Galectin-1 functions as a Th2 cytokine that selectively induces Th1 apoptosis and promotes Th2 function

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Galectin-1 functions as a Th2 cytokine that selectively induces Th1 apoptosis and promotes Th2 function.

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Abstract

Galectin-1 has been implicated in regulating T cell survival, function and Th1/Th2 balance in several mouse models, though the molecular and cellular basis of its immuno-modulatory activity has not been completely elucidated. Therefore, we examined galectin-1 expression and activity within differentiated murine Th1 and Th2 subsets. While galectin-1 specifically bound to both T cell subsets, Th1 and Th2 T cells expressed distinct constellations of galectin-1 reactive epitopes and were differentially responsive to galectin-1 exposure. Indeed, Th1 cells were more susceptible to galectin-1 induced death than Th2 cells. Th2 protection from apoptosis was correlated with expression of anti-apoptotic galectin-3. Further, galectin-1 diminished TCR-induced type 1 Th1 cytokine production, while it promoted TCR-induced type 2 cytokine production by Th2 cells. Differentiated Th2 cells constitutively expressed high levels of galectin-1, whereas comparable levels of galectin-1 in Th1 cells were only observed after re-stimulation. Co-culturing galectin-$1^{-/-}$ and galectin-$1^{+/+}$ Th1 and Th2 T cells demonstrated that Th2-derived galectin-1 induced Th1 apoptosis and antagonized Th1 cytokine production, whereas Th1-derived galectin-1 promoted Th2 cytokine production. These studies identify galectin-1 as a cross-regulatory cytokine that selectively antagonizes Th1 function and survival, while promoting TCR-induced Th2 cytokine production.

Introduction

Naïve CD4 T cells can differentiate into Th1 and Th2 cells that produce specific sets of cytokines capable of initiating different defense mechanisms against pathogens. Th1 cells secrete IL-2 and IFN-γ and play a role in mediating inflammatory responses, activating macrophages, and stimulating cellular immunity. Th2 cells produce IL-4, IL-5, IL-6 and IL-10 and activate B cells to mediate humoral immunity and are prominent mediators of allergic responses [1-3]. Uncontrolled Th1 and Th2 responses contribute to chronic inflammatory disease and allergies, respectively.

T helper precursors commit to either a Th1 or Th2 phenotype within a few days of activation. The strength of TCR signaling, antigen dose and route of administration, costimulation, the type of antigen presenting cell (APC) involved, and local cytokine milieu each provide cues which guide T helper differentiation [4, 5]. However, the presence of regulatory cytokines during the course of primary T cell activation has the greatest impact on CD4 Th1/Th2 lineage commitment. Indeed, IL-12 and IL-4 are the most potent inducers of Th1 and Th2 differentiation, respectively. Furthermore, Th subsets reciprocally regulate each other. Th1-derived IFN-γ inhibits the proliferation and function of Th2 cells; whereas Th2-derived IL-4 and IL-10 suppress Th1 cells. Once differentiated, variable regulation of cell survival and death in T cell subsets further influences the establishment and maintenance of a particular immune response [6], though regulators of these processes remain poorly characterized.

Regulation of cell death is an important function of the galectins, a family of lectins expressed in a wide variety of tissues. While secreted galectin-1 induces death in some T cells, intracellular galectin-3 can protect T cells from apoptosis [7, 8].

Additionally, recombinant galectin-1 can inhibit T cell proliferation [9, 10] and influence the persistence of T cell subsets [9-15]. Indeed, treatment of mice with recombinant galectin-1 has been reported to block the development of T cell mediated diseases, including autoimmune adjuvant induced arthritis [14], experimental autoimmune encephalitis (EAE) [15, 16], and myasthenia gravis [17]. Endogenous galectin-1 has also been implicated in tumor escape from immuno-surveillance [18] and protection from EAE [16]. In some instances, galectin-1 treatment skews the Th1 response toward a Th2 response, implicating galectin-1 in immune deviation [14], although the cellular and molecular basis of this activity has not been well elucidated.

The activation and differentiation of T cells results in altered expression of glycosyltransferases and glycosidases that regulate the generation of galectin-1 ligands [19-23]. Galectin-1 binds repeated units of galactose-β1-4-N-acetylglucosamine presented on N- and O- linked glycans with high avidity, although terminal sialic acid addition to these glycans can block galectin-1 binding [15]. During T cell activation, an increase in the number of lactosamine bearing core 2 O-glycans and N-glycans lacking terminal sialic acid are predicted to increase T cell susceptibility to galectin-1 binding and immunoregulation [21]. For example, murine T cells activated under either Th1 or Th2 skewing conditions decrease the sialyltransferase ST6Gal I and increase galactosyltransferase α1,3GalT expression [20], predicting that activation of either Th1 or Th2s might enhance galectin-1 binding and activity. Additionally, both Th1 and Th2 cells express β(1,6) N-acetylglucosaminyltransferase-I that catalyses the formation of lactosamine-bearing targets on O-linked glycoproteins. However, β-galactoside α2,3-sialyltransferase-I, the major inhibitor of core 2 O-glycan formation on CD43 and CD45

in T cells, is extensively expressed during Th2, but not Th1 differentiation [24]. Therefore, Th1 cells might display more galectin-1 binding sites than Th2 cells, rendering them preferentially sensitive to galectin-1 binding and immunoregulation. Which murine peripheral T cell populations are susceptible to specific galectin-1 binding, whether endogenous galectin-1 and galectin-3 are differentially expressed by distinct T cell subsets, and whether Th-derived galectins modulate death of murine T cell populations remains incompletely addressed.

Here, we characterize murine Th1 and Th2 subpopulations with regard to their relative level of galectin-1 expression, sensitivity to galectin-1-mediated death, and their ability to cross-regulate one another through secreted galectin-1. We found that differentiated Th2 cells expressed more galectin-1 and -3 prior to restimulation and were more resistant to galectin-1-mediated apoptosis than Th1 cells. Further, galectin-1 antagonized cytokine production by Th1 cells, whereas it promoted type 2 cytokine secretion by Th2 cells. Finally, Th2-secreted galectin-1 selectively induced apoptosis in neighboring Th1 cells, whereas Th1-derived galectin-1 promoted Th2 cytokine production in Th1/Th2 co-cultures. These findings identify endogenous galectin-1 as a cross-regulatory cytokine that impairs the survival of Th1 cells, while promoting Th2 function.

Results

Th2 cells are more resistant than Th1 cells to galectin-1-induced cell death.

Th1 and Th2 populations were generated *in vitro* by two rounds of antigenic stimulation under cytokine skewing conditions. Th1/Th2 effector cell differentiation was quantitated by intracellular cytokine staining and measuring levels of Th1- and Th2-type cytokines released into the culture supernatant after re-stimulation (Figure 1A and B). Consistent with previous reports, Th1 and Th2 populations differed in expression of core 2 *O*-glycan related epitopes on CD43 and CD45 [24]. A greater percentage of Th1 cells expressed significantly more of the 1B11 reactive glycoproteins compared with Th2 cells (Figure 1C).

To investigate the possibility that galectin-1 might differentially regulate cell survival in Th1 and Th2 populations, we compared the susceptibility of Th1 and Th2 cells to galectin-1-mediated death. Differentiated Th1 and Th2 populations were rested and subsequently exposed to recombinant galectin-1. The level of cell death was assessed using three independent measures: Annexin V binding and PI permeability (Figure 2A); hypodiploid DNA content (Figure 2B); and forward versus side scatter (Figure 2C).

In all three death assays, galectin-1 exposure of differentiated populations to recombinant galectin-1-induced significant cell death in Th1, but not Th2 subpopulations. Th1 cells responded to galectin-1 exposure with greater induction of Annexin V staining (52% versus 17%, Figure 2A); increased percentage of cells with hypodiploid DNA content (47.6% versus 13.6%, Figure 2B); and fewer cells within the FSC versus SSC live gate (17% versus 28% Figure 2C) relative to Th2 cells. When T cells were re-

activated with anti-CD3/CD28 eighteen hours prior to incubation with galectin-1, susceptibility to galectin-1-induced death was increased in both Th1 and Th2 populations (data not shown). Nonetheless, Th1 cells remained preferentially sensitive to galectin-1-induced death, even after activation. The specificity of galectin-1-induced death was confirmed using lactose to block its activity (Figure 2C).

Carbohydrate dependent binding of galectin-1 to resting and activated Th1 and Th2 populations.

It is possible that the differential susceptibility of murine Th1 and Th2 cells to galectin-1-mediated death reflects different galectin-1 binding capacities, in keeping with reports that Th1 cells preferentially express enzymes involved in the generation of cell surface galectin-1 counterligands [15, 24]. To directly test this hypothesis, we biotinylated recombinant galectin-1 and assayed its binding to Th1 and Th2 populations. As seen in Figure 3, biotin-labeled galectin-1 bound to unstimulated Th1 and Th2 cells. Binding was β-galactoside-specific since the presence of lactose reduced binding by >95%. Galectin-1 binding to unstimulated Th1 cells was slightly higher than to Th2 cells (MFI 91.65 for Th1 versus 79.65 for Th2, Figure 3 gray histogram). To determine how stimulation of Th1 and Th2 populations might modify galectin-1 binding, we activated cells using anti-CD3/CD28 antibodies for 18 h prior to biotin-galectin-1 staining (Figure 1C). In both T cell populations, we found an activation-induced increase in galectin-1 binding (Figure 3) with >90% of the cells from both cell populations showing high galectin-1 binding (Figure 3, M1). Therefore, galectin-1 can bind to carbohydrate ligand(s) on both Th1 and Th2 cell surfaces. However, as demonstrated by 1B11

staining, the upregulation of particular glycoprotein epitopes vary between Th1 and Th2 lineages (Figure 1).

