Short paper

Eric Muraille, Thibaut De Smedt, Jacques Urbain, Muriel Moser and Oberdan Leo

Laboratoire de Physiologie Animale, Département de Biologie Moléculaire, Université Libre de Bruxelles, Rhode-St-Genèse, Belgium

B7.2 provides co-stimulatory functions *in vivo* in response to staphylococcal enterotoxin B

Excessive T cell activation induced by bacterial superantigens plays an important role in the pathology associated with Gram-positive bacteremia. To gain insight into the early phases of T cell activation by bacterial enterotoxins *in vivo,* we investigated the ability of antibodies to well-defined co-stimulatory molecules to inhibit T cell activation and the subsequent toxic shock syndrome induced in BALB/c mice following the injection of staphylococcal enterotoxin B (SEB). We demonstrate here that a single dose of anti-B7.2 antibodies, but not anti-B7.1 antibodies, significantly inhibits T cell activation, as judged by lower systemic IL-2 release, blastogenesis and IL-2 receptor expression, and reduces the lethal effect of SEB in D-galactosamine-sensitized mice. These results demonstrate that co-stimulation through the B7.2 molecule plays an important role in the activation of T cells in response to SEB *in vivo* and suggest alternative therapies for septic shock caused by bacterial enterotoxins based on blocking antibodies to co-stimulatory molecules.

1 Introduction

A number of exotoxins, called superantigens (SAg), are secreted by certain Gram-positive bacteria such as *Staphylococcus aureus,* and have been implicated in septic shock. These molecules, such as staphylococcal enterotoxin B (SEB), are bifunctional agents, able to interact simultaneously with selected Vβ regions of the T cell receptor and with a conserved domain of the class II MHC molecule, causing T cell activation *in vitro* and *in vivo* (reviewed in [1]). In fact, injection of SEB in BALB/c mice rapidly induces activation and proliferation of $V\beta8^+$ T cells, which release cytokines into the circulation. There is compelling evidence that this excessive cytokine production may be one important cause of the pathophysiology associated with *Staphylococcus aureus* infection [2].

Numerous studies performed with conventional antigens have demonstrated that, in addition to TcR occupation, optimal $CD4^+$ T cell activation requires co-stimulatory signals delivered by the antigen-presenting cell (APC, for review see [3]). APC-associated molecules of the B7 family (B7.1/BB1 [4], B7.2 [5, 6]) which interact with counter-receptors CD28 and/or CTLA4, are thought to represent an important signaling pathway in T cell co-stimulation. While the role of the CD28/B7 pathways has been well documented with conventional antigens, there have been conflicting reports on the requirement for CD28-mediated co-stimulation in the immune response to bacterial SAg. Although numerous studies have indicated that signaling through CD28 enhances T cell responses to SAg [7–11], it is not clear to date whether CD28/B7 interactions represent an obligatory step for SAg-mediated T cell activation. Recent studies indicate, for example, that CTLA-4Ig [12, 13] or anti-B7.1 antibodies [14] have little or no effect on SAg-mediated activation of naive T cells. Recently, we demonstrated that a combination of monoclonal antibodies (mAb) to murine B7.1 and B7.2 molecules inhibits the response of naive T cells *in vitro* to the bacterial exotoxins staphylococcal enterotoxins A and B (SEA, SEB) and toxic-shock syndrome toxin (TSST)-1 [15], indicating that, as demonstrated for conventional antigens, SAg-mediated T cell activation *in vitro* requires APC-derived co-stimulatory functions. In this study, the response to SEB *in vivo* was analyzed using mAb to B7-related molecules as blocking reagents. Our data demonstrate that administration of anti-B7.2 mAb *in vivo* significantly reduces SAg-mediated T cell activation and consequent lethality, suggesting a potential therapeutical use of antibodies to co-stimulatory molecules in the treatment of septic shock caused by bacterial exotoxins.

2 Materials and methods

2.1 Mice

Female BALB/c mice, 6–8 weeks old, were purchased from Charles River Wiga (Sulzfeld, Germany) and maintained in a pathogen-free environment in our own animal facility.

