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Title: Interleukin-6 signaling mediates Galectin-8 costimulatory activity of antigen-specific CD4 T cell response

Short Title: Galectin-8 induces IL-6 secretion and signaling

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Abbreviations

APC: antigen-presenting cell

BMDC: bone marrow-derived dendritic cell

DC: dendritic cell

FMDV: Foot-and-Mouth-Disease virus

Gal: galectin

IL-6: interleukin-6

IL-6R: interleukin-6 receptor

KO: knock out

OVA: ovoalbumin

Tfh: follicular helper T cell

WT: wild type

Abstract

Galectin-8 (Gal-8) is a mammalian lectin endowed with the ability to costimulate antigen-specific immune responses. We have previously demonstrated that bone marrow-derived dendritic cells produce high levels of IL-6 in response to Gal-8 stimulation. Since IL-6 is a pleiotropic cytokine that has a broad effect on cells of the immune system, in the present study we aimed to elucidate whether IL-6 was involved in Gal-8-dependent costimulatory signals during antigen recognition by specific CD4 T cells. To this aim, splenocytes from DO11.10 mice were incubated with a low dose of the cognate ovoalbumin peptide in combination with Gal-8. IL-6 was found significantly increased in cultures stimulated with

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Gal-8 alone or Gal-8 plus cognate peptide. Moreover, IL-6 signaling was triggered during Gal-8-induced costimulation as determined by phosphorylation of STAT3. IL-6 blockade by neutralizing monoclonal antibody precluded Gal-8 costimulatory activity but did not affect the antigen-specific TCR activation. Different subsets of dendritic cells, as well as macrophages and B cells, were identified as the cellular source of IL-6 during Gal-8-induced costimulation. To confirm that IL-6 mediated the Gal-8 costimulatory effect, antigen-presenting cells from IL-6-deficient or wild-type mice were co-cultured with purified CD4 T cells from OTII mice in the presence of cognate peptide and Gal-8. Notably, Gal-8-induced costimulation but not the antigen-specific response, was significantly impaired in the presence of IL-6-deficient antigen-presenting cells. Even more, exogenous IL-6 fully restored Gal-8-induced costimulation. Taken together, our results demonstrate that IL-6 signaling mediates the Gal-8 immune-stimulatory effect.

Introduction

Galectins (Gals) constitute a family of mammalian lectins characterized by the presence of conserved carbohydrate-recognition domains (CRDs) that bind to *N*-acetyl-lactosamine-containing glycans on target cells. Several studies positioned Gals as cue mediators of the immune response since they are implicated in many different processes such as tumor escape, autoimmunity disorders, tolerance induction and host defense [1, 2]. Our group previously demonstrated that Gal-8 has a predominant activating role in the elicitation of primary adaptive immune response [3-5]. We found that Gal-8 costimulate borderline antigen-specific T cell responses by synergizing the TCR-specific signaling in the presence of a low dose of the antigen. From a mechanistic view, Gal-8 strengthens TCR signaling through activation of the same pathways triggered by the antigen [4].

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Although CD4 T cells were identified as target cells of the Gal-8 costimulatory effect, the simultaneous presence of antigen-presenting cells (APCs) and the antigen is required to reach the Gal booster of the T cell activation [4]. This observation unveils a role for Gal-8 on the cooperative link between APCs and CD4 T cells during antigen presentation. In line with this, we have recently reported that Gal-8 induces full activation of mouse bone marrow-derived dendritic cells (BMDCs) and splenic dendritic cells (DCs), which may represent one of the mechanisms involved in the elicitation of the adaptive immune response previously observed. Indeed, Gal-8-stimulated DCs evidenced an increased expression of MHCII, CD80 and CD86 molecules, an augmented capacity to activate antigen-specific T cell responses, and a strong production of IL-6, among other inflammatory cytokines [5].