Th2 cells express more galectin-3 than Th1 cells.

Previous reports indicate that galectin-1 can sensitize resting human T lymphocytes to Fas-mediated (CD95) cell death [25], and that Th2 cells are more resistant to both CD95L- and TRAIL-mediated apoptosis [26]. In addition, several genes related to apoptosis including *Trail*, *Bak-2*, and *Casp8* are preferentially expressed in Th1 cells, consistent with the increased susceptibility of Th1 cells to activation-induced death [27]. Therefore, we considered the possibility that galectin-1 might induce apoptosis through Fas and/or TRAIL in differentiated Th1 or Th2 cells. We observed that recombinant galectin-1 exposure to unstimulated or restimulated Th1 and Th2 cells increased the percentage of cells expressing CD95, CD95L or TRAIL (Figure 4A). However, the cells bearing these surface receptors were not enriched in the galectin-1-induced Annexin V positive subpopulations (not shown). Further, the addition of blocking antibodies against CD95L or recombinant DR5 were unable to block galectin-1-mediated cell death (not shown). These findings are consistent with previous reports characterizing galectin-1-mediated death as CD95 and caspase independent in T cells, rather involving rapid nuclear translocation of endonuclease G [8].

Intracellular expression of galectin-3 can inhibit extracellular galectin-1-mediated cell death in T and B cells [8]. Furthermore, galectin-3 expression is upregulated on some activated CD4+ T cells and is enhanced by IL-4, but not by IFN-γ [28], suggesting that preferential galectin-3 expression in Th2 versus Th1 cells might account for

decreased Th2 sensitivity to galectin-1-induced death. Accordingly, we find that Th2 cells express higher levels of galectin-3 mRNA than Th1 cells as measured by Q-PCR (Figure 4B). Consistent with higher levels of galectin-3 mRNA in Th2 cells, there was more galectin-3 staining in Th2 cells relative to Th1 cells when measured by flow cytometry (Figure 4C).

Galectin-1 promotes TCR-induced Th2 cytokine secretion and antagonizes Th1 cytokine secretion.

To determine the consequences of galectin-1 exposure on TCR/CD28-induced cytokine production, differentiated Th1 and Th2 cells were restimulated with plate bound anti-CD3 and soluble anti-CD28, alone or together with recombinant galectin-1. Supernatants were analyzed for the presence of Th1- or Th2- type cytokines. Supporting our findings that galectin-1 induces death in Th1 cells (Figure 2), recombinant galectin-1 prevented the accumulation of TCR/CD28-induced IL-2 and IFN-γ (Figure 5A). Conversely, galectin-1 promoted TCR/CD28-induced production of IL-4, IL-5 and IL-10 (Figure 5B). These findings are consistent with our data demonstrating that galectin-1 can bind Th2 cells (Figure 3), the relative resistance of Th2 cells to galectin-1-induced death (Figure 2), and our previous findings demonstrating that galectin-1 can selectively modulate TCR-induced functions, including activation-induced cell death and cytokine production [9].

Endogenous galectin-1 produced by Th2 cells binds Th1 cells and controls their survival.

Since Th1 cells are susceptible to galectin-1-mediated death, these cells could be targets of other cells secreting galectin-1. To determine whether endogenous galectin-1 produced by Th1 or Th2 cells acts as a regulatory cytokine contributing to the Th1/Th2 balance, we assessed endogenous galectin-1 expression, secretion, and regulatory activity in Th1 and Th2 populations. As shown in Figure 6A, unstimulated Th2 cells express more galectin-1 than unstimulated Th1 cells (MFI: 84.6 versus 54.1, respectively). Anti-CD3/CD28 stimulation increased intracellular galectin-1 expression after 24 h of activation in both subpopulations. Q-PCR analysis of galectin-1 mRNA levels similarly demonstrated increased galectin-1 mRNA in differentiated Th2 versus Th1 both prior to and in response to re-stimulation (Figure 6B).

To assay for secreted galectin-1, equivalent numbers of Th1 or Th2 cells were cultured in serum-free conditioned medium alone or in the presence of anti-CD3/CD28. Supernatants collected after 24 and 48 h of culture were analyzed by Western blot (Figure 6C). At every time point tested, Th2 cells secreted higher levels of galectin-1 than did Th1 cells (Figure 6C). Equivalent volumes of supernatants from equal number of galectin-$1^{-/-}$ Th1 and galectin-$1^{-/-}$ Th2 cells cultured for 24 h were processed in parallel and demonstrate the specificity of the galectin-1 band.

It is well established that Th1 and Th2 populations cross regulate one another by secreting cytokines that antagonize the development of the reciprocal population. Because Th2 cells secrete higher levels of galectin-1 and are relatively resistant to galectin-1-induced death, we next explored the possibility that Th2-derived galectin-1 might function as a cross-regulatory cytokine which promotes Th2 dominance through selective induction of Th1 death. To test this hypothesis, differentiated Th1 cells were

mixed with Th2 cells in a coculture assay in the absence or presence of stimulating anti-CD3/CD28 antibodies and the effects on cell death measured. Labeling the Th1 cells with CFSE prior to co-culture allowed us to discriminate Th1 from Th2 cells using flow cytometry, whereas utilization of wild type or galectin-1 null effectors allowed us to control for the source of endogenous galectin-1. Analysis of surface galectin-1 binding on Th1 ($CD4^+$ $CFSE^+$) and Th2 ($CD4^+$ $CFSE^-$) gated cells demonstrated that endogenous galectin-1 secreted by Th2 cells modestly binds to the surface of galectin-$1^{-/-}$ Th1 cells in the absence of stimulation, and to a greater extent in anti-CD3/CD28 restimulated cultures (Figure 7A, left panel). In contrast, when galectin-1 positive Th1 cells were included as the only source of galectin-1 in co-cultures, only modest levels of galectin-1 bound to galectin-$1^{-/-}$ Th2 cells (Figure 7A).

Galectin-1 secreted by stimulated Th2 cells significantly inhibited the survival of stimulated Th1 cells in co-culture experiments. Fewer live galectin-$1^{+/+}$ Th1 cells (Annexin V^-, 7-AAD^-) were present in co-cultures containing galectin-$1^{+/+}$ Th2 cells relative to those containing galectin-$1^{-/-}$ Th2 cells (Figure 7B). Conversely, galectin-1 secreted by Th1 cells was unable to influence galectin-$1^{+/+}$ Th2 survival (Figure 7B). Finally, supernatants from galectin-$1^{+/+}$ Th1 plus galectin-$1^{+/+}$ Th2 co-cultures contained less IFN-γ than those from galectin-$1^{+/+}$ Th1 plus galectin-$1^{-/-}$ Th2 co-cultures, whereas galectin-1 produced by Th1 promoted IL-5 production by galectin-$1^{+/+}$ Th2 (Figure 7C). These findings identify galectin-1 as a secreted cytokine that selectively antagonizes Th1 survival and function and promotes TCR-induced Th2 cytokine production.

Discussion

Galectin-1 can act as a negative immune regulator under pathological situations including autoimmunity [14], cancer [18], and infection [29]. *In vitro* experiments have implicated recombinant galectin-1 in selectively modulating TCR-induced functional outcome and survival [9]. In experimental models of chronic inflammation, administration of recombinant galectin-1 *in vivo* can skew towards a Th2-type cytokine response [14, 30, 31]. Nonetheless, it has yet to be determined which murine peripheral T cell populations produce galectin-1, which are susceptible to galectin-1 binding, and whether endogenous T cell galectin-1 expression can function to directly modulate Th1/Th2 survival and lymphokine production.

To address these issues, we differentiated Th1 and Th2 populations and first compared their susceptibility to galectin-1-mediated death. We found that recombinant galectin-1 exposure induced significantly more cell death in Th1 versus Th2 subpopulations both prior to and after TCR/CD28 engagement. These findings are in keeping with reports that human Th1 cell are more susceptible to galectin-1 death than human Th2 cells and that endogenous galectin-1 expression influences Th1 survival *in vivo* [15]. Further, we found that while both Th1 and Th2 cells produce galectin-1 and TCR/CD28 engagement increases galectin-1 expression, Th2 cells represent a greater source of galectin-1, particularly prior to TCR/CD28 restimulation. These findings predict that Th2-derived galectin-1 might regulate Th1 survival in situations where both subpopulations are present. Further, Th1-derived galectin-1 might function in an autocrine regulation of Th1 cell death and homeostasis after antigenic stimulation. That activation of Th2 cells can also increase their susceptibility to galectin-1-mediated death

upon activation predicts that galectin-1 may also play a role in regulating Th2 survival under some circumstances.

