.1 expression in the presence of *L. major* promastigotes. Furthermore, this hypothesis would also be consistent with a recent report showing that costimulation via B7.1 or B7.2 can lead to either Th1 or Th2 cytokine production, depending on the levels of the expression of these costimulatory molecules [27]. Finally, we also found in some experiments that inhibition of B7.1 signaling pathway using mAb led to a significant increase in IL-4 production by BALB/c CD4$^+$ T cells following stimulation with syngeneic epidermal cells (data not shown). These findings further support an indirect role for B7.1 in the induction of IL-4 production in our model.

To determine whether more than B7 costimulation on epidermal cells regulates cytokine production by CD4$^+$ *in vitro*, we used a congenic C3H-BALB/K model. We found that BALB/K T cells secreted IL-4 whether stimulated with *L. major*-pulsed BALB/K or C3H epidermal cells (Table 1). This suggests that a factor(s) in T cells in addition to B7 costimulation influences T cell development and cytokine secretion. For example, it is possible that, in addition to B7 expressed on epidermal cells, B7 molecules expressed on neighboring T cells could influence the secretion of IL-4 by *L. major*-specific CD4$^+$ T cells. It has been recently shown that B7.1 expressed on T cells can inhibit IL-4 production [40]. Moreover, the authors showed that levels of IL-4 produced regulate B7.1 expression on T cells. Thus, B7.1 expressed on bystander CD4$^+$ T cells could well be involved in the outcome of *L. major*-specific CD4$^+$ T cell priming by epidermal cells.

It should be noted that other factors are likely to be involved in the priming of IL-4- and IFN-γ-secreting CD4$^+$ T cells by LC. CD40-CD40L-mediated IL-12 production represents another pathway that can control the balance of Th1/Th2 responses [25, 26, 38]. This was demon-

strated in studies showing that CD40-deficient mice on a resistant background were unable to control infection with *L. major* [25, 26]. Thus, we compared levels of CD40 expression on BALB/c and C3H epidermal cells. Both strains of mice showed up-regulation of CD40 expression on NLDC 145^+ cells to equal maximum levels in the presence or absence of *L. major*. However, a broader range of CD40 expression was observed on C3H cells compared to BALB/c cells (Fig. 5). It is unclear how this difference in the population of $CD40^+$ epidermal cells impacts the outcome of T cell priming, but further study to examine this issue is warranted.

The selective down-regulation of B7.2 on Thy-1.2^+ positive C3H epidermal cells (Fig. 3D) argues for potential important roles played by Thy-1.2^+ epidermal cells, such as keratinocytes, in cutaneous leishmaniasis. Since keratinocytes do not migrate to lymph nodes draining the lesions, and do not present *L. major* antigens to T cells [28–30], the possibility that these cells participate as bystander APC in the secondary T cell responses *in situ* can not be excluded. This hypothesis is supported by previous findings demonstrating that maximal proliferation of memory murine $CD4^+$ T can be achieved via B7 costimulation delivered by bystander APC [41].

Epidermal cells displaying differential changes in B7 expression did not have to internalize the parasites, since we showed that Thy-1.2^+ epidermal cells differentially expressed B7.2. Thus, it is likely that other factors, such as the balance of cytokines in the epidermal cell environment, could influence B7.1 and/or B7.2 changes. For example, it has been shown that B7.1 expression on murine LC is up-regulated by either GM-CSF, TNF-α, IL-1α, or IL-1β in a dose-dependent manner [42]. On the other hand, IL-10 production inhibits the expression of B7–2 on LC [43]. Thus, cytokines secreted by LC or by neighboring cells such as keratinocytes during *L. major* infection can determine, at least in part, accessory molecule expression on LC.

It has been shown recently that *L. major* amastigotes, but not promastigotes, affected levels of expression of B7.1, B7.2, CD40 and MHC class II molecules on LC derived from disease-resistant C57BL/6 fetal skin [31]. These results do not agree with our findings showing that *L. major* promastigotes could influence B7 expression on epidermal cells. This discrepancy could be explained by the fact that we used LC-enriched epidermal cells instead of pure LC cultures. LC-enriched epidermal cells pulsed with *L. major* promastigotes primed antigen-specific $CD4^+$ T cells, as measured by the release of IL-4 and IFN-γ *in vitro*. This occurred in the absence of macrophages, which argues against an absolute requirement of macrophages to release amastigotes for LC to prime *L. major*-specific T cells. Therefore, although LC engulfed a low number of promastigotes, it appears that this could represent a crucial step toward the initiation of an immune response against *L. major* antigens.

4 Materials and methods

4.1 Mice and parasites

Female BALB/c, BALB/K and C3H/HeJ mice 8–12 weeks old were purchased from the Jackson Laboratories (Bar Harbor, ME) or were bred in the animal facilities at the Department of Pathology, Colorado State University. Stationary phase *L. major* promastigotes (LV39, MRHO/Sv/59/P) were used. Parasites were maintained as previously described [44].

4.2 Isolation of epidermal LC

Epidermal cells were isolated from mouse ears by trypsinization as previously described [45, 46]. Low-density epidermal cells, obtained after fractionation with dense BSA columns, contained 10–40% NLDC 145^+ LC by flow cytometry. These cells are referred to as LC-enriched epidermal cells. The variable purity of LC (10–40%) did affect the levels of cytokine production observed in the *in vitro* priming of $CD4^+$ T cell experiments, with higher percentage of LC present in the cultures yielding greater amounts of cytokines secreted by $CD4^+$ T cells.

