

Galectin-2 Suppresses Contact Allergy by Inducing Apoptosis in Activated CD8⁺ T Cells¹

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Galectins, a family of structurally related β -galactoside-binding proteins, are expressed by various cells of the immune systems and seem to be important for the regulation of immune responses and immune cell homeostasis. Since it has been demonstrated that galectin-2 regulates cell-mediated inflammatory bowel disease and colitis in mice, we intended to investigate the role of galectin-2 in inflammatory cutaneous T cell-mediated immune responses. To address this issue, groups of naive mice were sensitized to the contact allergen 2,4-dinitro-1-fluorobenzene and systemically treated with galectin-2 to analyze the effects of galectin-2 on contact allergy. Here we show that galectin-2 is expressed in murine skin and is up-regulated upon cutaneous inflammation. Interestingly, treatment of mice with galectin-2 significantly reduced the contact allergy response. This effect was long-lasting since rechallenge of galectin-2-treated mice after a 14-day interval still resulted in a decreased ear swelling. We were able to demonstrate that galectin-2 induced a reduction of MHC class I-restricted immune responses in the treated animals, which was mediated by the induction of apoptosis specifically in activated CD8⁺ T cells. Additionally, we report that the galectin-2-binding protein CD29 is up-regulated on the surface of activated CD8⁺ T cells compared with naive CD8⁺ T cells or CD4⁺ T cells, suggesting that increased galectin-2/CD29 signaling might be responsible for the proapoptotic effects of galectin-2 on activated CD8⁺ T cells. Taken together, these data indicate that galectin-2 may represent a novel therapeutic alternative for the treatment of CD8-mediated inflammatory disorders such as contact allergy. *The Journal of Immunology*, 2009, 182: 5419–5429.

Galectins are a growing family of evolutionarily conserved lectins characterized by their high affinity to β -galactosides and the presence of at least one carbohydrate-recognition domain of ~130 aa responsible for glycan binding (1). Galectins are ubiquitous and can be found in vertebrates, invertebrates, fungi, as well as viruses (2, 3). To date, 15 mammalian galectins have been identified in a wide variety of tissues from several species (4, 5).

Within the immune system galectins are expressed by activated macrophages, activated B cells, dendritic cells, as well as activated or regulatory T cells (6–8). Accordingly, it was shown that galectin expression seems to play a pivotal role during activation and differentiation of various leukocyte subpopulations. Galectins are up-regulated under pathological conditions such as inflammation, infections, or in the presence of cytokines and chemokines (reviewed in Refs. 9–13). Galectins

function as pleiotropic regulators of different immune functions by promoting anti- or proinflammatory effects, leading to the initiation or resolution of inflammatory responses. In this regard it has been recently hypothesized that the same galectin may exert pro- and antiinflammatory effects depending on the concentration in the inflammatory foci or the particular target cell (13). Additionally, galectins are involved in the regulation of cell growth as well as cell survival and they modulate cell adhesion (7).

Currently, galectin-1 is the best characterized galectin in humans and mice. It has been shown that galectin-1 acts as a negative regulator of immune responses by preventing the development of chronic inflammations in experimental models of graft-vs-host disease or colitis (14, 15). The antiinflammatory effects of galectin-1 are mediated by the regulation of effector T cell function (16–19). Since galectin-2 is structurally closely related to galectin-1 by sharing a 43% amino acid sequence identity with galectin-1 (20), we were interested to investigate the effects of galectin-2 on contact allergy in mice. Contact allergy is an ideal model to study the effects of galectin-2 on effector T cell function, as both MHC class I- and class II-restricted T cell responses are involved in cutaneous delayed-type hypersensitivity (21–24).

Here, we demonstrate that systemic treatment of mice with galectin-2 before elicitation of contact allergy significantly reduced the ear swelling. This effect was due to the regulation of MHC class I-restricted immune responses by galectin-2 and, importantly, galectin-2 treatment did not affect activated CD4⁺ T cells. Additionally, evidence is provided that the immunosuppressive effects of galectin-2 might be mediated via the β_1 integrin CD29, which is highly expressed on activated CD8⁺ T cells, in stark contrast to its expression on naive CD8⁺ T cells

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or activated CD4⁺ T cells. These results show that galectin-2 may represent a novel therapeutic alternative for the treatment of contact allergy.

Materials and Methods

Mice

Female C57BL/6 mice (Harlan) were used at the age of 8–12 wk and housed under specific pathogen-free conditions, and all experiments were performed according to institutional regulations (A55/2002 and G97/2005; Office for the Environment, Nature and Municipal Affairs of North Rhine-Westphalia). K14-CD40L transgenic (tg) mice have been described previously (25) and were used at the age of 16 wk.

Galectin treatment

C57BL/6 mice were sensitized to the hapten 2,4-dinitro-1-fluorobenzene (DNFB)³ at day 0 and injected i.p. on 6 consecutive days (days 0–5) twice per day with 10 μ g of recombinant human galectin-2 (R&D Systems) diluted in 100 μ l of 0.9% NaCl. Control groups received an equal amount of 0.9% NaCl. At day 5, contact allergy was elicited. Due to the severe autoimmune dermatitis (25) impeding the elicitation of contact allergies, CD40L tg mice were treated with similar amounts of galectin-2 without prior sensitization. For in vitro stimulation, cells were activated with mitogenic Abs (1 μ g/ml anti-CD3, clone 2c11, and 1 μ g/ml anti-CD28, clone 37.51; both BD Biosciences) and treated for 18–48 h with indicated concentrations of recombinant human galectin-2 or galectin-1 (both R&D Systems).

Blocking studies

To block galectin-2 effects, mice were injected i.v. with 0.5 mg of anti-galectin-2 (clone M-45; Santa Cruz Biotechnology) or rabbit IgG Ab (Abcam) 7 days before the start of galectin-2 treatment. To inhibit CD29 signaling, 50 μ g/ml anti-CD29 (clone Ha2/5; BD Biosciences) or hamster IgM Ab (Abcam) were added to cell cultures during the whole culture period. Caspase activity in cultured cells was blocked by adding 40 μ g/ml pan-caspase inhibitor Z-VAD (R&D Systems) alone or in combination with galectin-2.

UV-induced apoptosis

Purified CD8⁺ T cells were cultured in petri dishes and exposed to UV light (40 mJ/cm²) using a bank of four Philips UV TL40W/12 sunlamps, which have an emission spectrum from 280 to 350 nm. To inhibit CD29 signaling, 50 μ g/ml anti-CD29 (clone Ha2/5; BD Biosciences) or control Ab (Abcam) was added to cell cultures.

Immunofluorescence and TUNEL staining

Immunofluorescence stainings were performed on cryostat sections of murine skin (5 μ m) fixed in acetone according to standard methods (26). Slides were incubated in the appropriate dilutions of Abs (anti-CD8, clone 53-6.7; BD Biosciences; anti-galectin-2, clone M-45; Santa Cruz Biotechnology; or an isotype control; BD Biosciences) and subsequently incubated with an Alexa Fluor 594- or Alexa Fluor 488-labeled secondary Ab (Molecular Probes). For visualization of nuclei, slides were stained with 4',6-diamidino-2-phenylindole (DAPI; Roche) at a concentration of 10 μ g/ml for 1 h. TUNEL staining of murine skin for the detection of apoptotic cell death was performed using an in situ cell death detection kit (Roche) according to the manufacturer's instructions. Slides were examined with an Axiovert 200M laser scanning microscope and the LSM510 Meta software (Zeiss).

RT-PCR

Tissues were snap-frozen before RNA isolation and reverse transcription. RNA was extracted from frozen mouse skin biopsies using RNeasy columns (Qiagen) according to recent publications and the manufacturer's instructions (27). cDNA was synthesized from 1 μ g of total RNA using random hexanucleotide primers and a reverse transcription kit (Fermentas). Primers used were: galectin-2 forward, 5'-CTCGCTTCGATGAATCCAC CAT-3' and galectin-2 reverse, 5'-CCCACCCATGCTCAAGTAGT-3'; β -actin forward, 5'-GTGGGGCGCC-CCAGGCACCA-3' and β -actin reverse, 5'-CTCCTTAATGTCACGCACGATTTC-3'. Cycling profile was: 94°C for 4 min, (94°C for 1 min; 54°C for 1 min; 72°C for 1 min) $\times$ 35,

and 72°C for 5 min. Aliquots of PCR products were electrophoresed on 2% agarose gels and visualized by ethidium bromide staining.