To evaluate the possibility that the differential susceptibility of Th1 and Th2 cells to galectin-1-mediated death reflects different galectin-1 binding capacities, we assayed the ability of biotinylated recombinant galectin-1 to bind to Th1 and Th2 populations. We show that galectin-1 can bind to carbohydrate ligand(s) on both Th1 and Th2 cell surfaces, indicating that Th2 cells are protected from galectin-1-induced death through a mechanism other than generalized galectin-1 binding capacity. These findings contrast those characterizing galectin-1 in human Th1 and Th2 cells, where Th2 cells were found to be deficient at galectin-1 binding [15], highlighting species-specific aspects of galectin-1 immmuno-regulatory activity. To explore alternate mechanisms by which galectin-1 might differentially impact Th1 vs Th2 survival in murine T cells, we examined the relative expression of pro- or anti-apoptotic pathway effectors. While galectin-1 binding did lead to modest increases in the percentages of CD95L and TRAIL expressing cells in Th1 and Th2 populations, cells bearing these surface receptors were not enriched in the galectin-1-induced Annexin V positive subpopulation, nor did blocking these receptor ligand pairs protect from galectin-1-induced death. Therefore, increased Th1 cell sensitivity to galectin-1 could not be readily explained by differential CD95/CD95L or TRAIL expression and signaling. Alternatively, we found that anti-apoptotic galectin-3 [32, 33] is preferentially expressed in Th2 cells both prior to and after restimulation. Therefore, preferential upregulation of galectin-3 in Th2 cells may account for their increased resistance to galectin-1-induced apoptosis, though additional experimentation is necessary to establish galectin-3 anti-apoptotic activity in Th2 cells.

We also found that galectin-1 differentially affects TCR-induced cytokine production in Th1 and Th2 populations. Galectin-1 prevented the accumulation of TCR/CD28-induced IL-2 and IFN-γ produced by Th1 cells, while promoting IL-4, IL-5 and IL-10 production by surviving Th2 cells. These findings are consistent with our data demonstrating that galectin-1 binds to both Th1 and Th2 subpopulations, and our published findings that galectin-1 can selectively modulate TCR-induced functions including activation-induced cell death and cytokine production [9]. We have yet to determine whether the effects on Th1 cytokine production are secondary to galectin-1-mediated Th1 cell death or due to antagonism of TCR-induced cytokine production. Taken together, our findings elucidating galectin-1 immunoregulatory activity on murine Th1 and Th2 cells provide a cellular mechanistic basis for the ability of recombinant galectin-1 to antagonize inflammatory responses and skew toward Th2 responses in the context of autoimmune and cancer models.

Galectin-1 binding to different counterligands and/or induction of distinct glycolattice-based microdomains in Th1 and Th2 subpopulations might regulate its selective contributions to apoptosis and modulation of TCR-induced function in these discrete T cell subsets. Accordingly, we found that while galectin-1 specifically binds to the surface of both Th1 and Th2 subpopulations, expression of the glycosylation dependent CD43/CD45 epitopes required for galectin-1 binding to these particular glycoprotein counterligands is abundant on Th1, but not Th2 cells. Published studies demonstrate a role for galectin-1 binding in the formation of segregated glycolattice-based microdomains containing CD3/CD45 or CD43/CD7 in some T cells [34, 35].

Further, galectin-1 binding can interfere with polarized membrane lipid raft clustering at the site of TCR contact and modulate TCR signal transduction [13].

Glycolattice- and raft- based microdomains spatially organize receptors and signal transducers, coordinating translation of receptor engagement into functional outcome. Th1 and Th2 cells are known to utilize different microdomain organization schemes [36, 37]. Indeed, TCR signal transduction in Th1 cells depends on raft membrane microdomain partitioning and the immune synapse becoming organized into cSMAC/pSMAC supramolecular activation complexes which concentrate CD3, CD45 and CD43 into largely non-overlapping microdomains. Alternatively, Th2 cells appear to be less reliant on lipid raft integrity for TCR signal transduction and do not efficiently generate cSMAC/pSMACs [36] nor do they cluster CD45 in response to galectin-1 treatment [15]. Coupled with reports that Th1 and Th2 cells express different constellations of glycosyltransferases and glycosidases, it would seem likely that galectin-1 organizes distinct receptor based signalosomes on Th1 vs Th2 cells. Experiments are underway to determine if selective galectin-1-mediated microdomain organization is responsible for its ability to specifically promote Th1 apoptosis and enhance Th2 TCR-induced cytokine production.

We found that Th2 cells express and secrete higher levels of galectin-1 than Th1 cells when resting and during the first 24 hours post-TCR/CD28 re-stimulation. To test the hypothesis that Th2-derived galectin-1 might function as a cross-regulatory cytokine which promotes Th2 dominance through the selective induction of Th1 death and promotion of Th2 function, we utilized a co-culture system in which galectin-1 expression by each subpopulation was controlled through the addition of wild type or

galectin-1 deficient Th1 or Th2 subpopulations. These studies demonstrate that endogenous Th2-derived galectin-1 can bind to the surface of Th1 cells, modestly in the absence of anti-CD3/CD28 restimulation and to a greater extent in restimulated cultures. In contrast, only modest levels of Th1-derived galectin-1 could be found bound to the surface of Th2 cells, consistent with the low levels of galectin-1 secreted by Th1 cells after restimulation. In addition, galectin-1 secreted by stimulated Th2 cells selectively inhibited the survival and cytokine secretion of Th1 cells. Conversely, exogenous galectin-1 secreted by Th1 cells promoted TCR/CD28-induced production of IL-5 by Th2. Together, these findings identify galectin-1 as an endogenous cytokine that can selectively antagonize Th1 survival and function and promote TCR-triggered Th2 cytokine production. Furthermore, the increased secretion of endogenous galectin-1 by restimulated Th1 cells suggests an autocrine suicide mechanism by which Th1-secreted galectin-1 negatively regulates the Th1-mediated response.

Our findings demonstrate that galectin-1 is differentially expressed by Th cell subsets and can affect the Th1/Th2 balance by mediating Th1 cell apoptosis and helping Th2 cytokine secretion. The differential expression and regulation of galectin-1 coupled with developmentally regulated differences in susceptibility to galectin-1-mediated death and modulation of TCR-induced cytokine production combine to enable galectin-1 to function as a T cell derived cytokine which antagonizes Th1 cell survival and skews T cell responses toward Th2, ultimately impacting the balance between Th subpopulations.

Materials and Methods

Mice.

5CC7 TCR is specific for pigeon cytochrome C peptide (amino acids 81-104) presented by MHC-class II molecule H-2^k. 5CC7 mice, created by Dr. Barbara Fazekas de St. Groth, on the C57Bl/10.A genetic background were crossed to 129s/v galectin-$1^{-/-}$ mice [38]. Gal-$1^{-/-}$ 5CC7+ mice were backcrossed at least 4 times to B10.A. All experiments involving mice followed an approved protocol of the UCLA Chancellor's Animal Research Committee.

Antibodies and other reagents.

Abs used in cultures were from eBioscience, including neutralizing rat mAbs for murine IL-4 (11B11) and IFN-γ (R4-6A2). Recombinant IL-2, IL-4 and IL-12 were from R & D Systems. Abs for flow cytometry included FITC-labeled anti-IL-4 (BVD6-24G2, EBioscience), PE-labeled anti-IFN-γ (XMG1.2, EBioscience), R-PE-conjugated anti-mouse glycosylated CD43 (1B11, BD PharMingen), APC-labeled anti-CD4 (RM4-5, BD PharMingen), PE-labeled anti-CD95 (Jo2, BD PharMingen), PE-labeled anti-CD95L (MFL3, BD PharMingen) and biotin anti-mouse TRAIL (N2B2, eBioscience). Blocking antibody against CD95L, Annexin V-FITC, Annexin V-PE and 7-AAD, were from BD PharMingen. Recombinant mouse TRAIL R2 (DR5) was purchased from R & D Systems. PI was from Sigma-Aldrich.

Galectin-1 production.

Human galectin-1 was purified from *Escherichia coli* transformed with the expression vector pT7IML-1 as described [13]. The biotinylation of galectin-1 was performed as described by Perillo et al [39]. After purification, rgal-1 was dialyzed in 8 mM DTT in PBS. Dialysate solution was collected and used as DTT control in samples not receiving rgal-1.

Differentiation of T helper cells.

Splenocytes from 5CC7 mice and stimulated with 1μM PCC 88–104 under Th1-differentiating conditions (10 ng/ml IL-12, 10 μg/ml anti-IL-4, 40 U/ml IL-2) or Th2-differentiating conditions (20 ng/ml IL-4, 20 μg/ml anti-IFN-γ, 20 U/ml mIL-2). Three days after primary stimulation, cultures were expanded with complete RPMI supplemented with 20 U/ml IL-2. Because 5CC7 mice on a B10.A background is Th1-prone, two rounds of antigenic stimulation under Th2 cytokine skewing conditions were done. One week after primary stimulation, cells were washed and equal numbers were restimulated with platebound anti-CD3 (1 μg/ml) and soluble anti-CD28 (5 μg/ml) under differentiating conditions. Cells were expanded and maintained with complete RPMI supplemented with 20 U/ml IL-2. After treatment, >97% of cells were CD4+. Restimulated differentiated cells were first rested 2-3 days in complete RPMI without IL-2.

Cytokine measurement.

