Expression of Costimulatory Molecules in Antigen-Activated Peritoneal Macrophages Treated with either Ovalbumin or Palmitoyl-Ova Conjugates

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ABSTRACT

One of the mechanisms by which adjuvants are believed to promote T-cell activation and prevent induction of oral tolerance is by up-regulating the expression of co-stimulatory molecules on antigen presenting cells. Mice treated orally with palmitoyl-ovalbumin conjugates become immunized, while those treated with native ovalbumin (Ova) become tolerant. Cells from the peritoneal cavity of B6D2F1 mice were cultured in the presence of 0.01, or 0.1 mg/100μl of either Ova, or palmitoyl-Ova and tested for the presence of cell markers. PE-conjugated anti-mouse CD80, CD86, and CD11b antibodies as well as biotin-PE were used to stain the antigen-activated peritoneal cells. A significant increase in the expression of CD86 and CD80 was observed following in vitro stimulation with palmitoyl-Ova; additionally, both Ova and palmitoyl-Ova induced the basal expression of CD11b. These findings could be related with the strong T-cell proliferative response induced by palmitoyl-Ova.

Key words: Ovalbumin, Palmitic Acid, Cellular Immunity, CD86, CD80, CD11b

INTRODUCTION

Although oral administration of antigens offers significant advantages over the conventional parenteral routes in vaccination protocols, oral administration usually results in an immune unresponsiveness known as "oral tolerance" (Titus and Chiller 1981). Various strategies, such as the local production of secretory IgA and systemic immunization, have been examined to avoid the tolerance and to achieve stimulation. A complex interplay of mechanisms, including clonal deletion, clonal anergy, cytokine regulation and specific features of the mucosal tissues appears to govern the induction of oral tolerance (Friedman

and Weiner 1994). Several novel molecular mechanisms regulating oral tolerance have been proposed: active immune suppression by CD4+, CD25+Foxp3+ regulatory T cells that encompass self-reactive thymic-derived naturally occurring Tregs, and inducible Tregs generated from antigen-specific naïve CD4+CD25-Foxp3- T cells in peripheral areas under certain environmental conditions (Curotto de Lafaille and Lafaille 2009; Zhou et al. 2009). Thus, it is necessary to consider the differences in the nature and the dose of the antigenic stimulus, the involvement of specialized lymphoid structures and accessory cells in the host, and the activation of different antigen-induced populations of Tregs.

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One of the mechanisms by which adjuvants are assumed to promote the T-cell activation and prevent the induction of oral tolerance is through the upregulation of the expression of costimulatory molecules on antigen presenting cells (APCs), especially those belonging to the B7-28 family (Williansom et al. 1999). The classical B7-CD28 pathway is associated with two ligands, B7-1/CD80 and B7-2/CD86, on APCs and at least two receptors, CD28 and cytotoxic T-lymphocyte antigen 4, on T cells (Greenwald et al. 2005).

In an earlier studies, the nature of a soluble protein antigen (ovalbumin; Ova) was changed by coupling it to palmitoyl residues, which showed that this procedure did not interfere with the induction of immune responses in the animals that were injected with one, or another form of the antigen (Oliveira et al. 1998; Oliveira et al. 2002). However, when both the forms of the antigen were orally administered to mice, opposite effects were obtained, induction of oral toleranc by the native protein and development of humoral immune response by the lipid-conjugated protein (Oliveira et al. 1998).

The present study investigated the role of the accessory cells in the immune responses triggered by the oral route and focused on the pattern of expression of co-stimulatory molecules on Ova or palmitoyl-Ova-activated peritoneal macrophages and the induction of cellular immune responses.

MATERIALS AND METHODS

Animals

B6D2F1 mice, an abbreviation for (C57Bl6 × DBA2) F1 hybrids from both sexes, were obtained from the breeding unit at the Federal University of Minas Gerais (UFMG, Belo Horizonte, Brazil). Mice were used in the experiments only after they were 7–9 weeks old.

Antigens

Grade V chicken egg albumin (OVA) was purchased from the Sigma Chemicals Co. (St. Louis, MO, USA). The palmitoyl-Ova conjugates were prepared as previously published (Oliveira et al. 1998).

In vitro Treatment of Peritoneal Cells with Antigen

The peritoneal cavity (PerC) was injected with 5.0 ml of sterile phosphate-buffered saline (PBS).