Contact allergy

Mice were sensitized by painting 100 μ l of 0.5% DNFB (Sigma-Aldrich) in acetone/olive oil (4/1) on the shaved back. For elicitation of contact allergy responses, 12 μ l of 0.3% DNFB was painted on both sides of the left ear on day 5. Contact allergy was determined by the degree of ear swelling of the hapten-exposed left ear compared with the ear thickness of the unchallenged right ear and measured with a micrometer (Mitutoyo) at indicated time points after challenge. Mice that were ear challenged without prior sensitization served as negative controls. Rechallenge of mice was performed by applying 12 μ l of 0.3% DNFB on both sides of the left ear 14 days after the first challenge.

Cell preparation, stimulation, and flow cytometry

Single-cell suspensions of lymph nodes and spleens were prepared as described before (28). In some experiments purified CD4⁺ or CD8⁺ T cells were stimulated with galectin-2, galectin-1 (at indicated concentration), anti-CD29, Z-VAD (*N*-benzyloxycarbonyl valine-alanine-aspartate), or a combination of different stimuli. Expression of cell surface markers was analyzed by standard four-color flow cytometry on a FACSCalibur flow cytometer with CellQuest software (BD Biosciences). Cells were stained for FACS analysis in PBS containing 1% FCS with the following mouse mAbs purchased from BD Biosciences: FITC-conjugated anti-CD3 (clone 145-2C11), anti-CD44 (clone IM7), anti-CD103 (clone 2E7), anti-CD29 (clone Ha2/5), polyclonal goat anti-rabbit Ig; PE-conjugated anti-CD25 (clone PC61), anti-CTLA-4 (clone UC10-4F10-11), anti-CD127 (clone SB/199); PerCP-conjugated anti-CD19 (clone 1D3); allophycocyanin-conjugated anti-CD8 (clone 53-6.7), anti-CD4 (clone RM4-5), anti-CD11c (clone HL3). Mouse monoclonal anti-neuropilin-1 (clone H-286) and anti-mouse/rat galectin-2 (clone M-45) were purchased from Santa Cruz Biotechnology. Foxp3 stainings were performed using a Foxp3 staining kit (NatuTec). Isotype-matched control Abs were included in each staining. Apoptotic and necrotic cells were identified using an annexin V apoptosis detection kit (BD Biosciences) according to the manufacturer's instructions.

Proliferation assays

CD8⁺, total CD4⁺, CD4⁺CD25⁻, and CD4⁺CD25⁺ cells were sorted by MACS (Miltenyi Biotec). CD8⁺ and CD4⁺ T cells (1 $\times$ 10⁶/ml) were cultured in triplicates in 96-well round-bottom plates and stimulated with 1 μ g/ml anti-CD3 (clone 145-2C11) and 1 μ g/ml anti-CD28 (clone 37.51; both BD Biosciences). Proliferation assays were performed in a final volume of 200 μ l. [³H]thymidine (1 μ Ci/well) was added for the last 12 h of the experiment, and [³H]thymidine incorporation was measured by liquid scintillation counting. For some in vitro proliferation assays, CD4⁺CD25⁺ T cells from peripheral lymph nodes of galectin-2-treated mice or NaCl-treated controls were cultured alone or mixed with conventional CD4⁺CD25⁻ T cells from naive C57BL/6 mice at a 1:1 ratio.

Detection of cell death

The Cell Death Detection ELISA Plus from Roche was used to measure histone-bound DNA fragments of apoptotic cells. Culture supernatants were collected 18, 24, and 48 h after stimulation of cells and detection of cell death was performed according to the manufacturer's instructions.

Statistical analysis

The significance of differences between the mean values obtained was assessed by the Student's *t* test for unpaired data. A *p* value of <0.05 was regarded as being significant. Values are expressed as means $\pm$ SD.

Results

Galectin-2 is up-regulated in inflamed skin

Galectins are highly expressed on endothelial and epithelial cells (7). Hence, up-regulation of galectin-13 on placenta epithelia, galectin-9 on thymic epithelial cells, or galectin-3 as well as galectin-1 on tumor endothelial cells has been reported (7, 29). However, galectin-2 was predominantly found in epithelial cells of the stomach and the small intestine (20, 30). Since the skin represents one of the largest epithelial surfaces of the body, we analyzed whether galectin-2 is expressed in the skin. Whereas in normal murine skin we detected only low levels of galectin-2 mRNA,

³ Abbreviations used in this paper: DNFB, 2,4-dinitro-1-fluorobenzene; tg, transgenic; wt, wild type; Z-VAD, *N*-benzyloxycarbonyl valine-alanine-aspartate.

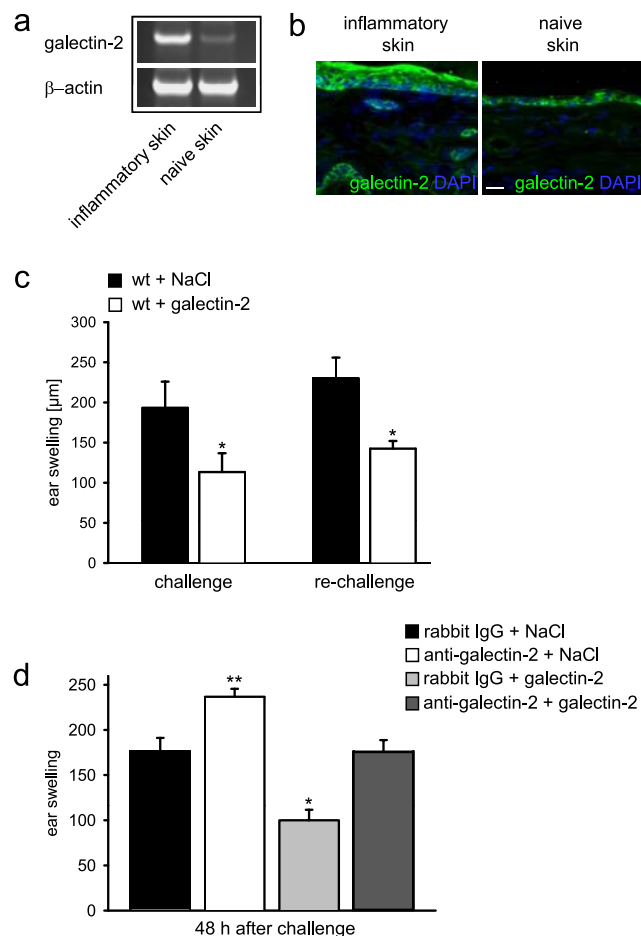


FIGURE 1. Galectin-2 is expressed in murine skin and is up-regulated during inflammation. *a*, Reverse transcription-PCR analysis of naive wt skin and inflamed skin from K14-CD40L tg mice. One representative out of three independent experiments is shown. *b*, Immunofluorescence stainings of murine skin from wt and K14-CD40L tg mice using an Ab directed against galectin-2. Original magnification, $\times 200$; scale bar 25 μm . *c*, Reduced contact allergy responses in galectin-2-treated mice. Ear swelling was assessed 48 h after challenge and re-challenge. Data are shown as mean ear swelling $\pm$ SD and are representative of 15 mice in three independent experiments. *, $p < 0.05$ by Student's *t* test. *d*, Reduced contact allergy is mediated by galectin-2. Mice were injected with anti-galectin-2 or a control Ab at day -7 , sensitized to DNFB at day 0, injected with galectin-2 from day 0 to day 5, and DNFB ear-challenged. Ear swelling as a measure of contact hypersensitivity responses was assessed 48 h after challenge. Data are shown as mean ear swelling $\pm$ SD and are representative of four mice per group. ** and *, $p < 0.05$ (Student's *t* test) between contact allergy responses in rabbit-IgG vs anti-galectin-2-treated mice and between galectin-2-injected animals vs controls, respectively.

inflammation due to severe autoimmune dermatitis resulted in a significant up-regulation of galectin-2 mRNA (Fig. 1*a*). Moreover, immunofluorescence stainings of naive mouse skin and cutaneous lesions from K14-CD40L tg mice (25) demonstrated that galectin-2 can be detected at low levels in normal skin, whereas we found strong galectin-2 expression in inflammatory skin of autoimmune-prone K14-CD40L tg mice (Fig. 1*b*), indicating that galectin-2 is up-regulated in murine skin during inflammation.