Cytokines were measured by ELISA per antibody manufacturer recommendations (BD PharMingen). Staining for intracellular IFN-γ and IL-4 was performed with

Cytofix/Cytoperm kit (BD Pharmingen) per manufacturer's recommendation. Samples were acquired using a FACScalibur flow cytometer (BD Biosciences), and analyzed using WinMDI 2.8 (J. Trotter, Scripps Research Institute, La Jolla, CA).

Flow cytometry for surface antigens and intracellular galectin-1 expression.

To determine surface antigen expression, Th1 and Th2 cells were washed with staining buffer, preincubated with blocking buffer (PBS, 5% mouse serum, 0.1% NaN_3) for 30 min at 4°C, stained with antibodies PE-labeled anti-CD43 (1B11); APC, FITC or PE-labeled anti-CD4; PE-labeled anti-CD95; PE-labeled anti-CD95L; biotin-labeled anti-TRAIL/tricolor-labeled streptavidin and analyzed by flow cytometry.

For determination of intracellular galectin-1 and galectin-3 expression, Th1 and Th2 cells were incubated for 30 min at 4°C with blocking buffer, stained with APC-labeled anti-CD4 antibody and permeabilized with Cytofix/Cytoperm solution (BD Biosciences) overnight at 4°C, incubated with rabbit polyclonal anti-galectin-1 (gift of Linda Baum, UCLA), rabbit polyclonal anti-galectin-3 antibody (BioLegend), or normal rabbit IgG (Santa Cruz Biotech) as isotype control for 30 min at 4°C, stained with 1:100 dilution of FITC-goat anti-rabbit IgG (Jackson Immunoresearch lab), and analyzed by flow cytometry. Similar procedure without fixation/permeabilization step was used to detect surface galectin-1.

Galectin-1 cell death assays.

Apoptosis was determined by evaluating hypodiploid DNA content. $1x10^6$ cells/ml were plated in 48-well plates with 10 μM galectin-1 in the presence of

platebound anti-CD3 (1μg/ml) and soluble anti-CD28 (5 μg/ml) with/without 50mM lactose for 5 h at 37°C. DNA content was determined as described [40]. After 5 h, cells were incubated for 30 min at 4°C with blocking buffer, stained with FITC-labeled anti-CD4 antibody, fixed in 1ml cold 70% ethanol, resuspended with hypotonic fluorochrome solution (50 μg PI/ml diluted in 4 mM sodium citrate, 0.3% NP-40), and kept in the dark for 18 h at 4°C. Frequency of apoptotic cells in CD4 population was assessed by evaluating percentage of subdiploid nuclei in the <2N DNA peak using flow cytometry.

To detect apoptosis, FITC-labeled Annexin V and PI staining were performed. After 5 h of stimulation, $1x10^5$ cells previously labeled for surface antigens (anti-CD4-APC and PE-labeled anti-CD95 or PE-labeled anti-CD95L or biotin/streptavidin-labeled anti-TRAIL) were transferred to V-bottom plates, diluted with equal volume of PBS containing 10 mM lactose (to dissociate galectin-1-agglutinated cell clumps), washed, and resuspended in 100 μl Annexin V-binding buffer plus 5 μl Annexin V-FITC for 15 min at room temperature. 380 μl of Annexin V-binding buffer and 10 μl of PI (500 μg/ml) was added to each tube prior to flow cytometry.

Determination of binding of biotinylated galectin-1.

$5x10^5$ cells Th1 and Th2 cells were suspended in PBS containing 10 μg of biotinylated galectin-1 in presence or absence of 50 mM lactose at 4°C for 1 h. After washing, cells were stained with streptavidin-Tri-color (Jackson Immunoresearch) and FITC-labeled anti-CD4 for 45 min at 4°C, washed, and analyzed by flow cytometry.

Western blot assay.

$1x10^6$ cells/well were incubated in 48-well plates with serum free medium with platebound anti-CD3 (1 μg/ml) and soluble anti-CD28 (5 μg/ml) for 24 or 48 h. 15 μl of supernatant was collected, resolved on a 15% polyacrylamide gel, transferred onto nitrocellulose membranes, probed with 1:4000 dilution of the anti-galectin-1 polyclonal antibody, followed by 1 μg/ml of horseradish peroxidase conjugated anti-rabbit IgG.

Q-PCR for galectin-1 and galectin-3.

RNA was isolated from Th1 or Th2 cells using Trizol and converted to cDNA using First Strand Synthesis System (Invitrogen). Q-PCR reactions were performed using fluorescein calibration dye (BioRad) and Sybergreen (Molecular Probes). Primer sequences for galectin-1 were from Santucci et al [31]. Primers for galectin-3: forward 5'-ACTGACGGTGCCCTATGACCT-3'; reverse: 5'GGTTTCACTGTGCCCATGATT-3'. Q-PCR products were normalized relative to L32.

Th1 and Th2 co-culture.

Th1 cells from galectin-$1^{+/+}$ or $^{-/-}$ mice were stained with CFSE (1 μl of 2.5 mM CFSE/$1x10^7$ cells in PBS for 10 min at 37°C and washed with complete medium). CFSE-labeled Th1 ($1x10^6$) and non-labeled Th2 ($1x10^6$) cells obtained from galectin-$1^{+/+}$ or $^{-/-}$ mice were cocultured in 1 ml in 24-well plate with plate bound anti-CD3 (1 μg/ml) plus anti-CD28 (5μg/ml) in complete RPMI. After 24 h, surface galectin-1 was detected on Th1 populations ($CD4^+$ $CFSE^+$ cells) or Th2 populations ($CD4^+$ $CFSE^-$ cells). To determine the number of Th1 or Th2 live cells/well after 24 h of co-culture, total number of live cells in each well was determined using trypan blue exclusion. $5x10^5$ cells of each

co-culture were transferred to V-bottom plates, washed, and resuspended in 100 µl Annexin V-binding buffer , 5 µl PE-labeled Annexin V, and 5 µl of 7-AAD for 15 min at room temperature. 380 µl of cold Annexin V-binding buffer was added, and percentage of live Th1 ($CFSE^+$, Annexin V^-, 7-AAD^-) or Th2 ($CFSE^-$, Annexin V^-, 7-AAD^-) cells were determined in the live gate (FSC vs SSC) by flow cytometry.

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References

1 **Abbas, A. K., Murphy, K. M. and Sher, A.,** Functional diversity of helper T lymphocytes. *Nature* 1996. **383**: 787-793.

2 **Constant, S. L. and Bottomly, K.,** Induction of Th1 and Th2 CD4+ T cell responses: the alternative approaches. *Annu Rev Immunol* 1997. **15**: 297-322.

3 **Glimcher, L. H. and Murphy, K. M.,** Lineage commitment in the immune system: the T helper lymphocyte grows up. 10.1101/gad.14.14.1693. *Genes & Dev.* 2000. **14**: 1693-1711.

4 **Constant, S., Pfeiffer, C., Woodard, A., Pasqualini, T. and Bottomly, K.,** Extent of T cell receptor ligation can determine the functional differentiation of naive CD4+ T cells. *J Exp Med* 1995. **182**: 1591-1596.

5 **Pfeiffer, C., Stein, J., Southwood, S., Ketelaar, H., Sette, A. and Bottomly, K.,** Altered peptide ligands can control CD4 T lymphocyte differentiation in vivo. *J Exp Med* 1995. **181**: 1569-1574.

6 **Bashyam, H.,** Th1/Th2 cross-regulation and the discovery of IL-10. 10.1084/jem.2042fta. *J. Exp. Med.* 2007. **204**: 237-.

7 **Yang, R. Y., Hsu, D. K. and Liu, F. T.,** Expression of galectin-3 modulates T-cell growth and apoptosis. *Proc Natl Acad Sci U S A* 1996. **93**: 6737-6742.

8 **Hahn, H. P., Pang, M., He, J., Hernandez, J. D., Yang, R. Y., Li, L. Y., Wang, X., Liu, F. T. and Baum, L. G.,** Galectin-1 induces nuclear translocation of endonuclease G in caspase- and cytochrome c-independent T cell death. *Cell Death Differ* 2004.

9 **Vespa, G. N., Lewis, L. A., Kozak, K. R., Moran, M., Nguyen, J. T., Baum, L. G. and Miceli, M. C.,** Galectin-1 specifically modulates TCR signals to enhance TCR apoptosis but inhibit IL-2 production and proliferation. *J Immunol* 1999. **162**: 799-806.

10 **Blaser, C., Kaufmann, M., Muller, C., Zimmermann, C., Wells, V., Mallucci, L. and Pircher, H.,** Beta-galactoside-binding protein secreted by activated T cells inhibits antigen-induced proliferation of T cells. *Eur J Immunol* 1998. **28**: 2311-2319.

11 **Pace, K. E., Hahn, H. P., Pang, M., Nguyen, J. T. and Baum, L. G.,** CD7 delivers a pro-apoptotic signal during galectin-1-induced T cell death. *J Immunol* 2000. **165**: 2331-2334.

12 **Perillo, N. L., Uittenbogaart, C. H., Nguyen, J. T. and Baum, L. G.,** Galectin-1, an endogenous lectin produced by thymic epithelial cells, induces apoptosis of human thymocytes. *J Exp Med* 1997. **185**: 1851-1858.