Cells were collected, washed three times with cold PBS and then re-suspended in RPMI 1640 supplemented with 2 mM L-glutamine (Sigma), 1 mM sodium pyruvate (Sigma), 5×10^{-5} M 2-mercaptoethanol (Merck-Schuchardt, München, Germany), 50 IU/ml penicillin, 50 µg/ml streptomycin (Sigma) and 10% heat-inactivated fetal calf serum (FCS–Interlab, São Paulo, Brazil). Cultures were run in Nunc Dishes (St. Louis, MO, USA) 96 U-bottomed tissue culture plates, where 3×10^5 cells/well were incubated in the presence of 0.01, or 0.1 mg/100 µL of either Ova or palmitoyl-Ova. After 3–4 h, cells were washed and examined for the presence of cell markers.

Flow Cytometry Analysis

PE-conjugated anti-mouse CD80, CD86, or CD11b antibodies (Southern Biotechnology, Birmingham, AL) as well as biotin-PE were used to stain the Ag-activated peritoneal cells and positive and negative control samples were run on every plate to standardize the assays. Antibodies were directly added to the wells, followed by incubation at 5°C and in the dark for 30 min. Subsequently, cells were fixed with glutaraldehyde and analyzed by flow cytometry using a FACScan flow cytometer (Becton Dickinson Mountain View, CA) using Cellquest.

Oral Treatment and Immunization

Mice were intubated with a urethral polyvinyl catheter calibrated to reach the stomach and given 20 mg of antigen (Ova or palmitoyl-Ova) in 0.5 mL of saline (0.15 M NaCl). The control groups received 0.5 mL of saline alone. One week after the oral treatment, mice were injected *ip* with 0.5 mL saline containing 10 µg of Ova mixed with 1 mg $Al(OH)_3$ as adjuvant, or with 100 µg of Ova in 50 µL of complete Freund's adjuvant (CFA) *sc*. Two weeks after the priming, mice were injected again with 0.5 mL saline containing 10 µg of Ova *ip*. One week after the booster, blood samples were collected from the animals.

Systemic DTH Responses

Three weeks after the primary immunization, systemic DTH was assessed using the footpad-swelling test. Mice were *sc* injected with 100 µg/30 µL of aggregated Ova in the right hind of the footpad, and with 30 µL PBS in the left hind of the footpad. Forty-eight hours after the challenge, the footpad thickness was measured using a dial thickness gauge (Teclock Corp., Tokyo, Japan)

and differences between the two measurements were used for group comparison.

Cellular Proliferation

Cell culture was performed as previously described (Oliveira et al. 2002). Splenocytes (5 × 10^6 cells/mL) were cultured in complete medium (RPMI 1640; Sigma) supplemented with 2 mM L-glutamine (Sigma), 1 mM sodium pyruvate (Sigma), 5 × 10^{-5} M 2-mercaptoethanol (Merck-Schuchardt, Müchen, Germany), 50 penicillin IU/mL, and 10% heat-inactivated fetal calf serum (FCS–Interlab, São Paulo, Brazil). Cultures were run in triplicate in 96-well flat-bottomed tissue culture plates (Nunc). Cells were stimulated with Ova (1 mg/mL), or Concanavalin A (ConA–200 µg/mL) and were pulsed on day 3 with 20 µL of [^{3}H]-thymidine with a specific activity of 2 Ci/mmol for another 12 h. Cells were then harvested and the total amount of incorporated thymidine was assessed using a β-counter.

Statistical Analysis

The difference between the experimental and control groups was assessed by the ANOVA and considered significant for $p < 0.05$. The data are from at least three separate experiments.

RESULTS AND DISCUSSION

Evaluation of the Expression of Costimulatory Molecules

It has been previously shown that the coupling of palmitoyl residues to Ova abolished the induction of oral tolerance in B6D2F1 mice (Oliveira et al. 1998; Oliveira et al. 2002). To determine whether this coupling might influence the activation of APCs, cells from PerC were cultured with Ova or palmitoyl-Ova at two different concentrations and evaluated for the expression of activation markers. Mouse PerC selectively attracts and maintains specialized immune cells, including PerC macrophages. Approximately 40% of PerC cells were B lymphocytes (78% B-1; 15% B-2), 55% were macrophages (90% large peritoneal macrophages; 10% small peritoneal macrophages), 2% were cells (DCs), and 3% were eosinophils, NK cells, and T lymphocytes (Ghosn et al. 2010).