Reduced contact allergy in galectin-2-treated mice

Galectins have recently emerged as important players during immune responses, and it has been demonstrated that galectin-2

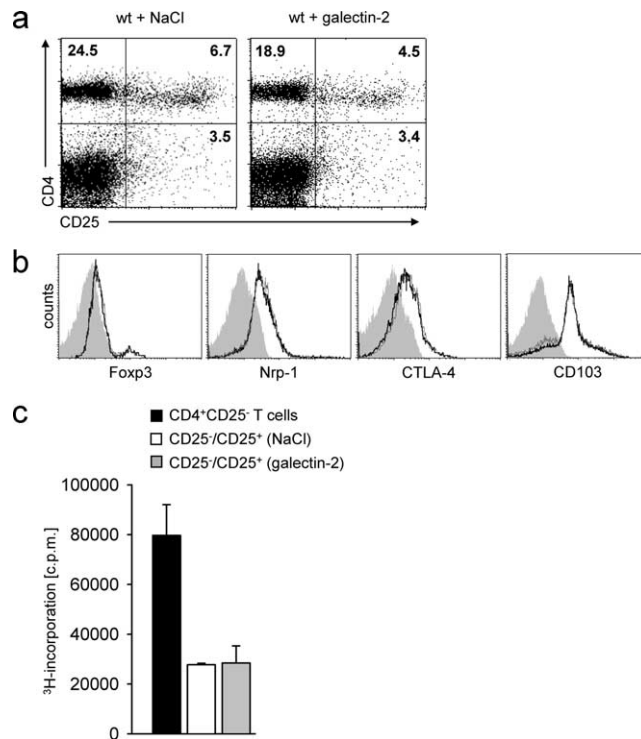
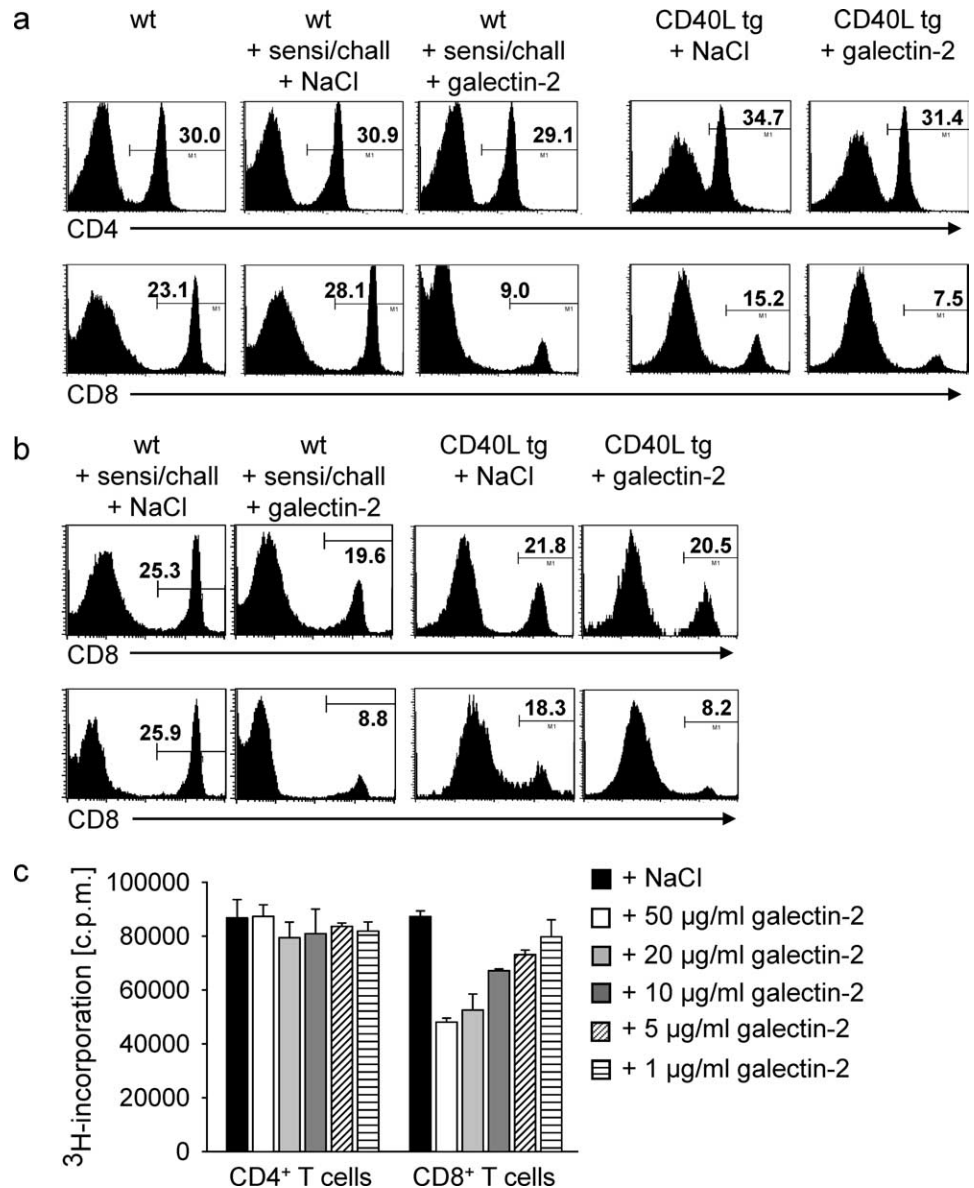


FIGURE 2. Galectin-2 treatment does not change the numbers and function of CD4⁺CD25⁺ regulatory T cells. *a*, Flow cytometric analyses of lymph node T cells from NaCl-treated ($n = 10$ animals analyzed) and galectin-2-treated ($n = 11$) mice 48 h after elicitation of contact allergy. Representative dot blots are shown for each experimental group. *b*, CD4⁺CD25⁺ T cells from NaCl- and galectin-2-treated mice show similar expression of surface markers characteristic for regulatory T cells. CD4⁺CD25⁺ T cells from sensitized/challenged NaCl- (black lines) and galectin-2-treated mice (gray lines) were analyzed by flow cytometry. CTLA-4 staining was performed after cell permeabilization. Isotype controls are shown in light gray shades. *c*, CD4⁺CD25⁺ T cells from galectin-2-treated mice are immunosuppressive. Proliferation assays were performed by stimulating naive CD4⁺CD25⁺ T cells with anti-CD3 and anti-CD28 Abs in the absence or presence of CD4⁺CD25⁺ T cells from NaCl or galectin-2-treated mice. Mean values of [³H]thymidine uptake $\pm$ SD are shown from one out of three independent experiments.

regulates cell-mediated inflammatory bowel disease and colitis in mice (20, 31). Since we have shown that galectin-2 is expressed in murine skin and, most importantly, is significantly up-regulated upon cutaneous inflammation (Fig. 1, *a* and *b*), we were interested to investigate the role of galectin-2 in inflammatory cutaneous T cell-mediated immune responses. To address this issue, groups of naive mice were sensitized to the contact allergen DNFB and systemically treated with galectin-2 to analyze the effects of galectin-2 on contact allergy. Interestingly, upon epicutaneous immunization followed by a local challenge at the ear, galectin-2-treated mice demonstrated a significantly decreased contact allergy response compared with controls (Fig. 1*c*). Even after rechallenge with the same hapten after a 2-wk period, galectin-2-injected mice mounted a reduced contact allergy response, possibly suggesting the induction of tolerance. The reduction of contact allergy in treated mice was mediated by galectin-2 signaling since blocking this pathway by injecting anti-galectin-2 increased the ear swelling response to control levels (Fig. 1*d*). These findings indicate that galectin-2 is able to suppress contact allergy responses in mice.

FIGURE 3. Decreased proliferation and reduced numbers of activated CD8⁺ T cells in skin-draining lymph nodes of galectin-2-treated and hapten-sensitized/challenged mice. *a*, Forty-eight hours after elicitation of contact allergy in C57BL/6 mice, lymph node cells from galectin-2-treated mice and controls were stained for CD4 and CD8 and analyzed by flow cytometry. FACS stainings of CD8⁺ and CD4⁺ T cells from peripheral lymph nodes of CD40L tg mice were performed 48 h after the last galectin-2 injection. Representative histograms from one out of three independent experiments are shown. *b*, Decreased numbers of activated CD8⁺ T cells in skin-draining lymph nodes of galectin-2-treated and sensitized/challenged C57BL/6 as well as CD40L tg mice. CD8⁺ CD44^{low} (upper) and CD8⁺ CD44^{high} T cells (lower) from NaCl-treated and galectin-2-treated C57BL/6 as well as CD40L tg mice were analyzed by flow cytometry. Representative histograms are shown for each experimental group. *c*, Reduced proliferation of CD8⁺ T cells after in vitro stimulation with galectin-2. CD4⁺ and CD8⁺ T cells from peripheral lymph nodes of naive C57BL/6 mice were treated on 2 consecutive days with galectin-2 at indicated concentrations. Proliferation assays were performed by stimulating CD4⁺ or CD8⁺ T cells with anti-CD3 and anti-CD28. Mean values of [³H]thymidine uptake $\pm$ SD are shown from one out of three independent experiments.