2.2 SEB treatment protocols

Mice were injected i.p. with the indicated doses of SEB (purified form from Toxin Technology, Sarasota, FL). Serum IL-2 content was determined by ELISA, using the rat anti-mIL-2 mAb S4B6 (available through the ATCC) as capture reagent and rabbit anti-mIL-2 serum (produced in

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Correspondence: Oberdan Leo, Laboratoire de Physiologie Animale, Université Libre de Bruxelles, 67 rue des Chevaux, B-1640 Rhode-Saint-Genèse, Belgium (Fax: 32-2-6509840)

Abbreviations: **SEB:** Staphylococcal enterotoxin B **SAg:** Superantigen

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Table 1. Effect of anti-B7.1 and anti-B7.2 mAb on SEB-mediated lethality in D-GalN-sensitized mice

Treatment[a]	Survival[b]		
Dose of SEB (μg/mouse):	Exp. 1 25 μg	Exp. 2 50 μg	Exp. 3 100 μg
1. PARSI-19 Asc	3/8 (37.5 %)	2/8 (25 %)	nd[c]
2. 16-10A1 (anti-B7.1) Asc	2/8 (25 %)*[d]	3/8 (37.5 %)*	nd
3. Control Asc	3/10 (30 %)	2/10 (20 %)	1/8 (12.5 %)
4. GL1 (anti-B7.2) Asc	8/8 (100 %)***	5/8 (62.5 %)**	4/8 (50 %)*
5. IR418 Pur	3/7 (42.8 %)	nd	nd
6. GL1 (anti-B7.2) Pur	7/7 (100 %)**	nd	nd
7. 16-10A1 Asc + GL1 Asc[e]	12/15 (80 %)	7/12 (58.3 %)	2/8 (25 %)

a) Mice were injected i.p. with antibodies as indicated (Asc: 400 μl of crude ascitic fluids; Pur: 700 μg of purified antibody) 1 h before injection of a mixture of SEB (see doses) and D-GalN (20 mg).
b) Number of mice surviving / number of mice treated. Percentage of survival is indicated in parentheses.
c) Not done.
d) Statistical analyses *vs.* the control group using Fishers' exact two-sample test: *$p < 0.6$ (not statistically significant) **$p < 0.04$ ***$p < 0.005$
e) Mice were treated with a mixture of 200 μl of 16-10A1 and 200 μl of GL1 ascites.

our laboratory) as revealing reagent. The lethal effect of SEB was studied by injecting mice i.p. with a mixture of SEB (see Sect. 3 and Table 1 for doses) and 20 mg of D-galactosamine (D-GalN) (Sigma, St. Louis, MO), in 200 μl PBS. Mortality was monitored after 72 h, after which time mortality did not increase. Data were analyzed for significance by Fisher's exact two-sample test. The following antibodies were used in this study: 16-10A1 (hamster anti-mB7.1 [16], GL1 (rat IgG2a anti-mB7.2) [5], PARSI-19 (hamster anti-ARS, produced in our laboratory), and IR 418 (rat IgG2a anti-DNP, kindly provided by H. Bazin, UCL, Brussels). Ascitic fluid induced by a non-secreting B cell hybridoma was used as control when indicated. Mice were injected i.p. with ascitic fluid or purified antibodies 1 h before SEB treatment.

2.3 Flow cytometry

Spleen cells were analyzed by flow cytometry with a FACScan cytometer (Becton Dickinson, Mountain View, CA). The cells were incubated with 2.4G2 (rat anti-mouse FcRII/III) for 10 min prior to staining to prevent antibody binding to FcR, and incubated with: FITC-coupled 7D4 (anti-IL-2R, purchased from Pharmingen), and biotin-coupled F23.1 (anti-Vβ8.1-2-3, mouse IgG2a, available through the ATCC and produced in our laboratory). In all cases, cells were gated according to size and scatter in order to eliminate dead cells and debris from analysis.

2.4 Responses *in vitro*

All immune responses *in vitro* were examined in serum-free medium containing RPMI 1640 (Seromed; Biochem KG, Berlin, Germany) supplemented with 2 % HY Ultroser (Gibco BRL), penicillin, streptomycin, nonessential amino acids, sodium pyruvate, 2-ME and L-glutamine (Flow ICN Biomedicals, Bucks, GB). An adequate number of spleen cells (see Sect. 3 for cell numbers) was stimulated by SEB in a total volume of 0.2 ml in 96-well U-bottom plates. Cultures were maintained at 37 °C in 7 % CO_2 in humidified air. Supernatants were collected after 18–24 h of culture, frozen and assayed for IL-2 content by a bioassay using a subclone of the CTLL cell line insensitive to murine IL-4 [17]. Proliferation was assayed by [^{3}H] thymidine incorporation.