It is well established that the induction and activation of T cells require the engagement of CD28 by CD80 and CD86 to provide a second potent costimulatory signal (Lenschow et al. 1996). APCs that express low levels of CD80 and CD86 appear to preferentially promote T-cell tolerance, while APCs expressing high levels of these molecules delivered stimulatory signals to the T cells (Perez et al. 1997). However, a paradigm has been established in many animal models that positive and negative costimulation by the members of the CD28 family is critical for the development of immune responses, and establishment and maintenance of peripheral tolerance. During the last decade, the complexity of costimulatory pathways has greatly increased due to its influence on regulatory T cells and APCs (Bour-Jordan et al. 2011). Importantly, costimulatory signals affect Treg development, homeostasis, and suppressive functions, independent of their effector T cells (Bour-Jordan; Bluestone 2009). Tregs have been reported to suppress the conventional T cells through the production of immunosuppressive cytokines and direct alterations of effector T cells or APCs (Sakaguchi et al. 2008).

CD11b/CD18 (also called Mac-1 or CR3) is a β2 integrin expressed predominantly in the activated macrophages and DCs, plays a significant role in the production of numerous genes (COX-2, IL-12 p35, IL-12 p40), and interferes with the induction of cellular responses (Wu et al. 2004). On the contrary, deficiency of CD11b worsens the inflammation and disease progression in several autoimmune models (Lee et al. 2005; Popov et al. 2006), suggesting that CD11b/CD18 is potentially involved in immune suppression rather than immune activation. Consistent with these findings, studies showed that the proportion of CD11c+CD11b+ DCs was higher than CD11c+CD8a+ DCs in the spleen of tolerant mice (Li et al. 2008). It is of interest that CD11+CD11b+ DCs induce the Th2 cytokines, namely IL-10 and IL-4, *in vivo* (Pulendran et al. 1999). CD11b-/- mice are defective in developing immune suppression upon low-dose Ag feeding, while CD11b deficiency does not affect immune the activation (Ehirchiou et al. 2007). IL-10 has been reported to play a major part in immune tolerance and its production by DCs is critical for the differentiation of Treg cells (Zhang et al. 2004).

Despite the presence of these two PerC macrophage subpopulations, two gates: R1 (which

specifically includes B-lymphocytes and small peritoneal macrophages) and R2 (large cells) were created and a shift to R2 was observed following

the antigen stimulation, specially by 1.0 mg/mL Ova and palmitoyl-Ova (Fig 1).
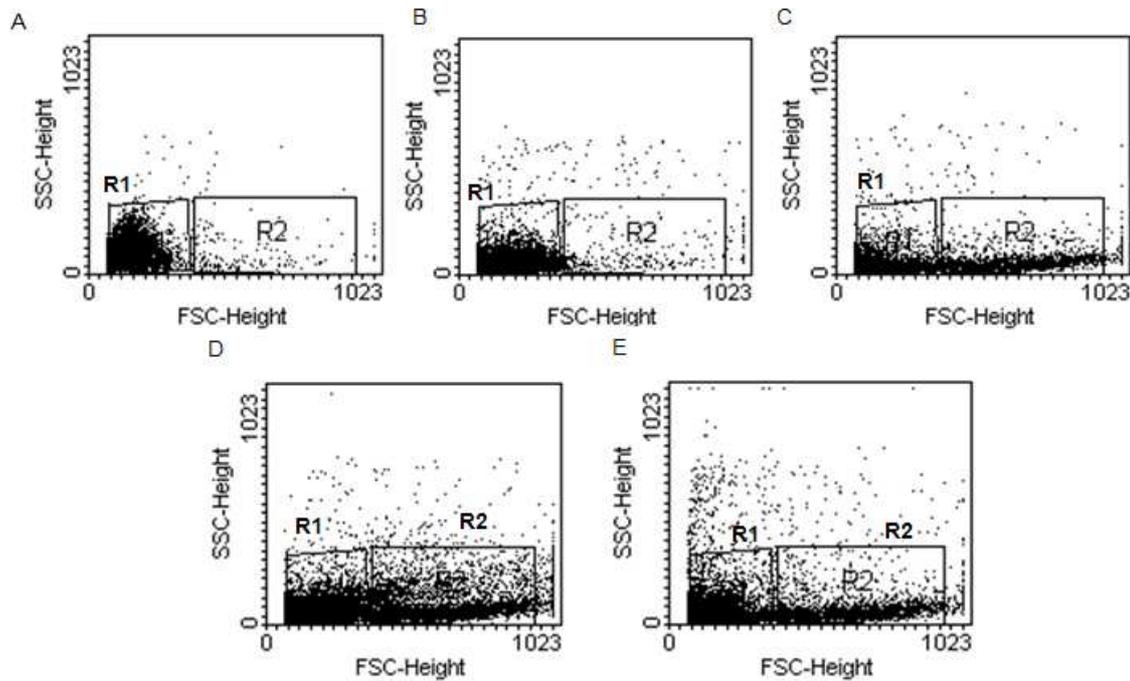
Figure 1 - Representative dot plots showing SSC-Height and FSC-Height for PerC cells by flow cytometry. A: Untreated (control); B: 0.1 mg/mL Ova-stimulated; C: 1.0 mg/mL Ova-stimulated; D: 0.1 mg/ml palmitoyl-Ova-stimulated; E: 1.0 mg/mL palmitoyl -Ova-stimulated. One of three representative experiments is shown.