Galectin-2 treatment did not change the numbers and function of regulatory T cells

Since contact allergy responses in mice can be controlled by CD4⁺CD25⁺ regulatory T cells (24, 26), we analyzed the numbers and function of regulatory T cells in regional lymph nodes after galectin-2 treatment (Fig. 2). Therefore, cervical and retroauricular lymph nodes were prepared from galectin-2-treated mice as well as controls and assessed for the presence of CD4⁺CD25⁺ regulatory T cells by multicolor flow cytometry. Galectin-2 did not change the peripheral numbers of regulatory T cells since mice systemically treated with galectin-2 for 6 days had similar numbers of CD4⁺CD25⁺ T cells compared with controls (Fig. 2a). We next addressed whether galectin-2 treatment would affect the expression of characteristic markers or the inhibitory function of regulatory T cells. As shown in Fig. 2b, CD4⁺CD25⁺ regulatory T cells from galectin-2-treated mice expressed comparable levels of typical markers such as the lineage specific transcription factor Foxp3, neuropilin-1 (Nrp-1), intracellular CTLA-4, or the integrin $\alpha_E\beta_7$ (CD103). To investigate whether regulatory T cells from galectin-2-treated mice are functionally suppressive, we isolated CD4⁺CD25⁺ T cells from skin draining lymph nodes of galectin-2- or

NaCl-treated mice 48 h after elicitation of contact allergy responses and cocultured them with freshly isolated naive CD4⁺CD25[−] effector T cells. Upon stimulation with anti-CD3/anti-CD28, the CD4⁺CD25⁺ T cells from galectin-2-treated and NaCl-treated mice suppressed the proliferation of CD4⁺CD25[−] conventional T cells to a similar extent (Fig. 2c), indicating that galectin-2 treatment did not change the numbers or inhibitory function of peripheral CD4⁺CD25⁺ regulatory T cells. These data suggest that CD4⁺CD25⁺ regulatory T cells played a rather minor role in the reduced contact allergy that we observed in mice systemically treated with galectin-2.

Reduced numbers of CD8⁺ T cells after galectin-2 treatment

Since contact allergy is a T cell-mediated disorder, we analyzed T cell numbers and T cell function in hapten-exposed skin and in skin draining lymph nodes of galectin-2-treated mice. Two days after challenge of mice with DNFB, the total numbers of CD4⁺ Th cells in regional lymph nodes were comparable between galectin-2- and NaCl-treated mice ($30.5 \pm 5.0\%$ vs $28.7 \pm 2.9\%$; Fig. 3a). Furthermore, we found similar numbers of CD4⁺ T cells in peripheral lymph nodes of autoimmune-prone CD40L tg mice

treated with either NaCl or galectin-2 ($32.9 \pm 4.8\%$ vs $29.7\% \pm 3.6\%$). Interestingly, the levels of total $CD8^+$ T cells were markedly decreased in regional lymph nodes of galectin-2-treated C57BL/6 and autoimmune-prone CD40L tg mice compared with NaCl-injected controls ($8.8 \pm 1.9\%$ vs $28.4 \pm 3.3\%$ in C57BL/6 mice and $6.9 \pm 2.4\%$ vs $15.6 \pm 3.5\%$ in CD40L tg mice; Fig. 3a). In contact allergy, topically applied hapten sensitizers are taken up by cutaneous APCs and are presented to naive T cells in skin-draining lymph nodes, leading to T cell activation (32). Upon challenge, hapten-activated $CD8^+$ T cells migrate to the challenged skin, whereas nonactivated or memory $CD8^+$ T cells remain in skin-draining lymph nodes (21). To analyze whether galectin-2 specifically reduced the number of a distinct subpopulation of $CD8^+$ T cells, total $CD8^+$ T cells were purified from regional lymph nodes after systemic galectin-2 treatment and elicitation of contact allergy. Subsequently, $CD8^+$ T cells were stained with anti-CD44 and anti-CD127 to identify naive, activated, and memory $CD8^+$ T cells. Strikingly, the galectin-2-induced reduction in cell numbers was even more pronounced in activated $CD44^{\text{high}}$ compared with naive $CD8^+$ T cell subsets as evidenced by flow cytometry (Fig. 3b). After sensitization and challenge, normal numbers of activated $CD44^{\text{high}}CD8^+$ T cells were detected in peripheral lymph nodes of control mice treated with NaCl ($25.4 \pm 3.1\%$ of total $CD8^+$ T cells). In contrast, mice that were systemically treated with galectin-2 showed strongly reduced levels of activated $CD8^+$ T cells ($8.3 \pm 2.7\%$) in lymph nodes draining DNFB-challenged skin areas (Fig. 3b). Likewise, systemic galectin-2 treatment of CD40L tg mice resulted in a pronounced reduction of $CD8^+CD44^{\text{high}}$ cells ($8.3 \pm 1.7\%$ vs $19.0 \pm 3.1\%$), whereas the numbers of $CD8^+CD44^{\text{low}}$ cells were not altered in galectin-2-treated CD40L tg mice compared with controls (Fig. 3b). To further characterize the effects of galectin-2 on the function of $CD8^+$ T cells, purified total naive $CD4^+$ and $CD8^+$ T cells were cultured in the presence of galectin-2. Galectin-2-treated $CD8^+$ but not $CD4^+$ T cells showed a significantly decreased proliferation upon TCR stimulation with mitogenic Abs (Fig. 3c). These data indicate that galectin-2 specifically reduced the proliferation and the number of activated $CD8^+$ T cells.

Galectin-2 induced apoptosis in activated $CD8^+$ T cells

Previous studies demonstrated that galectins, in particular galectin-1, galectin-3, and galectin-4, induced apoptosis in T cells (33, 34, 36). Therefore, the decreased numbers of activated $CD8^+$ T cells in peripheral lymph nodes of systemically galectin-2-treated and hapten-sensitized C57BL/6 and galectin-2 treated autoimmune-prone CD40L tg mice (Fig. 3b), as well as the reduced proliferation of activated $CD8^+$ T cells after in vitro stimulation with galectin-2 (Fig. 3c), might be mediated by the induction of apoptosis. To address this issue, naive mice were injected with galectin-2 on 6 consecutive days and subsequently sensitized to DNFB and ear-challenged. Afterward, single-cell suspensions from regional lymph nodes were stained for CD4, CD8, and annexin V to detect apoptotic cells. Annexin V expression was similar in $CD4^+$ T cells from galectin-2-treated mice and controls. However, the number of peripheral apoptotic $CD8^+$ and annexin V $^+$ T cells was significantly increased in sensitized/challenged and galectin-2-treated mice (Fig. 4a). To evaluate whether galectin-2 induced apoptosis of $CD8^+$ T cells not only in regional lymph nodes but also in cutaneous T cell infiltrates, sensitized mice were systemically treated with galectin-2 and challenged with DNFB. Two days later, skin samples were taken from the area of DNFB application and biopsies were stained for CD8 and for apoptotic cells using the TUNEL assay. Notably, the total number of apoptotic cells and

especially of apoptotic $CD8^+$ T cells was higher after systemic galectin-2 treatment (Fig. 4b).

Since the galectin-2 preparation contained 4 mM 2-ME, we sought to exclude that the reducing agent mediated the proapoptotic effects. Hence, activated $CD8^+$ T cells were cocultured for 18 h with either 4 mM 2-ME or 10 $\mu\text{g/ml}$ galectin-2 and analyzed for annexin V expression. Importantly, 2-ME alone did not alter the number of annexin V $^+$ cells, whereas we detected 8- to 10-fold increased levels of annexin V $^+$ propidium iodide $^-CD8^+$ T cells after treatment with galectin-2 (Fig. 4c).

