Redundant and Antagonistic Functions of Galectin-1, -3, and -8 in the Elicitation of T Cell Responses

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*J Immunol* published online 22 February 2012
http://www.jimmunol.org/content/early/2012/02/22/jimmunol.1102182

**Supplementary Material**
http://www.jimmunol.org/content/suppl/2012/02/23/jimmunol.1102182.2.DC1

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Galectins, a family of mammalian lectins, have emerged as key regulators of the immune response. We previously demonstrated that galectin (Gal)-8, from the tandem-repeat subgroup, exerts two well-defined effects on mouse naive peripheral CD4 T cells: Ag-specific costimulation and Ag-independent proliferation. These stimulatory signals on naive T cells have not been described for any other Gal. Therefore, we investigated whether Gal-1 and Gal-3, two prominent members of the Gal family, share the stimulatory effects exerted by Gal-8 on naive T cells. We found that Gal-1 costimulated Ag-specific T cell responses similarly to Gal-8, as evaluated in the DO11.10 TCRova-transgenic mouse model, by acting simultaneously on APCs and target CD4 T cells. In contrast, Gal-3 failed to costimulate Ag-specific T cell responses; moreover, it antagonized both Gal-1 and Gal-8 signals. We observed that both Gal-1 and Gal-3 were unable to induce Ag-independent proliferation; however, when two Gal-1 molecules were covalently fused, the resulting chimeric protein efficiently promoted proliferation. This finding indicates that Gal-1 might eventually induce proliferation and, moreover, stresses the requirement of a tandem-repeat structure. Remarkably, a single dose of recombinant Gal-1 or Gal-8 administered together with a suboptimal Ag dose to DO11.10 mice strengthened weak responses in vivo. Taken together, these findings argue for the participation of Gals in the initiation of the immune response and allow the postulation of these lectins as enhancers of borderline Ag responses, thus representing potential adjuvants for vaccine formulations.

The Journal of Immunology, 2012, 188: 000–000.

Gal-1 and -3 are the most abundantly expressed and extensively studied members of the Gal family. With regard to autoimmunity, chronic inflammation, infection, and tumor progression (4). Gal-1 and -3 are the most abundantly expressed and extensively studied members of the Gal family. With regard to T cell homeostasis, Gal-1 was shown to promote apoptosis of immature CD4+CD8+ and CD4+CD8- thymocytes, thus contributing to maintenance of self-tolerance (5). In the periphery, Gal-1 displayed anti-inflammatory effects by inducing apoptosis of activated T cells (6). Additionally, Gal-1 was shown to exert its immunoregulatory role by negatively modulating proinflammatory cytokine expression, skewing the balance from a Th1 toward a Th2 or T regulatory response (7).

Gal-3 is another key regulator of T cell homeostasis, with the peculiarity that it can induce T cell proliferation or apoptosis, depending on whether it acts intracellularly or extracellularly, respectively. When added exogenously, Gal-3 is able to induce apoptosis of several human T leukemia cell lines, as well as activated mouse cells (8). Moreover, this Gal also contributes to the maintenance of self-tolerance by triggering apoptosis of the CD4+CD8- thymocyte subset (9).

Gal-8, which belongs to the tandem-repeat group, is intrinsically a heterodimer, because it has an N-terminal CRD (N-CRD) and a C-terminal CRD (C-CRD) joined by a hinge linker peptide of variable length. It is expressed in different organs and tissues under physiological or pathological conditions, such as several human cancers (10, 11). We previously reported the expression of ordered cell surface Gal-glycan structures termed lattices, which engage specific cell surface glycoconjugates by traditional ligand–receptor interactions. These structures have been involved in the control of receptor endocytosis, host–pathogen interactions, and immune system homeostasis (3).

Abbreviations used in this article: C-CRD, C-terminal carbohydrate-recognition domain; CRD, carbohydrate-recognition domain; DC, dendritic cell; Gal, galectin; N-CRD, N-terminal carbohydrate-recognition domain; PKC, protein kinase C; PTPase, protein tyrosine phosphatase; TDG, thiodigalactoside.