Figures 1–7 summarize the data obtained from the flow cytometry analysis of the cells treated with fluorescent antibodies against CD80 (B7-1), CD86 (B7-2), and CD11b. As seen, PerC cells stimulated with 0.1 mg/mL Ova showed basal levels of costimulatory molecules, suggesting that the mechanisms of oral tolerance driven by 0.1 mg/mL Ova were based on the lack of signaling. T-cell activation in the absence of CD28-B7 signals could lead to a state of anergy. On the other hand, 1.0 mg/mL Ova induced all the costimulatory molecules to the same extent. These findings were in agreement with a study that showed an increase in CD11b-positive cells in the rectal mucosa of rabbits challenged with Ova (Bassan et al. 2005). CD11b deficiency abolished the establishment of oral tolerance to Ova by promoting the Th17 immune deviation (Ehirchiou et al. 2007). Th17 cells and cytokine IL-17 produced a variety of allergic autoimmune diseases, including rheumatoid arthritis, multiple

sclerosis, inflammatory bowel disease and asthma (Fujino et al. 2003; McKenzie; Kastelein; Cua 2006).
Liu et al. (1999) also demonstrated the role of CD86 in the induction of oral tolerance; they observed that the induction of oral tolerance at a low dose was abrogated by anti-CD86 treatment. However, when a high dose of Ag was fed (25 mg Ova–five times), tolerance was induced. The dominant role of CD86, not CD80, in the induction of immune response could be due to the fact that CD86 was rapidly induced whereas CD80 expression could not be detected until 2–3 days after B-cell activation (Lenschow et al. 1995). On the other hand, CD86 was not required for the development of oral tolerance in the experimental autoimmune encephalomyelitis (Gonella et al. 2006). Taken together, these findings showed that oral tolerance was the result of a complex immunoregulatory strategy for each antigen.
