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Inducible costimulator (ICOS) blockade inhibits accumulation of polyfunctional T helper 1/T helper 17 cells and mitigates autoimmune arthritis

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Abstract

Objectives *Inducible costimulator (ICOS) and its ligand (ICOSL) regulate T and B cell responses. Glucose-6-phosphate isomerase (G6PI)-induced arthritis requires T and B lymphocytes. It was hypothesised that blocking ICOS/ICOSL interactions ameliorates G6PI-induced arthritis and reduces G6PI-specific B and T lymphocyte responses.*

Methods *DBA/1 mice were injected with a blocking, non-depleting anti-ICOSL monoclonal antibodies (mAbs) during the induction or effector phase of G6PI-induced arthritis. G6PI-specific antibody responses were measured by ELISA. G6PI-specific T helper (Th) cell responses were assayed by polychromatic flow cytometry.*

Results *Transient blockade of ICOS/ICOSL interactions profoundly reduced the severity of G6PI-induced arthritis. ELISA and proliferation assays showed no clear ex vivo correlates of protection. Polychromatic flow cytometry revealed two major findings: the absolute number of G6PI-specific Th cells was markedly diminished in secondary lymphatic organs from mice with blocked ICOS/ICOSL interactions. Within the pool of G6PI-specific Th cells the frequency of interleukin 17 (IL17), interferon γ or tumour necrosis factor α producers or polyfunctional Th cells (expressing two or more of these cytokines) was higher in treated than in control mice.*

Conclusions *ICOS costimulation is not mandatory for the differentiation of Th1 or Th17 cells. Instead, the lack of ICOS costimulation results in reduced survival of G6PI-specific Th cells irrespective of their functional differentiation. This study demonstrates that a thorough examination of the quantity and the quality of antigen-specific immune responses is useful to determine ex vivo correlates of efficacy for immunomodulating treatments.*

Introduction

Clinical and experimental data suggest that rheumatoid arthritis (RA) is an autoimmune disease and recent clinical trials indicate a role for T and B lymphocytes.1 2 In murine models of RA, immunisations with cartilage constituents or systemic alterations in the immune system can induce arthritis.

Immunisation of DBA/1 mice with the ubiquitous autoantigen glucose-6-phosphate isomerase (G6PI) or a self-peptide derived from the G6PI sequence induces symmetric polyarthritis with a rapid onset and a high incidence,3 4 Importantly, B cells and T cells are critically required for the disease, demonstrated by the inability to induce the disease in B cell-deficient mice and furthermore by the preventive and therapeutic effectiveness of CD4 cell depletion.3 5 Severity of G6PI-induced arthritis

can be inhibited by blockade of tumour necrosis factor α (TNFα),3 interleukin 17 (IL17)6 and injection of cytotoxic T lymphocyte antigen 4 (CTLA4) Ig.7

Interactions between inducible costimulator (ICOS) (CD278) and its ligand (ICOSL) (CD275) regulate adaptive immune responses. ICOS is expressed at low levels by resting T cells, strongly upregulated upon activation8 and highly expressed on T follicular helper (T_{FH}) cells within germinal centres.9 ICOSL is widely and constitutively expressed in lymphoid tissue and can be induced in non-lymphoid tissues under inflammatory conditions.10 11 Blockade of ICOS/ICOSL interactions using monoclonal antibodies (mAbs) against ICOSL has been shown to be beneficial in collagen-induced arthritis (CIA)12 13 and murine models of lupus.13 14 In experimental autoimmune encephalomyelitis (EAE), however, treatment during the induction phase exacerbated the disease, whereas later treatment blocked the disease15 and ICOS-deficient mice are more susceptible to EAE than wild-type mice.16 17 The unpredictable outcome of ICOS blockade in vivo together with the pathogenetic relevance of T helper (Th) cells and T/B interaction in G6PI-induced arthritis prompted us to investigate the role of ICOS/ICOSL interaction in G6PI-induced arthritis.

