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Galectin-1 triggers an immunoregulatory signature in T helper cells functionally defined by IL-10 expression

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Abstract

Galectin-1 (Gal-1), aβ-galactoside-binding protein, can alter fate and effector function of T helper (Th) cells; however, little is known about how Gal-1 induces Th cell differentiation. Here, we show that both uncommitted and polarized Th cells bound by Gal-1 expressed an immunoregulatory signature defined by IL-10. IL-10synthesis was stimulated by direct Gal-1 engagement to cell surface glycoproteins, principally CD45, on activated Th cells and enhanced by IL-21 expression through the c-Maf/aryl hydrocarbon receptor (AhR) pathway, independent of antigen-presenting cells. Gal-1-induced IL-10^{+} Tcells efficiently suppressed T cell proliferation and T-cell-mediated inflammation and promoted the establishment of cancer immune-privileged sites. Collectively, these findings show how Gal-1 functions as a major glycome determinant regulating Th cell development, inflammation and tumor immunity.

Introduction

Type 1 regulatory Th cells (Tr1) have received increased attention due to their immunosuppressive potential mediated by expression of IL-10 (1). Tr1 cells can be generated *in vitro* using exogenous cytokines, such as IL-10, TGF-β and IL-27, among other mechanisms (2–4). *In vivo*, similar tolerogenic inputs (IL-10, IL-27 and IL-21), elaborated by components of the immunologic interface through antigen-presenting cells (APCs), promote Tr1 cell formation and expansion that effectively control T cell-mediated inflammatory processes (2, 5). While Tr1 cell differentiation through regulatory cytokines, notably IL-27, has been extensively studied (6), recent evidence showing formation of regulatory T cells by antibody-mediated stimulation of heavily glycosylated transmembrane glycoproteins, CD45 (7) and CD46 (8), has suggested a potential role of lectins as regulators of T cell differentiation.

Galectin-1 (Gal-1), the prototype S-type lectin, has been shown to play a profound role in modulating adaptive immune responses by altering the fate and phenotype of T cells (9–12). Immune profiles of malignancies with enhanced Gal-1 expression (13), and experimental models of autoimmune diseases where recombinant Gal-1 (rGal-1) was used therapeutically (14), have suggested that IL-10 expression is a major component of Gal-1's immunomodulatory signature. Recently, Ilarregui et al. showed that rGal-1 and native Gal-1 induce APCs to produce IL-27, which subsequently promotes the formation of IL-10^{+} Tr1-

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like cells (15). Interestingly, due to oxidative inactivation and monomer/dimer equilibrium problems inherent to the use of rGal-1 *in vitro*, T cell cultures incubated with rGal-1 in the absence APCs have failed to demonstrate direct Gal-1-mediated enhancement of IL-10 expression (9, 16). However, Gal-1 preparations stabilized via leucine zipper approaches (16) or engineered to constitutively dimerize through the hinge region of Fc immunoglobulin domains (9, 17, 18), have been shown to significantly induce IL-10 synthesis in human and mouse T cells in the absence of APCs. While the mechanisms of direct Gal-1-mediated expression of IL-10 have not yet been explored, tangential data show that T cell stimulation of CD45 via *O*-linked carbohydrate-specific anti-CD45RO (A6) mAb (19) induces a Tr1-like phenotype with suppression of IFN-γ production and increased levels of IL-10 (7). Since Gal-1 engagement to CD45 on effector T cells has been shown to mediate apoptosis-related morphological features when used at micromolar concentrations (20); we hypothesized that dimeric Gal-1 used at non-apoptotic concentrations may induce IL-10 synthesis and other immunoregulatory activity, in part, by CD45 engagement.

In this report, using a new Gal-1 preparation that forms stable and functional dimers at physiologic concentrations, we show that Gal-1 binds to a heavily glycosylated isoform of CD45 on the surface of unpolarized activated Th cells that helps facilitate a molecular circuit resulting in IL-10 production and immunosuppressive activity. Signaling analyses revealed that Gal-1 binding to undifferentiated Th cells and polarized Th1 and Th17 cell subsets increases STAT1/STAT3/STAT6 phosphorylation and up-regulates IL-21 expression via transcriptional regulation of c-MAF and the aryl hydrocarbon receptor (AhR), concomitant with elevated IL-10 synthesis. These Gal-1-induced IL-10^{+} T cells are decorated by PD-1 and CTLA-4, and, while negative for FoxP3, resemble Tr1 cells by effectively suppressing T cell proliferation, ameliorating contact allergic dermatitis, and promoting immune escape of tumors in an IL-10-dependent manner. Collectively, we provide mechanistic evidence of how Gal-1 activates the development of immunoregulatory Tr1-like T cells, cementing Gal-1's role in establishing the immune privilege of tumors and in resolving inflammatory responses.

Materials and Methods

Mice, Cell Lines, Antibodies and Chemicals

C57BL/6 wild type (wt) mice, IL-10-eGFP reporter mice, and IL-$10^{-/-}$mice in the C57BL6 background were obtained from the Jackson Laboratory (Bar Harbor, ME). C57BL/6 mice deficient in CD43 were kindly provided by Dr. Hermann J. Ziltener (University of British Columbia, British Columbia, Canada). IL-$21R^{-/-}$mice on C57BL/6 background were generated as previously described (2). IL-$27R^{-/-}$mice on C57BL/6 background were obtained from Dr. Chris Saris (Amgen, Thousand Oaks, CA). J558L plasmacytoma cells producing the Gal-1hFc and non binding mutant dmGal-1hFc, each containing the IL-2 signal secretion sequence and non FcRγ-binding hFc variant, were generated as previously described (9, 17, 18, 21). B16F10 melanoma cells were a kind gift from Dr. Hans Widlund (Brigham and Women's Hospital). Cell cultures were maintained in RPMI 1640/10% FBS/ 1% penicillin/streptomycin (Invitrogen, Inc., Carlsbad, CA). Antibodies included: anti-mouse/human Gal-1 (N16), anti-CD45 (AF114), c-Maf (M-153), anti-goat IgG isotype, and anti-mouse β-actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); alkaline phosphatase (AP)-conjugated anti-goat IgG, anti-rat IgG and anti-human IgG (Southern biotech, Inc., Birmingham, AL); allophycocyanin (APC)-conjugated anti-human Fc (Jackson Immunoresearch, Inc., West Grove, PA); anti-mouse CD3 (145-2C11), anti-mouse CD28 (37.51), FITC-labeled anti-mouse CD4, PE-labeled anti-mouse CD3, Alexa Fluor® 488 conjugated anti-CD45, APC-conjugated anti-mouse CD25, anti-mouse CTLA-4, PD-1, IL-4, -IFN-γ, -IL-10, FoxP3, IL-17, and IL-21 (BL25168) (Biolegend, Inc., San Diego, CA); anti-mouse IL-21 (FFA21) for neutralization assays (eBioscience, San Diego, CA); Alexa Fluor®

488 donkey anti-mouse IgG, Alexa Fluor® 594 donkey anti-rabbit IgG, Alexa Fluor® 350 donkey anti-goat IgG (Invitrogen, Carlsbad, CA). CD45 phosphatase inhibitor (EMD Chemicals, Gibbstown, NJ); TRIzol, DEPC-treated water, carboxyfluorescein succinimidyl ester (CFSE), Platinum® PCR SuperMix High Fidelity, FBS, Zeocin, OPTI-MEM, Lipofectamine 2000, and protein-G agarose were also from Invitrogen.

Gal-1 Stability Assay

100ng of recombinant Gal-1 (rGal-1) (R&D, Minneapolis, MN) or Gal-1hFc were resuspended in 30μl of PBS and incubated at 37°C for 0, 8h and 48h in the absence of reducing agents. Samples were separated by non-reducing 4–20% SDS-PAGE gradient gels, transferred and immunoblotted with anti-mouse Gal-1 (N16) Ab.

Th1, Th17 and Treg Polarization

Naïve $CD4^+$ T cells ($CD62L^+/CD44^{low}$) were isolated from 6-week old C57BL/6 mouse spleens by immunomagnetic bead selection, and polarized into Th1, Th17 and Treg subsets as previously described (9, 22). Briefly, cells were resuspended (1×10^6 cells/ml), and stimulated for 48h with plate-bound anti-CD3 mAb (2μg/ml) and soluble anti-CD28 mAb (1μg/ml). For Th1 polarization, 10ng/ml IL-12 and 20μg/ml anti-IL-4 (11B11) mAb were added; for Th17 polarization, 20ng/ml IL-6, 5ng/ml TGF-β1, 20 μg/ml anti-IFN-γ (XMG1.2) mAb, and 20 μg/ml anti-IL-4 (11B11) mAb were added. Tregs were polarized using 3ng/ml TGF-β1 and 25U/ml IL-2. Th1 cells were expanded with RPMI-1640 supplemented with 25U/ml IL-2; and Th17 cells were expanded with 10ng/ml IL-23 for 4 additional days. All cytokines were purchased from Peprotech, Inc (Rocky Hill, NJ), and Abs were purchased from BD Biosciences (San Diego, CA).