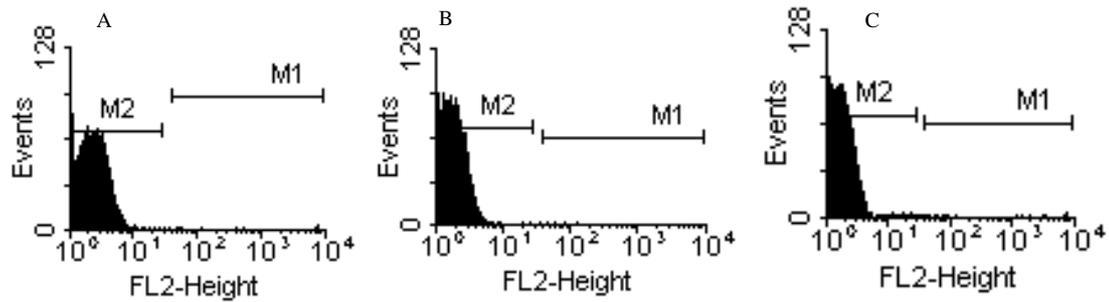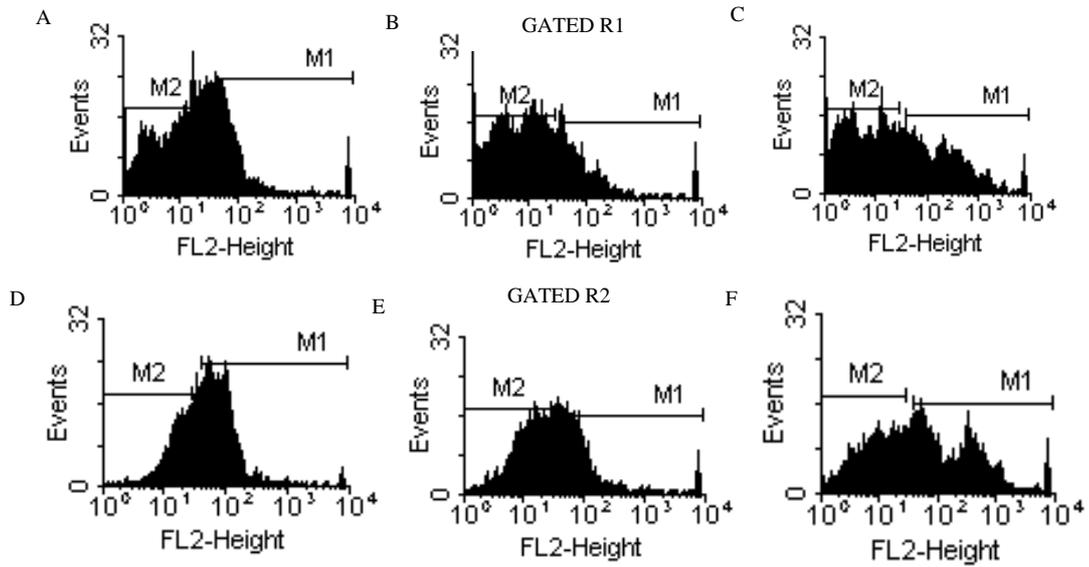
Figure 2 - Representative flow cytometry histogram of the immunomarker profiles of unstimulated PerC cells (Ungated). A: PE-conjugated anti-mouse CD80; B: PE-conjugated anti-mouse CD86; C: PE-conjugated anti-mouse CD11b. One of three representative experiments is shown.
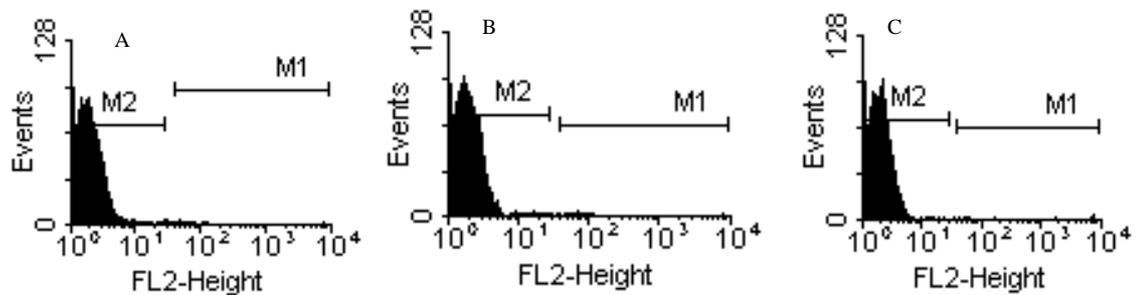
Figure 3 – Representative flow cytometry histogram of the immunomarker profiles of 0.1 mg/mL Ova-stimulated PerC cells (Ungated). A: PE-conjugated anti-mouse CD80; B: PE-conjugated anti-mouse CD86; C: PE-conjugated anti-mouse CD11b. One of three representative experiments is shown.
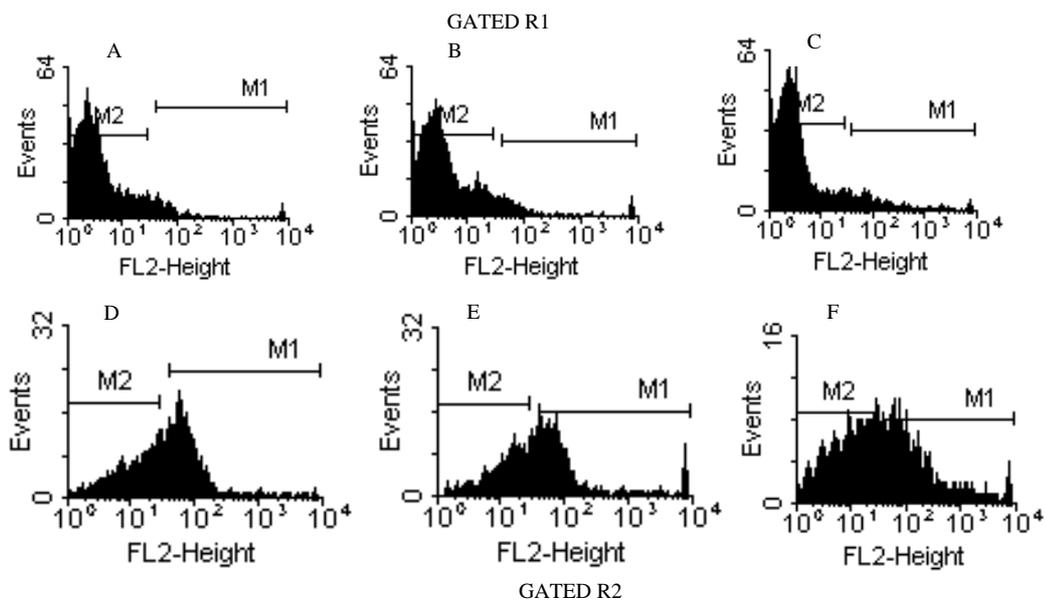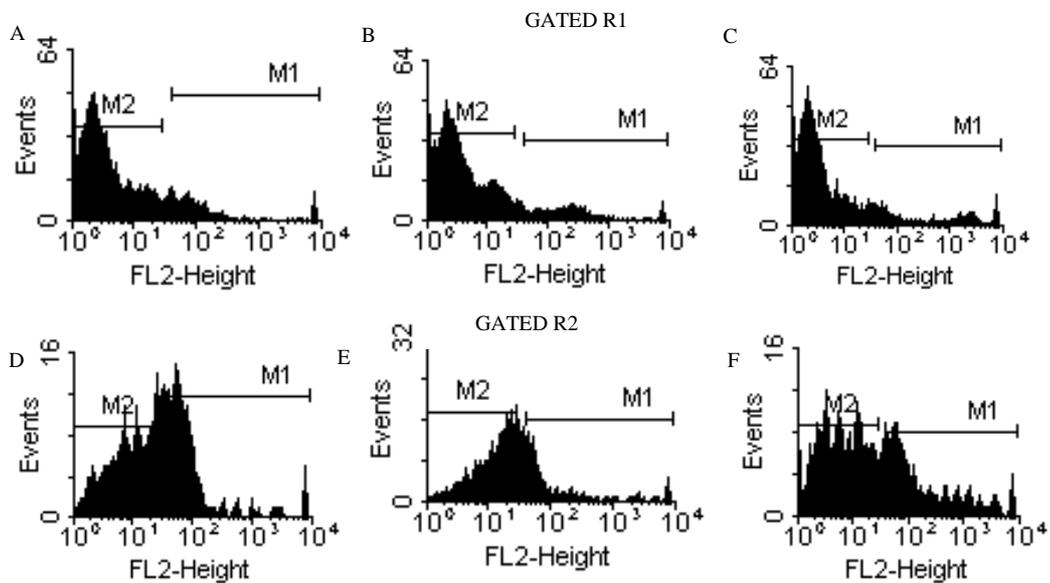