3 Results and discussion

3.1 Anti-B7.2 antibodies prevent SEB-induced lethality

Whereas SEB or D-GalN injected separately were not toxic at doses used in this study, co-injection induced acute toxicity and lethality, as previously shown ([18] and Table 1). Injection of control xenogeneic (rat or hamster) mAb or ascitic fluid failed to alter toxicity of SEB in this experimental protocol. Administration of mAb to B7.2 (ascitic fluid or purified) prior to SEB injection rescued, in a SEB dose-dependent manner, a significant number of animals from death (100 % survival at low doses of SEB, Table 1, groups 4 and 6). Antibodies to B7.1 were found to be ineffective in providing protection (Table 1, group 2), and also failed to synergize with anti-B7.2 mAb in reducing lethality following SEB administration (Table 1, group 7). Overall, mice receiving mAb to B7.2 (Table 1, groups 4, 6 and 7), were significantly protected when compared to control mice (Table 1, groups 1, 3 and 5, 27/30 surviving mice versus 9/25, $p < 0.0001$). These observations strongly suggest that SEB-mediated toxicity *in vivo* requires selective B7.2-mediated co-stimulation.

3.2 Anti-B7.2 antibodies inhibit T cell activation by SEB *in vivo*

SEB-induced lethal shock involves a massive cytokine release following hyperacute activation of T cells [1]. The role of co-stimulatory molecules on SAg-mediated T cell activation was studied by evaluating the effect of anti-B7 mAb treatment on the production of IL-2 *in vivo* following SEB injection. As shown for lethality, only mAb to B7.2 were effective in reducing cytokine production *in vivo*, suggesting that B7.2 represents the major co-stimulatory molecule involved in the acute T cell response of naive

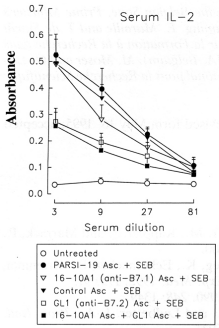

Figure 1. Effect of anti-B7 mAb on IL-2 production induced by SEB *in vivo*. Animals (8–10 per group) were pretreated with mAb (400 µl ascites i.p. when a single mAb was used, or a mixture of 200 µl anti-B7.1 and 200 µl anti-B7.2 ascites) 1 h before SEB administration (50 µg i.p.) and bled 4 h later. Serum IL-2 activity was tested by ELISA. Results are expressed as the mean ± SD of individual determinations.

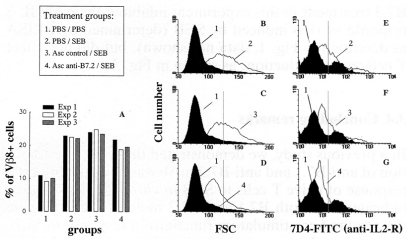

Figure 2. Effect of anti-B7.2 mAb on T cell activation induced by SEB *in vivo*. Four groups of animals were treated on day 0 as indicated in the insert and analyzed on day 2 for parameters of T cell activation. A: Expression of the Vβ8 marker by unfractionated spleen cells. Results represent the mean value of three individual determinations from three independent experiments. B–F: Pooled cells from three individuals in each group were analyzed by two-color immunofluorescence following staining with biotin-labeled Vβ8-specific mAb and FITC-labeled anti-IL-2R mAb and SA-PE-avidin. Forward scatter (B–D) and IL-2R expression (E–F) of Vβ8$^+$ cells from a single representative experiment are shown.

mice to SEB *in vivo* (Fig. 1). The effect of mAb to B7.2 on Vβ8-specific cell size increase and IL-2 receptor (IL-2R) expression was also characterized. Mice were pretreated with control ascites or anti-B7.2-containing ascites and injected with SEB 1 h later. Based on a preliminary study aimed at determining the kinetics of T cell activation *in vivo*, spleen cells of control and experimental animals were analyzed by flow cytometry 48 h following SEB injection. Anti-B7.2 mAb did not alter the early increase in frequency of Vβ8$^+$ cells among total splenic T cells (see Fig. 2). However, anti-B7.2 treatment affected both Vβ8-specific blastogenesis (compare forward scatter of control and anti-B7.2 injected mice, Fig. 3) and IL-2R expression induced by SEB. In particular, the frequency of Vβ8$^+$ cells expressing high levels of IL-2R in response to SEB was drastically reduced in anti-B7.2 treated animals. A similar reduction in cell size and IL-2R expression was found when CD4$^+$ and CD8$^+$ cells were assayed separately (data not shown).