Generation of Gal-1-induced Tr1-like T cells

Naïve sorted Th cells (wt or $IL\text{-}27R^{-/-}$) were stimulated for 48h with plate-bound anti-CD3 mAb (2μg/ml) and soluble anti-CD28 mAb (1μg/ml) followed by 24–72h incubation with 0.25μM Gal-1hFc or dmGal-1hFc. Neutralization of CD45 PPase activity was performed adding 2μM CD45 PPase inhibitor or PBS control for 48h during Gal-1hFc incubation. To neutralize IL-21 activity, 5μg/ml anti-mouse IL-21 (FFA21) was added during Gal-1hFc incubations.

Flow Cytometry

Cells were first stimulated with PMA/ionomycin and brefeldin A for 5h to aid in cytokine detection, stained for surface markers (CD4, PD-1, CD25, CTLA-4 and Gal-1 ligands), fixed and permeabilized using Fix/Perm kit (BD Biosciences) and then stained for intracellular cytokines and transcription factors (IL-4, IL-10, IFN-γ, IL-17, IL-21 and FoxP3). Flow cytometry analyses were performed using a FACSCanto (BD Biosciences) and FlowJo 8.1 software (Tree Star, Ashland, OR).

Western Blotting and Immunoprecipitation

Cell lysates or immunoprecipitates were separated by reducing 4–20% SDS-PAGE gradient gels and transferred to immunoblot membrane (Bio-Rad, Hercules, CA). Following a 1h blocking step, membranes were incubated overnight with anti-mouse STAT1, STAT2, STAT3, STAT5 or STAT6 antibodies (Cell Signalling Technology, Danvers, MA) individually, followed by a 30min incubation with AP-anti-goat IgG, extensive washing and development with Western Blue (Promega, Inc., Madison, WI). For Gal-1 ligand detection, membranes were incubated overnight with 10μg/ml Gal-1hFc in TBS at 4°C, followed by 30min incubation with AP-anti-human Fc, extensive washing and development with Western Blue. Gal-1hFc blotting in the presence of 50mM lactose and blotting with

dmGal-1hFc were also performed to control for β-galactoside-dependent staining activity. For CD45 immunoprecipitation, 60μg of precleared lysate was incubated with 1μg of anti-mouse CD45 mAb or isotype control Ab and 30μl of protein-G agarose overnight on a rotator at 4°C as described (9, 23).

Immunofluorescence

Unpolarized activated T cells were treated with 0.25μM Gal-1hFc or dmGal-1hFc for 30min in RPMI-1640/10% FBS/1% penicillin/streptomycin. Cytospin preparations were fixed with 4% paraformaldehyde (Sigma) for 10 min, permeabilized with 1% Triton-X-100 (Sigma) for 10 min, blocked with 2% FBS for 30 min, and stained with Alexa-Fluor 488 anti-mouse CD45 (1:100) and counterstained with DAPI-containing mounting medium Prolong Gold Antifade (Invitrogen). Immunofluorescence imaging was performed using a Nikon eclipse Ti microscope supplied with a Nikon FDX-35 digital camera, and images were analyzed using SPOT Advanced Software (Spot Imaging Solutions, Sterling Heights, MI).

Immunohistochemistry

Tumors were fixed in 10% formalin, embedded in paraffin and sectioned (4μm). Following antigen retrieval with EDTA (pH 8), slides were treated with hydrogen peroxide for 5 min, protein block for 20 min and then stained with rabbit anti-GFP polyclonal Ab (1:1500) (Abcam, San Francisco, CA) for 1 h at room temperature. Slides were washed and incubated for 30 min with HRP-conjugated anti-rabbit IgG (1:200) (Invitrogen, Carlsbad, CA), followed by standardized Permanent red chromagen® (Invitrogen) development and counterstained with hematoxylin. Images were acquired using a Nikon eclipse Ti microscope and a Nikon FDX-35 digital camera.

T cell suppression assay

Gal-1-induced IL-10$^+$ T cells were isolated by using the IL-10 Miltenyi® cytokine secretion assay (Miltenyi Biotec, Inc), followed by flow cytometry sorting. Sorted IL-10$^+$ Th cells were cultured using 2 different concentrations of plate-bound anti-CD3 mAb (0.5μg/ml) and soluble anti-CD28 mAb (0.5μg/ml) in the presence of carboxyfluorescein succinimidyl ester (CFSE)-labeled sorted naïve CD4$^+$ T cells (CD62L$^+$/CD44^{low}) at the following T suppressor (T_{sup})/ T responder (T_{resp}) ratios (0:1, 1:1, 1:2, 1:4). T_{resp} proliferation was determined by CFSE dilution via flow cytometry fluorescence analysis on days 1 and 6. IL-10 was neutralized by including 20μg/mL of anti-IL-10 mAb (JES5-2A5) in the culturing conditions from day 1.

Adoptive transfer of Gal-1-induced IL-10$^+$ T cells into a mouse model of hapten-mediated contact hypersensitivity

Sorted naïve CD4$^+$ T cells from wt or IL-10$^{-/-}$mice were activated *ex vivo* with α-CD3/CD28 mAbs for 48h and treated with Gal-1hFc for additional 72h. Unsorted Gal-1-induced IL-10-competent or -deficient T cells (1×10^6) were injected intravenously into wt mice (n=8) immediately after sensitization of abdominal skin with 2% oxazolone as previously reported (9). On day 5 post-sensitization, ears were challenged with 0.5% oxazolone (right) or vehicle (left), and re-measured after 24h. Inguinal lymph nodes were harvested and analyzed by intracellular cytokine flow cytometry. Alternatively, IL-10-eGFP reporter mice were inoculated with 2×10^5 B16 Gal-1^{hi} cells, and on day 15-post injection, tumor-draining lymph nodes and spleens were harvested. Single cell suspensions were stained with APC-conjugated anti-mouse CD3 and flow sorted for CD3$^{+/}$GFP$^+$ expression. CD3$^+$/GFP$^+$ or CD3$^+$/GFP$^-$ cells (3×10^5) were injected intravenously into oxazolone-sensitized mice, and oxazolone-challenged ears and immune profiles were analyzed as described above.

Silencing Gal-1 and overexpressing Gal-1 in B16 melanomas

B16 Gal-1^{hi} and B16 Gal-1^{KD} were engineered as previously described (18).

Production of Gal-1hFc

Gal-1hFc and non-β-galactoside-binding mutant (dmGal-1hFc) were prepared as described (9, 17, 18).

Assaying of Tumor Growth and IL-10^{+} Cells in Tumors

B16 melanoma Gal-1^{hi} or Gal-1^{KD} cells (2×10^5 cells) were injected subcutaneously into the flank of wt, IL-10-eGFP reporter, IL-$10^{-/-}$, and IL-$21R^{-/-}$mice (10 mice/group/3 experiments), and tumor volume was assayed with a micrometer caliper every other day. Tumors were harvested on day 15 post-inoculation and tumor-draining lymph nodes were harvested, minced and single cell suspensions were analyzed by flow cytometry.

Quantitative Real Time RT-PCR

Th1/Th17 cells were treated with Gal-1hFc or dmGal-1hFc, and total RNA extraction and cDNA conversion were performed as previously described (23). RT was performed using cDNA mastermix (Bio-Rad). SYBR green quantitative PCR (qPCR) was performed using the following primers: IL-21 Forward: 5' TGCCGCTGCTTTACTCATTG 3', Reverse 5' TGCCGCTGCTT TACTCATTG; c-MAF Forward: 5' CCCCTGGCCATGGAATATG 3', Reverse 5' CGCACTGGCTGATGATGC 3'; AhR Forward: 5' CCGTCCATCCTGGAAATTCGAACC 3', Reverse: 5' CCTTCTTCATCCGTTAGCGGTCTC 3', and β-actin Forward: 5' GCACCGTCAGCTTTCAGAGA 3', Reverse 5' AGCGCAAGTACTCTGTGTGG. Samples were analyzed in triplicate and normalized to β-actin expression.

Statistical Analyses

Statistical significance was ascertained between groups using a paired Student's *t*-test.

Results

Gal-1 binding to unpolarized activated Th cells drives IL-10 expression, in part, through CD45 engagement

Using recombinant Gal-1 (rGal-1) in research has led to significant advances to the understanding of Gal-1 and its pro-apoptotic activity (10, 24). However, due to rapid oxidative inactivation, rGal-1 requires addition of reducing agents (e.g. DTT) to maintain a functional carbohydrate recognition domain. In addition, rGal-1 concentrations need to exceed 7μM in order to prevent dissociation into monomeric forms, limiting its use at low more physiologic concentrations (25). Thus, to ensure functional dimerization in biologic assays, we recently generated a constitutively dimeric form of Gal-1 fused to the Fc domain of human IgG (Gal-1hFc) and its non-β-galactoside binding mutant control (dmGal-1hFc) that can be used in the absence of reducing agents to explore Gal-1-mediated immunomodulatory effects other than apoptosis (9, 18, 26). Here, under non-reducing conditions, we showed that the functional dimeric form of Gal-1hFc is preserved for up to 48h at concentrations below the 7μM threshold, whereas rGal-1 formed oligomers after only 8h in identical conditions(Figures S1a and b) (27). Consequently, Gal-1hFc can be used at non-pro-apoptotic concentrations in T cell cultures to explore heretofore ill-defined biologic effects, such as cytokine modulation and T cell differentiation, which have been technically challenging using rGal-1.