IL-6 is a pleiotropic cytokine that provides key early signals to shape innate immune response [6], CD4 and CD8 T cell effector responses, as well as memory formation [7-13]. In particular, IL-6 has been identified as a key driver of follicular helper T (Tfh) cell differentiation, which support high affinity antibody production by germinal center B cells[14-16]. At molecular level, IL-6 positively regulates cathepsin S expression in DCs, which leads to a more diverse repertoire of Tfh cells [17]. Recently, Brahmakshatriya *et al* [18] demonstrated that high levels of IL-6 directly delivered from TLR-pre-activated DCs are a central factor for generating optimal helper T cell responses that drives an effective humoral immunity in aged mice. In this regard, we have recently demonstrated that a single dose of inactivated Foot-and-Mouth-Disease virus (FMDV) formulated with soluble Gal-8 triggers a neutralizing humoral response that enhanced protection against homologous viral challenge. Remarkably, the Gal-8-induced anti-FMDV response was preceded by a peak of IL-6 and IFN-γ production at 48 h post-immunization, which returned to basal levels at day 5 after immunization, suggesting that Gal-8 circumscribed its effects to the very beginning of the immune response induction [5]. Considering that inactivated virus-pulsed DCs secrete IL-6,

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which is crucial for the rapid anti-FMDV antibody response [19], we postulated that Gal-8-induced DC-derived IL-6 could be involved in the stimulation of humoral response in the FMDV experimental model.

Despite the accumulating data regarding IL-6 production during the immune-stimulatory activity exerted by Gal-8, the actual involvement of IL-6 in the Gal-8-induced adaptive response still remains to be investigated.

Methods

Mice

C57BL/6J, C.Cg-Tg(DO11.10)10Dlo/J (DO11.10), B6.Cg-Tg(TcraTcrb)425Cbn/J (OTII) and B6.129S2-Il6^{tm1Kopf}/J (IL6KO) breeding pairs were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and bred in our facilities. The Ethical Committee Boards of the Universidad Nacional de San Martín and CIBICI-CONICET approved all procedures involving animals.

Galectins

Recombinant Gal-8 was obtained as described previously [5]. Briefly, Gal-8L mouse isoform (GenBankEF524570) was expressed in *Escherichia coli* BL-21 and purified by Lactosyl-Sepharose (Sigma-Aldrich, St. Louis, MO, USA) followed by immobilized metal affinity chromatography. Lectin activity was tested by hemagglutination as reported elsewhere [20].

Splenocytes and CD4$^+$ T cell purification or depletion

Mouse splenocytes were obtained as described previously [4]. For CD4$^+$ T cell purification, MojoSort Mouse CD4 Naïve Cell Untouched Isolation kit was used, following the manufacturer's instructions (Biolegend, San Diego, CA, USA). Cell purity (>90%) was

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checked by flow cytometry. For CD4 T cell depletion, MiniMacs columns and anti-CD4-coupled paramagnetic particles (Miltenyi Biotec, Auburn, CA, USA) were used, following the manufacturer's instructions. Depletion was confirmed by flow cytometry.

Costimulation assays

For costimulatory assays, splenocytes ($3x10^5$ cells) from DO11.10 mice were cultured for 48 h at 37˚C in 5% CO2 in flat-bottom, 96-well plates in 0.2 ml RPMI 1640 medium (Thermo Fisher Scientific, Waltham, MA, USA), in the presence of 10% FCS (Gibco, Thermo Fisher Scientific, Massachusetts, USA), 2 mM glutamine, and 5 mg/ml Gentamicin (complete medium), in the presence of the cognate $OVA_{323–339}$ peptide (Genscript, Piscataway, NJ, USA) at the indicated concentrations and 0.2 μM of recombinant Gal-8. For IL-6 neutralization, 2 μg/ml of IL-6 neutralizing monoclonal antibody (Clone: MP5-20F3) or matching isotype control (Biolegend) were used. Two different approaches were carried out to obtain IL6KO and C57BL/6J mice-derived APCs. For Mitomycin-APCs, splenocytes were pretreated with 200 mg/ml Mitomycin C (Sigma) in RPMI 1640 media for 1 h on ice, and cells were washed three times with ice cold PBS. For CD4 T cell-depleted APCs, CD4 T cells were depleted from splenocytes as described above. Complete blocking of cell division was checked by inhibition of Con A-activated proliferation. To assess proliferation, 1 μCi [^{3}H] methylthymidine (New England Nuclear, Newton, MA, USA) was added to each well 18 h before harvesting. Thiodigalactoside (Sigma) was added 30 min before the stimuli. Recombinant mouse IL-6 (Biolegend) was added together with the stimuli. Unstimulated cells' basal response ranged from 200 to 1000 cpm and was subtracted in all experiments. Assays were performed in quadruplicate.

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Cytokines quantification

IL-6 and IFN-γ levels were quantified in costimulation-assay supernatants by commercial ELISA (Biolegend) following manufacturer's instructions, using recombinant cytokines-standard curves.