Having demonstrated that galectin-2 exhibits proapoptotic effects, we intended to analyze whether galectin-2 induced apoptosis only in activated $CD8^+$ T cells. Therefore, $CD4^+$ and $CD8^+$ T cells were purified from peripheral lymph nodes and activated by stimulation with anti-CD3/anti-CD28. Subsequently, cells were cocultured for 18–24 h with galectin-2 at indicated concentrations and analyzed for the presence of apoptotic cells by propidium iodide and annexin V staining. The data show that galectin-2 treatment does not alter the number of annexin V $^+$ propidium iodide $^-CD4^+$ T cells, whereas increased levels of annexin V $^+$ propidium iodide $^-CD8^+$ T cells were detected after stimulation of cells in the presence of galectin-2 (Fig. 4, d and e). Strikingly, the proapoptotic effect of galectin-2 on activated $CD8^+$ T cells was even more pronounced after 18 h of stimulation compared with 24 h (Fig. 4d). Similar to galectin-2, treatment of activated T cells with the closely related galectin-1 increased the numbers of annexin V $^+$ propidium iodide $^-CD8^+$ T cells significantly, whereas the levels of apoptotic $CD4^+$ T cells were not altered (Fig. 4, d and e). In contrast to the increased number of apoptotic cells, the total number of propidium iodide $^+$ necrotic $CD8^+$ T cells was not altered in galectin-2-treated cultures compared with controls. These data indicate that galectin-2 indeed induced apoptosis specifically in TCR-activated $CD8^+$ T cells. Cellular turnover is associated with the exposure of surface phosphatidylserine resulting in positive annexin V staining (35). However, recent studies indicated that surface phosphatidylserine exposure cannot be equated with apoptosis (35). Therefore, we performed cell death detection ELISA analyses to quantify DNA fragmentation by assessing the levels of histone-complexed DNA fragments. Stimulation of $CD4^+$ T cells with various concentrations of galectin-2 did not induce DNA fragmentation exceeding the baseline level of NaCl-treated cells (Fig. 4f). In contrast, in culture supernatants of $CD8^+$ T cells stimulated for 18 h with galectin-2 in a concentration of 5, 10, or 20 $\mu\text{g/ml}$, we detected significantly increased numbers of histone-associated DNA fragments compared with NaCl-stimulated control cells, demonstrating that galectin-2 treatment indeed induced apoptosis in activated $CD8^+$ T cells (Fig. 4f). Likewise, the closely related galectin-1 elicited apoptotic cell death in activated $CD8^+$ T cells, as shown by the increased DNA fragmentation (Fig. 4f). To investigate whether galectin-2 would also induce DNA fragmentation in in vivo-activated autoreactive $CD8^+$ T cells, we cocultured $CD8^+$ T cells from autoimmune-prone CD40L tg mice with galectin-2 for 18 h and subsequently performed cell death detection ELISA analyses. However, compared with wild types (wt), we observed less DNA fragmentation in $CD8^+$ T cells from CD40L tg mice (Fig. 4g), suggesting that autoreactive T cells might exhibit a reduced sensitivity to galectin-2-induced apoptosis.

Galectin-2-induced apoptosis of activated $CD8^+$ T cells can be blocked by the pan-caspase inhibitor Z-VAD

Having documented the proapoptotic effects of galectin-2 on activated $CD8^+$ T cells, we intended to investigate whether proapoptotic caspases are involved. To address this point, $CD8^+$ T cells

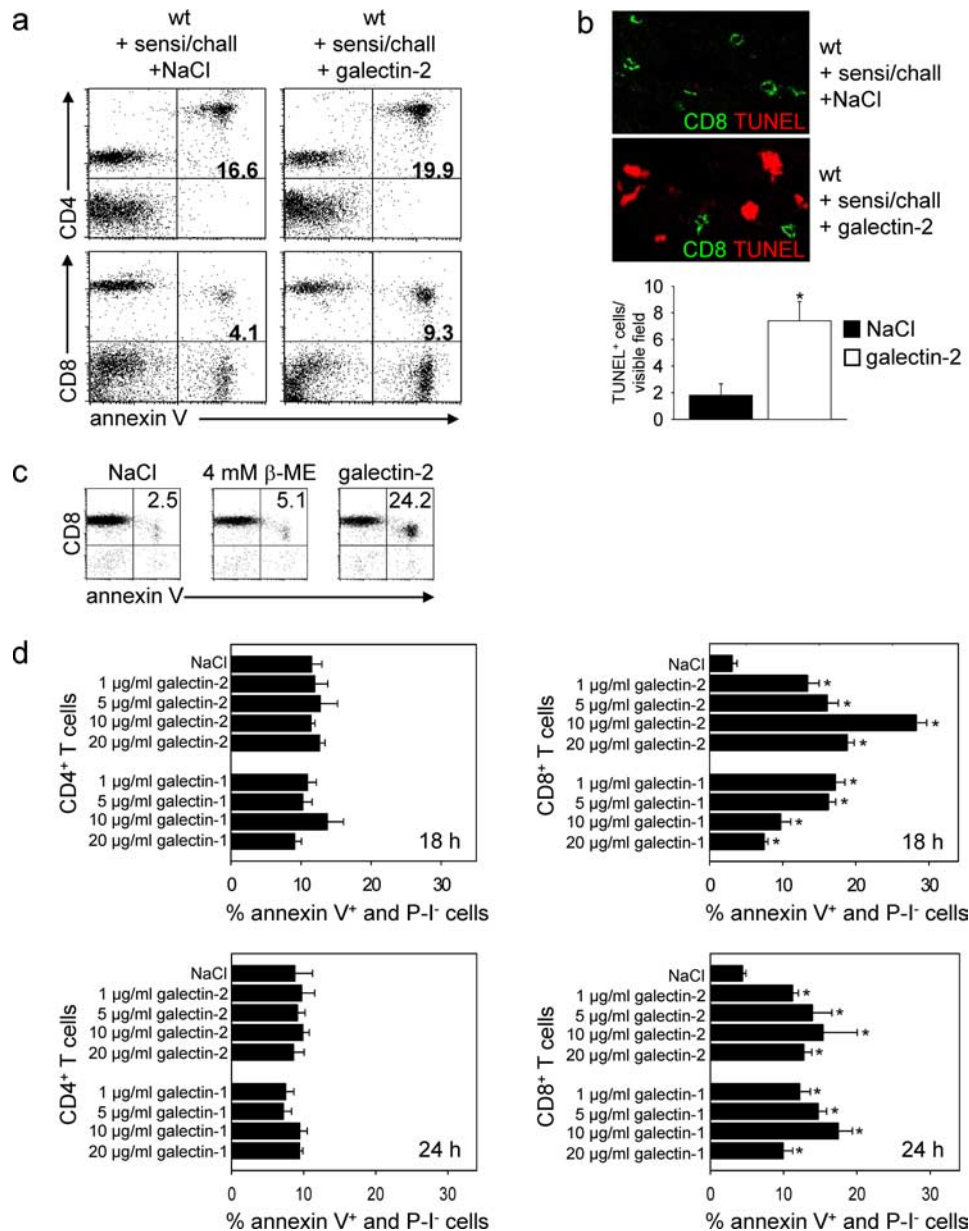


FIGURE 4. Galectin-2 treatment induced apoptosis in CD8⁺ T cells. *a*, Lymph node cells from DNFB-sensitized/challenged mice that had been treated with either NaCl or galectin-2 were stained for annexin V and subjected to flow cytometry analysis. Representative dot plots from one out of three independent experiments are shown for each experimental group. *b*, Immunofluorescence analysis of ear skin from NaCl- or galectin-2-treated and sensitized/challenged mice. Two days after challenge of mice with DNFB, cryosections of treated skin areas were stained for CD8 (green) and for apoptotic cells (TUNEL assay, red). Original magnification, $\times 400$; scale bar 25 μ m. One representative immunofluorescence staining is shown, and the bar graph summarizes the results of five mice in each group. Data are shown as mean number of apoptotic cells in each visible field, and five visible fields have been analyzed per mouse. *, $p < 0.05$ by Student's *t* test. *c*, The reducing agent 2-ME does not mediate galectin-2-induced apoptosis. CD8⁺ T cells were stimulated for 18 h with either 4 mM 2-ME or 10 μ g/ml galectin-2, stained for annexin V and propidium iodide (P-I), and subjected to flow cytometry. Representative dot plots gated for propidium iodide⁻ cells are shown. *d*, In vitro treatment of CD8⁺ T cells with galectin-2-induced apoptosis. CD4⁺ and CD8⁺ T cells from naive C57BL/6 mice were stimulated for 18 (*upper*) or 24 h (*lower*) with indicated concentrations of galectin-2 or galectin-1. Subsequently, cells were stained for annexin V and propidium iodide and subjected to flow cytometry analysis. Mean percentages of annexin V⁺ propidium iodide⁻ (P-I⁻) cells are shown. *, $p < 0.05$ (Student's *t* test) between T cells stimulated with NaCl vs galectin. *e*, Representative dot plots for each experimental group 18 h after galectin stimulation are shown. *f*, Detection of DNA fragmentation. Histone-associated DNA fragments in culture supernatants of CD4⁺ and CD8⁺ T cells stimulated for 18 h with indicated concentrations of galectin-2 or galectin-1 were quantified using in situ cell death detection ELISA. *g*, CD4⁺ and CD8⁺ T cells from C57BL/6 and CD40L tg mice were stimulated for 18 h with 10 μ g/ml galectin-2 or NaCl and, subsequently, histone-associated DNA fragments in culture supernatants were quantified using in situ cell death detection ELISA.