Materials and methods

Mice and induction of arthritis

DBA/1 mice (6–12 weeks old) were immunised subcutaneously at the base of the tail with 400 µg recombinant human G6PI,3 emulsified in 100 µl complete Freund's adjuvant (CFA; Sigma-Aldrich, Taufkirchen, Germany). Animals were scored for clinical signs of inflammation (redness, swelling) on a 0–3-point scale for each paw, giving a total maximum score of 12. Histopathological assessment was performed as described previously3 by a pathologist (LM) who was blinded with respect to the experimental groups. Mice were treated three times with 100 µg anti-ICOSL antibody (MIL-5733) at the indicated time points after immunisation. MIL-5733 is a non-depleting, blocking mAb (see supplementary figure 1 for details) generated by immunising rats with ICOSL transfectants as described.18

Anti-G6PI-Ig ELISA

Titres of G6PI-specific antibodies were measured by ELISA as previously described.3 The titre was defined as the last dilution that gave an optical density (OD) that was higher than mean+3SD than the background.

Proliferation assay

Single cell suspensions from draining lymph nodes were prepared at day 9 after immunisation and cultured with 10 µg/ml G6PI for 72 h or left unstimulated. [^{3}H]-thymidine (1 µCi) per well was added for the last 18 h. Incorporated radioactivity was measured using a γ scintillation counter (Perkin Elmer, Rodgau-Jügesheim, Germany).

Cytokine ELISA

Lymph node cells were cultured with 10 µg/ml G6PI for 48 h. Interferon γ (IFNγ) and IL17 content in culture supernatants was measured by sandwich ELISA using matched antibody pairs from BD or an IL17 DuoSet ELISA development Kit (R&D Systems, Wiesbaden, Germany).

Flow cytometry

Cells from draining lymph nodes (1×10^7 cells/ml) were cultured for 6 h with 20 µg/ml G6PI or left unstimulated. For the last 4 h, Brefeldin A (Sigma) at 5 µg/ml was added to all samples. Subsequently cells were washed and incubated with the fixable amine-reactive Aqua viability stain (Invitrogen) AU: please give address details for Invitrogen, eBiosciences and Miltenyi according to manufacturer's instructions. After fixation (2% paraformaldehyde) and permeabilisation with 0.5% Saponin/0.5% bovine serum albumin/0.02% NaN_3 in phosphate buffered saline, non-specific binding of antibodies was blocked by preincubation of the cells with anti-CD16/32 (2.4G2) and rat IgG, followed by staining with fluorochrome-conjugated mAbs against CD4, IFNγ, TNFα, IL17, IL10 (eBiosciences) and CD154 (Miltenyi Biotech). Early expression of CD154 is highly dependant on T cell receptor (TCR) stimulation and is used to identify Ag-specific Th cells.19,–,21 2.5 million events were acquired using a BD LSRII flow cytometer. Data were analysed using FlowJo 8.1.1 (Tree Star, Ashland, Oregon, USA). Gates for CD154 were set using unstimulated control samples and gates for cytokine$^+$ cells were set using fluorescence –1 controls for the respective cytokine.

Statistics

Data were analysed using the non-parametric Mann–Whitney U test with SPSS 15.0 (SPSS, Chicago, Illinois, USA) unless otherwise indicated. Graphs were generated using SigmaPlot 10.0 (Systat Software, Chicago, Illinois, USA). For analysis of boolean-gated cells, data were exported from FlowJo and analysed using PESTLE 1.5.4 and SPICE 4.1.6 software suites (kindly provided by Dr M Roederer, Vaccine Research Center, NIAID/NIH, Bethesda, Maryland, USA). Data are presented as means±SEM unless otherwise indicated.