We have previously demonstrated that Gal-1hFc can directly induce undifferentiated mouse $CD4^+$ T cells to synthesize IL-10 and Th2 cytokines in the absence of APCs (9). Similarly, an independent study shows that antibody-mediated stimulation of *O*-linked carbohydrate epitopes on CD45RO can promote the formation of IL-10^+ T cells (7). Therefore, to determine whether Gal-1 engagement of CD45 could facilitate IL-10 synthesis, we first analyzed the Gal-1 ligand repertoire on unpolarized activated Th cells. In Gal-1hFc blots of unpolarized activated Th cell lysates and of anti-CD45 immunoprecipitates, we indeed found that CD45 at ~180kDa was a major Gal-1-reactive protein (Figure 1a). In fact, when unpolarized activated Th cells were treated with non-apoptotic concentrations of Gal-1hFc, immunofluorescence analyses showed CD45 polarization and reorganization into multiple microdomains as early as 30 min post-incubation (Figure 1b). This effect was not evident when a lectin mutant, dmGal-1hFc, was used as control (Figure 1b). Furthermore, when CD45 phosphatase activity was abrogated with a specific CD45 phosphatase inhibitor, Gal-1hFc-mediated IL-10 expression on Th cells was significantly diminished (Figures 1c and S1c) (Student's paired *t*-test, **$p<0.001$). These data revealed, however, the possibility that cell surface glycoproteins other than CD45 contributed to IL-10 synthesis. Since others have shown that Gal-1 binding to CD43 on APCs induces a tolerizing circuit, which promotes the expansion of IL-10^+ Tr1-like cells (15), we treated $CD43^{-/-}$Th cells with non-apoptotic concentration of Gal-1hFc and found that $CD43^{-/-}$activated Th cells avidly bound Gal-1 and produced a similar level of IL-10 as wt control cells (Figures 1d and S1d). Although our Th cell cultures had purity greater than 98%, contaminant APCs (dendritic cells and macrophages) could be a potential source of IL-27, obscuring the direct role of Gal-1 in IL-10 synthesis. To completely rule out the influence of IL-27 in our system, we incubated unpolarized activated Th cells from IL-27 receptor null mice (IL-$27R^{-/-}$) with Gal-1hFc and evaluated IL-10 synthesis. Notably, Gal-1-mediated expression of IL-10 was unaltered in IL-$27R^{-/-}$Th cells compared with their wt counterpart (Figure 1e and S1e). These data suggested that Gal-1 binding to cell surface glycoproteins, namely CD45, on activated Th cells can initiate IL-10 synthesis in activated Th cells independent of IL-27-mediated amplification.

Gal-1-induced IL-10^+ T cells show increased phosphorylation of STAT1, STAT3 and STAT6, but do not co-express IL-4 or FoxP3

IL-10 is a regulatory cytokine produced by many different cell types. Monocytes (28), B cells (29), $CD8^+$ T cells (30) and most of the $CD4^+$ Th subsets express IL-10 under certain stimuli, depending on particular signaling activities in the JAK-STAT pathway (31). To elucidate the signaling events associated to the synthesis of Gal-1-mediated IL-10^+ T cells, we incubated unpolarized activated Th cells with Gal-1hFc or dmGal-1hFc for 4h and analyzed the phosphorylation status of different STAT molecules in the absence of exogenous cytokines. Notably, Gal-1hFc-treated cells showed presence of phosphoSTAT1 and phosphoSTAT6, and greater phosphorylation status of STAT3 compared to control-treated cells (Figure 2a). Importantly, STAT1 and STAT3 phosphorylation have previously been linked to the synthesis of Tr1 cells and expression of IL-10 in both, mouse and human (32–34). Conversely, STAT6 phosphorylation has been shown to trigger IL-4 synthesis and Th2 differentiation (35); biological effects previously associated to Gal-1 on lymphocytes (9, 36). Therefore, to further characterize the phenotype of Gal-1-induced IL-10^+ cells, we performed intracellular staining for IL-4 and FoxP3 and found that, after 24h incubation with Gal-1hFc, IL-10^+ T cells rarely co-expressed either IL-4 or the transcription factor FoxP3 (Figures 2b and c and S2a).

Gal-1-induced IL-10$^+$ Th cells express regulatory surface markers and suppress T cell proliferation through an IL-10-dependent mechanism

Gal-1 is highly expressed during the peak and resolution phases of inflammatory immune responses, similar to other immunomodulatory molecules, such as programmed death -1 (PD-1) and its ligand, PD-1L (15). To investigate whether Gal-1 directly enhanced the synthesis of surface-bound immunomodulatory molecules, we analyzed the expression of CD25 and members of the CD28 family, cytotoxic T-lymphocyte antigen 4 (CTLA-4) and PD-1, in T cells 24h post-incubation with Gal-1hFc or dmGal-1hFc control. Remarkably, Gal-1-induced IL-10$^+$ Th cells co-expressed a high level of CD25 with significant amounts of CTLA-4 and PD-1 (Figures 3a and b) (Student's paired *t*-test, **p<0.001). Of note, we also investigated the level of inducible costimulator (ICOS) after Gal-1hFc treatment; and found no difference compared to dmGal-1hFc treatment control (data not shown).

To determine the immunosuppressive activity of Gal-1-induced IL-10$^+$ Th cells, we produced and purified these cells (Figure S2a) and co-cultured them with naïve T cells (T_{resp}) at ascending ratios in the presence of plate-bound anti-CD3 and soluble anti-CD28 mAbs. Gal-1-induced IL-10$^+$ Th cells efficiently suppressed T_{resp} cell proliferation in a concentration-dependent manner, and like Tr1 cells(1), their suppressive potential was neutralized with an anti-IL-10 blocking mAb (Figures 3c). To demonstrate that Gal-1-induced IL-10$^+$ Th cells resemble Tr1 suppressive activity *in vivo*, we incubated activated Th cells from wt or IL-10$^{-/-}$mice with Gal-1hFc , and injected 1×10^6 cells intravenously into mice sensitized with oxazolone on their abdominal skin. Changes in thickness of oxazolone-challenged ear skin along with immunologic profiles of skin-draining lymph nodes were evaluated. Transfer of IL-10-competetent but not IL-10-deficient Gal-1-treated Th cells efficiently suppressed both afferent and efferent phases of oxazolone-dependent hypersensitivity responses, as reflected by smaller oxazolone-draining abdominal lymph nodes (Figure S2b), which contained significantly lower levels of IFN-γ and IL-17$^+$ T cells (Figures 3d and S2c), and by markedly reduced ear swelling after oxazolone challenge (Figure 3e) (Student's paired *t*-test, p=0.039). These data reinforced the role of Gal-1-induced IL-10$^+$ Th cells as efficacious suppressors of inflammation.

Transcription factor c-Maf and the aryl hydrocarbon receptor regulate Gal-1-induced IL-10$^+$ Th cell production via expression of IL-21

Tr1 cell development in mouse and human is the result of an orchestrated series of events involving tolerogenic inputs, transcriptional activation and amplification steps controlled by autocrine growth factors. IL-27, a heterodimeric cytokine of the IL-12 family, has been widely characterized as an immunosuppressive cytokine produced by APCs, which provides the initial input for Tr1 differentiation and expansion (2, 15, 33, 34). IL-27 directly induces the transcription factor c-Maf to transactivate *IL10* and *IL21* promoters, generating Tr1 cells *in vitro* and *in vivo* (37). Furthermore, IL-27 is a potent inducer of the costimulatory receptor ICOS, which itself, also facilitates the expression of IL-10 (2). Similarly, IL-21 has been shown to play immunomodulatory effects *in vitro* and *in vivo* via enhancement of IL-10 synthesis (31). To determine whether Gal-1-induced IL-10 synthesis follows a similar induction pathway as Tr1 cell development, we analyzed c-Maf and IL-21 expression at the transcriptional and protein level following Gal-1hFc-binding. Activated Th cells incubated in the presence of Gal-1hFc expressed significantly higher levels of c-Maf than dmGal-1hFc-treated cells, starting at 4h post-incubation and increasing over a 24h period, as demonstrated by real-time RT-PCR (Figures 4a and Figure S2d) (Student's paired *t*-test, **p<0.001) and by c-Maf immunoblots (Figure 4b). Interestingly, 24h post Gal-1 treatment, we also observed a significant increase in the mRNA levels of Jun-B (Student's paired *t*-test, p<0.001), a transcription factor that mediates IL-10 expression in Th2 cells (38) (Figure S2e). Additionally, we confirmed that Gal-1-driven IL-10$^+$ Th cells express high levels of

IL-21 (~15kDa) (Figure 4c), and that neutralizing IL-21 activity in Gal-1hFc-containing Th cultures, diminished the synthesis of IL-10 by 85%, while showing a slight enhancement in IL-4 expression (Student's paired *t*-test, **p<0.001 and **p<0.01 respectively) (Figures 4d and e).

Although the concepts of plasticity and commitment are still under debate, Th1 and Th17 cell subsets can be directed to express IL-10 under special circumstances, which could alter their capacity to facilitate inflammatory responses. Indeed, IL-21 has been shown to modify the plasticity of committed Th1 and Th17 cells by inducing IL-10 synthesis via STAT3 phosphorylation (31). While previous reports have shown that Th1 and Th17 subsets are highly sensitive to Gal-1-mediated apoptosis at concentrations >7μM (22), it is unclear if, at low Gal-1 concentrations, the phenotype of *ex vivo*-polarized Th1 and Th17 cells (Figure S3a) can be altered. Importantly, our results showed that Th1 and Th17 cells share a similar Gal-1 ligand profile, in which CD45 appeared to be a major immunoreactive protein (Figure S3b), and that using 0.25μM Gal-1hFc significantly enhanced their c-Maf expression (Figures 4f and g).