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two Gal-8 isoforms in mouse thymus and spleen, which differed only in the length of the linker region. Both isoforms were able to induce apoptosis of the immature CD4<sup>hi</sup>CD8<sup>hi</sup> thymocyte subpopulation involving caspase pathway activation, suggesting that this Gal could also participate in central negative selection (12). At the periphery, we found that Gal-8 exerts two actions on naive CD4 T cells: at high concentrations, it induces strong Ag-independent proliferation, whereas at low concentrations, it costimulates T cells in the presence of APCs and the corresponding Ag. These activities are mediated by interaction with the T cell surface glycoprotein CD45 and involve the activation of ZAP70- and ERK signaling pathways (13). The molecular requirements for both of Gal-8’s stimulatory effects were recently assessed; its tandem-repeat structure is only essential for proliferation and not for costimulation (14).

Interestingly, Gal-8’s proliferative and costimulatory signals on primary T cells have not been described for any other Gal and suggest a possible involvement of this lectin in inflammatory and autoimmune processes. Moreover, these stimulatory activities differentiate Gal-8 from other Gals, particularly Gal-1 and Gal-3, which are known to display immunoregulatory functions (2). As discussed above, the ability to kill activated peripheral T cells endowed Gal-1 and Gal-3 with anti-inflammatory properties, which were preactivated with PHA or CD3/CD28 stimulators (14). Thus, the effector phase of ongoing responses could be limited, not only by Gal-1 and Gal-3, but also by Gal-8.

The analysis of the existence of redundant or antagonistic functions between Gals is a major concern, because these proteins can converge under normal or pathological conditions, such as inflammatory foci or certain tumors (16–19). The proapoptotic functions between Gals is a major concern, because these proteins were preactivated with PHA or CD3/CD28 stimulators (14). Therefore, Gal-8 is able to induce strong proliferation of freshly naive CD4 T cells: at high concentrations, it induces strong Ag-independent proliferation, whereas at low concentrations, it costimulates T cells in the presence of APCs and the corresponding Ag. These activities are mediated by interaction with the T cell surface glycoprotein CD45 and involve the activation of ZAP70- and ERK signaling pathways (13). The molecular requirements for both of Gal-8’s stimulatory effects were recently assessed; its tandem-repeat structure is only essential for proliferation and not for costimulation (14).

Recombinant Gals

Recombinant Gal-1-8-1 chimera was designed on a synthetic construction by connecting two sequences of full-length mouse Gal-1 with the 9-aa linker peptide from mouse Gal-8 isoform (12). Mouse Gal-1 was cloned using mRNA from thymic epithelial cells as a template (for details see Ref. 12), and mouse Gal-3 was obtained by a synthetic gene construction (GenScript, Piscataway, NJ). Conditions for protein expression and purification by lactosyl-Sepharose (Sigma), followed by immobilized metal-affinity chromatography (GE Healthcare, Uppsala, Sweden), were as described for mouse Gal-8 (12). After the first step of purification, Gal-1 was alkylated in the presence of 0.1 M iodoacetamide (Sigma) overnight at 4°C. The use of alkylated Gal-1 prevents oxidation that leads to a loss of activity and, at the same time, overcomes the need to use reducing agents, such as DTT, which is known to induce deleterious effects in cell culture (20, 21). Gal-8 was biotinylated using EZ-Link Sulfo-NHS-Biotin (Pierce, Rockford, IL), following the manufacturer’s instructions. Lectin activity of these recombinant proteins was tested by hemagglutination assays.

Binding assays

Proliferation assays on mouse cells were performed as described previously (13). Briefly, splenocytes (5 × 10<sup>5</sup> cells) from C57BL/6J mice were cultured at 37°C in 5% CO<sub>2</sub> for 48 h in flat-shaped, 96-well plates in 0.2 ml RPMI 1640 medium in the presence of 10% FBS (Invitrogen), 2 mM glutamine, and 5 μg/ml gentamicin (complete medium). For costimulatory assays, splenocytes (3 × 10<sup>5</sup> cells) from DO11.10 mice were cultured for 72 h in 0.2 ml medium in the presence of the cognate OVA323–339 peptide at 1 μg/ml (Genscript). When BALB/c splenocytes were used as APCs, they were pretreated with 100 μg/ml Mitomycin C in RPMI 1640 medium for 1 h on ice, using siliconized propylene tubes to avoid binding of adherent cell subpopulations, and cells were washed three times with ice-cold PBS. Complete blocking of cell division was checked by inhibition of Con A-activated proliferation. Gal treatment was performed by incubating APCs or purified CD4 T cells with the indicated amounts of Gal-1 or Gal-8 in RPMI 1640 complete media for 30 min on ice, and cells were washed to eliminate unbound lectin. In all proliferation assays, 1 μCi [3H]methyl-thymidine (New England Nuclear, Newton, MA) was added to each well 18 h before harvesting. TDG- and signaling pathway-specific inhibitors were added 30 min before the addition of recombinant proteins, with the exception of the U0126 inhibitor, which was added 1 h before. Unstimulated cells’ basal response ranged from 200 to 1000 cpm and was subtracted in all experiments. Assays were performed in quadruplicate.