Results

Blockade of ICOS/ICOSL interactions ameliorates G6PI-induced arthritis

G6PI-immunised DBA/1 mice were left untreated or injected with 100 µg anti-ICOSL mAb (MIL-5733, a blocking and non-depleting mAb; see supplementary figure 1 for details) either at days 2, 4 and 6 or at days 13, 15 and 17 after immunisation. Mice treated early developed profoundly less severe arthritis as judged by clinical and histological parameters (figure 1A–C). Treatment at the peak of clinical disease (days 13, 15 and 17) resulted in only marginally reduced clinical arthritis severity (figure 1A) suggesting that ICOS/ICOSL interactions are critical for the induction but less important for the perpetuation of G6PI-induced arthritis.

Blockade of ICOS/ICOSL interactions does not alter G6PI-specific Ig production

B lymphocytes and FcγR$^+$ effector cells are central elements in the pathogenesis of G6PI-induced arthritis.3 5 Since ICOS/ICOSL interactions are crucial for germinal centre formation, antibody production and isotype switching, we investigated whether the preventive effect of the anti-ICOSL mAb treatment was due to a reduced production of anti-G6PI antibodies. ELISAs for G6PI-specific IgM, IgG1, IgG2a, IgG2b and IgA did not reveal statistically significant differences between treated and control mice 9 days after immunisation (figure 2A) or at later time points (data not shown). Therefore, the preventive effect of short-term ICOS blockade is not mediated *via* inhibition or alteration of anti-G6PI antibody production.

Impaired proliferation but unaltered cytokine production in lymph node cells from anti-ICOSL-treated mice

Next, we investigated if blockade of ICOS costimulation influences the G6PI-specific T cell response. At 9 days after G6PI immunisation total lymph node cellularity and T cell numbers were similar in anti-ICOSL mAb-treated and control mice (figure 2B and data not shown). Using bulk assays of G6PI-restimulated lymph node cells, we found that proliferation was reduced in anti-ICOSL mAb-treated mice (figure 2C). The concentrations of IFNγ and IL17 in culture supernatants of these restimulated lymph node cells however were only slightly lower in treated mice as compared with controls (figure 2D). Taken together, these analyses employing whole organ cultures failed to reveal a clear cut ex vivo correlate for the clinical effects of ICOS blockade.

Reduced number but unimpaired Th1/Th17 differentiation of G6PI-specific Th cells in anti-ICOSL-treated mice

We next analysed the G6PI-specific Th cell response by polychromatic flow cytometry. Cells from draining lymph nodes were briefly restimulated with G6PI. G6PI-specific T cells were identified by staining for CD4 and CD154 (see supplementary figures 2 and 3A for details) and further analysed for cytokine production as described in the Materials and methods section. The total number of CD4 T cells was not reduced in the treated mice (data not shown). In contrast, the frequency of G6PI-specific T cells among all CD4 T cells was significantly reduced in mice treated with anti-ICOSL mAb (figure 3A; 0.52±0.07% vs 1.32±0.16% in control mice, mean±SEM, $p<0.008$). Therefore, the anti-ICOSL-treated mice harboured only approximately half the number of G6PI-specific Th cells than the control mice. To assess the functional differentiation of the G6PI-specific Th cells, we investigated their expression of IFNγ, TNFα, IL17 and IL10. The pattern of cytokine expression of G6PI-specific T cells was roughly similar between control and anti-ICOSL-treated mice with approximately 22% versus 26% TNFα producing, 19% versus 25% IL17 producing and 9% versus 13% IFNγ producing cells in the controls and the anti-ICOSL-treated group, respectively (figure 3B). IL10-producing G6PI-specific T cells were undetectable in both groups of mice (data not shown). Thus, although the number of G6PI-specific Th cells was strikingly reduced in anti-ICOSL-treated mice, differentiation towards Th1 or Th17 phenotypes was unimpaired or even slightly enhanced. In treated and control mice the majority of IFNγ-producing G6PI-specific Th cells also produced TNFα (figure 3B, left panel). Similarly, about half of the IL17 producing cells coproduced TNFα (figure 3B, middle panel). Remarkably, approximately 50% of the IFNγ producers coproduced IL17 (figure 3B, right panel).