were activated using mitogenic Abs and cocultured either with galectin-2, the pan-caspase inhibitor Z-VAD, or a combination of both. To detect the number of apoptotic cells, cultures were stained for annexin V and propidium iodide. The proapoptotic effects of galectin-2 on activated CD8⁺ T cells could be reversed by addition

of Z-VAD, as evidenced by the reduced annexin V expression in TCR-stimulated CD8⁺ T cells treated with galectin-2 and Z-VAD ($6.2 \pm 2.9\%$) compared with only galectin-2-treated controls ($12.4 \pm 1.7\%$; Fig. 5*a*). Blocking caspase activity with the pan-caspase inhibitor Z-VAD furthermore restored the proliferative

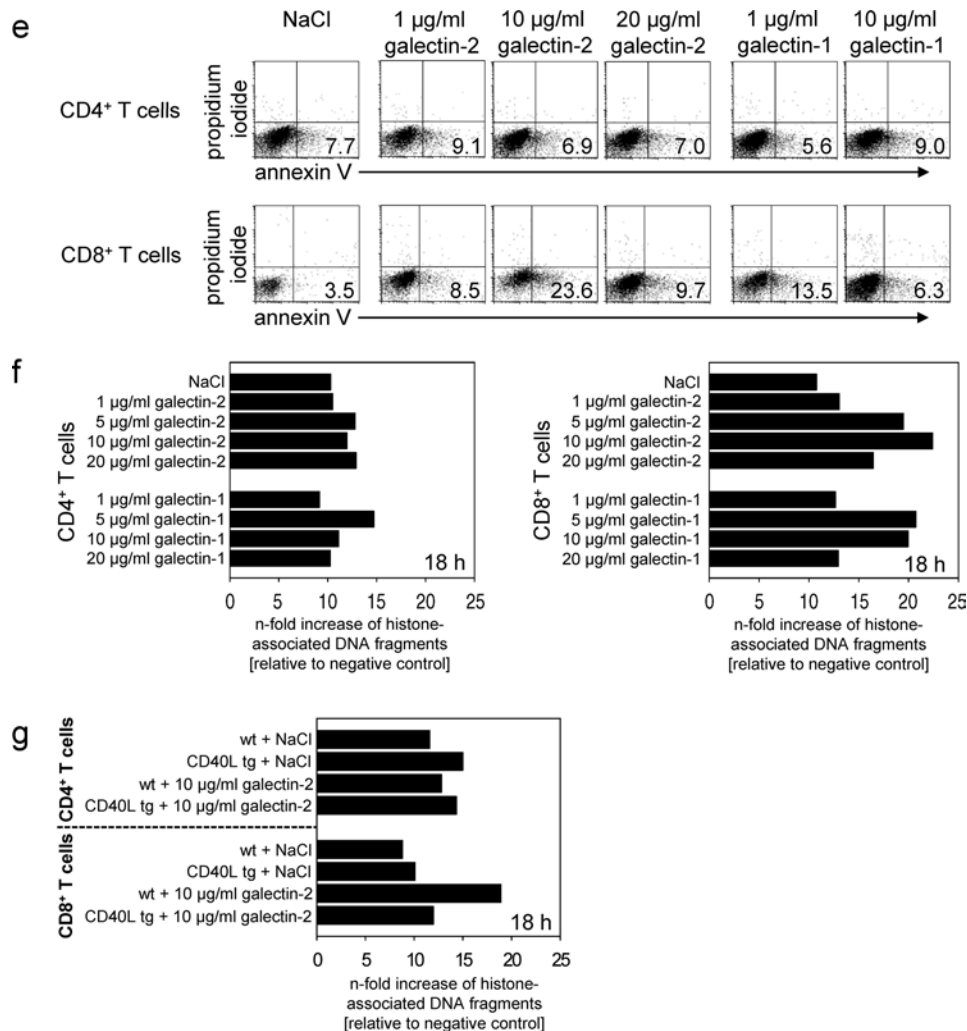


FIGURE 4. (continued)

response of TCR-activated CD8⁺ T cells (Fig. 5*b*). Thus, participation of caspases in the galectin-2-mediated induction of apoptosis in activated CD8⁺ T cells was confirmed.

Increased expression of the galectin-2-binding protein CD29 on activated CD8⁺ T cells

It has been shown that galectin-3 induces apoptosis in T cells via binding to the β_1 integrin CD29 (36), and Sturm et al. described that galectin-2 is also able to bind CD29 (20). Therefore, we speculated that CD29 might function as a receptor for galectin-2 on activated CD8⁺ T cells in our model as well, and we furthermore hypothesized that the β_1 integrin might play a role in galectin-2-mediated induction of apoptosis in activated CD8⁺ T cells. To analyze the relevance of CD29, we first quantified the β_1 integrin expression in lymphocytes from DNFB-sensitized and -challenged mice. As evidenced by flow cytometry, CD8⁺ T cells showed an increased expression of CD29, whereas only a small subpopulation of CD4⁺ T cells expressed the β_1 integrin (Fig. 6*a*). Strikingly, the CD29 expression was significantly higher in activated CD44^{high}CD8⁺ T cells than in nonactivated CD44^{low}CD8⁺ T cells (Fig. 6*b*). These data demonstrate that the β_1 integrin CD29 is up-regulated in CD8⁺ T cells during Ag-specific activation in contact allergy and may suggest that the induction of apoptosis specifically in activated CD8⁺ T cells might be mediated by the increased expression of the galectin-2-binding protein CD29 in this cell population. To analyze whether the β_1 integrin indeed affects the apoptosis-

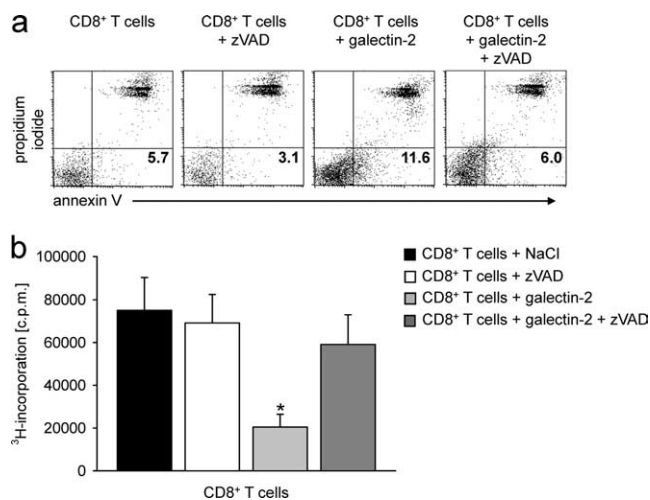


FIGURE 5. The pan-caspase inhibitor Z-VAD is able to block galectin-2-induced apoptosis of activated CD8⁺ T cells. *a*, CD8⁺ T cells from peripheral lymph nodes of naive mice were stimulated with 20 $\mu\text{g/ml}$ galectin-2 and/or 40 $\mu\text{g/ml}$ Z-VAD. Subsequently, cells were stained for annexin V and propidium iodide and subjected to flow cytometry analysis. Representative dot plots for each experimental group are shown. *b*, Z-VAD treatment restores the proliferation of galectin-2-stimulated CD8⁺ T cells. CD8⁺ T cells from peripheral lymph nodes of naive mice were treated with 20 $\mu\text{g/ml}$ galectin-2 and/or 40 $\mu\text{g/ml}$ Z-VAD. Proliferation assays were performed by stimulating CD8⁺ T cells with anti-CD3 and anti-CD28. Mean values of [³H]thymidine uptake $\pm$ SD are shown from one out of three independent experiments. *, $p < 0.05$ by Student's *t* test.