Inhibition of cell proliferation

For T cell activation, splenocytes (5 × 10<sup>5</sup>) from C57BL/6J mice were cultured, as described in proliferation assays (see above), in the presence of 2.5 μg/ml Con A for 24 h. Then, preactivated cells were treated with the indicated amounts of Gals for an additional 24 h. Inhibition of cell proliferation was assessed by adding 1 μCi [3H]methyl-thymidine to each well 7 h before harvesting.

In vivo costimulation

Initially, to determine suboptimal Ag dose, 6–8-week-old female DO11.10 mice were immunized i.p. with decreasing amounts (from 5 to 0.05 μg) of OVA (Sigma) in 0.2 ml PBS; spleens were collected 5 d after immunization. In vitro restimulation of splenocytes was performed basically as for costimulation assays (see above), using two doses of OVA<sub>323–339</sub> cognate.
peptide (0.1 and 0.25 μg/ml). For in vivo costimulation, mice received 0.5 μg OVA i.p. in combination with 10–25 μg Gal-8 (OVA + Gal-8) or 25 μg Gal-1 (OVA + Gal-1). Control groups consisted of mice that received Ag only (OVA), vehicle (PBS), or Gals (Gal-1 and Gal-8). In vitro restimulation was performed in the presence of 0.25 μg/ml OVA cognate peptide.

**Flow cytometry**
A FlowMax cytometer PASIII (Partec, Münster, Germany) and WinMdi 2.9 software were used.

**Statistical analysis**
The Student t test was used; p values < 0.05 were considered significant.

**Results**

**Gal-1 costimulates Ag-specific T cell response**
In our previous work, Gal-8 costimulatory activity on naive T cells became evident when splenocytes from DO11.10 mice were incubated in the presence of 0.1–0.2 μM of Gal-8, together with suboptimal doses of OVA cognate peptide (13). We first investigated whether Gal-1 was able to display a similar activity in this model. Remarkably, when Gal-1 was tested in costimulation assays, a dose-dependent effect similar to that of Gal-8 was observed (Fig. 1A). However, 100 times more Gal-1 was needed to equal the activity of Gal-8. Preincubation with TDG almost completely prevented Gal-1–induced costimulation, indicating that this effect relies upon lectin–glycan interaction (Fig. 1B). In the search for a synergistic effect in costimulatory activity, we tested the simultaneous addition of Gal-1 and Gal-8; a similar response was observed to that obtained with Gal-8 alone. Moreover, when lectins were added separately with a 1-hour delay, only a minor, if any, additive effect was observed (Fig. 1C). These findings suggest that these Gals probably recognize similar receptors at the T cell surface to induce the same activation pathways. In fact, we observed that the presence of Gal-1 reduced biotin-labeled Gal-8 binding to CD4 T cells, independently from the addition order, indicating that these lectins actually compete for their binding at the cell surface (Supplemental Fig. 1A).

Because this was the first incidence, to our knowledge, of T cell costimulatory activity described for Gal-1, and it seemed to contradict its previously reported immunoregulatory function, we assessed whether both functions are mutually exclusive. For this purpose, we incubated splenocytes in the presence of the T cell mitogen Con A for 24 h and exposed cells to different concentrations of Gal-1. Gal-8 was also tested, because we previously demonstrated that it is able to inhibit cell proliferation of PHA or anti-CD3/anti-CD28–stimulated human PBMCs (14). As shown in Fig. 1D, both Gals inhibited the proliferation of activated T cells, an effect prevented by the addition of TDG, thus supporting the involvement of Gals and cell–glycan interactions. Unexpectedly, this antiproliferative effect could not be ascribed to cell death induction, because no increment of apoptotic cells was observed after Gal-1 or Gal-8 treatment of activated splenocytes, as assessed by propidium iodide incorporation or caspase-3 activation (data not shown).

Taken together, these results support a dual role for Gal-1 and Gal-8 in the immune response by enhancing initial T cell responses but limiting those that become exacerbated.