For a more detailed analysis of Th cell cytokine coexpression, we used a boolean combination of gates22 to identify among the G6PI-specific CD4CD154 cells those that produced either one of the analysed cytokines alone, any possible combination of two cytokines, or all three cytokines. Thus, seven different possible subsets of cytokine-expressing cells can be identified (figure 3C and supplementary figure 3B). Due to their lower number of G6PI-specific Th cells (figure 3A), the treated mice also harboured fewer G6PI-specific cytokine producers than the controls (figure 3C). The overall pattern of cytokine coproduction differed little between the two groups of mice. Therefore, these data reveal that ICOS costimulation is not critically required for T cell differentiation into specific effector subsets, but rather regulates the magnitude of a T cell response.

Double producers of IFNγ and IL17 ($IFN\gamma^{+}IL17^{+}TNF\alpha^{-}$) were very infrequent in either group of mice. Boolean gating revealed that the vast majority of IFNγ and IL17 coexpressing cells were triple positive, producing IFNγ together with IL17 and TNFα ($IFN\gamma^{+}IL17^{+}TNF\alpha^{+}$, figure 3C). This could not have been detected from the two-dimensional dot plots shown in figure 3B.

ICOS costimulation is not essential for early proliferation of antigen-specific T cells

Having established that the blockade of ICOS costimulation strongly reduced the number of G6PI-specific T cells (figure 3A), but did not impair the functional differentiation of the G6PI-specific Th cells, we next aimed to investigate whether this reduced magnitude of the G6PI-specific Th cell response was mediated by decreased proliferation. Currently available techniques prohibit the tracking of G6PI-specific Th cells in vivo. Therefore, we adoptively transferred carboxyfluorescein succinimidyl ester (CFSE)-labelled ovalbumin $(OVA)_{323–339}$/$I\text{-}A^{b}$-specific T cells from TCR-transgenic OT-II mice into

C57BL/6 recipients, which were subsequently immunised with OVA and treated with anti-ICOSL mAb. As shown in figure 4A, 3 days after transfer there was no difference in the percentage of transgenic T cells among all CD4 T cells between anti-ICOSL-treated mice and controls, whereas by day 7 the number of transgenic T cells in treated mice was reduced by half. This reduction of antigen-specific T cells could not be attributed to arrested proliferation because at day 3 the frequency of OT-II cells in the CD4 compartment in every generation was similar in both groups of mice (figure 4B). By day 7, there was still a low but similar frequency of cells in the first five generations in both groups. In generations 6 and later, however, there were significantly fewer cells in the anti-ICOSL mAb-treated group, compared to controls. Our data using anti-ICOSL mAb therefore confirm and extend our previous results using ICOSL-deficient mice as recipients of TCR transgenic cells and thereby demonstrate that our treatment approach effectively blocked ICOS costimulation.8 Of note, since the OVA-specific T cells were identified by their expression of the transgenic TCR chains this also excludes the possibility that the reduced number of G6PI-specific T cells, detected by CD154 expression, was due to a defective CD154 upregulation in mice treated with anti-ICOSL.

We also examined the cytokine production of the transgenic T cells in this adoptive transfer system. As shown in figure 4C, there was no difference in the expression of IFNγ, TNFα and IL17 between both groups at day 3. At day 7, however, we found a higher proportion of cells producing either cytokine in the antigen-specific T cell compartment of the mice treated with anti-ICOSL mAb (figure 4C). This confirms our findings in the arthritis model that ICOS costimulation is not pivotal for the acquisition of particular T cell effector functions. The net effect of the greatly diminished numbers of antigen-specific T cells with similar or even slightly enhanced proportions of cytokine producing cells is a significant reduction of antigen-specific cells producing effector cytokines in the mice treated with the anti-ICOSL antibody.