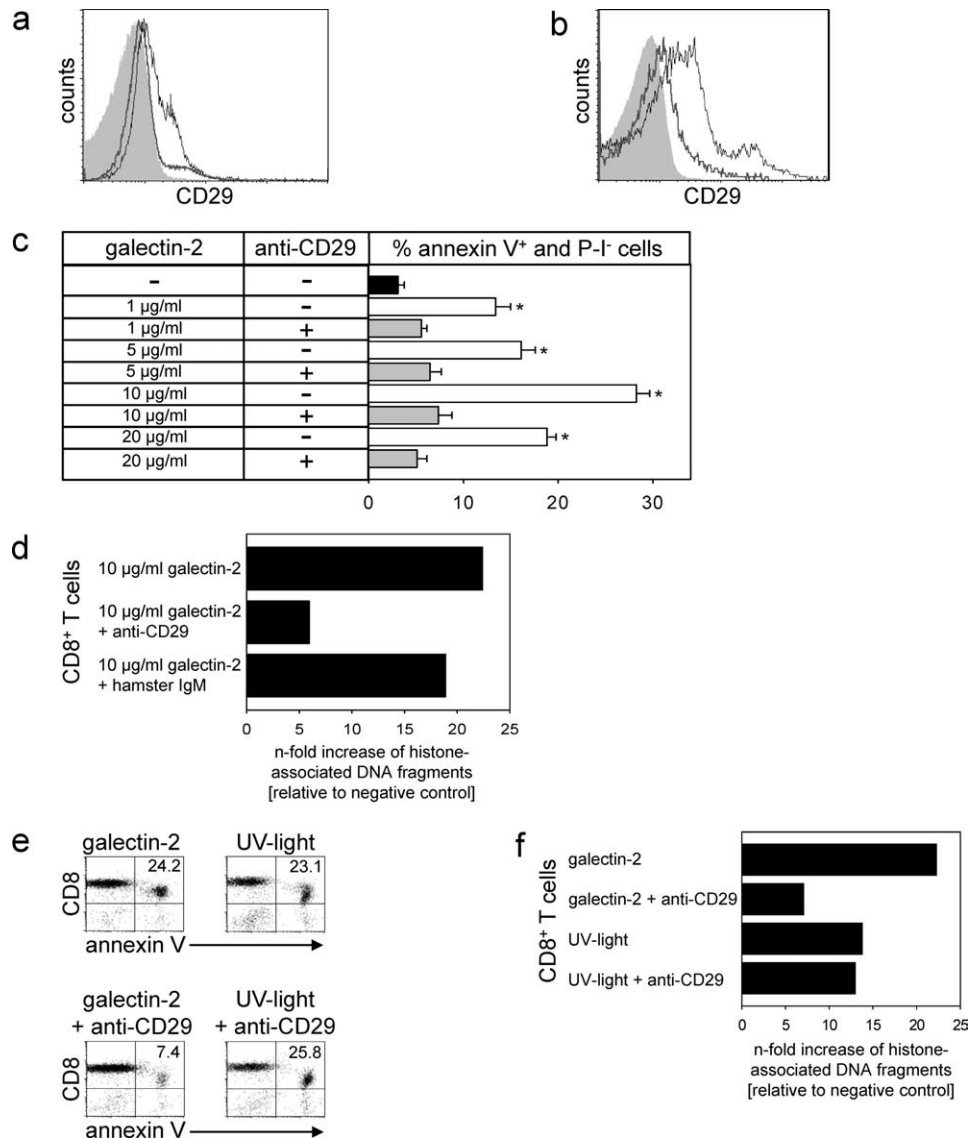


FIGURE 6. Increased expression of the galectin-2-binding protein CD29 in activated CD8⁺ T cells. *a*, CD4⁺ (gray line) and CD8⁺ T cells (black line) from DNFB-sensitized and -challenged mice were stained for the β_1 integrin CD29 and analyzed by flow cytometry. Isotype controls are shown in light gray shades. *b*, CD29 expression is up-regulated in activated CD8⁺ T cells. CD8⁺ T cells were isolated from skin-draining lymph nodes of DNFB-sensitized and -challenged mice, stained for CD44 and CD29, and analyzed by flow cytometry. Cells were gated for CD8⁺CD44^{low} (gray line) or CD8⁺CD44^{high} (black line). Isotype controls are shown in light gray shades. *c*, Galectin-2-induced apoptosis in activated CD8⁺ T cells is dependent on CD29 signaling. CD8⁺ T cells from C57BL/6 mice were activated with mitogenic Abs and stimulated for 18 h with indicated concentrations of galectin-2 and 50 µg/ml anti-CD29 or an equal amount of a hamster IgM control Ab. Subsequently, cells were stained for annexin V and propidium iodide and subjected to flow cytometry analysis. Mean percentages of annexin V⁺ propidium iodide⁻ (P-I⁻) cells are shown. *, $p < 0.05$ (Student's *t* test) between CD8⁺ T cells treated with anti-CD29 vs hamster IgM. *d*, Activated CD8⁺ T cells from C57BL/6 mice were stimulated for 18 h with 10 µg/ml galectin-2 in the presence of 50 µg/ml anti-CD29 or a hamster IgM control Ab. Subsequently, histone-associated DNA fragments in culture supernatants were quantified using an in situ cell death detection ELISA. *e*, UV-induced apoptosis in CD8⁺ T cells is independent from CD29 signaling. Activated CD8⁺ T cells from C57BL/6 mice were treated with 50 µg/ml anti-CD29 or a control Ab and were either stimulated for 18 h with 10 µg/ml galectin-2 or were UV irradiated (40 mJ/cm²). Subsequently, cells were stained for CD8, annexin V, as well as propidium iodide and subjected to flow cytometry. Representative dot plots gated for propidium iodide⁻ cells are shown. *f*, Activated CD8⁺ T cells from C57BL/6 mice were stimulated for 18 h with 10 µg/ml galectin-2 or were UV irradiated (40 mJ/cm²) in the presence of 50 µg/ml anti-CD29 or a control Ab. Subsequently, histone-associated DNA fragments in culture supernatants were quantified using an in situ cell death detection ELISA.

inducing activity of galectin-2, activated CD8⁺ T cells were stimulated with galectin-2 and cultured in the presence of anti-CD29. Interestingly, blocking galectin-2/CD29 signaling resulted in reduced numbers of apoptotic cells, as evidenced by annexin V and propidium iodide staining (Fig. 6c). Treatment of activated, galectin-2-stimulated CD8⁺ T cells with anti-CD29 furthermore decreased the amount of DNA fragmentation to 25% from normal levels in cells cultured with a hamster IgM control Ab (Fig. 6d).

Next, we were interested to investigate whether anti-CD29 treatment specifically inhibits galectin-2-induced apoptosis or whether this Ab also affects proapoptosis induced by other apoptotic stimuli. Therefore, purified CD8⁺ T cells were cocultured with anti-CD29 or a control Ab and irradiated with 40 mJ/cm² UV light since this UV dosage has been described to induce apoptosis in T cells (37). Subsequently, the numbers of apoptotic cells were quantified by annexin V staining and cell death detection ELISA. As shown in Fig. 6, *e* and *f*, blocking CD29 signaling in UV-irradiated

CD8⁺ T cells did not reduce the level of annexin V expression or the amount of DNA fragmentation, clearly indicating that anti-CD29 treatment did not affect UV-induced apoptosis. However, anti-CD29 treatment inhibits galectin-2-mediated apoptosis, possibly by abrogating galectin-2 binding to CD29 (Fig. 6, c–f).

Taken together, these data demonstrate that galectin-2 is a potent inducer of apoptosis specifically in activated CD8⁺ T cells and possibly mediated by the increased galectin-2/CD29 signaling in this cell population.

Discussion

Galectins are a family of soluble glycan-binding proteins, which have been shown in recent years to play an increasingly important role in the modulation of cellular immune responses. Most results have been presented on the effects of galectin-1 and galectin-3 in various tumor, infection, and inflammation models (11, 12, 38, 39). In contrast, much less is known about the modulation of immunity by galectin-2. The data presented herein indicate that systemic application of galectin-2 is able to inhibit especially MHC class I-restricted effector T cell function during allergic contact allergy. Contact allergy is one of the most common acquired skin disorders, which is treated so far topically or systemically with rather broad unspecific immunosuppression-inducing corticosteroids. Therefore, blocking CD8-mediated contact allergy responses is of particular practical relevance. Several other galectins have been shown to block inflammation in different mouse models. Toscano et al. demonstrated that systemic treatment of mice with 50 μ g of galectin-1 per day for 3 days reduced experimental autoimmune uveitis significantly (40), and similar amounts of galectin-9 suppressed allergic asthma (41). However, even higher concentrations of galectin-4 (60 μ g/day for 8 days) were used to inhibit dextrane sulfate-induced colitis (34). Here we demonstrate that contact allergy can be blocked by i.p. injection of 20 μ g of galectin-2 per day for 6 days. These data suggest that various galectins might exhibit similar effects but seem to vary in their activity. Furthermore, the differences in galectin concentrations required to efficiently block inflammatory disorders might also be attributed to different kinds of administration (e.g., galectin-9 and galectin-1 were injected i.v., whereas galectin-4 and galectin-2 were given i.p. (34, 40, 41)).