**Gals activate TCR downstream signaling pathways to induce costimulation**
To better characterize Gal-1 and Gal-8 costimulation activities, we performed assays in the presence of Ick (Src)-, PI3K-, PKC-, p38 MAPK-, and ERK–specific inhibitors, as representative TCR downstream signal transducers. We also tested CD45 PTPase inhibitor, because we previously determined that Gal-8 interacts with CD45 and that its PTPase activity is involved in Gal-8–induced costimulation and proliferation (13). As expected, inhibition of TCR signaling pathways resulted in a decreased OVA-induced response (Fig. 2). The Gal-8 and Gal-1 costimulatory effect was strongly affected in the presence of these inhibitors, thus supporting that both Gals may reinforce the same intracellular pathways, activated by the corresponding Ag, to promote T cell costimulation.

**Gals induce costimulation by simultaneously acting on APCs and CD4 T cells**
To gain insight into the mechanism by which Gals mediate their costimulatory activity, we performed a set of experiments to determine whether these lectins are actually acting on APCs, T cells, or both. First, we designed costimulation assays in which Mitomycin C-treated splenocytes from BALB/c mice were used as APCs, and highly purified CD4 T cells from TCR<sub>OVA</sub> DO11.10 mice were used as target. As observed in Fig. 3A, Gal-1 and Gal-8 readily stimulated CD4 T cells in the presence of OVA Ag, confirming that these cells are targets for the costimulatory effect.
Next, APCs and CD4 T cells were separately preincubated with Gal-8 or Gal-1 for 30 min on ice to allow Gal binding to the cell surface and then unbound lectins were washed out. Interestingly, when Gals were bound to only one of the populations involved, costimulation was no longer achieved. Furthermore, costimulation was also absent when APCs and T cells treated separately with Gals were mixed together. These findings indicate that to completely costimulate the Ag response, Gals need to bind on both APCs and T cells, at the very same time, during the Ag-presenting process. To confirm these observations, we repeated this assay using CD4 T cell-depleted splenocytes from DO11.10 mice (Fig. 3B). Again, Gal treatment on either APCs or T cells was ineffective in inducing costimulation.

A tandem-repeat Gal-1 chimera displays Ag-independent proliferative activity

In addition to the costimulatory activity, Gal-8 induces strong proliferation of mouse resting CD4 T cells in the absence of antigenic stimulus, when used at relatively high concentrations (13). To test whether Gal-1 also shares this effect, we incubated C57BL/6J mouse splenocytes in the presence of increasing amounts of Gal-1. Even at the highest concentrations used (20 μM), Gal-1 was unable to induce the strong cell proliferation observed with 2 μM of Gal-8 (Fig. 4A). It is important to point out that, although it homodimerizes under certain conditions, Gal-1 contains only one CRD, whereas Gal-8 is a tandem of two CRDs fused by a linker peptide, thus being intrinsically a dimer. This difference could explain why Gal-1 can only display costimulatory, but not proliferative, activity on T cells (14). In fact, we recently demonstrated that both Gal-8 single N-CRDs and C-CRDs...
are sufficient to costimulate T cell response to cognate peptide but, in strong contrast, they are unable to trigger Ag-independent proliferation. In contrast, chimeric homodimers N-CRD–N-CRD and C-CRD–C-CRD triggered proliferation, indicating that Gal-8 tandem-repeat structure is essential for Ag-independent proliferation but not for costimulation induction (14). This prompted us to investigate whether the stable dimerization of Gal-1 could overcome its inability to trigger T cell proliferation. For this purpose, we designed a chimeric protein in which two Gal-1 CRDs are covalently fused by the Gal-8L “long” linker peptide (12), Gal-1-8-1. Gal-1-8-1 retained lectin activity and exhibited 2-fold greater hemagglutinating activity than did Gal-1 (6.25 and 12.5 μg/ml, respectively). Remarkably, Gal-1-8-1 displayed a significant proliferative effect from 10 μM, which was inhibited by preincubation with the Gal inhibitor, TDG (Fig. 4C). Of note, a 10-fold greater amount of chimeric Gal-1-8-1 was needed to equal Gal-8’s proliferative activity (20 and 2 μM, respectively). Moreover, Gal-1-8-1 induced costimulation at a similar rate as did Gal-1 (Fig. 4D), in agreement with the fact that Gal-8 monomeric CRDs are sufficient to trigger this effect (14). Taken together, these results indicate that stable linkage of two CRDs provides Gal-1 with a proliferative ability, although it does not necessarily imply an increase in its costimulatory activity. Alternatively, differences in Gal-1 and Gal-8 potency might be explained by a fine distinction among CRDs’ specificity rather than Gal molecular requirements. In this regard, the induced cellular phenotype of cultured splenocytes was completely different in the presence of Gal-1 or Gal-8: although Gal-8 induced an adhesive and spread phenotype, both Gal-1 and Gal-1-8-1 promoted strong cell agglutination, without adhesive or spread phenotype (Fig. 4E). This is in agreement with the fact that Gal-8 contains two CRDs with different glycan specificities that enables it to promote cell adhesion by linking the extracellular matrix and the cell surface (22). The strikingly different cellular phenotypes induced by Gal-1-8-1 and Gal-8 suggest that they might involve distinct mechanisms of cellular activation to induce the proliferation of T cells. 

