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B7RP-1 Is Not Required for the Generation of Th2 Responses in a Model of Allergic Airway Inflammation but Is Essential for the Induction of Inhalation Tolerance

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The recently described ICOS-B7RP-1 costimulatory pathway has been implicated in the generation of effector Th2 responses and, hence, has become an attractive therapeutic target for allergic diseases. In the present study, we used B7RP-1-deficient mice to investigate the role of B7RP-1 in the generation and maintenance of Th2 responses in a model of mucosal allergic airway inflammation. We found that exposure of B7RP-1 knockout mice to aerosolized OVA in the context of GM-CSF leads to airway eosinophilic inflammation. This response was long lasting because rechallenge of mice with the same Ag recapitulated airway eosinophilia. Moreover, significant expression of T1/S2 on T cells and production of Th2-affiliated cytokines (IL-5, IL-4, and IL-13) and Igs (IgE and IgG1) conclusively demonstrate the generation of a Th2 response in the absence of B7RP-1. In addition, expression of two major Th2-associated costimulatory molecules—CD28 and ICOS—indicates T cell activation in the absence of B7RP-1 signaling. Finally, B7RP-1 knockout mice are resistant to the induction of inhalation tolerance as indicated by the sustained eosinophilia in the lung and IL-5 production. In summary, our results demonstrate that in a model of mucosal allergic sensitization, the ICOS-B7RP-1 pathway is redundant for the generation of Th2 responses but essential for the induction of inhalation tolerance.

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Abbreviations used in this paper: Ad, adenoviral; KO, knockout; i.p., intraperitoneally; i.n., intranasally; BAL, bronchoalveolar lavage; WT, wild type; DC, dendritic cell.
suggest the impairment in the maintenance of lung homeostasis. Indeed, B7RP-1 KO mice were unable to establish inhalation tolerance and instead developed eosinophilia and produced IL-5. In summary, our data indicates the redundancy of B7RP-1 in the establishment of Th2 responses in a mucosal model of allergic inflammation but the essential role of this molecule interaction with its ligand for the generation of inhalation tolerance.

Materials and Methods

Animals

B7RP-1-deficient mice and control littersmates were generated as described before (13) and obtained from Dr. T. Mak (University of Toronto, Toronto, Ontario, Canada). Mice were housed in a specific pathogen-free environment following a 12-h light-dark cycle. All experiments performed were approved by the Animal Research Ethics Board of McMaster University.

Model of respiratory mucosal allergic sensitization

As previously described, a replication-deficient human type 5 Ad construct encoding murine GM-CSF cDNA in the E1 region of the viral genome (Ad/GM-CSF) was delivered intranasally (i.n.) 24 h before the first OVA exposure. Ad/GM-CSF was administered i.n. at a dose of 3 × 10^6 PFU in 30 μl of PBS vehicle (two 15-μl administrations, 5 min apart) into anesthetized animals. Over a period of 10 consecutive days (days 0–9), mice were placed in a Plexiglas chamber (10 × 15 × 25 cm) and exposed for 20 min daily to aerosolized OVA (1% w/v in 0.9% saline). OVA aerosol was generated by a Bennet nebulizer at a flow rate of 10 L/min. Inhalation tolerance was induced by exposing animals to OVA only. For in vivo rechallenge with OVA, mice were re-exposed to a 1% OVA aerosol for 20 min daily for 3 consecutive days, following complete resolution of initial airways inflammation.

Tolerance induction

Mice were placed in a Plexiglas chamber, as described before, and exposed for 20 min daily over a period of 10 consecutive days to aerosolized OVA (1% w/v in 0.9% saline) (17, 19). Control mice were exposed to saline only. Two days after tolerance induction, mice were subjected to respiratory mucosal allergic sensitization.