Additionally, recent reports highlight the role of the aryl hydrocarbon receptor (AhR) as a synergistic binding partner of c-Maf, which potentiates transactivation of the *IL10* and *IL21* promoters and consequent enhancement of Tr1 cell differentiation (37, 39). Hence, we explored AhR expression 8h post-Gal-1hFc treatment, and showed a significant up-regulation in unpolarized activated Th cells or in Th1 or Th17 cell subsets compared to dmGal-1hFc control (Figure 4h) (Student's paired *t*-test, *p<0.01, **p<0.001 or ***p<0.0001).

Gal-1 induces IL-10 synthesis in Th1 and Th17 cells subsets via IL-21 expression

To verify whether Gal-1hFc-induced changes in c-Maf and AhR expression in Th1 and Th17 subsets resulted in enhanced IL-10 synthesis, we incubated Gal-1hFc or dmGal-1hFc in the presence of Th1 or Th17 polarizing cytokines during the activation (priming) phase of naïve $CD4^+$ T cells. Surprisingly, in conditions used to polarize Th1 and Th17 cell subsets, Gal-1hFc induced Th1 and Th17 cells to express significant levels of IL-10 (Student's paired *t*-test, **p<0.001) (Figures 5a and S3c). Interestingly, the high fluorescence intensity of IL-17/IL-10 double producers suggested the involvement of IL-21, which is know to induce both IL-10 and IL-17 synthesis (31). Indeed, assaying IL-21 expression by flow cytometry (Figures 5b and S3d) and real-time RT-PCR (Figure S3e) showed a direct role of IL-21 in differentiation/expansion of these dual IL-10 and IFN-γ or IL-17-producing subsets. Since Gal-1 enhanced IL-10 production during Th1 and Th17 cell priming, we then ascertained whether fully committed Th1 and Th17 cells might also exhibit Gal-1-mediated IL-10 production in an IL-21-dependent manner. Following *ex vivo* polarization for 3 days, Th1 and Th17 cells were treated with Gal-1hFc or dmGal-1hFc control for 48h in the presence or absence of neutralizing anti-IL-21 mAb. FACS analysis revealed that Gal-1hFc and not dmGal-1hFc increased IL-10 expression in committed Th1 and Th17 cells (Figure 5c). Importantly, Gal-1-induced IL-10 expression was significantly reduced when IL-21 was neutralized with a blocking mAb (Student's paired *t*-test, p<0.001) (Figure 5c and S3f). Of note, IL-21 neutralization minimally affected IFN-γ expression, whereas IL-17 expression was markedly suppressed (Figure 5c).

Gal-1-induced IL-10^+ Th cells favor tumor growth by preventing effective anti-tumor responses in murine B16 melanoma

Recent evidence indicates a key role of Gal-1 in the establishment of immune privilege in cancer (18, 40). Gal-1 can induce apoptosis of anti-tumor immunocytes, thereby dampening the production of anti-tumor cytokine, IFN-γ, and favoring the proliferation of Th2 and

regulatory T cells (13, 18, 40, 41). In fact, abrogation of tumor-derived Gal-1 results in suppression of tumor growth and effective anti-tumor responses mediated by increased levels of IFN-γ (40). To help demonstrate that Gal-1-induced IL-10^{+} Th cells play an important role in tumor immune escape, we inoculated B16 melanoma cells overexpressing (B16 Gal-1^{hi}) or lacking Gal-1 (B16 Gal-1^{KD}) , and analyzed the presence of IL-10^{+} tumor-infiltrating cells in syngeneic IL-10-eGFP reporter mice. Immunoanalysis of GFP expression revealed that B16 Gal-1^{hi} tumors had 8-fold more IL-10^{+} (GFP^{+}) infiltrating cells than B16 Gal-1^{KD} tumors (Student's paired *t*-test, ***p<0.0001) (Figures 6a and S3g). To demonstrate the immunosuppressive phenotype of these *in vivo*-generated Gal-1-induced IL-10^{+} Th cells, we flow-sorted CD3^{+}/GFP^{+} (IL-10^{+}) and CD3^{+}/GFP^{-}cells from B16 Gal-1^{hi} tumor-draining lymph nodes and spleens, and transferred them intravenously into syngeneic oxazolone-sensitized mice. Adoptive transfer of GFP^{+} Th cells but not GFP^{-} Th cells significantly suppressed hapten-mediated skin inflammation in recipient mice, as evidenced by smaller oxazolone-draining lymph nodes (Figures S4a) with lower levels of IFN-γ $^{+}$ T cells (Student's paired *t*-test, **p<0.001) and IL-17^{+} T cells (Student's paired *t*-test, *p<0.01) (Figures S4b and c). Concomitantly, transfer of Gal-1-induced IL-10^{+} Th cells generated *in vivo*, ameliorated efferent inflammatory responses by diminishing ear swelling after oxazolone challenge (Figure S4d) (Student's paired *t*-test, *p=0.047).

To further elaborate on the role of Gal-1 induction of IL-10 and its significance in tumor growth, we injected B16 Gal-1^{hi} or B16 Gal-1^{KD} cells in wt mice or in mice deficient in IL-10 or in the IL-21 receptor. Notably, IL-10$^{-/-}$and IL-21R$^{-/-}$mice grew significantly smaller tumors than wt mice, independent of their Gal-1 expression (Figures 6b and c) (Student's paired *t*-test, **p<0.001), pointing out their role in this novel Gal-1-related immunomodulatory pathway. Strikingly, 100% of IL-21R$^{-/-}$mice (n=10) inoculated with B16 Gal-1^{KD} cells rejected tumor formation (Figures 6c). Similar percentages of tumor rejection had been reported previously for IL-21R$^{-/-}$mice inoculated with E.G7 lymphoma cells (42), highlighting the potential immunosuppressive role of endogenous IL-21 in cancer immune evasion. Complementarily, immune profiles in T cells were analyzed in tumor-draining lymph nodes of all groups, where IFN-γ levels in CD3^{+} gated cells were highly elevated in all mouse groups injected with B16 Gal-1^{KD} cells, compared to mice bearing B16 Gal-1^{hi} tumors(Figures 6d and S4e) (Student's paired *t*-test, **p<0.001). In contrast, CD3^{+} gated cells in tumor-draining lymph nodes from wt mice inoculated with B16 Gal-1^{hi} cells exhibited higher IL-10 expression than in mice bearing B16 Gal-1^{KD} tumors(Figures 6e and S4e) (Student's paired *t*-test, **p<0.001). However, CD3^{+} gated cells from IL-21R$^{-/-}$mice inoculated with B16 Gal-1^{hi} cells expressed negligible IL-10 levels comparable to those in cells from control IL-10$^{-/-}$mice inoculated with B16 Gal-1^{hi} tumors.

Discussion

Gal-1, an evolutionarily-conserved S-type lectin, is increasingly appreciated as a potent immunomodulatory molecule. As a homodimer, it is known to trigger pro-apoptotic activity in effector leukocytes and induce tolerogenic features of APCs (10, 15). While rGal-1 has offered valuable insights into the mechanisms of Gal-1-mediated cell death, other immunologic features attributed to rGal-1 efficacy *in vivo* have been technically difficult to reproduce in the laboratory. Nevertheless, we previously demonstrated that a stable Gal-1 dimer consisting of a bridging human Fc immunoglobulin domain (Gal-1hFc), can promote Th2 skewing and expansion of IL-10^{+} T cells in *ex vivo* T cell culturing experiments (9, 18). Similar findings were reported by using a leucine-zippered dimeric Gal-1 preparation, suggesting a direct role of dimeric Gal-1 in the synthesis of IL-10 in human T cells(16). Nevertheless, a defined mechanism by which dimeric Gal-1 induces T cells to express IL-10 and exhibit immunosuppressive activity has not been established. While APC-derived IL-27, itself, can drive IL-10^{+} Tr1 formation, we believe that our results provide an alterative

scenario by which Gal-1 can directly trigger a Tr1-like phenotype. This mechanism is distinct from the another model of Gal-1-mediated immunosuppression, wherein Gal-1 triggers APCs through CD43 engagement to secrete IL-27 expression and encourage Tr1 cell differentiation (15). Alternatively, we show that Gal-1 engagement to activated Th cell glycoproteins, in particular CD45, can drive the synthesis of IL-10 through amplification steps mediated by IL-21 and the c-Maf/AhR pathway.

Previous reports have shown that Ab-mediated T cell stimulation through *O*-linked carbohydrate determinants present in CD45RO glycoform results in higher IL-10 expression, anergy, and enhanced immunosuppressive capacity (7). Additional reports have linked reduced CD45 expression with enhanced anti-viral responses to Ebola virus infection through diminution of IL-10 synthesis (43). Similarly, jacalin, a plan lectin with high affinity for O-linked glycans, has been implicated in CD45-mediated cytokine synthesis (44). While the mechanism of Tr1 cell differentiation via stimulation of *O*-glycosylated CD45RO epitopes has not been fully addressed, we hypothesized that stimulation via a CD45-binding lectin (e.g. Gal-1) might help facilitate similar effects in a physiologic setting. Indeed, in T cell leukemic cell lines, CD45 has been shown to be a *bona fide* Gal-1 ligand, which, at high concentrations, mediates pro-apoptotic activity and phagocytic turnover of dying cells, but has never been ascribed to cytokine modulation per se (20, 45, 46). Nevertheless, Gal-1's role in driving cytokine synthesis and T cell differentiation has remained ill-defined.