Gal-3 antagonizes Ag-specific T cell response

Next, we investigated whether Gal-3, another prominent member of the Gal family, was also able to exert costimulation of naive T cells. In contrast to Gal-1 and Gal-8, Gal-3 was unable to costimulate Ag-specific responses at any of the concentrations tested (5–10 μM, data not shown), and it inhibited the OVA response when the cognate peptide dose was ≤1 μg/ml (Fig. 5A). It was reported that the lattice formed by Gal-3 negatively regulates the TCR response by restricting receptor lateral motility (23–25). These reports support the lack of costimulation in the presence of Gal-3, as well as its inhibitory effect on the Ag-specific T cell response. Subsequently, we investigated the addition of Gal-3 on Gal-1 and Gal-8 costimulatory activity using low (1 μg/ml) and high (2 μg/ml) OVA peptide concentrations. Under low-Ag conditions, at which Gal-3 inhibits the OVA response per se, it also prevented Gal-1 and Gal-8 costimulatory effects (Fig. 5B, 5C). Gal-3 inhibited Gal-1’s effect, despite whether the lectins were added simultaneously or were separated by 1 hour and independently of the addition order. The costimulatory effect of Gal-8 was inhibited when Gal-3 was added simultaneously or 1 h before, but not when added 1 h after (Fig. 5B). Under high-Ag conditions, even when Gal-3 was unable to affect the OVA response per se, it was able to prevent either Gal-1 or Gal-8 costimulation when added simultaneously. However, Gal-3 was unable to affect either Gal-1– or Gal-8–induced costimulation when added separately (Fig. 5D, 5E). These results suggest that Gal-3 negatively reg-

FIGURE 4. Gal-1 stable dimerization confers proliferative activity. (A) Splenocytes from C57BL/6J mice were incubated for 48 h in the presence of 20 μM Gal-1 or 2 μM Gal-8. (B) Schematic representation of the recombinant chimera Gal-1-8-1. (C) Proliferation assays were performed with the indicated amounts of Gal-1-8-1 or Gal-8. (D) Splenocytes from DO11.10 mice were cultured for 48 h in the presence of the cognate OVA peptide (1 μg/ml) together with increasing amounts of Gal-1 or Gal-1-8-1. Con A was used at 2.5 μg/ml as positive control. TDG was used at 30 mM. (E) Phase-contrast microscopy images of cultured mouse splenocytes (original magnification ×200). After purification, 0.5 × 10⁶ cells were plated onto 96-well plates and stimulated for 18 h in complete media with the indicated amounts of Gal-1, Gal-8, or Gal-1-8-1. Assays are representative of more than three independent experiments and were carried out each time with different recombinant protein preparations. *p < 0.05, **p < 0.005, ***p < 0.0001.
Gal-1 and Gal-8. However, Gal-8 seemed to trigger a rapid and strong cell activation, regardless of whether Gal-1 was present. This effect was observed in both in vitro and in vivo experiments, indicating that Gal-8 may have a more prominent role in costimulation.