Intracellular IL-6 expression

To analyze IL-6 intracellular expression, costimulation assays were performed as described before; and for the last 6 h, Monensin and Brefeldin A (Biolegend) were added to the cultures together with 1 μg/ml of LPS (Sigma). To label surface antigens, $4x10^6$ cells were incubated in 100 μl of cold PBS-azide plus anti-Fcγ receptor monoclonal antibody (CD16/32, Clone: 93) for 20 min at 4°C. Then anti-CD11c (Clone: N418), anti-CD11b (Clone: M1/70), anti-B220 (Clone: RA3-6B2), anti-F4/80 (Clone: BM8), anti-CD4 (Clone: GK1.5), or anti-MHCII (Clone: M5/114.15.2) monoclonal antibodies conjugated to different fluorochromes, were added at the recommended concentrations. After 30 min, cells were washed and fixed with Cytofix/Cytoperm Fixation/Permeabilization Solution (BD, Franklin Lakes, New Jersey, USA) for 20 min at 4°C. Then, IL-6 intracellular labeling was performed following the manufacturer's instructions, using anti-IL-6 (Clone: MP5-20F3). All monoclonal antibodies and their isotype controls were from Biolegend. IL-6-positive cells were determined by isotype and *fluorescence minus one* (FMO), negative controls.

STAT3 phosphorylation

For Western blot analysis, costimulation assays were performed for different time periods (2, 4 and 7 h). Then, cells were washed with ice-cold PBS containing 2 mM Sodium orthovanadate, and lysed in cracking buffer added with protease and phosphatase inhibitor cocktails from Sigma, followed by ultrasonication. Cell extracts were run in 10% SDS–

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PAGE and then transferred to nitrocellulose membranes (GE Healthcare Limited, Amersham Place, Little Chalfont, Buckinghamshire, UK). Blots were probed with anti-pSTAT3 antibody (Cell Signaling Technology, Beverly, MA, USA), followed by goat anti-rabbit IRDye800 CW secondary antibody (LiCor Biosciences, Lincoln, Nebraska, USA). Fluorescence emission was detected with an Odyssey clx infrared imaging system, and signal intensity was analyzed using Image Studio Lite software (LiCor Biosciences). Stripped blots were re-probed with anti-STAT3 antibody (Cell Signaling Technology) and revealed as for pSTAT3.

Flow cytometry analysis

FlowMax cytometer PASIII (Partec, Münster, Germany) and FlowJo software (FLOWJO, LLC) were used throughout this work.

Statistical

ANOVA test was used; except for the analysis shown in Figure 2, where Student's *t* test was used. *p* values <0.05 were considered significant.

Results and Discussion

When naïve splenocytes from DO11.10 mice are incubated with low doses of $OVA_{323-339}$ cognate peptide (OVA) in the presence of Gal-8, a synergistic CD4 T cell response defined as Gal-8-induced costimulation, is observed [3, 4, 21, 22]. In the present work, we used this established and well-characterized *in vitro* model, to assess whether IL-6 was involved in the elicitation of antigen-specific responses induced by Gal-8. First, we analyzed IL-6 secretion during Gal-8-induced costimulation. Thus, splenocytes from DO11.10 mice were cultured for 48 h in the presence of OVA alone, or in combination with Gal-8, and IL-6 levels

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were determined in the supernatants by ELISA. As observed in Figure 1A, IL-6 levels were significantly increased in those cultures stimulated with Gal-8 alone or in combination with the antigen. However, no differences were recorded between antigen-stimulated and control cells, indicating that in this limited-antigen condition, IL-6 secretion was triggered specifically in the presence of Gal-8. Remarkably, the amount of IL-6 was significantly higher in the supernatants from Gal-8 plus OVA compared to Gal-8-alone stimulated cells, indicating that cognate TCR activation potentiated IL-6 secretion induced by Gal-8. Furthermore, IL-6 levels mirror the synergistic effect of Gal-8 on TCR activation, evidenced by an augmented cell proliferation and IFN-γ secretion (Figure 1B), suggesting its involvement in the Gal-8 costimulatory effect. It should be stressed that IL-6 level is not a mere reflect of cell proliferation rate, since it was also increased in Gal-8-alone treated cells, where proliferation was similar to basal level. Conversely, in proliferating OVA-treated cells the amount of secreted-IL-6 remained scarce (Figure 1A and 1B). Pre-incubation with thiodigalactoside, a Gal inhibitor, prevented the IL-6 increment induced by Gal-8 alone or in combination with the antigen, thus highlighting both Gal-8 specificity and the dependence of lectin-glycan interaction at the cell surface. It is well known that pathogen-recognition or damage-associated molecular patterns activate specific danger receptors like TLRs, to stimulate a range of signaling pathways including NF-kB, which enhances IL-6 production [23-25]. In this regard, we have previously demonstrated that Gal-8 induces NF-kB activation and IL-6 secretion in human endothelial cell line HMEC-1 [26]. Thus, it is plausible to consider that Gal-8 could directly interact with TLRs to positively regulate IL-6 transcription. In line with this, Gal-3 was recently described as an endogenous ligand for TLR4 that promotes neuroinflammation [27]. Considering that TLR4 activation leads to Gal-8 secretion in endothelial cells, B cells and BMDCs [5, 26, 28], the presence of a positive feedback among TLR-signaling, Gal-8 and IL-6 that amplifies the inflammatory response, can be postulated.

