[illegible]

M. La[illegible] Mb[illegible], G[illegible] K. D[illegible] K[illegible] a[illegible] R[illegible] c[illegible] a[illegible] d G. T[illegible]

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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 *Le*[illegible] 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. *I*[illegible] *v*[illegible] T cell priming experiments, using syngeneic epidermal cells as antigen-presenting cells (APC), showed that the production of IFN-[illegible] 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. ma*[illegible] antigens by congenic BALB/K epidermal cells. Conversely, C3H epidermal cells supported the priming of BALB/K CD4$^+$ T cells for IL-4 production [illegible]. Thus, the differential expression of B7 molecules on epidermal cells may not represent the sole factor governing the polarization of *L. ma*[illegible]-specific CD4$^+$ T cells [illegible].

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K[illegible] d[illegible]: Rodent / Dendritic cell / Costimulatory molecule / Protozoan parasite

1 [illegible]

The protozoan parasite *Le*[illegible] induces cutaneous leishmaniasis. It exists as a flagellated promastigote in its insect vector, the sand fly. The vertebrate host becomes infected with *L. ma*[illegible] 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

[I 21025]

Abb[illegible]: **LC:** Langerhans cells **L[illegible]:** *Le*[illegible] *ma*[illegible] **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]. Fur-

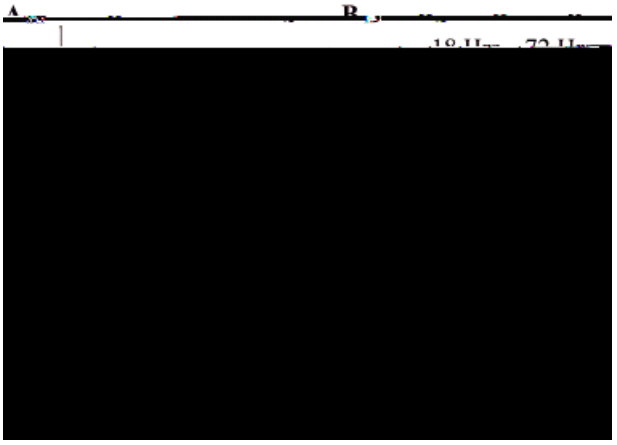
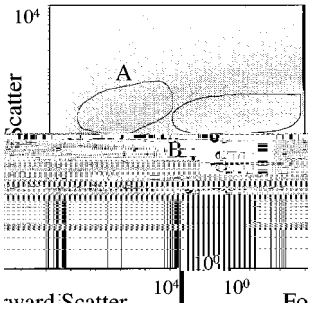
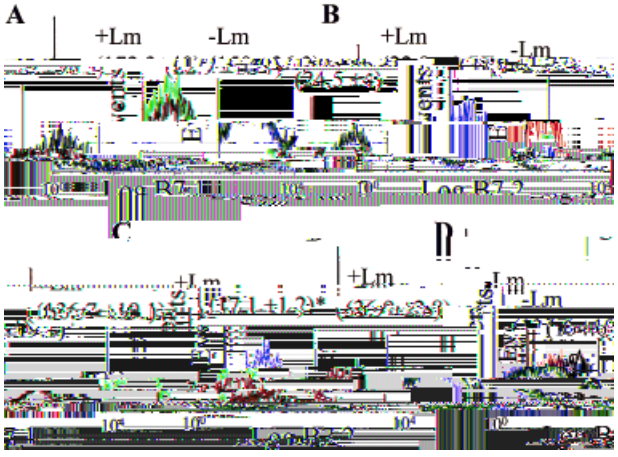


Figure Up-regulation of B7 molecules on epidermal cells cultured in the absence of *L. major*. Epidermal cells from (A, C) BALB/c and (B, D) C3H mice were analyzed for B7.1 using PE-conjugated anti-B7.1 and B7.2 expression using FITC-conjugated anti-B7.2 at 18 h (blue lines) and 72 h (black lines). Red 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.

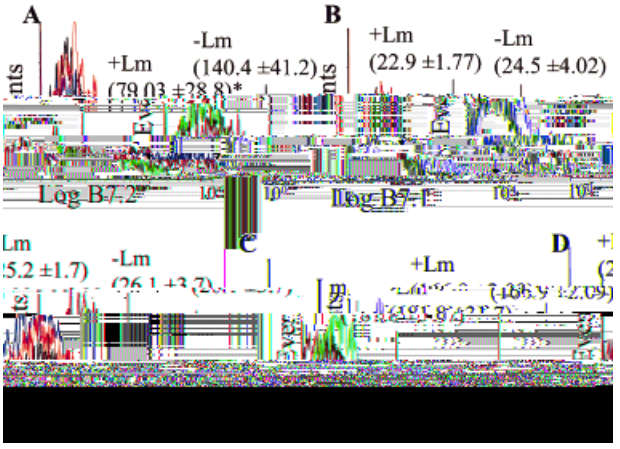


F Light scatter gates of NLDC 145 and Thy1.2$^+$ BALB/c epidermal cells. Epidermal cells were double-stained with NLDC-145 and anti-Thy1.2 mAb. The gates shown enclose NLDC 145$^+$ (A) and Thy1.2$^+$ (B) epidermal cells. No NLDC 145$^+$/Thy1.2 double-positive cells were identified. The light scatter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