4.3 mAb

The following mAb were used: anti-mouse CD24 (heat-stable antigen, clone J11d, rat IgM, ATCC TIB-183), anti-mouse CD11b (Mac-1, clone M1/70, rat IgG2b, ATCC TIB-128), anti-mouse dendritic cell (clone 33D1, rat IgG2b; ATCC TIB-227), anti-mouse CD45R (B220, clone RA3–6B2, rat IgG2a), anti-mouse CD8 (clone H35–17.2, rat IgG2b) [34], anti-mouse MHC class II (clone N22, hamster IgG; ATCC HB-225).

Anti-mouse CD80 (B7.1, clone 1G10, rat IgG2a) and anti-mouse CD86 (B7.2, clone GL1, rat IgG2a) Ab were previously described [24]. Anti-mouse Fcγ II/III receptor (rat IgG2b, clone 2.4G2), PE-conjugated anti mouse CD4 (clone GK1.5), PE-conjugated anti-CD80 (clone 16–10A1, hamster IgG), anti-mouse CD90 (Thy1.2, clone 53–2.1, rat IgG2a) conjugated with PE or fluorescein isothiocyanate (FITC) and FITC-conjugated anti-CD86 (clone GL1, rat IgG2a) were purchased from PharMingen (San Diego, CA). Biotinylated anti-mouse CD40 (clone 3/23, rat IgG2a) and FITC-conjugated goat $F(ab')_2$ anti-rat IgG were purchased from Caltag Laboratories (Burlingame, CA). Anti-mouse LC (clone NLDC 145, rat IgG2a) was obtained from Bachem Bioscience, Inc. (King of Prussia, PA).

4.4 *In vitro* cytokine production

Freshly isolated LC-enriched epidermal cells were resuspended in DMEM (Gibco BRL) supplemented with 10% heat-inactivated FBS (Hyclone, Logan, UT), 0.1 mM nonessential amino acids (Gibco), 0.05 mM 2-mercaptoethanol, 10 mM Hepes (Gibco), 1% penicillin-streptomycin, 50 μg/ml gentamycin (Sigma, St. Louis, MO) and aliquoted into 96-well round-bottom plates (2×10^4 cells/well in a total volume of 100 μl) in the presence or absence of *L. major* promastigotes at a ratio 2:1 (*L. major*: epidermal cell) and incubated at 37°C for 3 days. The infection rate of epidermal cells was determined by incubating LC-enriched epidermal cells with a mixture of acridine orange (5 μg/ml) and ethidium bromide (50 μg/ml) as previously described [30].

Syngeneic splenic $CD4^+$ T cells were added to epidermal cell cultures (2×10^5) to a final volume of 200 μl/well, and incubation was continued for another 5 days. $CD4^+$ T cells were obtained from spleen cell populations by negative selection. Cells were incubated with a cocktail of mAb (J11d, B220, H35–17, M5/114, 33D1) followed by anti-rat IgG-coupled magnetic beads, and then passed through MiniMACS columns (Miltenyi Biotec, Auburn, CA). $CD4^+$ T cells were routinely enriched up to 94% as determined by flow cytometry. The enriched $CD4^+$ populations were not contaminated by APC because they did not express MHC class II, as determined by flow cytometric analysis (data not shown).

In some experiments, neutralizing anti-B7.1 (clone 1G10) and/or B7.2 mAb (clone GL1) were added (50 μg/ml each) to the cultures 30 min before adding $CD4^+$ T cells. The optimal concentration of neutralizing anti-B7.1 and/or B7.2 mAb was determined in pilot experiments in which various concentrations of neutralizing Ab were tested in their ability to inhibit IL-4 and IFN-γ production. Isotype-matched Ab were used as negative controls. Culture supernatants were collected 5 days later and analyzed for the presence of IL-4 and IFN-γ by ELISA.

4.5 Cytokine ELISA

Culture supernatants were assayed for IL-4 and IFN-γ by standard sandwich ELISA with published protocols [16] or manufacturer's instructions.

4.6 Flow cytometry

Cells were collected from *in vitro* cultures and washed in cold PBS-1%BSA, 0.05% sodium azide. For double staining with B7.1 and B7.2 mAb, cells were incubated with anti-mouse Fc receptor for 20 min, followed by PE-conjugated anti-B7.1 and FITC-conjugated anti-B7.2 mAb. All incubations were carried out on ice for 30 min.

To predetermine light scatter gates for NLDC-145^+ and Thy1.2^+ epidermal cells, separate samples were incubated in PBS with 5% FBS to block nonspecific sites, then stained with NLDC 145 followed by FITC-conjugated goat anti-rat IgG and PE-conjugated anti-Thy1.2 mAb. Cells were fixed with 1% paraformaldehyde in PBS and analyzed on a Coulter XL flow cytometer (Coulter Corp., Hialeah, FL). Dead cells and debris were excluded from analysis using a list-mode gate on forward and light scatter events. At least 10,000 events per sample were collected.

4.7 Statistical analysis

Significant differences between experimental groups were determined by ANOVA followed by all pairwise comparison *t* tests. *p* values of less than or equal to 0.05 were considered significant.

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