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Next, we asked which cells were actually responsible for IL-6 production in response to Gal-8 costimulatory activity. For this purpose, splenocytes from DO11.10 mice were stimulated with Gal-8 plus OVA for 24 h and IL-6 intracellular levels were determined in different cell subpopulations by flow cytometry. As shown in Figure 2, $CD11b^-$ and $CD11b^+$ conventional DCs (cDCs) and plasmacytoid DCs (pDCs) as well as macrophages, B cells and CD4 T cells significantly increased IL-6 production in response to Gal-8 induced-costimulation. As expected, a large proportion of cDCs were induced to express IL-6 upon Gal-8 costimulation (about 23-44 %), being $CD11b^+$ cDCs the subpopulation that expressed higher levels of this cytokine (MFI >10). A lower frequency (between 9 to 18%) of pDCs, macrophages and B cells produced IL-6 in response to Gal-8 plus OVA, and among these subpopulations, pDCs produced the highest level of this cytokine (MFI =10). Cytokine production was almost marginal in both stimulated and unstimulated CD4 T cells. These findings are in agreement with our previous observations where both $MHCII^{int}CD11b^{high}$ and $MHC^{high}CD11b^{int}$ BMDCs subpopulations, each of which resembles cDCs and monocyte-derived macrophages respectively, produce IL-6 in response to Gal-8 stimulation. Moreover, IL-6 secretion was impaired in BMDCs differentiated from Gal-8 knock out (Gal-8KO) mice, suggesting a physiological role for endogenous Gal-8 in the regulation of IL-6 production [5]. Regarding B cells, and also in line with our findings, Tsai *et al* [28] have reported that despite both Gal-8 and Gal-1 play important roles in the generation of plasma cells, only Gal-8 induces IL-6 expression on B cells. Taken together, our results indicate that Gal-8 stimulates different splenic subpopulation of APCs to produce and secrete IL-6 during antigen-specific CD4 T cell costimulation.

To test our hypothesis by which IL-6 signaling is involved in Gal-8-dependent costimulatory effect during antigen recognition by specific CD4 T cells, we repeated the assay depicted in Figure 1B, but in the presence of an IL-6-neutralizing monoclonal antibody

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(anti-IL-6). Notably, IL-6 neutralization inhibited Gal-8-induced costimulation, being this reduction specifically dependent on IL-6 blockade since no significant differences were observed in the presence of the isotype control antibody (Figure 3A). It should be noted that lymphocyte proliferation in response to OVA was independent of IL-6, since no changes in T cell activation were recorded when anti-IL-6 was added to antigen alone-stimulated cells. This observation correlated with those results described in Figure 1A and 1B where OVA-stimulated cells proliferated and produced IFN-γ in the absence of IL-6. These results indicate that the costimulation induced by Gal-8, but not the antigen-response itself, depends on IL-6.

The multiple functions of IL-6 are initiated upon its binding to the IL-6 receptor (IL-6R) system, which comprises the IL-6Rα and the gp130 signal-transducing chain. Cytokine binding induces the homodimerization of IL-6R gp130 chains, which in turn, triggers the activation of downstream Janus-activated kinase (JAK)-STAT3 pathway [29]. Various sets of IL-6-responsive genes are induced by the activation of STAT3 [25]. Having demonstrated that IL-6 is increased during Gal-8-induced costimulation of CD4 T cell response (Figure 1A), we next asked whether IL-6 signaling was actually triggered in the presence of Gal-8. For this purpose, DO11.10 mouse splenocytes were stimulated with OVA, Gal-8 or OVA plus Gal-8 for different time-points and then the phosphorylation of STAT3 (pSTAT3) was determined by western-blot. As shown in Figure 3B, Gal-8 stimulation triggered STAT3 phosphorylation independently of the presence of the antigen after 2 h of treatment. Notably, a synergistic effect on the transcription factor activation was observed in the presence of both Gal-8 and OVA. Similar results were obtained after 4 h (data not shown) and 7 h of treatment (Figure 3B right). Gal-8-induced pSTAT3 was inhibited in the presence of the anti-IL-6, indicating a tight dependence on interleukin signaling. Conversely, antigen-induced TCR activation was independent of IL-6 signaling, since no differences in transcription factor phosphorylation