Collection and measurement of specimens

At various time points, mice were killed and bronchoalveolar lavage (BAL) was performed according to a standard protocol (16). Briefly, the lungs were dissected and the trachea was cannulated with a polyethylene tube (BD Biosciences). The lungs were lavaged twice with PBS (0.25 ml followed by 0.2 ml); ~0.3 ml of the instilled fluid was recovered consistently. Total cell counts were determined using a hemocytometer. Cell pellets were resuspended in PBS, and smears were prepared by cytocentrifugation (Thermo Shandon) at 300 rpm for 2 min. Hema 3 (Biochemical Sciences) was used to stain all smears. Differential counts of BAL cells were determined from at least 500 leukocytes using standard hemocytological criteria to classify the cells as neutrophils, eosinophils, lymphocytes, or macrophages/monocytes. Additionally, blood was collected by retroorbital bleeds. Serum was obtained by centrifugation after incubating whole blood for 30 min at 37°C. Finally, lung tissue was fixed in 10% formalin and embedded in paraffin. Three-micrometer thick sections were stained with H&E.

Splenocyte culture

Spleens were harvested into sterile tubes containing sterile HBSS (Invitrogen Life Technologies). Tissue was triturated between the ends of sterile, frosted slides, and the resulting cell suspension was filtered through nylon mesh (BSH Thompson). RBC were lysed with ACK lysis buffer (0.5 M NH₄Cl, 10 mM KHCO₃, and 0.1 mM Na₂EDTA at pH 7.2–7.4). Remaining splenocytes were washed twice with HBSS and then resuspended in RPMI supplemented with 10% FBS (Invitrogen Life Technologies), 1% l-glutamine, and 1% penicillin/streptomycin. Cells were cultured in medium alone or with 40 μg of OVA/well at 8 × 10^6 cells/well in a flat-bottom, 96-well plate (BD Biosciences). After 5 days of culture, supernatants were harvested for cytokine measurements.

Cytokine and Ig measurement

ELISA kits for murine IL-13, IL-4, IFN-γ, and IL-5 were purchased from R&D Systems. Each of these assays has a threshold of detection of 3–5 pg/ml. Levels of OVA-specific IgE and IgG1 were measured using a previously described Ag-capture ELISA method (16).

Flow cytometric analysis

Flow cytometric analysis was performed on lung cells isolated as previously described with slight modifications (16). Briefly, total lung mononuclear cells were obtained by collagenase digestion (collagenase type III; Invitrogen Life Technologies) followed by discontinuing gradient centrifugation in 30 and 60% Percoll (Pharmacia Biotech). The interface containing mononuclear cells was collected, washed twice with PBS, and stained with a panel of Abs. The following Abs were purchased from BD Pharmingen: anti-CD3 (PE-conjugated 145-2C11), anti-CD4 (biotin-conjugated L3T4), and anti-CD28 (FITC-conjugated 37.51), T1/ST2 (3E10) and ICOS Ab were provided by Millennium Pharmaceuticals and were FITC-labeled in-house. To minimize nonspecific binding, 10^5 cells were preincubated with FcBlock (CD16/CD32; BD Pharmingen). For each Ab combination, 10^5 cells were incubated with mAbs at 0–4°C for 30 min; the cells were then washed and treated with second stage reagents. Streptavidin-cy5 (BD Pharmingen) was used as a second step reagent for detection of biotin-labeled Abs. Titration was used to determine the optimal concentration for each Ab. Cells were fixed in 1% paraformaldehyde, counted on a FACScan, and analyses were performed using WinMDI software (The Scripps Research Institute). Twenty thousand to 30,000 events were acquired.

Data analysis

Data are expressed as mean ± SEM. Statistical interpretation was performed using ANOVA with Fisher post hoc test or Student t test. Differences were considered statistically significant when p < 0.05.