13 **Chung, C. D., Patel, V. P., Moran, M., Lewis, L. A. and Miceli, M. C.,** Galectin-1 induces partial TCR zeta-chain phosphorylation and antagonizes processive TCR signal transduction. *J Immunol* 2000. **165**: 3722-3729.

14 **Rabinovich, G. A., Daly, G., Dreja, H., Tailor, H., Riera, C. M., Hirabayashi, J. and Chernajovsky, Y.,** Recombinant galectin-1 and its genetic delivery suppress collagen-induced arthritis via T cell apoptosis. *J Exp Med* 1999. **190**: 385-398.

15 **Toscano, M. A., Bianco, G. A., Ilarregui, J. M., Croci, D. O., Correale, J., Hernandez, J. D., Zwirner, N. W., Poirier, F., Riley, E. M., Baum, L. G. and**

Rabinovich, G. A., Differential glycosylation of T(H)1, T(H)2 and T(H)-17 effector cells selectively regulates susceptibility to cell death. *Nat Immunol* 2007. **8**: 825-834.

16 **Offner, H., Celnik, B., Bringman, T. S., Casentini-Borocz, D., Nedwin, G. E. and Vandenbark, A. A.,** Recombinant human beta-galactoside binding lectin suppresses clinical and histological signs of experimental autoimmune encephalomyelitis. *J Neuroimmunol* 1990. **28**: 177-184.

17 **Levi, G., Tarrab-Hazdai, R. and Teichberg, V. I.,** Prevention and therapy with electrolectin of experimental autoimmune myasthenia gravis in rabbits. *Eur J Immunol* 1983. **13**: 500-507.

18 **Rubinstein, N., Alvarez, M., Zwirner, N. W., Toscano, M. A., Ilarregui, J. M., Bravo, A., Mordoh, J., Fainboim, L., Podhajcer, O. L. and Rabinovich, G. A.,** Targeted inhibition of galectin-1 gene expression in tumor cells results in heightened T cell-mediated rejection; A potential mechanism of tumor-immune privilege. *Cancer Cell* 2004. **5**: 241-251.

19 **Morgan, R., Gao, G., Pawling, J., Dennis, J. W., Demetriou, M. and Li, B.,** N-Acetylglucosaminyltransferase V (Mgat5)-Mediated N-Glycosylation Negatively Regulates Th1 Cytokine Production by T Cells. *J. Immunol.* 2004. **173**: 7200-7208.

20 **Comelli, E. M., Sutton-Smith, M., Yan, Q., Amado, M., Panico, M., Gilmartin, T., Whisenant, T., Lanigan, C. M., Head, S. R., Goldberg, D., Morris, H. R., Dell, A. and Paulson, J. C.,** Activation of Murine CD4+ and CD8+ T Lymphocytes Leads to Dramatic Remodeling of N-Linked Glycans. *J. Immunol.* 2006. **177**: 2431-2440.

21 **Daniels, M., Hogquist, K. and Jameson, S.,** Sweet 'n' sour: the impact of differential glycosylation on T cell responses. *Nat Immunol* 2002. **3**: 903-910.

22 **Amano, M., Galvan, M., He, J. and Baum, L. G.,** The ST6Gal I sialyltransferase selectively modifies N-glycans on CD45 to negatively regulate galectin-1-induced CD45 clustering, phosphatase modulation, and T cell death. *J Biol Chem* 2003. **278**: 7469-7475.

23 **Blander, J. M., Visintin, I., Janeway, C. A., Jr. and Medzhitov, R.,** Alpha(1,3)-fucosyltransferase VII and alpha(2,3)-sialyltransferase IV are up-regulated in activated CD4 T cells and maintained after their differentiation into Th1 and migration into inflammatory sites. *J Immunol* 1999. **163**: 3746-3752.

24 **Grabie, N., Delfs, M. W., Lim, Y. C., Westrich, J. R., Luscinskas, F. W. and Lichtman, A. H.,** Beta-galactoside alpha2,3-sialyltransferase-I gene expression during Th2 but not Th1 differentiation: implications for core2-glycan formation on cell surface proteins. *Eur J Immunol* 2002. **32**: 2766-2772.

25 **Matarrese, P., Tinari, A., Mormone, E., Bianco, G. A., Toscano, M. A., Ascione, B., Rabinovich, G. A. and Malorni, W.,** Galectin-1 Sensitizes Resting Human T Lymphocytes to Fas (CD95)-mediated Cell Death via Mitochondrial Hyperpolarization, Budding, and Fission. 10.1074/jbc.M409752200. *J. Biol. Chem.* 2005. **280**: 6969-6985.

26 **Zhang, X., Zhang, L., Devadas, S., Li, L., Keegan, A. and Shi, Y.,** Reciprocal expression of TRAIL and CD95L in Th1 and Th2 cells: role of apoptosis in T helper subset differentiation. *Cell Death Differ* 2003. **10**: 203-210.

27 **Rogge, L., Bianchi, E., Biffi, M., Bono, E., Chang, S., Alexander, H., Santini, C., Ferrari, G., Sinigaglia, L., Seiler, M., Neeb, M., Mous, J., Sinigaglia, F. and Certa, U.,** Transcript imaging of the development of human T helper cells using oligonucleotide arrays. *Nat Genet* 2000. **25**: 96-101.

28 **Joo, H. G., Goedegebuure, P. S., Sadanaga, N., Nagoshi, M., von Bernstorff, W. and Eberlein, T. J.,** Expression and function of galectin-3, a beta-galactoside-binding protein in activated T lymphocytes. *J Leukoc Biol* 2001. **69**: 555-564.

29 **Rabinovich, G. and Gruppi, A.,** Galectins as immunoregulators during infectious processes: from microbial invasion to the resolution of the disease. *Parasite Immunol* 2005. **27**: 103-114.

30 **Ilarregui, J. M., Bianco, G. A., Toscano, M. A. and Rabinovich, G. A.,** The coming of age of galectins as immunomodulatory agents: impact of these carbohydrate binding proteins in T cell physiology and chronic inflammatory disorders. 10.1136/ard.2005.044347. *Ann Rheum Dis* 2005. **64**: iv96-103.

31 **Santucci, L., Fiorucci, S., Rubinstein, N., Mencarelli, A., Palazzetti, B., Federici, B., Rabinovich, G. A. and Morelli, A.,** Galectin-1 suppresses experimental colitis in mice. *Gastroenterology* 2003. **124**: 1381-1394.

32 **Yu, F., Finley, R. L., Jr., Raz, A. and Kim, H. R.,** Galectin-3 translocates to the perinuclear membranes and inhibits cytochrome c release from the mitochondria. A role for synexin in galectin-3 translocation. *J Biol Chem* 2002. **277**: 15819-15827.

33 **Acosta-Rodriguez, E. V., Montes, C. L., Motran, C. C., Zuniga, E. I., Liu, F. T., Rabinovich, G. A. and Gruppi, A.,** Galectin-3 mediates IL-4-induced survival and differentiation of B cells: functional cross-talk and implications during Trypanosoma cruzi infection. *J Immunol* 2004. **172**: 493-502.

34 **Hernandez, J. D., Nguyen, J. T., He, J., Wang, W., Ardman, B., Green, J. M., Fukuda, M. and Baum, L. G.,** Galectin-1 Binds Different CD43 Glycoforms to Cluster CD43 and Regulate T Cell Death. *J. Immunol.* 2006. **177**: 5328-5336.

35 **Pace, K. E., Lee, C., Stewart, P. L. and Baum, L. G.,** Restricted receptor segregation into membrane microdomains occurs on human T cells during apoptosis induced by galectin-1. *J Immunol* 1999. **163**: 3801-3811.

36 **Balamuth, F., Leitenberg, D., Unternaehrer, J., Mellman, I. and Bottomly, K.,** Distinct patterns of membrane microdomain partitioning in Th1 and th2 cells. *Immunity* 2001. **15**: 729-738.

37 **Miceli, M. C., Moran, M., Chung, C. D., Patel, V. P., Low, T. and Zinnanti, W.,** Co-stimulation and counter-stimulation: lipid raft clustering controls TCR signaling and functional outcomes. *Semin Immunol* 2001. **13**: 115-128.

38 **Poirier, F. and Robertson, E. J.,** Normal development of mice carrying a null mutation in the gene encoding the L14 S-type lectin. *Development* 1993. **119**: 1229-1236.

39 **Perillo, N. L., Pace, K. E., Seilhamer, J. J. and Baum, L. G.,** Apoptosis of T cells mediated by galectin-1. *Nature* 1995. **378**: 736-739.

40 **Nicoletti, I., Migliorati, G., Pagliacci, M. C., Grignani, F. and Riccardi, C.,** A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J Immunol Methods* 1991. **139**: 271-279.