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were observed in the presence of anti-IL-6 or the isotype control. These observations are in agreement with the absence of IL-6 secretion and signaling in OVA alone-treated cells (results depicted in Figure 1A and 3A, respectively). Until here, our results strongly suggest that Gal-8 induces APCs to produce and secrete IL-6, which in turns signals through IL-6R in antigen-specific CD4 T cells to promote Gal-8 costimulatory activity.

To further confirm our hypothesis, in which APC-derived IL-6 is responsible for the costimulatory activity, antigen presentation assays were performed using either Mitomycin-C-treated or CD4 T cell-depleted splenocytes from IL-6 knock out (IL-6KO) or wild type (WT) mice as APCs source, co-cultured with purified CD4 T cells from OTII mice. As observed in Figure 3C (upper panels), Gal-8-induced costimulation was significantly impaired when Mitomycin-APCs were unable to produce IL-6, evidenced by cell proliferation (right) and IFN-γ secretion (left). Similar results were obtained when CD4 T cell-depleted splenocytes from IL-6KO mice were used as APCs (Figure 3C, lower panels). Once again, antigen-specific CD4 T cell stimulation was not affected in the absence of IL-6, since no differences in T cell proliferation were observed in OVA-alone stimulated cells in the presence of WT or IL-6-KO APCs. Additionally, this experimental design allowed demonstrating that antigen-specific CD4 T cells are target for IL-6-mediated effect upon Gal-8 stimulation. Finally, to confirm that the reduction in the Gal-8 costimulatory effect was dependent on IL-6 deficiency, the assay depicted in Figure 3C was performed with the exogenous addition of recombinant IL-6. Proliferation fold increase was calculated as the ratio: cpm (OVA + Gal-8)/cpm (OVA) of co-cultures using IL-6KO or WT APCs. As depicted in Figure 3D**,** exogenous IL-6 restored the proliferation rate of IL-6KO APC:CD4 T cell co-cultures, demonstrating that the presence of IL-6 was sufficient to rescue Gal-8-induced costimulation. Altogether, our findings demonstrate that Gal-8 stimulates APCs to

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produce IL-6, which signaling synergizes the TCR activation during antigen recognition on CD4 T cells, resulting in the Gal-8 costimulatory activity.

In summary, the *in vitro* model used throughout this work allowed us to evidence the participation of IL-6 signaling in Gal-8-induced costimulation during the elicitation of the antigen-specific CD4 T cell response. Since IL-6 is a key cytokine involved in the differentiation of Tfh cells and germinal center formation, our findings support a plausible mechanism by which Gal-8 stimulates the antigen-specific protective humoral response previously observed in a viral vaccine model [5]. Interestingly, aged CD4 T cells require higher levels of IL-6 produced during the CD4 T cell:APC cognate interaction compared to young CD4 T cells, to effectively induce IL-6 signaling, read out as IgG antibody production [10, 18]. Gal-8, by activating IL-6 production from APCs during cognate interaction with naïve CD4 T cells, could circumvent the insufficient response of aged cells, thus becoming an attractive candidate to add in vaccines formulation for the compromised populations.

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J.C., C.A.P., O.C. and M.V.T. conceived the project and designed experiments. J.C., C.A.P. and L.M.S performed the experiments. J.C., C.A.P., L.M.S., M.P.A., O.C. and M.V.T. analyzed data, discussed results and wrote the manuscript. O.C. and M.V.T. supervised work. J.C. and L.M.S. are fellows and M.P.A., O.C. and M.V.T are researchers from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET; Argentina); C.A.P is fellow from Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT, Argentina). This work was supported by ANPCyT; grant numbers PICT 2012-0216, PICT 2015-2587. Technical assistance in animal care, provided by Mr. Fabio Fraga, is highly appreciated.

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Conflict of Interest

Authors declare no conflict of interest.