Figure 4 – Representative flow cytometry histogram of the immunomarker profiles of 1.0 mg/mL Ova-stimulated peritoneal macrophages (Gated R1 A-B-C; Gated R2 D-E-F). A-D: PE-conjugated anti-mouse CD80; B-E: PE-conjugated anti-mouse CD86; C-F: PE-conjugated anti-mouse CD11b. One of three representative experiments is shown.

Figure 5 - Representative flow cytometry histogram of the immunomarker profiles of 0,1 mg/mL palmitoyl-Ova-stimulated PerC cells (Gated R1 A-B-C; Gated R2 D-E-F). A-D: PE-conjugated anti-mouse CD80; B-E: PE-conjugated anti-mouse CD86; C-F: PE-conjugated anti-mouse CD11b. One of three representative experiments is shown.

Figure 6 – Representative flow cytometry histogram of the immunomarker profiles of 1.0 mg/mL palmitoyl-Ova-stimulated PerC cell (Gated R1 A-B-C; Gated R2 D-E-F). A-D: PE-conjugated anti-mouse CD80; B-E: PE-conjugated anti-mouse CD86; C-F: PE-conjugated anti-mouse CD11b. One of three representative experiments is shown.
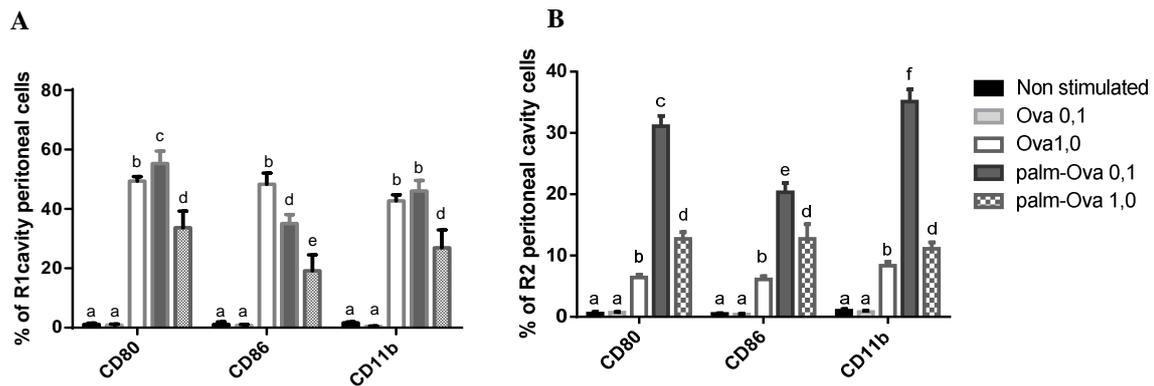
Figure 7 – The CD80, CD86, CD11b immunomarker profiles of PerC cells (Unstimulated; 0,1
mg/mL Ova-stimulated; 1,0 mg/mL Ova-stimulated; 0,1 mg/mL palmitoyl-Ova-
stimulated; 1,0 mg/mL palmitoyl-Ova-stimulated): A (Gated R1); B (Gated R2). The
median ± SD of three experiments is shown. Statistics: p (a/b/c/d/e/f) < 0.01.

Palmitoyl-Ova, when tested at both
concentrations, induced the expression of CD80,
CD86, and CD11b at higher levels. The
underlying mechanisms by which lipopeptides,
mucosal adjuvants and antigen delivery systems
elicit both local and systemic immune responses
without an adjuvant are only partially understood.
The mechanism of action is assumed to be a
complex multistep phenomenon resulting from the
physiological conditions and direct interactions of
antigens with different cell types at the presumed
site of uptake and immune induction (Alves et al.
2005; Oliveira et al. 2007). An elegant study,
performed by two-photon excitation, characterized
the antigen-specific CD4+ T cells in vivo in real
time during the induction of oral priming versus
oral tolerance (Zinselmeyer et al. 2005). Dramatic
changes in T-cell behavior were simultaneously
observed in local (MLN) and peripheral (CLN)
lymphoid organs after the oral administration of
the antigen, with, or without an adjuvant. The
investigators suggested that these changes could
be a consequence of variations in the expression of
the associated signaling molecules such as
chemokines, cytokines, costimulatory molecules,
and their receptors. Lipopeptides have been shown
to increase the expression of CD11b, CD11c, and
costimulatory molecules (BenMohamed et al.
2002) and to stimulate innate immunity by specific
interaction with the Toll family receptor (TLR) on
APCs. Robinson et al. (1992) have shown that
lipopeptides exhibited stronger interactions with
specific T-cell clones and APCs than native
peptides.
Similar mechanisms were also identified in oral
immunization models. Treating mice with the Flt3