Figure Legends

Figure 1. Th1 and Th2 effector populations differentiated from 5CC7 TCR transgenic mice. Th1 and Th2 cells were generated by two rounds stimulation and cytokine skewing conditions as described in the Material and Methods. An equivalent number of cells were restimulated for 4 h with plate bound anti-CD3 and soluble anti-CD28 in the presence of 1μl/ml GolgiStop. A) Density plots of IL-4 versus IFN-γ expression in the CD4+ gated population (>97% of the cells) of Th1 (left) and Th2 (right) differentiated cells. B) Cytokine levels of Th1 (open) and Th2 (filled) differentiated cell cultures restimulated with platebound anti-CD3 and soluble anti-CD28. Error bars represent standard deviations of triplicate samples per cytokine. Results are representative of at least four independent experiments. ND: not detectable. C) Histograms of unstimulated Th1 (left) and Th2 (right) populations stained with the 1B11 antibody (filled), isotype (solid line) or no staining (dotted line). The line indicates high 1B11 staining; the number above the line represents the percentage of cells, while the number below represents the MFI of 1B11 staining within the marker. Results are typical of 5 experiments.

Figure 2. Th2 cells are more resistant to galectin-1 induced cell death than Th1 cells. Unstimulated differentiated Th1 or Th2 cells were incubated with recombinant galectin-1 (10 μM). A) Annexin V versus PI dot plots of Th1 or Th2 cells after 5 hours of exposure to recombinant galectin-1 or DTT control. B) After recombinant galectin-1 exposure, unstimulated Th1 or Th2 cells were double-stained with FITC-labeled anti-CD4 and propidium iodide. Hypodiploid DNA content in CD4+ gated cells was assessed. The percentage of CD4+ cells in sub-G0/G1 as denoted by marker M1 is shown. C) Forward

versus side scatter dot plots of unstimulated Th1 (top) or Th2 cells (bottom) in presence of DTT control (left), recombinant galectin-1 (middle), or galectin-1 plus 50 mM lactose (right). The percentage of cells in the gate is listed. Greater than 90% of Th1 and Th2 cells were FSC^{low} resting cells prior to galectin-1 treatment. These graphs are representative of at least five individual experiments.

Figure 3. Binding of galectin-1 to differentiated Th1 and Th2 cells prior to and after TCR/CD28 restimulation. Flow histograms of Th1 (top) or Th2 (bottom) cells unstimulated (filled) or restimulated with anti-CD3/CD28 (thick line) and stained with biotin-galectin-1. Unstimulated cells stained with biotin-galectin-1 in the presence of 50 mM lactose (thin line) to ascertain carbohydrate-dependent binding or control isotype (dotted line) are also shown. M1 indicates the cells demonstrating high levels of galectin-1 binding. Histograms are representative of three individual experiments.

Figure 4. Galectin-1-mediated apoptosis does not rely on CD95-CD95L or TRAIL-TRAIL-R expression, though protection is correlated with galectin-3 expression. Restimulated Th1 or Th2 populations were incubated for 4 h with galectin-1 (10 μM) or DTT control. A) Percentage of recombinant galectin-1 (filled) or DTT control (open) treated Th1 (left) or Th2 (right) cells expressing CD95, CD95L and TRAIL. The illustrated plots are representative of three individual experiments yielding similar results. B) Galectin-3 mRNA expression in unstimulated, 24 h, and 48 h TCR/CD28 restimulated Th1 (left) and Th2 cells (right). Expression was normalized to the L32 housekeeping gene. The graph is representative of two individual experiments. c) Fold

increase of MFI of galectin-3 staining in unstimulated Th1 (open) or Th2 (filled) cells over unstimulated Th1 cells. Results are representative of two independent experiments.

Figure 5. Galectin-1 antagonizes Th1- and promotes Th2- cytokine secretion. A) IL-2 and IFN-γ levels in cultures of differentiated Th1 cells restimulated with anti-CD3/CD28 in the presence of 10 μM recombinant galectin-1 (filled) or DTT control (open). B) IL-4, IL-5, IL-10, and IL-2 levels in cultures of differentiated Th2 cells restimulated with anti-CD3/CD28 in the presence of 10 μM recombinant galectin-1 (filled) or DTT control (open). Error bars represent standard deviation of triplicate samples per cytokine. Results are representative of three independent experiments. * represents statistical significance, $p < 0.05$

Figure 6. Galectin-1 expression and secretion by Th1 and Th2 cells. A) Galectin-1 staining on differentiated Th1 (top) or Th2 (bottom) cells after 0 (filled), 24 (thin line), or 48 (dotted line) h of TCR/CD28 restimulation. The MFI of each histogram and corresponding isotype control are shown (right panel). Results are representative of three independent experiments. B) Galectin-1 mRNA expression in differentiated Th1 and Th2 cells after 0, 24 h or 48 h of TCR/CD28 restimulation. Expression levels were normalized to the L32 housekeeping gene. The graph is representative of two individual experiments. C) Galectin-1 protein levels in supernatants of resting, 24 h and 48 h restimulated differentiated Th1 (filled) or Th2 (open) cells. Serum free conditioned medium from galectin-$1^{-/-}$ Th1 or galectin-$1^{-/-}$ Th2 cells cultured for 24 h served as negative controls. The position of monomeric (14.5 kDa) form of galectin-1 is shown on

the left. The immunoreactive protein bands were quantified by densitometry and expressed as relative units (lower panel). The results are representative of two individual experiments.

Figure 7. Galectin-1 produced by Th2 cells binds Th1 cells and controls their survival. $1x10^6$ galectin-$1^{+/+}$ or $^{-/-}$ Th1 cells were labeled with CFSE and mixed with an equal number of galectin-$1^{+/+}$ or $^{-/-}$ Th2 cells in a 24 well plate and restimulated with anti-CD3/CD28 for 24 h. A) Average MFI of galectin-1 staining observed on Th1 cells ($CD4^+CFSE^+$ gated cells, left) or Th2 cells ($CD4^+CFSE^-$ gated cells, right) normalized to the MFI of galectin-1 staining obtained in the galectin$^{-/-}$ Th1 plus galectin-$1^{-/-}$ Th2 coculture. Normal rabbit IgG was used as isotype control of galectin-1 staining. B) The number of live galectin-$1^{+/+}$ Th1 ($CD4^+CFSE^+$Annexin V^-7-AAD^-, left) or Th2 ($CD4^+CFSE^-$Annexin V^-7-AAD^-, right) cells per well is shown for each co-culture combination. The bars represent the average and SD of duplicate values obtained in the same experiment. One representative of four experiments is shown. * represents statistical significance, p=0.03. C) Th1- and Th2-type cytokine levels from supernatants of the mixed co-cultures were quantitated using ELISA. One representative of four experiments is shown. *p=0.01

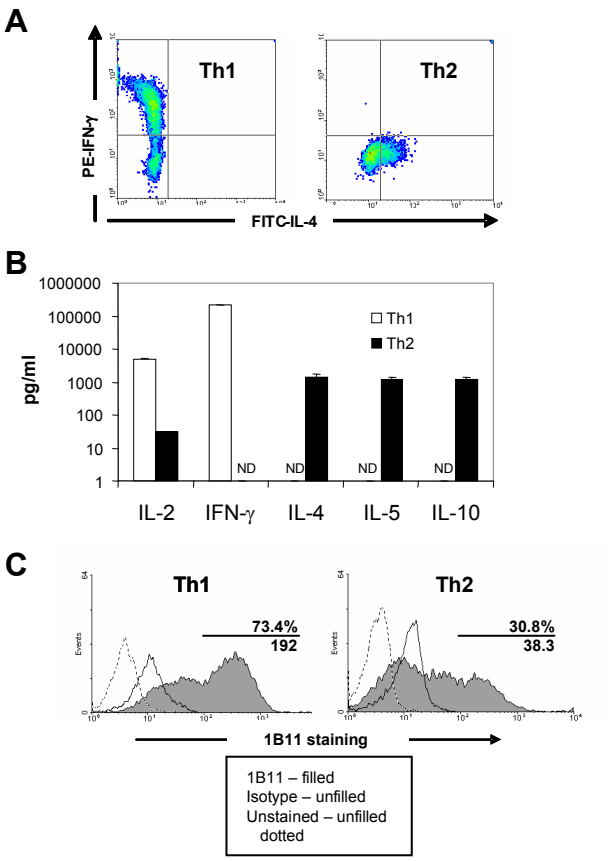
A
Th1
Th2
PE-IFN-γ
FITC-IL-4
B
pg/ml
1000000
100000
10000
1000
100
10
1
Th1
Th2
ND
IL-2
IFN-γ
IL-4
IL-5
IL-10
C
Th1
Th2
73.4%
192
30.8%
38.3
1B11 staining
1B11 – filled
Isotype – unfilled
Unstained – unfilled dotted