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Figure Legends

Figure 1. Gal-8 induces IL-6 secretion during costimulation of antigen-specific CD4 T cell response A) Quantification of IL-6 by ELISA in supernatants from Gal-8-induced costimulation cultures. **B)** Quantification of IFNγ by ELISA in supernatants (left) and cell proliferation (right) of Gal-8-induced costimulation cultures. For all assays, splenocytes ($3x10^5$ cells) from DO11.10 mice were cultured for 48 h in the presence of 0.1 μg/ml of $OVA_{323–339}$ peptide (OVA), and/or 0.2 μM of Gal-8. Thiodigalactoside (TDG, 30 mM) was added 30 min before the stimulus. TDG had no effect on OVA response (not shown). ND, not detected. Depicted assays are representative of at least three independent experiments and were carried out, each time, with different recombinant protein preparations. $^{**}p<0.01$; $^{***}p<0.001$; $^{****}p<0.0001$.

Figure 2. APCs produce IL-6 during Gal-8-induced costimulation. IL-6 intracellular expression was determined by flow cytometry during Gal-8-induced costimulation. Splenocytes ($4x10^6$) from DO11.10 mice were cultured for 24 h in the presence of 0.1 μg/ml of $OVA_{323–339}$ peptide and 0.2 μM of Gal-8 (OVA + Gal-8), or left untreated (Control). Cells were labeled with specific monoclonal antibodies for surface antigens (MHCII, CD11b, CD11c, F4/80, CD4 and B220) and for intracellular IL-6. IL-6^+ population is box enfolded in the counter plots. Bars indicate percentage of IL-6^+ cells and the mean fluorescence intensity (MFI). FSC, forward side scatter. Depicted assays are representative of two independent experiments and were carried out, each time, with different recombinant protein preparations. $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

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Figure 3. Gal-8-induced CD4 T cell costimulation is dependent on IL-6. A) IL-6 neutralization. Splenocytes ($3x10^5$ cells) from DO11.10 mice were cultured in the presence of 0.1 μg/ml of $OVA_{323–339}$ peptide (OVA), and/or 0.2 μM of Gal-8, in the presence or absence of IL-6 neutralizing monoclonal antibody (anti-IL-6) or isotype control (Iso). After 48 h, proliferation was assessed. **B)** STAT3 activation. Splenocytes ($9x10^6$ cells) from DO11.10 mice were stimulated for 2 or 7 h with 0.1 μg/ml of OVA, and/or 0.2 μM of Gal-8, in the presence or absence of IL-6 neutralizing monoclonal antibody (anti-IL-6) or isotype control (Iso). For positive control, cells were incubated for 15 min with 100 ng/ml of recombinant IL-6 (rIL-6). Protein expression levels of pSTAT3 and total STAT3 were detected by western blot analysis. Bars depict pSTAT/STAT3 fluorescence signal ratio. **C)** Gal-8-induced costimulation in the presence of IL-6-deficient APCs. IL6KO and C57BL/6J mice-derived APCs ($2x10^5$): Mitomycin-APCs (upper panels) or CD4 depleted-APCs (lower panels), were co-cultured with CD4 T cells ($1X10^5$) purified from OTII mice, in the presence of 0.05-0.1 μg/ml of OVA, and/or 0.2 μM of Gal-8. After 48 h, cell proliferation (right), and quantification of IFNγ by ELISA in supernatants (left) were assessed. **D)** Same as **C)** with Mitomycin-APCs, and with addition of 5 ng/ml of rIL-6 to the co-cultures containing IL-6KO APCs. Fold increase was calculated as the cpm (OVA+Gal-8)/cpm (OVA) ratio. Depicted assays are representative of three (**A**) and two (**B**, **C** and **D**) independent experiments and were carried out, each time, with different recombinant protein preparations. *p<0.05; ***p<0.001.