Discussion

In this study, we show that a brief treatment with a blocking anti-ICOSL antibody profoundly reduces the severity of G6PI-induced arthritis. Protection from arthritis was associated with a sharply reduced number of G6PI-specific Th cells. Although surprising, given the large role of ICOS/ICOSL interactions in humoral immune responses, we did not find reduced serum concentrations of anti-G6PI IgM, IgG and IgA antibodies in anti-ICOSL-treated mice. However, our findings are in line with earlier studies, which demonstrated that the reduced production of IgG1 and IgG2a antibodies in ICOS-deficient mice could be overcome by using CFA as adjuvant.23 In studies on CIA, ICOSL blockade was associated with an ameliorated course of arthritis and a reduction in collagen type II (CII)-specific IgG1, IgG2a and IgG2b antibodies.12 13 One important difference between the CIA model and G6PI-induced arthritis investigated in our study is the much earlier onset of G6PI-induced arthritis. The reduced serum titres of anti-CII antibodies in the studies on ICOSL blockade in CIA12 13 were detected between day 28 and day 43 after the primary immunisation, that is, much later than in our investigation. Hence, it remains hypothetically possible that a reduction of G6PI-specific Igs in the treated mice would be detectable at later time points. However, approximately 30 days after immunisation G6PI-induced arthritis is usually resolved (figure 1 and Schubert *et al*3), further emphasising the pathogenetic differences between G6PI-induced arthritis and CIA. Consequentially, the mechanisms mediating the protective effects of ICOS blockade may differ between G6PI-induced arthritis and CIA.

Another distinguishing feature of G6PI-induced arthritis is that Th cells are obligatory in the induction and the effector phase of the disease. CD4 depletion cures established G6PI-induced arthritis in DBA/1 mice.3 Given this prominent role of Th cells in G6PI-induced arthritis, we used CD154 expression to identify G6PI-specific Th cells and examined their cytokine production directly. CD4 T cells rapidly and transiently upregulate expression of CD154 upon TCR stimulation.19 20 CD154 expression is therefore highly indicative of antigen-specific CD4 T cell stimulation during the 6 h of in vitro culture (supplementary figure 2 and references by Chattopadhyay *et al*, Frentsch *et al*, Mittrücker *et al*, Huaman *et al*, Gaucher *et al* and Tokoyoda *et al*19 20 24,–,27).

In mice treated with anti-ICOSL mAb, the most prominent effect on the pathogenic T cell response was the markedly reduced number of G6PI-specific T cells. This finding was not peculiar to the G6PI-induced arthritis model but was also confirmed for OVA-specific T cells in the OT-II TCR transgenic T

cell adoptive transfer model. Our data presented here, support our earlier work and strongly suggest that ICOS costimulation is not critical for early proliferation (ie, first five generations). Instead, the most likely explanation for the reduced number of G6PI-specific or OT-II cells in the absence of ICOS costimulation is reduced survival after unaffected priming and early proliferation. Thus, a reduced G6PI-specific T cell response due to decreased survival of these cells is the most likely mechanism for the clinical effect of the treatment on arthritis development and explains why our short course treatment had long lasting effects. Additionally, since the peak of the T cell response precedes the onset of clinical disease in G6PI-induced arthritis3 this explains why the late treatment with anti-ICOSL had very little effect on arthritis severity.