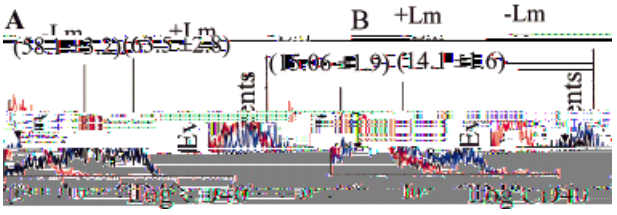
(light scatter gates of the different epidermal cell populations are shown in Fig. 2). Both B7.1 and B7.2 were equally up-regulated on NLDC 145$^+$ epidermal cells (LC) from C3H mice in the presence or absence of ***L. maj*** (Fig. 3A, B; ***p*** values in each figure were greater than 0.2). However, ***L. maj*** 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. maj*** compared to 79±28.8 in the presence of ***L. maj***, $p=0.05$]. In contrast, B7.2 expression on BALB/c NLDC 145$^+$ was not affected in cultures containing ***L. maj*** (Fig. 4B). Our results also showed that ***L. maj*** 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. maj*** (Fig. 3D; MFI of 36±9.2.87 in the absence of ***L. maj*** compared to a MFI of 17.1±1.2 in the presence of ***L. maj***, $p=0.03$). ***L. maj*** did not alter



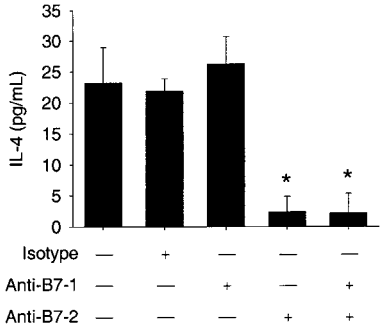
F selectively down-regulates B7.2 expression on Thy1.2$^+$ epidermal cells from C3H mice. Epidermal cells were isolated and cultured with (black lines) or without (blue lines) ***L. maj*** (Lm) promastigotes and analyzed by flow cytometry 3 days later by gating on (A, B) NLDC 145$^+$ or (C, D) Thy-1.2$^+$ 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. *$p=0.03$.



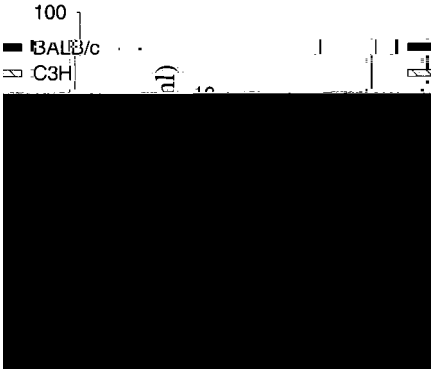
F selectively down-regulates B7–1 expression on BALB/c NLDC 145$^+$ LC. Epidermal cells were isolated and cultured with (black lines) or without (blue lines) ***L. maj*** (Lm) promastigotes and analyzed by flow cytometry 3 days later by gating on (A, B) NLDC 145$^+$ or (C, D) Thy-1.2$^+$ BALB/c epidermal 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. *$p=0.05$.



F 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. maj***. Epidermal cells derived from (A) C3H and (B) BALB/c mice were cultured in the presence (black lines) or absence (blue lines) of ***L. maj*** (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.



F B7.2, but not B7.1, on BALB/c epidermal cells costi- ***i***
mulates for IL-4 production by ***L. maj*** -specific $CD4^+$ T cells ***r***
i



Tab 1. 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. * $p < 0.05$.

Tab 2. production of IFN- using a congenic C3H-BALB/K mouse model[a)]

APC-T cells	IFN- (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 BALB/K-C3H

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- production . 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 . These results suggest that costimulation for IL-4 production by $CD4^+$ T cells 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- 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- 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- (Table 2).

3

The T cell cytokine profile elicited in the host represents a crucial factor in determining disease outcome in mice infected with *L. maj* [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 repre-

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 (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

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 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-¼IL-1¼or IL-1, 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-. 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 cells

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 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 Abs

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 Fc 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* culture of epidermal cells

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).

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