Figure 1

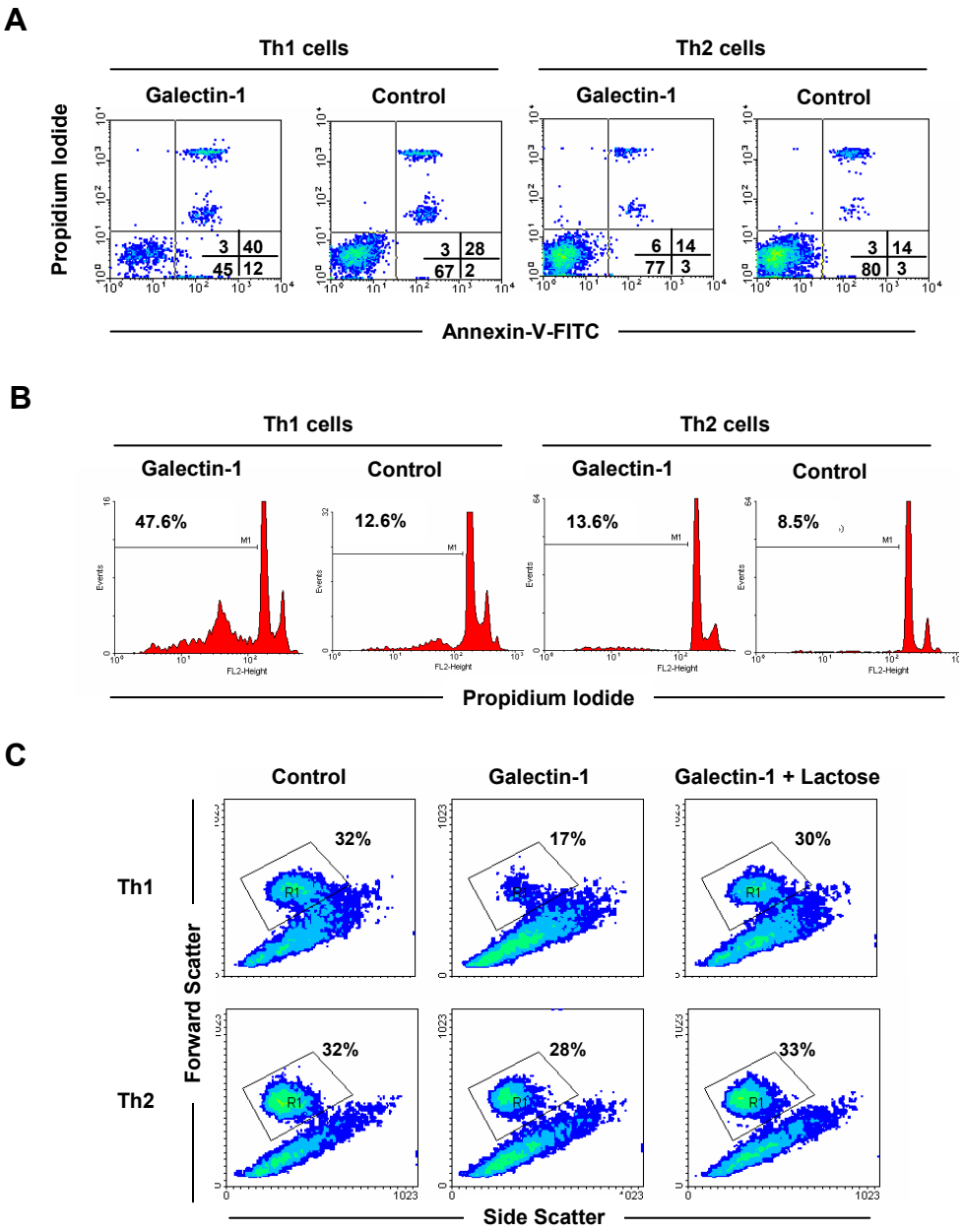
A
Th1 cells
Th2 cells
Galectin-1
Control
Galectin-1
Control
Propidium Iodide
3
40
45
12
3
28
67
2
6
14
77
3
3
14
80
3
Annexin-V-FITC
B
Th1 cells
Th2 cells
Galectin-1
Control
Galectin-1
Control
47.6%
12.6%
13.6%
8.5%
M1
Events
FL2-Height
Propidium Iodide
C
Control
Galectin-1
Galectin-1 + Lactose
Th1
Th2
32%
17%
30%
32%
28%
33%
R1
Forward Scatter
Side Scatter
0
1023

Figure 2

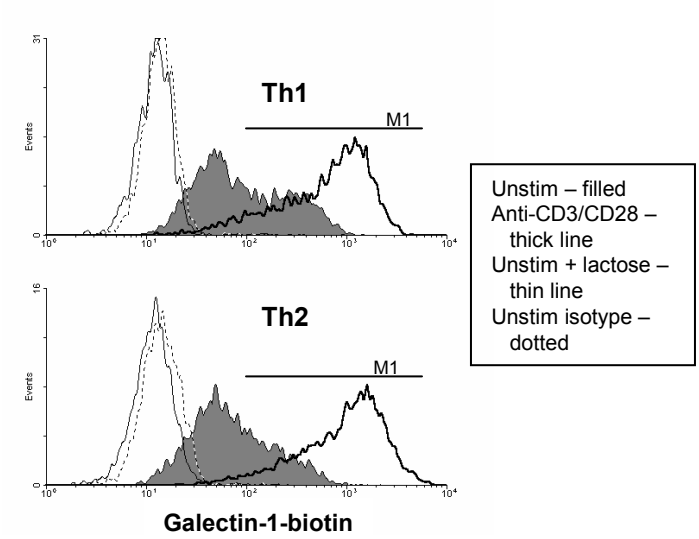
Th1
M1
Events
Th2
M1
Events
Galectin-1-biotin
Unstim – filled
Anti-CD3/CD28 – thick line
Unstim + lactose – thin line
Unstim isotype – dotted

Figure 3

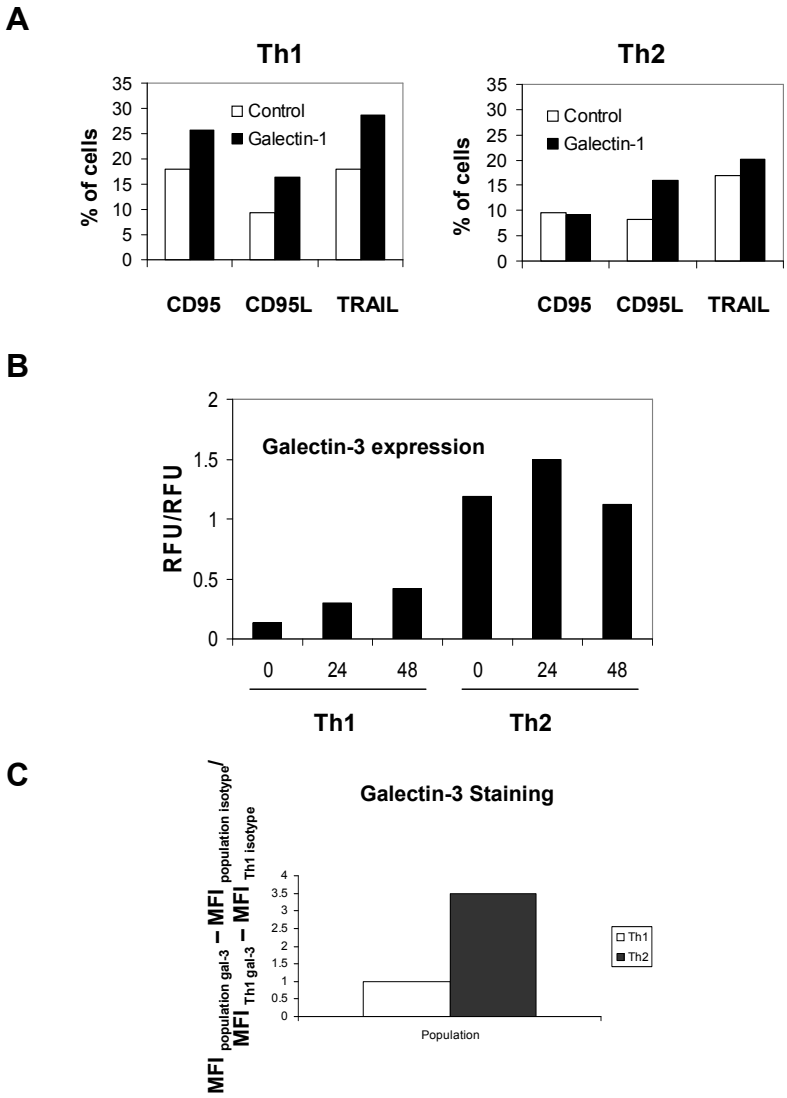
A
Th1
Th2
% of cells
Control
Galectin-1
CD95
CD95L
TRAIL
B
Galectin-3 expression
RFU/RFU
Th1
Th2
C
Galectin-3 Staining
MFI population gal-3 – MFI population isotype / MFI Th1 gal-3 – MFI Th1 isotype
Population
Th1
Th2

Figure 4

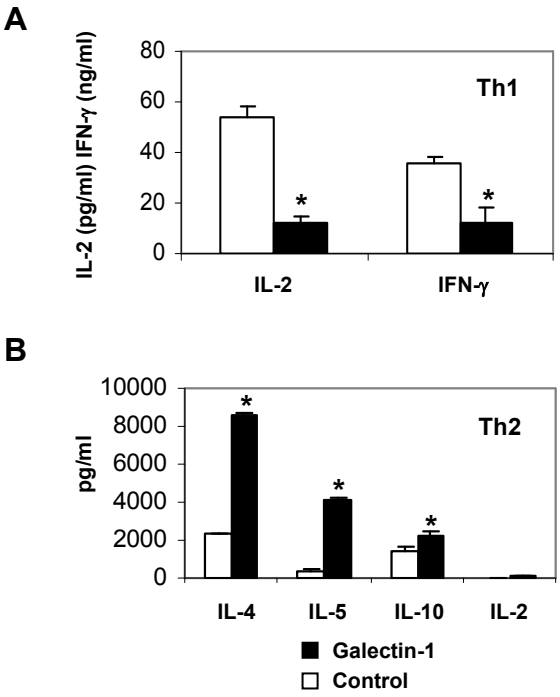
A
IL-2 (pg/ml) IFN-γ (ng/ml)
80
60
40
20
0
Th1
*
*
IL-2
IFN-γ
B
pg/ml
10000
8000
6000
4000
2000
0
Th2
*
*
*
IL-4
IL-5
IL-10
IL-2
Galectin-1
Control