**FIGURE 5.** Gal-3 inhibits both Gal-1– and Gal-8–induced costimulation. (A) Splenocytes from DO11.10 mice were cultured for 48 h in the presence of increasing amounts of the cognate OVA peptide (OVA), together with Gal-3. The inhibitory effect of Gal-3 on Gal-1– and Gal-8–induced costimulation was tested using two concentrations of peptide: 1 μg/ml (B and C) or 2 μg/ml (D and E). Combinations of Gal-3 and Gal-8 (B and D) or Gal-3 and Gal-1 (C and E) were added either simultaneously (from a mixture containing both Gals, +) or separately (1 h incubation between gals, –>). (A–E) Gal-1 and Gal-3 were used at 10 μM, and Gal-8 was used at 0.2 μM. (F) Splenocytes from C57BL/6j mice were incubated for 48 h in the presence of 10 μM Gal-3, 2 μM Gal-8, or a combination of both Gals. Assays are representative of at least three independent experiments. *p < 0.01, **p < 0.001.

**Discussion**

Gals have arisen as key mediators of the innate and adaptive immune response that participate in several processes, such as host–pathogen interaction, inflammatory and autoimmune disorders, host-versus-graft disease, fetal–maternal tolerance, and self-tolerance maintenance. Therefore, how Gals control immune cell homeostasis and the underlying mechanisms are crucial. Given that some Gals can be simultaneously present in the same microenvironment, the cross-talk among these lectins is another key aspect that is now emerging. In the present work, we demonstrated that Gal-1, as well as Gal-8, can costimulate borderine Ag-specific T cell responses. Blockade of established TCR signaling pathways (Lck, PI3K, PKC, p38 MAPK, and ERK) prevented Gal-1– and Gal-8–induced costimulation, indicating that these Gals actually enhance weak signals from borderline Ag-TCR engagement. CD45, an abundant and heavily glycosylated cell surface mucin, is a common ligand for Gal-1 and Gal-8. CD45 PTPase activity was previously shown to be involved in Gal-8–induced proliferation and costimulation, probably by lowering the TCR activation threshold by dephosphorylation of the Lck Tyr505 inhibitory site. As shown in Fig. 2A, CD45 PTPase activity was also involved in Gal-1–induced costimulation. Although CD45 is a well-established Gal-1 counterreceptor,
In agreement, Gal-3 failed to induce phosphatidylinerse exposure in resting T cells, indicating that T cell activation is required to sensitize cells to Gal-3–induced apoptosis (21). Although the negative effect of Gal-3 on TCR activation was absent in the presence of a higher dose of Ag, Gal-3 was still able to inhibit Gal-1– and Gal-8–induced costimulation only when they were added simultaneously. Given that it was previously shown that Gal-3 partially inhibits Gal-1 binding to the T cell surface (9), we reasoned that Gal-3 might displace Gal-1 or Gal-8 more efficiently when present simultaneously. However, we observed that Gal-3 partially reduced Gal-8 binding on T cells independently of the addition order, not fully explaining our findings. Alternatively, differences in the quaternary structures and cross-linking activities might explain the inhibitory effects of Gal-3 on the activities of Gal-1 and Gal-8. In this regard, the observation that exogenously added Gal-3 antagonizes the apoptotic effects of Gal-1 in susceptible T cells was previously related to a greater avidity of the Gal-3 pentamer compared with the Gal-1 homodimer for glycoprotein receptors on the cell surface (27).

The fact that 100-fold more Gal-1 is required to equal Gal-8 costimulatory activity could be explained by the observation that tandem-repeat Gals, which have two CRDs stably fused by a linker peptide, are more potent effectors than monomeric Gals. In fact, it has been demonstrated that two covalently linked Gal-1 CRDs induce apoptosis more efficiently than the monomeric form (28–30). However, chimeric Gal-1-8-1 did not induce greater costimulation than native Gal-1, indicating that structure is not the only important factor; rather, a fine distinction among CRDs’ specificity may account for the differences observed.

We previously established several molecular requirements for Gal-8 to induce its costimulation and T cell-proliferation properties; although the tandem-repeat structure is essential for Ag-independent proliferation, both single N-CRDS and C-CRDS are able to induce Ag-specific costimulation (14). We also determined that Gal-8’s single N-CRD is sufficient to trigger platelet activation (31). In agreement, in this study we showed that Gal-1 costimulated T cells in the presence of the cognate peptide, but it was unable to trigger proliferation of naive splenocytes, even when tested at a high concentration (20 μM). Notably, Gal-1 stable dimerization stabilized with Gal-8 peptide linker (Gal-1-8-1) overcame its inability to trigger proliferation in the absence of Ag, although 10-fold more Gal-1-8-1 was still needed to equal Gal-8 activity. Again, differences in potency could be related to distinct CRD specificity among these Gals, because we observed that proliferating splenocytes displayed an adhesive and spread phenotype in the presence of Gal-8, whereas cells were only agglutinated in the presence of Gal-1-8-1.