By exploiting Gal-1hFc as a glycobiological tool to characterize the β-galactoside repertoire of activated Th cells, we demonstrate that a heavily glycosylated form of CD45 functions as a major Gal-1 ligand. Nevertheless, while Gal-1 engagement to CD45 facilitated the synthesis of IL-10, treatment with a CD45-specific phosphatase inhibitor did not completely abrogate IL-10 synthesis, suggesting that other Gal-1 glycoprotein ligands and/or the non-enzymatic C-terminal domain of CD45, which can also elicit Gal-mediated effects (20), are capable of triggering IL-10 synthesis.

IL-10 has been extensively described as a regulatory cytokine that modulates innate and adaptive immune responses (47). In this report, we show that Gal-1-induced IL-10^+ T cells effectively abrogate the synthesis of pro-inflammatory cytokines, IFN-γ and IL-17, ameliorating skin inflammation in a mouse model of allergic contact dermatitis and promote immune evasion of murine melanomas. Importantly, interference of IL-10 activity via neutralizing mAb or knockout approaches completely eliminates their suppressive potential, providing firm evidence that Gal-1-induced IL-10^+ Th cells exhibit a Tr1-like phenotype dependent on IL-10 (1, 48).

Based on phenotypical and functional similarities between Gal-1-induced IL-10^+ Th cells and Tr1 cells (1, 48), we hypothesized that both regulatory Th subsets might share analogous induction pathways. Similar to other Tr1-inducing molecules IL-21 and IL-27, Gal-1-treated Th cells exhibit increased phosphorylation of two molecules previously associated to Tr1 formation, pSTAT1 and pSTAT3 (32) . Our data also suggest that expression of the transcription factors c-Maf and AhR is upregulated during the induction stages of Gal-1-mediated IL-10 synthesis. c-Maf is a proto-oncogene with crucial activity in Tr1 cell differentiation (2), which, directly or coupled to AhR, transactivates the *IL10* and *IL21* promoters (37, 39). Moreover, we demonstrate that IL-21 is the pivotal immunoregulatory molecule that links Gal-1 binding to IL-10 expression and immunoregulatory function.

Previous reports suggest that Gal-1 promotes the formation of immune-privileged sites by inducing apoptosis on IFN-γ-producing cells, and by skewing the microenvironment towards

a Th2 cytokine profile (13, 40). Our data, using IL-10-eGFP reporter mice inoculated with B16 melanoma variants engineered to over-express (B16 Gal-1^{hi}) or lack Gal-1 (B16 Gal-1^{KD}), reveal that IL-10^{+} tumor-infiltrating cells are abundantly found in Gal-1-competent tumors that promote tumor growth and progression, as IL-$10^{-/-}$mice inoculated with B16 Gal-1^{hi} cells show impaired tumor development, likely derived from effective IFN-γ-mediated anti-tumor responses.

Importantly, the role of IL-21 in cancer still remains controversial. While the use of exogenous recombinant IL-21 has been shown to enhance anti-tumor immune responses in many different cancer models and is currently used in clinical trials for metastatic melanoma (49); endogenous IL-21 seems to control opposing immunoregulatory activities (42). In this regard, Sondergaard et al. show that mice deficient in IL-21 or IL-21R, reject immunogenic syngeneic E.G7 lymphomas (53% and 94% respectively) by limiting the expansion of $CD8^{+}$ T cells (42). In parallel, recent evidence demonstrates that native IL-21 up-regulates the synthesis of IL-10 in $CD4^{+}$ and $CD8^{+}$ T cells via STAT3 phosphorylation (31), and that IL-21 is required for IL-10 production in Tr1 cells (2). Our data support these findings suggesting that IL-21 is a critical cytokine induced by dimeric Gal-1 that regulates the development and expansion of Gal-1-induced IL-10^{+} T cells. Furthermore, our data show that IL-21 helps establish immune-privileged sites in tumors, whereby IL-$21R^{-/-}$mice bearing B16 Gal-1^{hi} melanomas, exhibit low levels of IL-10^{+} T cells in their tumor-draining lymph nodes and attenuated tumor growth. These effects are amplified in IL-$21R^{-/-}$mice inoculated with B16 Gal-1^{KD} cells, wherein the tumors are readily rejected and immune profiles show decreased levels of IL-10 and high numbers of IFN-γ^{+} T cells in tumor-draining lymph nodes.

Another interesting feature of our data is that dimeric Gal-1 can trigger IL-10 synthesis during differentiation or after commitment of Th1 and Th17 cells through the c-Maf/AhR/IL-21 pathway. These results suggest that Gal-1 can elicit different effects on pro-inflammatory Th cell subsets depending on the local concentrations in tissues, that is, high concentrations may favor the hallmark pro-apoptotic activity, while lower concentrations may induce immunoregulation activity via IL-21 and IL-10 expression.

In conclusion, our data provide a detailed molecular mechanism by which Gal-1 directly induces the development of IL-10^{+} Th cells independent of APC-IL-27 collaboration. Gal-1-induced IL-10^{+} Th cells resemble Tr1-like cells, as they are potent suppressors of inflammation and encourage tumor immune evasion. These findings highlight the importance Gal-1-mediated IL-21 and IL-10 as key immunomodulators of T cell responses against tumors and reinforce the critical role of Gal-1 – Gal-1 ligand interactions as glycome regulators of T-cell-mediated inflammation and anti-tumor immunity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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F.C.L designed the research, performed the experiments, analyzed the data and wrote the paper. M.O. performed the experiments and analyzed the data. S.R.B. designed the research, performed the experiments and analyzed the data. C.J.D. conceived the study, designed the research, analyzed the data, wrote the paper and supervised all experimentation. V.K.K provided the reagents.

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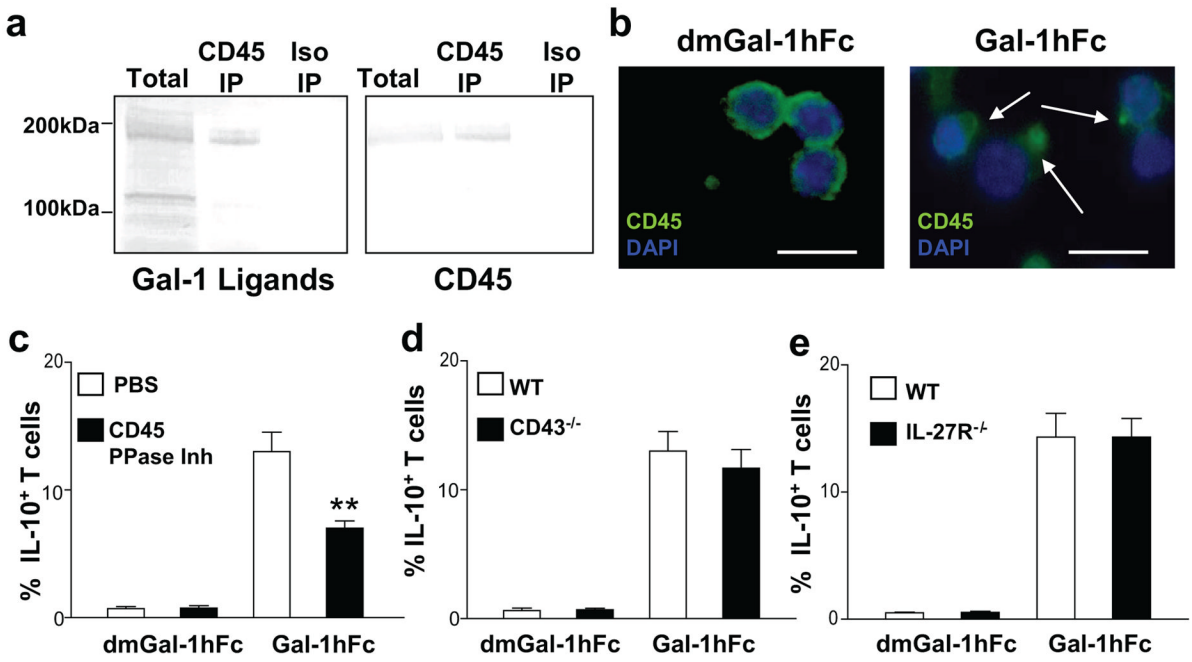

Figure 1. Gal-1hFc is a stable dimer that recognizes CD45 on activated Th cells and facilitates IL-10 expression

(a) Total lysate from unpolarized activated Th cells or anti-CD45/isotype control immunoprecipitates were Western blotted with Gal-1hFc (left panel) or anti-CD45 mAb (right panel). Representative blots of three independent experiments. **(b)** Unpolarized activated Th cells were treated with dmGal-1hFc or Gal-1hFc for 30min, and cytospins were analyzed for CD45 expression (green) by immunofluorescence. Scale bars=20μM. Arrows indicate formation of microdomains. **(c)** Unpolarized activated Th cells were incubated with Gal-1hFc or control dmGal-1hFc for 48h in the presence of CD45 phosphatase (PPase) inhibitor or PBS control, followed by flow cytometry analysis of intracellular IL-10. Graphical representation of data from 3 independent experiments is shown as mean± SD % IL-10^+ Th cells. Statistically significant difference compared with control-treated Th cells (**p<0.001). **(d)** Sorted naïve Th cells from wt or $CD43^{-/-}$mice were activated *ex vivo* with anti-CD3/CD28 mAbs for 48h and incubated in the presence of Gal-1hFc or dmGal-1hFc for additional 24h. Expression of IL-10 and expression of surface Gal-1 ligands were evaluated by flow cytometry. Graphical representation of data from 3 different experiments is shown as mean± SD % IL-10^+ T cells. **(e)** Sorted naïve Th cells from wt or IL-$27R^{-/-}$mice were activated *ex vivo* with anti-CD3/CD28 mAbs for 48h and incubated in the presence of Gal-1hFc or dmGal-1hFc for additional 24h. Expression of IL-10 and expression of surface Gal-1 ligands were evaluated by flow cytometry. Graphical representation of data from 3 different experiments is shown as mean± SD % IL-10^+ T cells.