Results

Impact of the B7RP-1 deficiency on the generation of primary Th2 responses

B7RP-1 KO mice and control littersmates subjected to our protocol of respiratory mucosal sensitization were sacrificed 48 h after the last OVA aerosol challenge, and the BAL content was assessed. Both strains mounted an overall inflammatory response in the lungs of a similar degree. The absolute number and percentage of eosinophils (43 ± 6% B7RP-1KO vs 27 ± 4% wild type (WT)) in the BAL of B7RP-1 KO mice were in fact greater than that in WT mice and statistically different between groups (Fig. 1, a and b). Upon histopathological examination, the extent of the lung eosinophilic inflammatory infiltrate was, in agreement with the BAL findings, greater in B7RP-1KO than in control littersmate mice.
Sections were stained with H&E. Panels represent WT (Ad/GM-CSF). Tissues were obtained 48 h after the last OVA exposure. Additionally, to assess the ability of B7RP-1 KO mice to develop Th2 responses, we restimulated splenocytes collected 48 h after last OVA exposure with OVA Ag. Effector Th2-associated cytokines were measured in the supernatants of these cultures. As shown in Fig. 3, the levels of IL-4, IL-5, and IL-13 detected were in fact greater in B7RP-1 KO mice as compared with control littermates. Thus, the absence of the ICOS-B7RP-1 interaction in mice mucosally sensitized not only does not preclude Th2 differentiation but insinuates even greater Th2 polarization. A compromised IFN-γ production that would counterbalance Th2 responses (20) could potentially explain the particularly elevated production of Th2 cytokines. However, as demonstrated in Fig. 3d, the levels of IFN-γ were similar in B7RP-1 KO and control littermates, indicating that another yet unidentified mechanism is responsible for the increased production of Th2 cytokines in B7RP-1 KO.

Impact of the B7RP-1 deficiency on the generation of memory responses

The importance of costimulatory pathways in the generation of memory T cell responses is controversial (21). Therefore, although not playing a major role in Th2 differentiation, the ICOS-ICOSL interaction might be important for the generation of Th2 effector memory. To investigate this aspect, mice sensitized to OVA were left for 35 days to allow a complete resolution of the acute inflammatory response and were then re-exposed to aerosolized OVA on 3 consecutive days. Seventy-two hours after the last exposure, mice were sacrificed and the BAL cellular response was assessed. As shown in Fig. 5a, B7RP-1 KO and control littermate mice mounted an eosinophilic airway inflammatory response that was quantitatively similar. To determine whether the infiltration of T cells reflected a preferential accumulation of Th2 cells, lung mononuclear cells were subjected to flow cytometric analysis. T1/ST2, a putative marker of Th2 effector cells (22), was expressed on CD3/CD4 cells in both B7RP-1 KO and control mice (Fig. 5b). In addition, the expression of T1/ST2 in B7RP-1 KO was significantly higher than in WT controls.

Expression of costimulatory molecules in lung T cells

We next evaluated the expression of two costimulatory molecules, namely CD28 and ICOS, on lung T cells. To this end, lungs were subjected to enzymatic digestion followed by the isolation of mononuclear cell fraction. As shown in Table I, the expression of CD28 and ICOS was similar in both mouse strains, indicating activation of T cells, which is independent on signaling generated by B7RP-1.

Generation of inhalation tolerance

The increased responses that we observed in B7RP-1 KO mice as compared with control littermates could be explained by the decreased ability to control homeostatic conditions in the lung. Therefore, we examined the induction of inhalation tolerance that constitutes the major controlling mechanism of Th2 responses in the airways (19). Fig. 6 demonstrates that although the tolerance was induced in control mice (diminished eosinophilia and IL-5 production), B7RP-1 KO mice presented sustained eosinophilic response and production of IL-5, indicating inability to mount inhalation tolerance.
Discussion

It has been postulated that ICOS is important for the generation of Th2 cells because ICOS mRNA is expressed at high levels on in vitro-differentiated Th2 cells (6), ICOS ligation mediates IL-4 expression (7), and IL-4 expression is deficient in ICOS-KO mice (8, 9). Thus, the prediction was that the absence of ICOS ligand, namely B7RP-1 and, therefore, the disruption of the ICOS-ICOSL costimulatory pathway, would compromise the generation of Th2-associated responses. However, our experiments with mice genetically engineered for the absence of B7RP-1 (B7RP-1KO) demonstrate that efficient Th2 sensitization and effector function occur in the absence of this costimulatory pathway.

B7RP-1-deficient mice subjected to a model of mucosal allergic sensitization responded efficiently to OVA and generated productive Th2 response at both the cellular and humoral levels. Indeed, OVA aerosolization in the context of GM-CSF led to the accumulation of eosinophils in both airways and lung parenchyma and Th2-affiliated cytokine production (IL-4, IL-5, and IL-13). Interestingly, the extent of cellular lung infiltration, the levels of cytokine production, and T1/ST2 expression on T cells were greater in B7RP-1KO than in control mice. We considered whether decreased IFN-γ production in B7RP-1KO could explain these heightened Th2 responses; however, B7RP-1KO and control littermate mice produced similar amounts of IFN-γ in agreement with a recent report by Nurieva et al. (14). Therefore, our data indicate another unidentified mechanism that is responsible for the increased production of Th2 cytokines in B7RP-1KO mice.