Galectin-2 treatment inhibited TCR-activated CD8⁺ T cells both in vivo and in vitro. We speculate that this inhibition of activated CD8⁺ T cells significantly contributed to the protective effects of galectin-2 on contact allergy. These findings are in agreement with reports demonstrating that galectin-2 is expressed in the intestinal mucosa and is able upon injection to prevent the development of experimentally induced colitis. In that model, Ag-specific MHC class II-restricted T cells were transferred into naive recipients, which were subsequently challenged with the cognate Ag. In colitis, galectin-2 was able to inhibit the effector function of CD4⁺ T cells (20, 31). In contrast to experimental colitis, galectin-2 suppressed, in the model of contact allergy, activated CD8⁺ T cells rather than CD4⁺ T cells. Obviously, MHC class II-restricted immunity plays an important role during colitis development, whereas MHC class I-restricted responses induce cutaneous delayed-type hypersensitivity. Collectively, the results from both models show that galectin-2 can control inflammation at both interfaces, that is, the skin and the intestine. Perhaps intestinal galectin-2 expression plays an additional role in tissue homeostasis since it was demonstrated that galectin-2 is able to regulate the function of lamina propria lymphocytes (31).

Within the galectin family, galectin-1 shares a 43% amino acid sequence identity to galectin-2 (20). Galectin-1 has been shown to exert similar inhibitory effects on Ag-primed or mitogen-stimu-

lated CD8⁺ T cells. In this context, galectin-1 induced cell cycle arrest and/or apoptosis in leukemia T cells or thymocytes, suggesting a role for galectin-1 in T cell homeostasis (19, 42, 43). Our results show that besides galectin-1, galectin-2 is able to induce apoptosis in vivo, especially in activated CD8⁺ T cells in mice. These data are congruent with the results obtained from studying the effects of galectin-2 on human T cells. It was shown that similar to galectin-1, galectin-2 induced apoptosis in human T cells, possibly by modulating the ratio of proapoptotic Bax vs antiapoptotic Bcl-2 expression (20, 33). Our finding that the pan-caspase inhibitor Z-VAD was able to prevent the inhibitory effects of galectin-2 on TCR-stimulated CD8⁺ T cell proliferation supports the hypothesis that the suppressive effects of galectin-2 on T cells are mediated by the induction of apoptosis in activated T cells. In other reports it was demonstrated that galectin-1, galectin-2, and galectin-4 induced the surface expression of phosphatidylserine on human neutrophils and T cells, which had been activated under specific experimental culture conditions (35). Phosphatidylserine mediates the tethering of apoptotic cells to phagocytes for removal. In brief, these investigations suggest that different galectins induce in various leukocyte subpopulations signaling pathways involved in apoptosis or removal of cells, which may be important for leukocyte turnover. Whether galectin-2 is able to stimulate the expression of phosphatidylserine on CD8⁺ T cells in mice independently of the induction of apoptosis is under investigation. Moreover, Stowell et al. demonstrated that galectin-1 binds to activated human T cells but induces apoptosis in those cells only in the presence of a reducing agent (35). Since the galectin-2 preparation used in this study contains 4 mM 2-ME as a reducing agent, we are currently analyzing if 2-ME is required for the proapoptotic effects of galectin-2 or is at least facilitating galectin-2-mediated apoptosis in activated CD8⁺ T cells.

Galectins can also act in the intracellular milieu via protein-protein interactions to regulate RNA splicing and/or cell cycle progression (44–47). After extracellular secretion, galectins recognize β -galactoside-containing glycoconjugates of extracellular matrix structures and cellular adhesion molecules. Human galectin-2 was shown to bind to the β_1 integrin (CD29), but not to CD3 or CD7, on human peripheral blood lymphocytes (20). In mice, our data demonstrate that CD29 was expressed on most activated CD8⁺ T cells, whereas only a small fraction of activated CD4⁺ T cells expressed CD29 during contact allergy. Since galectin-2 induced apoptosis in activated CD8⁺ T cells, we hypothesize that apoptosis signaling might be mediated by binding of galectin-2 to CD29 on TCR-activated CD8⁺ T cells. This hypothesis is supported by the observation that treatment of galectin-2-stimulated CD8⁺ T cells with anti-CD29 abrogated the apoptosis-inducing effects of galectin-2. Therefore, our data on galectin-2 are congruent with the findings by other groups demonstrating that soluble galectin-3 can induce T cell death via binding to CD29 and thereby triggering the apoptosis signal through mitochondria, resulting in caspase activation (36, 38). However, the observation that CD29-deficient T cells are still sensitive to galectin-3-induced apoptosis indicated that this receptor is not essential for the proapoptotic effects of galectin-3. Whether CD29 signaling is crucial for galectin-2-induced apoptosis in activated CD8⁺ T cells has to be investigated in detail. Taken together, our findings suggest that galectin-2 binding to CD29 on CD8⁺ T cells might mediate apoptosis induction, which is involved in the regulation of inflammatory contact allergy.

For the induction of Ag-specific immune responses, presentation of haptens by APCs to naive T cells is required. In particular, galectin-3 has been shown to regulate the T cell activation induced by the interaction of APCs and T cells via impairing TCR complex

formation (48, 49). Similarly, galectin-1 has been demonstrated to inhibit the production of IL-2 by TCR-activated lymphocytes (50). In our investigation, treatment of mice with galectin-2 appeared not to induce apoptosis in subsets of Ag-presentation cells such as dendritic cells, macrophages, and B cells (data not shown). After galectin-2 treatment, similar numbers of APCs were detectable upon flow cytometry in skin-draining lymph nodes (not shown). The results presented point to a rather inferior role of galectin-2 on APCs. Our data provide strong evidence that the reduction of contact allergy responses induced by galectin-2 injections was primarily mediated by impaired CD8⁺ T cell effector function.

T cell-mediated autoimmune or delayed-type hypersensitivity responses can be suppressed by regulatory CD4⁺CD25⁺ T cells (47). Indeed, galectin-1 treatment reduced the development of experimental autoimmune uveitis in mice (40). This protective effect induced by galectin-1 was associated with the increased expression of IL-10 and TGF- β , two important factors for the induction of regulatory T cells. Accordingly, adoptive transfer of galectin-1-induced regulatory T cells was able to inhibit autoimmune uveitis in recipient animals (8, 40). Our findings, however, suggest that regulatory CD4⁺CD25⁺ T cells in galectin-2-treated mice showed similar numbers and suppressor function compared with controls. Perhaps the low expression of CD29 on CD4⁺ T cells rendered these cells unresponsive to the immunomodulatory effects of galectin-2.

Another biological function of galectins is the regulation of cell adhesion. Cell adhesion studies using galectin-2-treated human T cells from peripheral blood or intestinal lamina propria and collagen type I or fibronectin as matrix compounds showed differential effects. Galectin-2 reduced the binding of both human T cell subpopulations to collagen matrix; in contrast, binding to fibronectin was significantly enhanced (20, 31). It was proposed that galectin-2 might be able to bridge glycan chains of matrix compounds and cell surface glycoproteins. Since normal numbers of naive CD4⁺ or CD8⁺ lymph node, peripheral, or splenic T cells were detectable in galectin-2-treated mice, our data suggest a rather weak role of galectin-2 on T cell adhesion to extracellular matrix compounds *in vivo*. In support of this hypothesis, analysis of contact dermatitis ear specimens showed reduced T cell infiltrates in the vicinity of the basal membrane or dermis (Fig. 4b and data not shown). In brief, the reduced number of activated CD8⁺ T cells and increased apoptosis in CD8⁺ T cells detected in galectin-2-treated mice strongly suggest that galectin-2 decreased contact allergy responses via affecting T cell homeostasis rather than cell adhesion.

Collectively, our results provide new insights into the intrafamily diversity of galectins by demonstrating that galectin-2 inhibited the development of contact allergy by regulation of MHC class I-restricted immunity. Since contact allergy represents one of the most common skin disorders, these data suggest a future possible treatment alternative by employing an endogenous regulator of inflammation to restore T cell homeostasis in situations of unwanted immune responses.

Disclosures

The authors have no financial conflicts of interest.

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