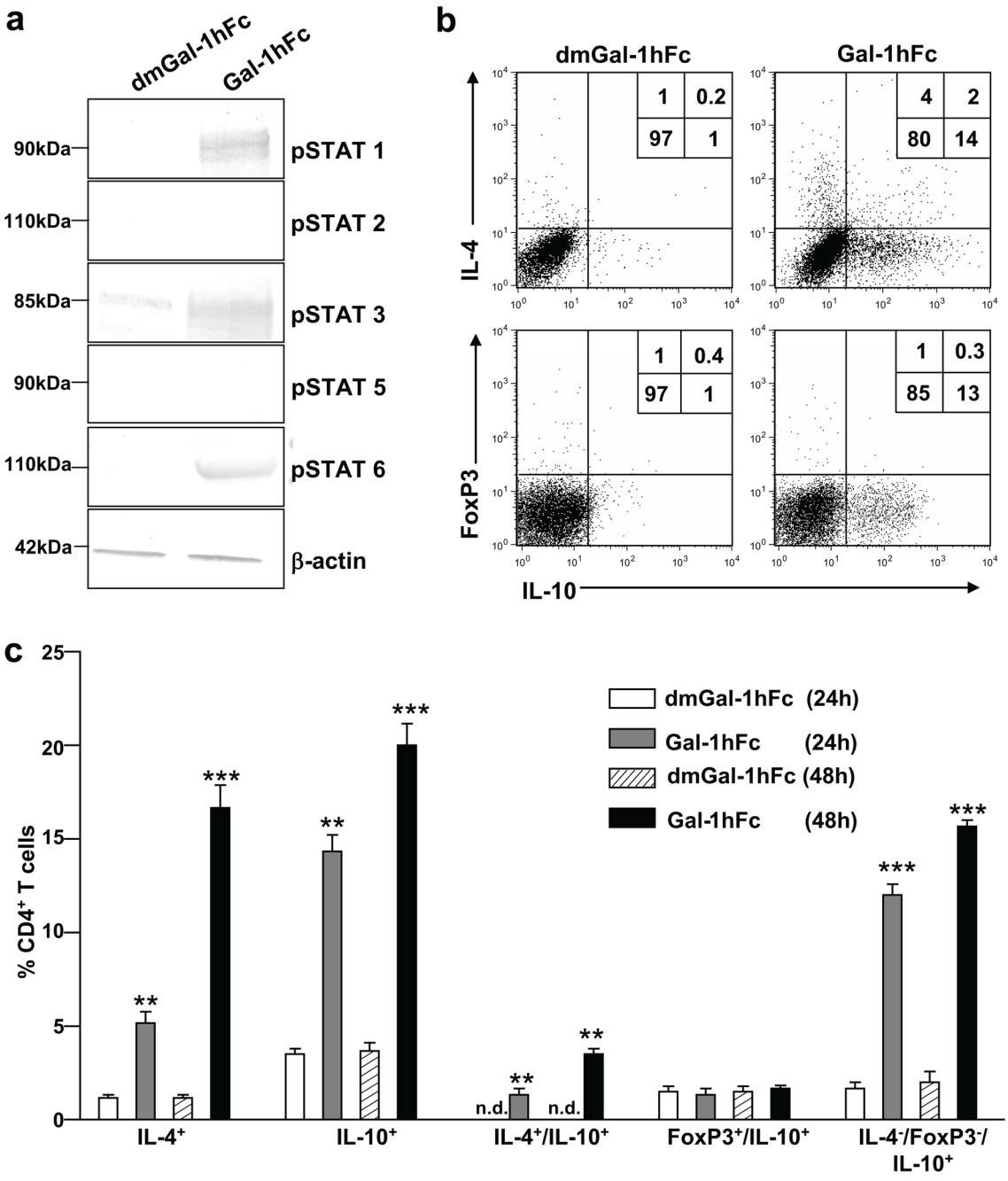

Figure 2. Gal-1 incubation triggers increased phosphorylation of STAT1, STAT3, and STAT6 and elevated IL-4 and IL-10 synthesis in uncommitted Th cells

(a) Lysates of activated Th cells treated with 0.25μM Gal-1hFc or dmGal-1hFc for 2h were Western blotted with anti-STAT1, 2, 3, 5 or 6 mAbs or anti-β-actin mAb. Representative blot of 3 independent experiments is shown. **(b)** Sorted naïve Th cells were activated *ex vivo* with anti-CD3/CD28 mAbs for 48h and incubated in the presence of Gal-1hFc or dmGal-1hFc for additional 24h–48h. Expression of IL-4, FoxP3 and IL-10 was evaluated by flow cytometry. Representative FACS plots after 24h Gal-1hFc or control incubations are shown. **(c)** Graphical representation of data from 3 different experiments is shown as mean±

SD % $CD4^+$ T cells. Statistically significant difference compared with dmGal-1hFc-treated Th cells (*p<0.01, **p<0.001, *** p<0.0001).

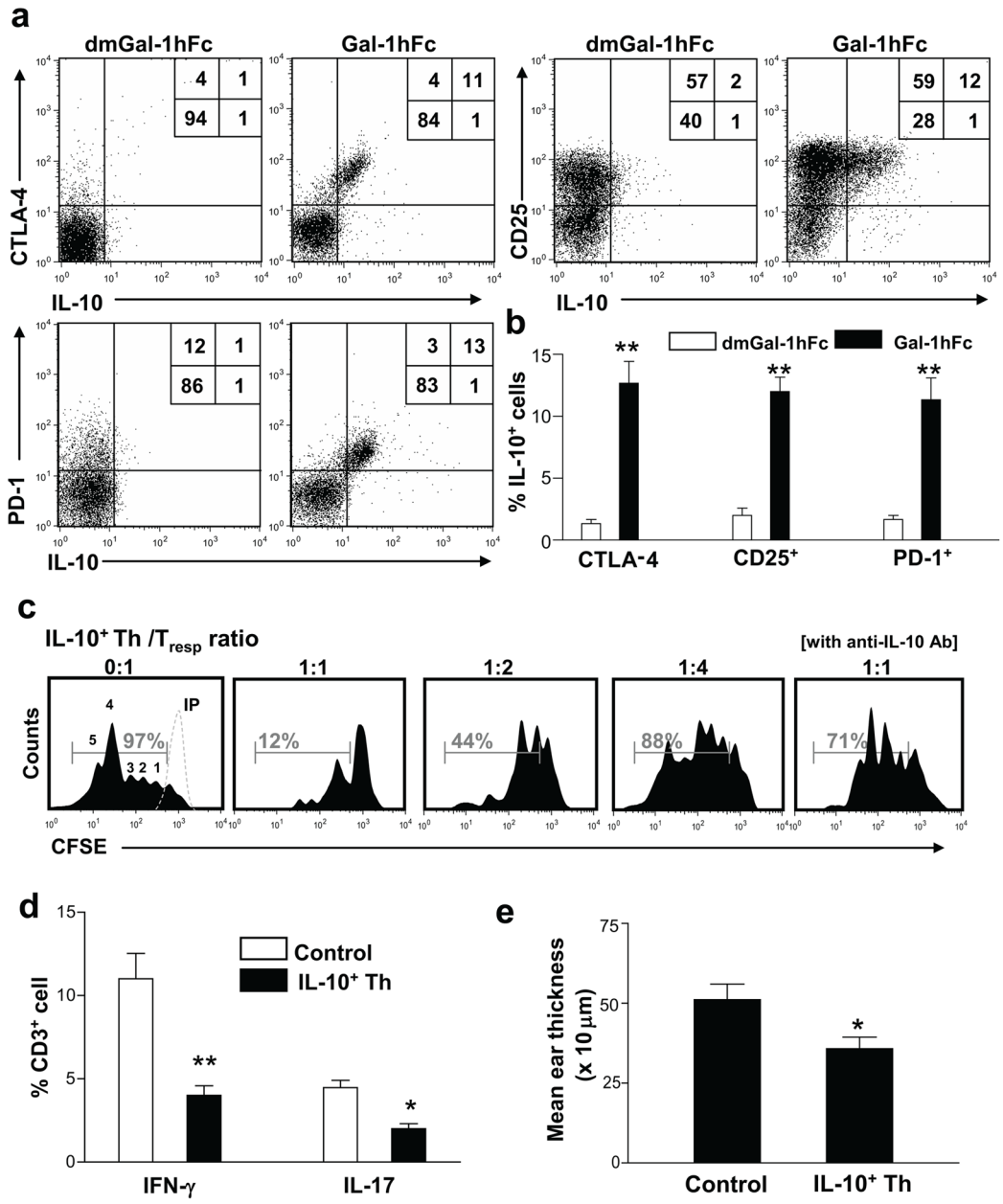

Figure 3. Gal-1 incubation creates CD4$^+$ IL-10$^+$ FoxP3$^-$ Th cells decorated by CTLA-4 and PD-1 that suppress T cell proliferation through IL-10