The ICOS-B7RP-1 pathway appears to be important in humoral immunity as ICOS-deficient mice subjected to different immunization protocols revealed deficits in IgG1, IgG2a, and IgE levels (8, 23–25). Our data demonstrate an intact production of Ag-specific immunoglobulins (IgG1 and IgE). Although apparently at

\begin{table}
\centering
\begin{tabular}{lcc}
\hline
 & WT & KO \\
\hline
CD28 & 90.5 ± 0.6 & 90.2 ± 2.9 \\
ICOS & 55.5 ± 1.9 & 68.8 ± 3.8 \\
\hline
\end{tabular}
\caption{Flow cytometric analysis of T cells (CD3⁺ CD4⁺) obtained from lungs of control littermates (WT) and B7RP-1-deficient (KO) micea}
\end{table}

a Lung mononuclear cell fraction was obtained by enzymatic digestion of whole lung, and, expression of CD28 and ICOS was examined on lung T cells. Thirty thousand events were collected. Data are representative of four independent measurements (n = 4/group) for individual lungs (mean ± SEM).

FIGURE 5. In vivo rechallenge of control littermates (WT) and B7RP-1-deficient (KO) mice exposed to OVA in the context of GM-CSF. Data were obtained 48 h after last exposure to OVA. a, Total cell number acquired in BAL fluid (mean ± SEM; n = 4). b, Percentage and cell number of eosinophils present in the BAL (mean ± SEM; n = 4). c, Levels of IL-5 production by splenocytes (mean ± SEM; n = 4).

FIGURE 6. Induction of inhalation tolerance in control littermates (WT) and B7RP-1-deficient (KO) mice. Mice were exposed to saline (Th2) or OVA (tolerance (TOL)) for 10 consecutive days, and 1 wk later, they were re-exposed to OVA in the context of GM-CSF. Data were obtained 48 h after last exposure to OVA. a, Total cell number acquired in BAL fluid (mean ± SEM; n = 4). b, Percentage and cell number of eosinophils present in the BAL (mean ± SEM; n = 4). c, Levels of IL-5 production by splenocytes (mean ± SEM; n = 4).
variance with the results observed in ICOS KO mice, it must be
noted that the impairment in Ab production observed in ICOS-
deficient mice can be overcome by the use of a strong adjuvant
such as CFA (25). Similarly, administration of polyethylene glycol-
glycol-GM-CSF in B7RP-1KO led to partial rescue of IgG1 produc-
tion (13). In this regard, the presence of intact humoral responses
in B7RP-1 KO in our mucosal model can be explained by the
nature of a protocol that involves repeated exposure to Ag (OVA)
in the context of a GM-CSF-rich airway microenvironment.

That the generation of primary and memory Th2 responses is
completely intact in B7RP-1 KO mice suggests that the importance
of the ICOS costimulatory pathway is redundant under our exper-
imental conditions. Our findings are at variance with those recently
reported by Mak et al. (13). The discrepancy can be explained, at
least in part, by the nature of the experimental models used in both
studies. Whereas Mak et al. (13) used a conventional model in-
volving two i.p. injections of OVA/Alum, followed by OVA aero-
sol challenge, we used a model of mucosal allergic sensitization
that entails delivery of OVA directly to airways through aerosol-
ization in the context of locally expressed GM-CSF. We have elab-
orated recently in detail on the importance of GM-CSF to allergic
airway inflammation (18). More specifically, that GM-CSF is in-
volved in the expansion of a particular Th2-associated dendritic
(cell (DC) subset (myeloid DC; DC2) is documented extensively in
the literature. Of direct relevance to the work presented here, Mak
et al. (13) showed that delivery of polyethylene glycol-GM-CSF to
B7RP-1-deficient mice led to robust expansion of DC2 and resto-
ration of IgG1 production in 50% of mice. Unfortunately, no other
components of Th2 responses were evaluated in these mice.