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Figure 1

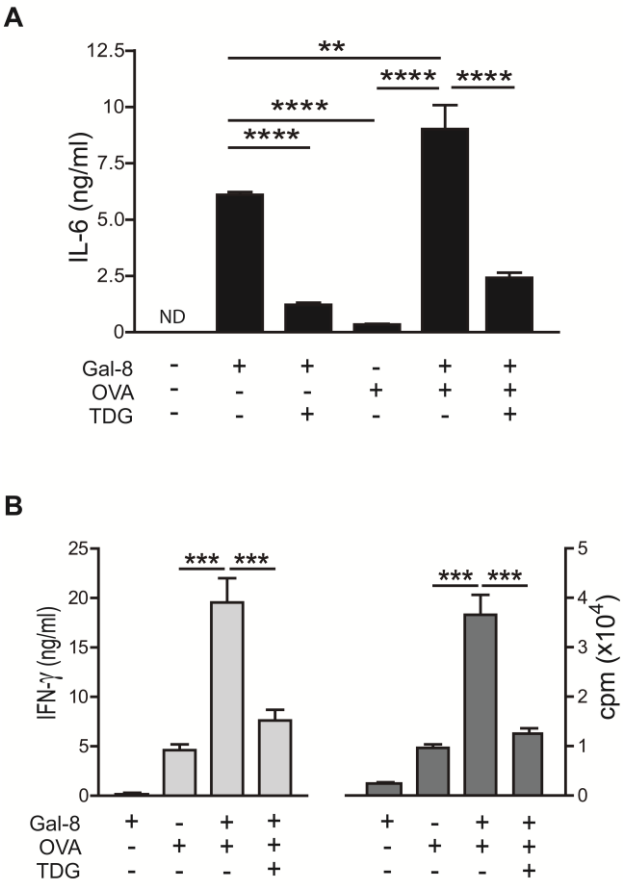

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Figure 2