ligand, a DC growth factor, and immunization
with cholera toxin (CT), was found to convert the
tolerant DCs into immunogenic APCs, probably
due to the up regulation of CD80 and CD86
(Willianson et al. 1999). Cong et al. (1997) have
shown that the role of CT as a mucosal adjuvant
involves the selective up regulation of CD86
expression on bone marrow-derived macrophages.
An interesting correlation demonstrated that CD86
could be critical for the secretion of TGF-β as well
as the production of IgA antibodies (Liu et al.
1999). It has also been shown that CD86 may be
more critical for the induction of IL-4-producing
cells, and IL-4 is a differentiating factor of TGF-β-
producing Th-3 cells. Taken together, these
findings could be associated with the present
observation that the oral administration of
palmitoyl-Ova produced systemic and local
immune responses. The present results suggested
that the kinetics and the level of expression of the
costimulatory molecules on the APCs in turn
regulated the oral tolerance, or the systemic and
local immune responses. In addition, palmitoyl-
Ova-mediated changes in the interaction between
APCs and T cells could induce the stimulatory
signals and determine the characteristics of the
effector response.

Evaluation of Cellular Immune Responses
Since the in vitro stimulation with palmitoyl-Ova
strongly induced the B7.1 (CD80), B7.2 (CD86)
and CD11b molecules, the effect of this form of
the Ag on both in vivo and in vitro T-cell
mediated responses was further studied. The
response of mice orally treated with saline, Ova,
or, palmitoyl-Ova, and immunized with the Ag in

CFA, was assayed by Ag-induced footpad swelling. Footpad swelling did not differ between the mice pretreated with palmitoyl-Ova (experimental group), or saline (immune group) but was significantly suppressed in mice pretreated with soluble Ova (data not shown).

The effect of the in vitro stimulation of spleen cells obtained from the mice that had been orally treated with palmitoyl-Ova, before being immunized with Ova adsorbed in CFA or Al(OH)3 was also studied. The profiles obtained with both the adjuvants were quite similar, although a higher stimulation index was reached when the animals were immunized with CFA. Spleen cells obtained from the mice that had been orally pretreated with palmitoyl-Ova proliferated as much as cells from the immune group (pretreated with saline), but much higher than the cells from the tolerant group (data not shown).

The control of T-cell expansion by B7/CD28 signals is both a direct consequence of entry and progression into the cell cycle, and an indirect outcome of an increased production of the T-cell growth factor IL-2 (Appleman et al. 2000). In this way, a strong T-cell proliferative response induced by palmitoyl-Ova may be indirectly related to high CD80 and CD86 expression and production of IL-2. Additionally, ISCOMS-Ova, an antigen entrapped in a lipid structure, also primed antigen-specific proliferative PLNs responses and produced a significant amount of IL-2 when administered orally (Maloy et al. 1995).

While palmitoyl-Ova increased B7 expression at both the concentrations, only high dose Ova induced the expression of CD80 and CD86. These results suggested that varying doses of Ova triggered different mechanisms that regulated the induction of oral tolerance by the B7 delivery signals. This postulation was supported by the results of an array examining the B7-CD28 functions. Blocking the B7-CD28 interaction promoted the transplantation tolerance that was initially driven by massive T-cell inhibition (Wells 1999). In addition, T-cell activation in the absence of B7-CD28 signals can lead to a state of anergy (Wells et al. 2003). In agreement with the previous reports, the effector phase of the oral tolerance, subsequent to low dose Ag feeding, was related to a decrease in IL-2 expression in the present work. Furthermore, an ip injection of the anti-CD86 mAb concurrent with a low dose feeding regimen, did not inhibit the production of IL-2 indicating a possible role in the induction of IL-2 producing cells (Liu et al. 1999).

Thus, these factors appeared to affect the cellular proliferative response against low dose Ova. On the other hand, a high dose of Ova further induced the expression of costimulatory molecules when compared to untreated cells. Importantly, CTLA-4 also binds to the ligands CD80 and CD86, thereby blocking TCR proximal signaling and consequently attenuating cell cycle progression, cytokine production, and proliferation (Kumar et al. 2013). Thus, the immune response delivered by APC lymphocytes is a complex interplay of costimulatory signals.

CONCLUSIONS

Taken together, the results suggested a direct effect of palmitoyl-Ova in the increased expression of CD86, CD80 and CD11b in the peritoneal cavity adherent cells through the induction of systemic and local immune responses.

ACKNOWLEDGMENTS

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