It is known that expression of ICOS on T cells is dependent on
TCR and CD28 signals and that absence of CD28 results in di-
minished levels of ICOS (7). To this end, we examined the ex-
pression of CD28 and ICOS on CD3/CD4 cells isolated from the
lungs in our recall protocol. As shown on Fig. 6, both molecules
are expressed in B7RP-1 KO. Two potential explanations can emerge
from our studies: either the CD28-B7 pathway can fully substi-
tute for the absence of a secondary signal, namely ICOS-
ICOSL, or there is a second ligand for ICOS, which is distinct
from B7RP-1, that interacts with ICOS expressed on T cells. Pre-
vious studies using either CD28KO or B7/1/2 antagonists have
shown that the CD28-B7 pathway is absolutely necessary for the
generation of Th2 responses, supporting the former notion (26). In
addition, current literature postulates that blockade of the ICOS-
ICOSL pathway with ICOS-Ig does not prevent Th2 differentia-
tion but can reduce acute airways inflammation (11, 27). In addi-
tion, evidence of eosinophilic infiltration in a model of allergic airway
inflammation in ICOS KO (9) supports the notion that CD28/B7 is
the primary pathway for Th2 responses, whereas ICOS-ICOSL
serves as an enhancing arm.

The issue that remains unresolved is the potential role of
B7RP-1 in T cell priming. Although it is clear that B7RP-1 is
expressed on resting B cells and macrophages, it is still contro-
versial whether this molecule is also expressed on murine DC.
Human DC express B7RP-1, which is down-regulated upon stim-
ulation with LPS or TNF-α (3). Similarly, airway exposure to
OVA leads to the decrease of B7RP-1 expression on lung DC (28,
29). Both studies imply that activated DC might not use B7RP-1
for the purpose of activating naive T cells but rather use the
B7RP-1 pathway to sustain homeostasis. Indeed, many peripheral
tissues, among them endothelial cells, constitutively express low
levels of B7RP-1 and presumably can interact with ICOS+ T cells
producing regulatory IL-10 (29). The involvement of ICOS/
ICOSL pathway in regulating homeostasis remains, however, un-
resolved and requires additional investigations. That mice trans-
genetic for B7RP-1Fc develop T cell hyperplasia, plasmacytosis,
and hypergammaglobulinemia suggest that the contribution of
B7RP-1 involves primarily a direct interaction between T and B
cells rather than with DC (24). However, the presence of Ag-spe-
cific Abs in our model supports the notion that stimulation through
CD40-CD40L is sufficient in the absence of in vivo ICOS signal-
ing to execute an efficient humoral response (25).

The increased Th2 deviation observed in the B7RP-1 KO was
quite perplexing, especially in light of evidence claiming that
ICOS-B7RP-1 pathway is important for the generation of Th2 re-
sponses. Therefore, we have decided to examine whether the en-
hancement of Th2 responses in our mucosal model might result
from the interference with ICOS/B7RP-1-dependent regulatory
mechanisms in the lung. To this end, we tested whether B7RP-
1KO mice are able to generate inhibition tolerance in response to
OVA. Under these experimental conditions, exposure of mice to
OVA leads to the establishment of tolerance because the subse-
quent exposure to OVA in the context of GM-CSF results in di-
minished airway eosinophilia and negligible levels of Th2-associ-
cated cytokines (17). Our data show that B7RP-1KO mice, unlike
the control animals, did develop airway eosinophilia and were able
to produce IL-5. Our findings are in agreement with those by Ak-ar{b}ari et al. (15), who postulated that intact ICOS-ICOSL pathway
is necessary for the effective generation of regulatory T cells and
inhalation tolerance. Therefore, the absence of B7RP-1 signaling
could lead to the impairment of homeostatic regulation in the lung
that results in the generation of default Th2 responses.

In summary, our data shows that we could efficiently trigger
Th2-associated cellular and humoral responses in B7RP-1-defi-
cient mice in a model of allergic airway sensitization. In sharp
contrast, ICOS-B7RP-1 signaling is absolutely essential for the
induction of inhalation tolerance. That the absence of this signaling
pathway leads to the enhancement of Th2 responses indicates the
importance of costimulatory pathways in the balancing the devel-
opment of specific immune responses.

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Disclosures
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