Figure 5

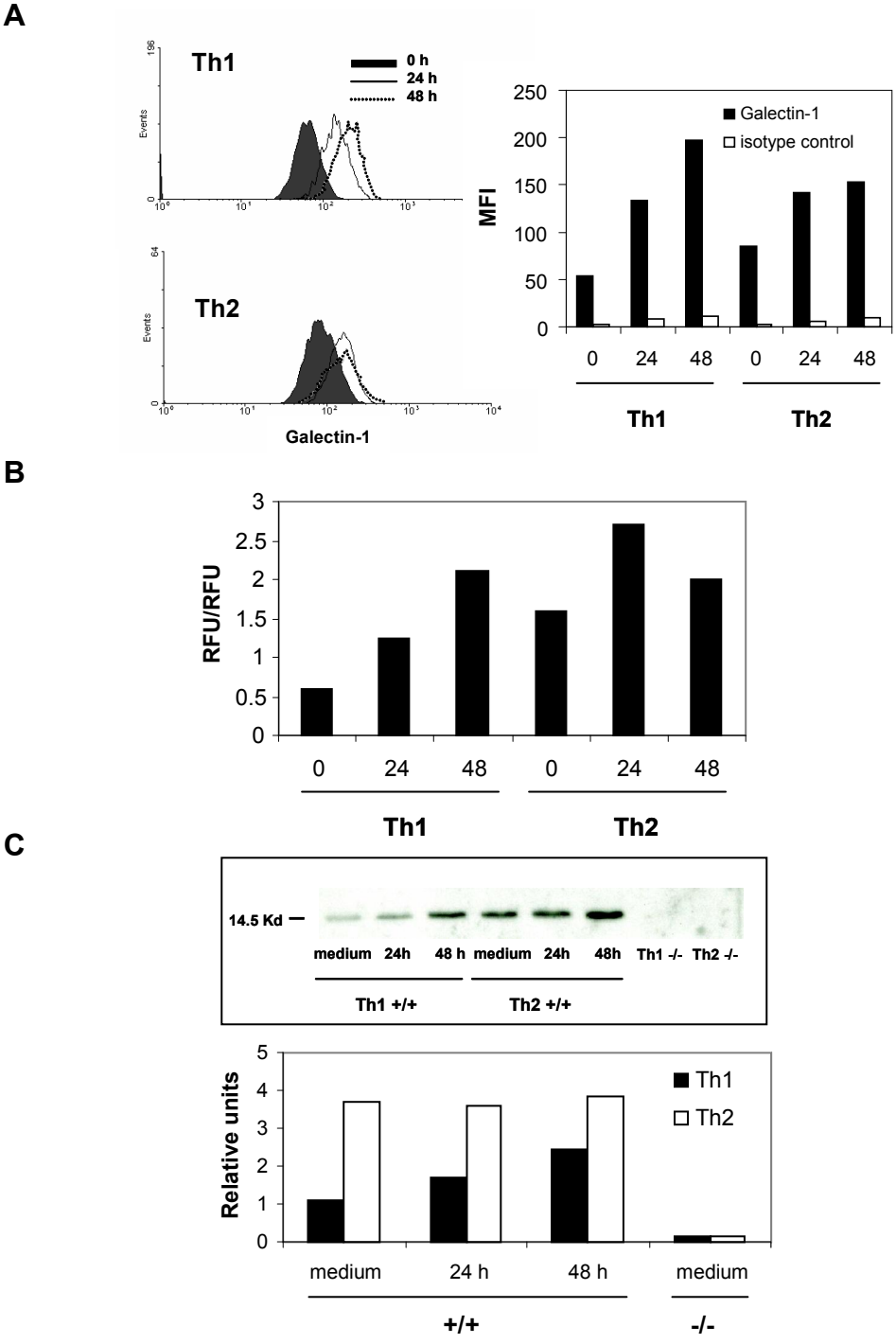
A
Th1
0 h
24 h
48 h
Events
Th2
Galectin-1
MFI
Galectin-1
isotype control
0
24
48
Th1
Th2
B
RFU/RFU
C
14.5 Kd
medium
24h
48 h
Th1 -/-
Th2 -/-
Th1 +/+
Th2 +/+
Relative units
Th1
Th2
+/+
-/-

Figure 6

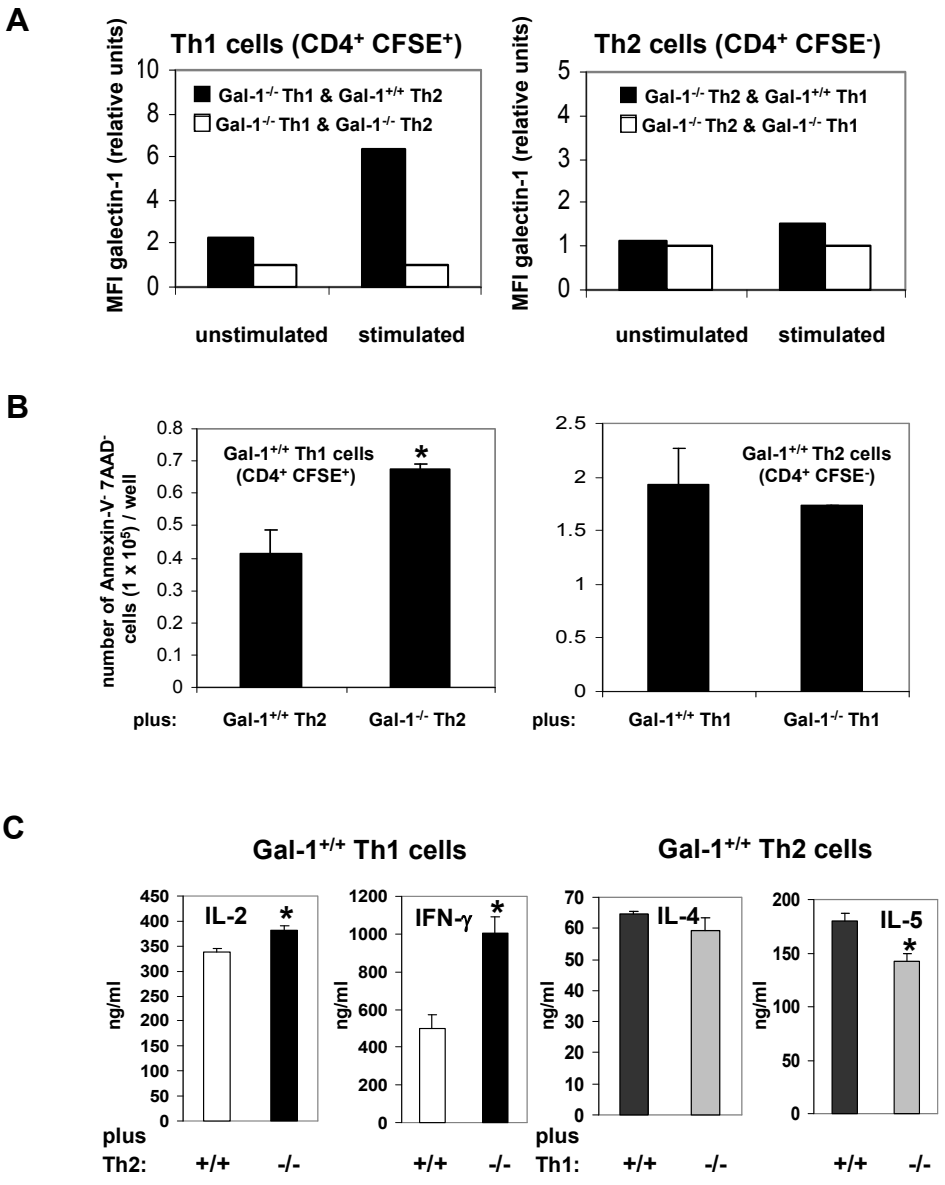
A
Th1 cells (CD4+ CFSE+)
MFI galectin-1 (relative units)
Gal-1-/- Th1 & Gal-1+/+ Th2
Gal-1-/- Th1 & Gal-1-/- Th2
unstimulated
stimulated
Th2 cells (CD4+ CFSE-)
Gal-1-/- Th2 & Gal-1+/+ Th1
Gal-1-/- Th2 & Gal-1-/- Th1
B
number of Annexin-V- 7AAD- cells (1 x 10^5) / well
Gal-1+/+ Th1 cells (CD4+ CFSE+)
Gal-1+/+ Th2 cells (CD4+ CFSE-)
plus: Gal-1+/+ Th2
Gal-1-/- Th2
plus: Gal-1+/+ Th1
Gal-1-/- Th1
C
Gal-1+/+ Th1 cells
Gal-1+/+ Th2 cells
IL-2
IFN-γ
IL-4
IL-5
ng/ml
plus Th2: +/+ -/-
plus Th1: +/+ -/-

Figure 7