Although the data presented here indicate that the B7.2 molecule provides co-stimulatory functions during a response to SEB, it is of note that anti-B7.2 mAb did not completely block T cell activation *in vivo*. Whether partial inhibition results from inability of the mAb to saturate all B7.2 molecules *in vivo* or from the existence of a B7.2-independent pathway of T cell activation is presently not known. In favor of the latter hypothesis, it has been demonstrated that mice genetically deficient for CD28 [19] or expressing a trangenic CTLA-4Ig construct [20] display residual T cell competence.

3.3 Anti-B7.2 antibodies do not prevent T cell anergy induction by SEB

To assay the immunocompetence of Vβ8-expressing T cells following challenge with SEB *in vivo*, spleen cells were restimulated *in vitro* with SEB. As previously reported (for review see [1]), T cells from SEB-injected mice become refractory to subsequent stimulation *in vitro* by SEB. Anti-

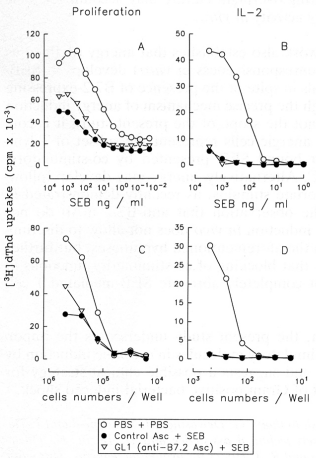

Figure 3. Anti-B7.2 mAb do not affect anergy induction by SEB. Controls and anti-B7.2-treated animals were injected with 50 µg of SEB i.p. and their immunocompetence tested *in vitro* 7 days later. Unfractionated splenic cells were cultured with SEB and proliferation (A and C) and IL-2 production (B and D) were assayed as described in Sect. 2.5. Cells (5×10^5) were stimulated by graded doses of SEB in panels A and B, whereas graded doses of responder cells were stimulated by 500 ng/ml of SEB in panels C and D. Values represent the mean of triplicate wells.

B7.2 treatment in this experiment inhibited the early IL-2 response *in vivo* induced by SEB (determined by ELISA as described in Fig. 1, data not shown), but did not affect T cell anergy induction, as shown in Fig. 3.

3.4 Concluding remarks

In a previous study, we demonstrated that only a combination of anti-B7.1 and anti-B7.2 mAb was able to inhibit the response of naive T cells to SEB *in vitro* [15]. This finding indicates that both B7.1 and B7.2 molecules can provide the required co-stimulatory functions in response to SEB. In contrast, the present study establishes that B7.2 represents the major co-stimulatory molecule *in vivo* in response to the same bacterial exotoxin. We believe that the difference in the response kinetics to SEB *in vitro* and *in vivo* may explain this apparent discrepancy. The response to SEB *in vitro* displays slow kinetics (16 to 24 h), while the response *in vivo* is characterized by very rapid lymphokine production: serum levels of several cytokines can be detected as early as 1 to 2 h following i.v. injection of bacterial exotoxins. A recent report indicates that B7.2 represents the major co-stimulatory molecule found to be constitutively expressed in lymphoid and nonlymphoid tissues, whereas detectable B7.1 expression is only observed following culture *in vitro* [21]. Based on these observations, we would suggest that the lack of B7.1-mediated co-stimulation *in vivo* results from the weak expression of this molecule on naive APC. In contrast, up-regulation of B7.1 molecules during overnight culture may account for their co-stimulatory activity *in vitro*.

The present work also establishes that anergy (defined as Vβ8-specific unresponsiveness *in vitro*) develops in SEB-treated animals in spite of the presence of B7.2-expressing cells. Although the precise mechanism of anergy induction by SEB was not the scope of the present study, it is conceivable that anergic cells represent the subset of T lymphocytes that bound SEB presented by co-stimulatory-deficient APC. Alternatively, anergy may develop following an appropriate stimulus, as recently demonstrated *in vitro* [22]. The observation that anti-B7.2 mAb do not affect anergy induction *in vivo* does not allow to discriminate between the aforementioned hypotheses, but further demonstrates that blocking of co-stimulatory functions *in vivo* does not completely abrogate SEB-mediated T cell signaling.

In conclusion, the present study underscores the importance of co-stimulatory molecules in cytokine induction by SAg *in vivo*, and suggests a possible clinical strategy for the treatment of Gram-positive bacterial-induced shock.

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