In the present work, Gal-1–stimulating activity on resting T cells is supported by demonstration of Gal-1–induced costimulation and chimeric Gal-1-8-1–induced proliferation. This constitutes a novel role for this Gal, because the majority of previous reports focused on its proapoptotic role on immature or activated, but not naive, T cells. In agreement with our results, Perillo et al. (6) demonstrated in an early report that Gal-1 induces apoptosis on PHA-activated PBMCs, but it is unable to exert the same effect on naive peripheral human T cells. In addition, it was shown that, although Gal-1 induces apoptosis of 2-d Con A-activated T cells, it promotes the survival of resting T cells without inducing proliferation (32). In contrast to our observations and those from other investigators, Matarese et al. (33) observed that Gal-1 sensitized human resting T lymphocytes to Fas-mediated cell death and that, at high doses, it was capable of inducing apoptosis in these primary cells. Discrepancies might reflect the different species used (human versus mouse), different protein preparations, or the method used to assess cell death, because phosphati-
dyserine exposure is not necessarily associated with Gal-induced apoptosis (34).

The observation that Gal-1 and Gal-8 stimulated naive T cells, but displayed an antiproliferative effect when these cells became activated (Fig. 1), strongly suggests a dual role for these lectins: enhancing normal or physiological immune responses, especially when stimulus is limited, and restraining the effector phase of ongoing or exacerbated responses. However, the antiproliferative effect could not be ascribed to cell death induction, because apoptosis was only observed when activated splenocytes were incubated in the presence of Gal-3 and not with Gal-1 or Gal-8. Of note, our results are in line with a previous report that Gal-3, but not Gal-1, induces apoptosis of primary activated T cells (21). Therefore, further studies are necessary to properly address the mechanism by which these Gals are exerting their antiproliferative effect on activated T cells.

A single dose of 25 µg of Gal-1 or Gal-8, administered with a suboptimal dose of Ag in mice, was sufficient to increase the T cell response, demonstrating that Gals can prime resting T cells in vivo to enhance borderline Ag responses. Although this outcome could be mediated by Gal-induced costimulation processes observed in vitro, it should be considered that Gals can exert different functions on many cell types in vivo. For example, Gals might also be stimulating different APCs to sustain T cell activation after priming. In this regard, it was reported that dendritic cells (DCs) engineered to express transgenic Gal-1 displayed an enhanced ability to stimulate naive T cells and, conversely, induced apoptosis of activated T cells (35). These Gal-1–expressing DCs exhibited a mature phenotype, as reflected by the increase in MHC and costimulatory molecule expression, as well as enhanced levels of proinflammatory cytokines. It was also demonstrated that Gal-1 stimulates T cell proliferation directly through its ability to promote DC maturation, because pretreatment of DCs with recombinant Gal-1 was sufficient to trigger T cell stimulation. Furthermore, Gal-1–treated human DCs displayed an enhanced migration through extracellular matrix, suggesting that it could participate in initiating the immune response (36, 37). In line with this evidence, it was reported very recently that mature and immature human DCs constitutively express Gal-8 protein (38); moreover, we observed that Gal-8 treatment induced the expression of activation markers on murine bone marrow-derived DCs (J. Carabelli, M.V. Tribulatti, and O. Campetella, unpublished observations). With regard to B cells, it was reported that both Gal-1 and Gal-8, but not Gal-3, promoted plasma cell differentiation (39), probably contributing to the humoral response as well. Nevertheless, we cannot discount a direct effect of Gals on naive T cells during the Ag response in vivo, because Gal-8 was demonstrated to induce purified CD4 T cell proliferation (13) and, as described in this article, chimeric Gal-1-8-1 also induced splenocyte proliferation.

Finally, the findings reported in this article allow us to postulate these lectins as enhancers of borderline Ag responses, thus representing suitable candidates as additives for vaccines preparations, among other applications.

Acknowledgments
We thank Dr. Philippa Marrack (Howard Hughes Medical Center, Denver, CO) for providing DO11.10 T cell hybridoma and Dr. Alejandro Cassola for carefully reading the manuscript. Technical assistance with animal care provided by Fabio Fraga is highly appreciated.

Disclosures
The authors have no financial conflicts of interest.

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