(a) Sorted naïve Th cells were activated *ex vivo* with anti-CD3/CD28 mAbs for 48h, rested for 24h, and incubated in the presence of Gal-1hFc or dmGal-1hFc for additional 24h. Expression of IL-10, CTLA-4, CD25 and PD-1 was evaluated by flow cytometry. Representative FACS plots are shown. **(b)** Graphical representation of data from 3 independent experiments is shown as mean± SD % IL-10$^+$ T cells. Statistically significant difference compared with dmGal-1hFc-treated Th cells (**p<0.001). **(c)** Gal-1-induced IL-10$^+$ Th cells were flow sorted and cultured at the indicated ratios with CFSE- labeled naïve CD4 cells (T_{resp}), under the stimulus of anti-CD3 (1μg/ml) /CD28 (0.5μg/ml) mAbs. T_{resp} proliferation was determined by CFSE dilution on day 6. Representative histograms of 3 independent experiments are shown. **(d)** Cytokine profile analysis of CD3$^+$ cells in skin-

draining lymph nodes harvested 6 days post-oxazolone sensitization in mice injected with $1X10^6$ IL-10^- or IL-10^+ Thcells. Graphical representation of data is shown as mean± SD % $CD3^+$ cells. Statistically significant difference compared to control-treated mice (**p<0.001, *p<0.01). **(e)** Ears from oxazolone sensitized mice treated with Gal-1-induced IL-10^+ Th cells or control T cell transfer (n=8), were challenged with 0.5% oxazolone or vehicle on day 5 post-sensitization, and measured after 24h. Graphical representation of data is shown as mean± SD ear thickness (×10μm). Statistically significant difference compared with control-treated mice (*p=0.039).

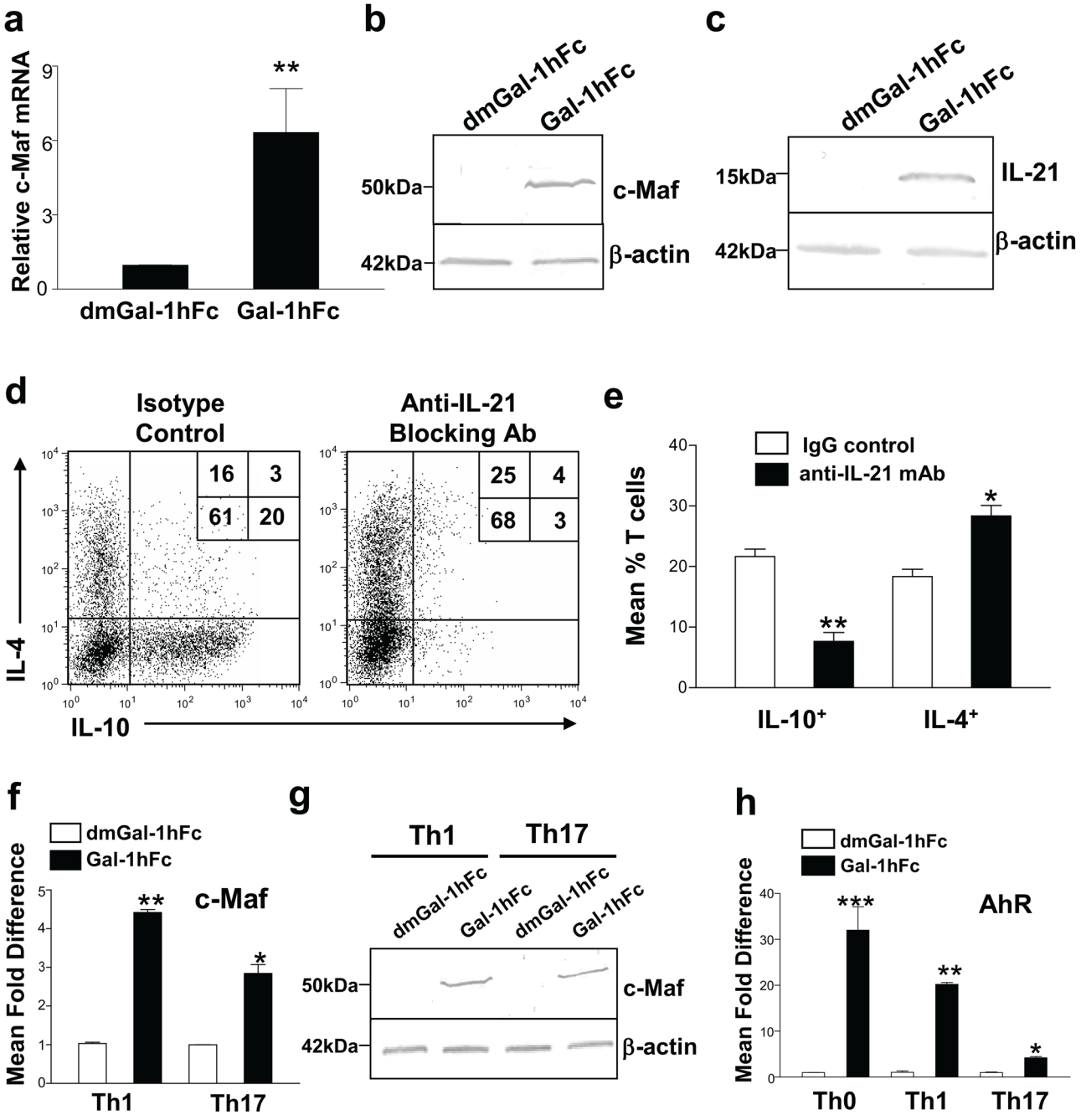

Figure 4. Gal-1 incubation induces c-Maf and the aryl hydrocarbon receptor (AhR) in Th cells concomitant with IL-21-dependent IL-10 expression

(a) *Ex vivo* unpolarized activated Th cells were treated with Gal-1hFc or dmGal-1hFc for 6h, and total RNA was extracted to evaluate differences in c-Maf expression by quantitative RT-PCR. Graphical representation of 3 experiments normalized to β-actin mRNA expression. Statistically significant difference compared with dmGal-1hFc control (**p<0.001). **(b)** Unpolarized activated Th cells were treated with Gal-1hFc or dmGal-1hFc for 24h and Western blotted with anti-c-Maf or -β-actin mAb. **(c)** Unpolarized activated Th cells were treated with Gal-1hFc or dmGal-1hFc for 24h, followed by PMA/ionomycin/brefeldin A stimulation for 5h, and Western blotted with anti-IL-21 or -β-actin mAb. **(d)** Unpolarized

activated Th cells were treated with 0.25μM Gal-1hFc for 48h in the presence or absence of 5μg/ml anti-IL-21 neutralizing mAb, and further analyzed for IL-4 and IL-10 expression by flow cytometry. **(e)** Graphical representation of data from 3 independent experiments is shown as mean± SD % T cells. Statistically significant difference compared with anti-IL-21 mAb neutralization (**p<0.001). *Ex vivo*-polarized Th1 and Th17 cells were treated with 0.25μM Gal-1hFc or dmGal-1hFc for 24h and c-Maf mRNA relative to β-actin levels was quantified by real-time RT-PCR **(f)** or by anti-c-Maf immunoblotting **(g). (h)** Unpolarized activated Th cells or *ex vivo*-polarized Th1 or Th17 cells were treated with 0.25μM Gal-1hFc or dmGal-1hFc for 24h and AhR mRNA levels relative to β-actin levels were assayed by real-time RT-PCR. Statistically significant difference compared with dmGal-1hFc-treated control (*p<0.01, **p<0.001, *** p<0.0001).