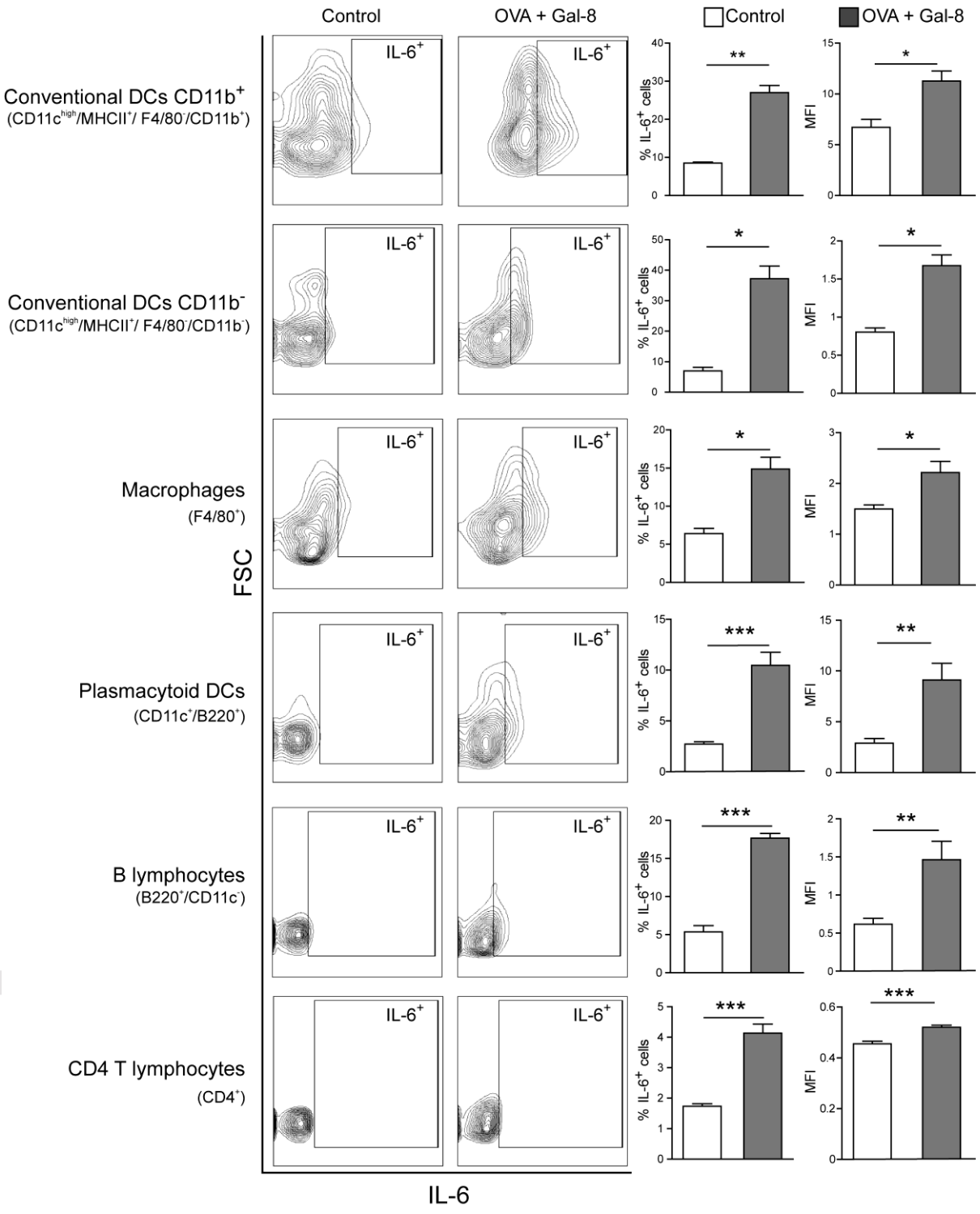

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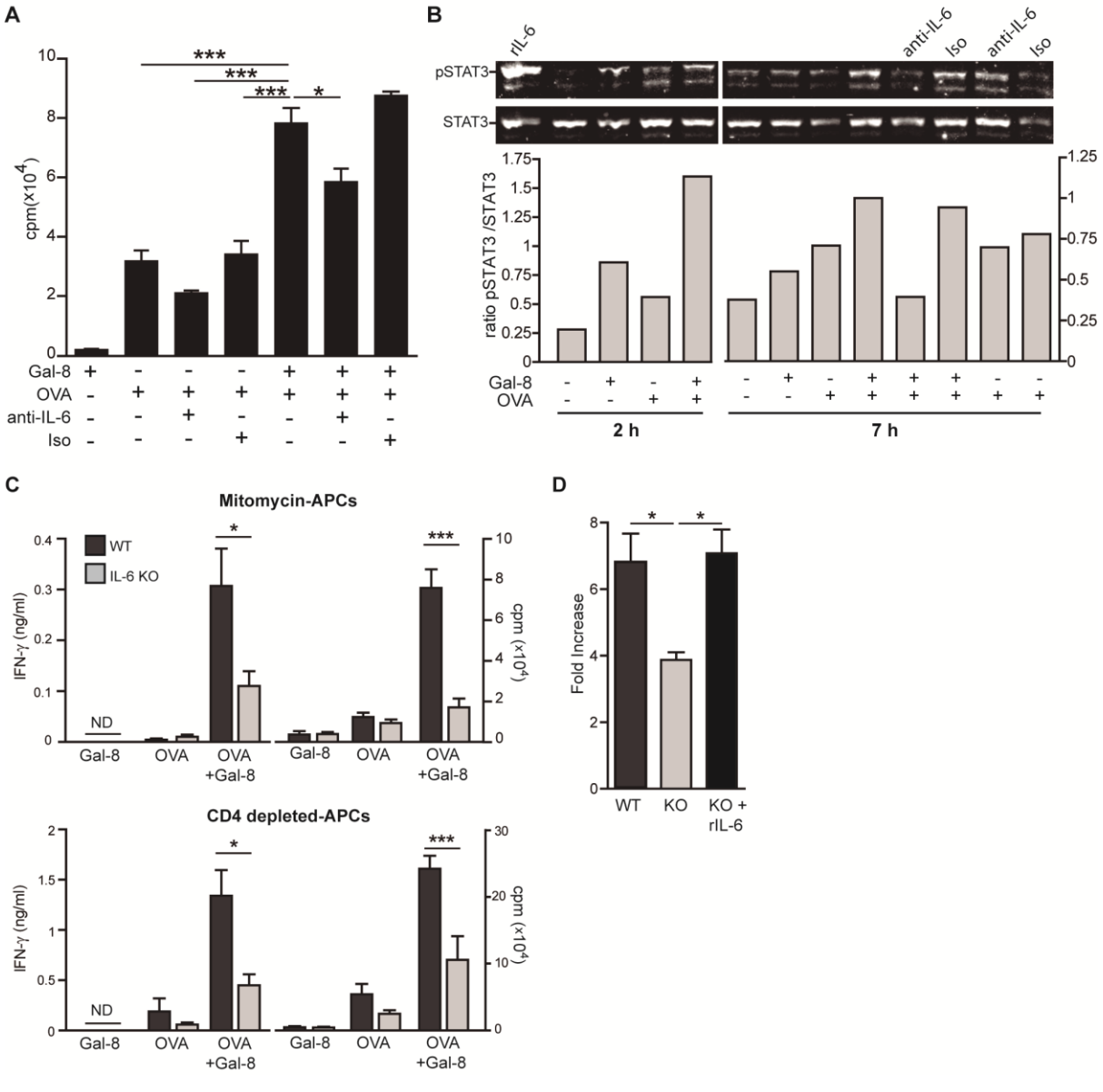
Figure 3



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