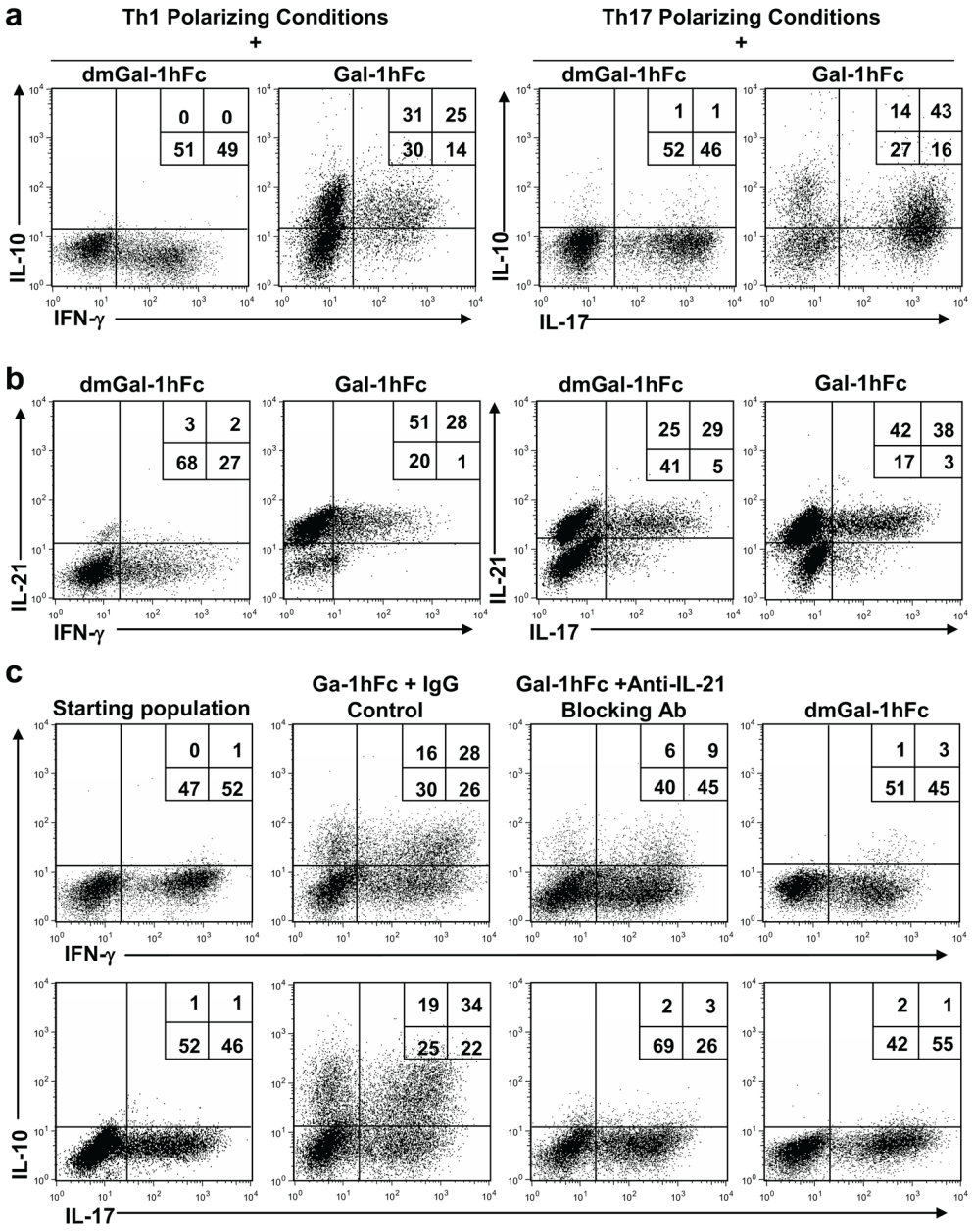

Figure 5. Gal-1 incubation up-regulates IL-10 in Th1 and Th17 cells in an IL-21-dependent manner

Naïve Th cells were activated for 72h under Th1 or Th17 polarizing conditions with either 0.25μM Gal-1hFc or dmGal-1hFc, and expression of IFN-γ, IL-17 and IL- 10 **(a)** or IL-21 **(b)** were assessed by flow cytometry. **(c)** *Ex vivo*-polarized Th1 and Th17 cells were subsequently treated for 48h in the presence of 0.25μM Gal-1hFc or dmGal-1hFc +/−5μg/ml anti-IL-21 neutralizing mAb. Expression of IFN-γ, IL-17 and IL-10 were assessed by flow cytometry. Representative plots of 3 independent experiments are shown.

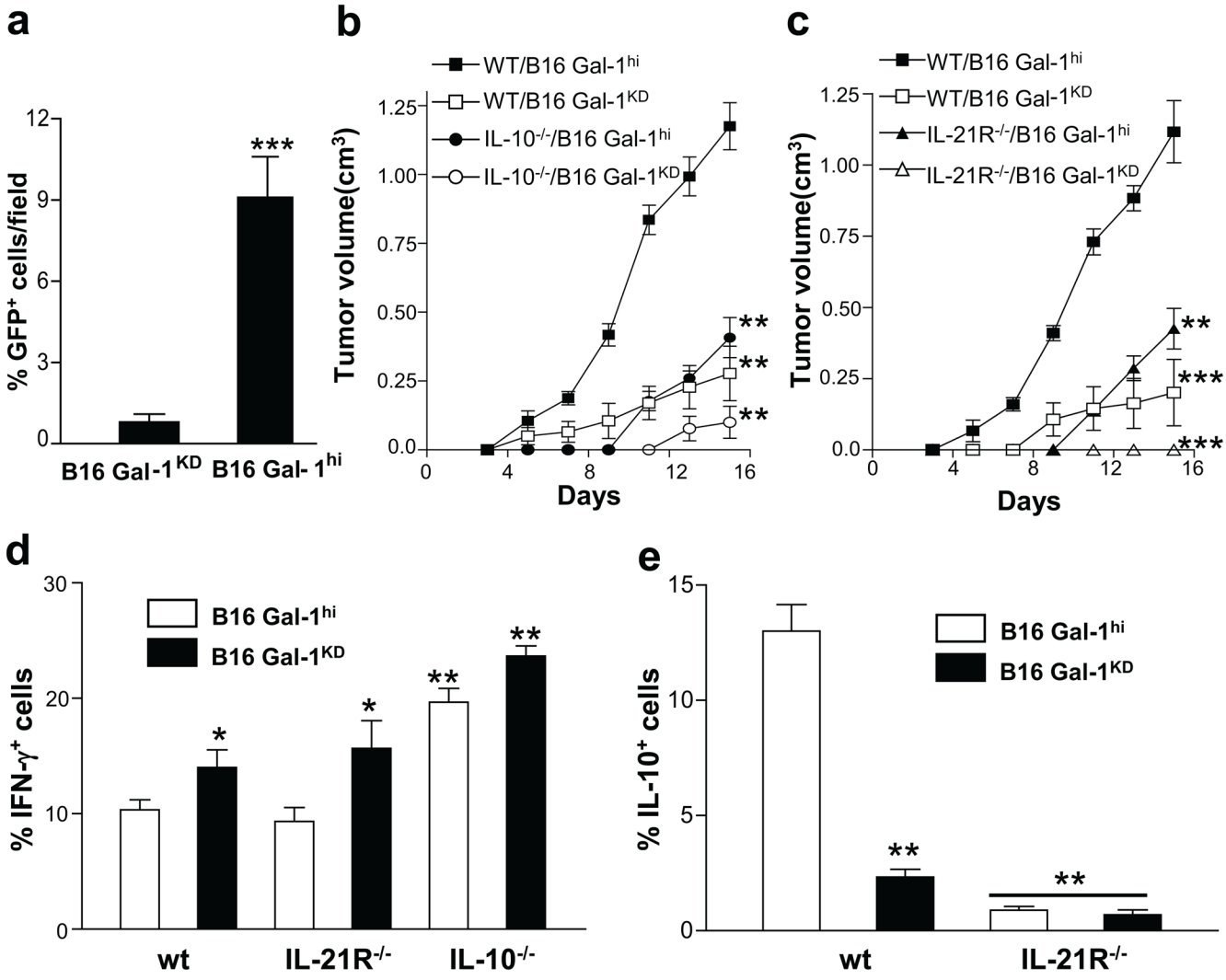

Figure 6. Tumor immune escape is favored by Gal-1-mediated expansion of IL-10$^+$ T cells in an IL-21-dependent manner

(a) IL-10-eGFP reporter mice were inoculated subcutaneously with 2×10^5 B16 Gal-1^{hi} or B16 Gal-1^{KD} cells, and on day 15 post-injection, tumors were harvested, and analyzed for GFP$^+$ tumor-infiltrating cells by immunohistochemistry. GFP$^+$ cells were counted in 50 individual/non-overlapping 20× fields from B16 Gal-1^{hi} or B16 Gal-1^{KD} tumors (n=4 tumors/group). Statistical significance compared with B16Gal-1^{KD} tumors (***p<0.0001). **(b)** Tumor growth in wt and IL-10$^{-/-}$mice inoculated with 2×10^5 B16 Gal-1^{hi} or B16 Gal-1^{KD} cells (n=10) was assayed. Statistically significant differences compared with B16 Gal-1^{hi} tumors in wt mice (**p<0.001). **(c)** Tumor growth in wt and IL-21R$^{-/-}$mice inoculated with 2×10^5 B16 Gal-1^{hi} or B16 Gal-1^{KD} cells (n=10) was assayed. Statistically significant differences compared with B16 Gal-1^{hi} tumors in wt mice (**p<0.001, ***p<0.0001). Tumor-draining lymph nodes from mice (wt, IL-10$^{-/-}$, IL-21R$^{-/-}$) bearing B16 Gal-1^{hi} or B16 Gal-1^{KD} tumors were harvested on day 15 post-inoculation, minced and washed, and CD3$^+$ T cells were analyzed for IFN-γ and IL-10 expression by flow cytometry (**d** and **e**, respectively). Graphical representation of data is shown as mean±SD % CD3$^+$ cells. Statistically significant difference compared with wt mice inoculated with B16Gal-1^{hi} melanoma cells (*p<0.01, **p<0.001).