The published data as to whether ICOS specifically supports the differentiation of a particular Th subset is highly contradictory.16 17 28,–,30 Most reports were based on observations of reduced expression of signature cytokines after blockade of ICOS costimulation or in ICOS-deficient mice. Several groups, including ours, have already shown in earlier work that ICOS expression is not linked to the expression of particular cytokines.8 31 32 Bauquet *et al* recently reported that Th17 development was unimpaired in ICOS-deficient mice. However, after several days of in vitro culture in the presence of cytokines including IL23, the frequency of IL17-producing T_{FH} cells was lower in ICOS-deficient mice than in wild-type controls.17 Our data presented here demonstrate that ICOS costimulation is critically required for the maintenance of the antigen-specific Th cell pool in vivo but not mandatory for Th1/Th17 differentiation. Therefore, our data are compatible with the idea that earlier reports on reduced expression of Th1 or Th17 cytokines in the absence of ICOS costimulation reflect the lower absolute number of antigen-specific T cells rather than a diminished intrinsic propensity of these cells to differentiate into particular Th effector cell subsets. Importantly, these conclusions could be drawn only from complex simultaneous assessment of the number and cytokine production of antigen-specific T cells using polychromatic flow cytometry. Further illustrating this point is the fact that the reduced absolute number of G6PI-specific cytokine producing Th cells was not detected by cytokine measurements in culture supernatants by ELISA. A number of variables including cytokine production CD4 T cells which are not specific for G6PI (ie, bystander activation), cytokine production by cells which are not CD4 T cells and the consumption of cytokines released into the culture, could explain the discrepancy between the flow cytometric analyses of cytokine expression by G6PI-specific T cells and the cytokine ELISA.

A substantial proportion of the G6PI-specific Th cells coexpressed combinations of two or even three cytokines simultaneously and was thus polyfunctional. The frequency of such polyfunctional T cells rather than the total numbers of IFNγ producing cells has been associated with the ability to control or prevent chronic infections in a series of recent studies in mice and man.21 22 26 33,–,39 The relevance of polyfunctional T cells for protective or pathogenic T cell responses cannot be assessed directly with current technologies, which do not permit the isolation of distinct viable cell populations producing different combinations of cytokines. Still, our data suggest that such polyfunctional T cells might be also relevant in autoimmunity. Similar to the infection studies21 22 26 33,–,39 we also noted a higher per cell cytokine production of cells producing combinations of two or all three cytokines examined (data not shown). Consequently, a small reduction of such polyfunctional T cells could have an overproportional effect compared to reductions of cells that produce only one single cytokine. As exemplified in this report, less complex assessment of T cell functions harbours significant risks of underestimation or overestimation of alterations in the magnitude and quality of antigen-specific Th cell response. Therefore polychromatic flow cytometry could become one useful tool for the evaluation of therapeutic effects of immunological interventions in autoimmunity and transplantation.

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Figures

Figure 1 Anti-ICOSL treatment prevents G6PI-induced arthritis. A. DBA/1 mice were immunised with 400 µg G6PI in complete Freund's adjuvant and treated with 100 µg anti-ICOSL monoclonal antibody (mAb) MIL-5733 either at days 2, 4 and 6 (circles) or at days 13, 15 and 17 after immunisation (squares). Control mice (inverted triangles) were immunised but otherwise left untreated. Clinical arthritis severity was scored at the indicated time points on a 0–3 scale for each limb, giving a total maximum score of 12. Each group consists of at least five mice and the data shown are representative for two separate experiments. B. Severe synovitis in control mice (upper panel) with inflammatory infiltrates (black arrows), pannus formation with abundant activated fibroblasts (white arrows), severe destruction of bone and cartilage (stars) and extension of the inflammation into the adjacent skeletal muscle (circle). Normal joint architecture and absence of inflammation in mice treated with anti-ICOSL mAb at days 2, 4 and 6 (lower panel). The cartilage surfaces are smooth and intact (stars), the synovial lining is not enlarged (arrows) and no inflammatory cells can be detected. Representative photomicrographs of joint sections prepared 40 days after immunisation with 400 µg G6PI (H & E staining; original magnification ×50 (left) or ×200 (middle and right), respectively). C. Semiquantitative scoring of the histopathological changes in the affected paws showed significant reduction of histological arthritis severity by anti-ICOSL treatment at days 2, 4 and 6 (n=5, open bars) compared to control mice (n=5, filled bars, $p<0.05$). G6PI, glucose-6-phosphate isomerase; ICOS, inducible costimulator; ICOSL, ICOS ligand.

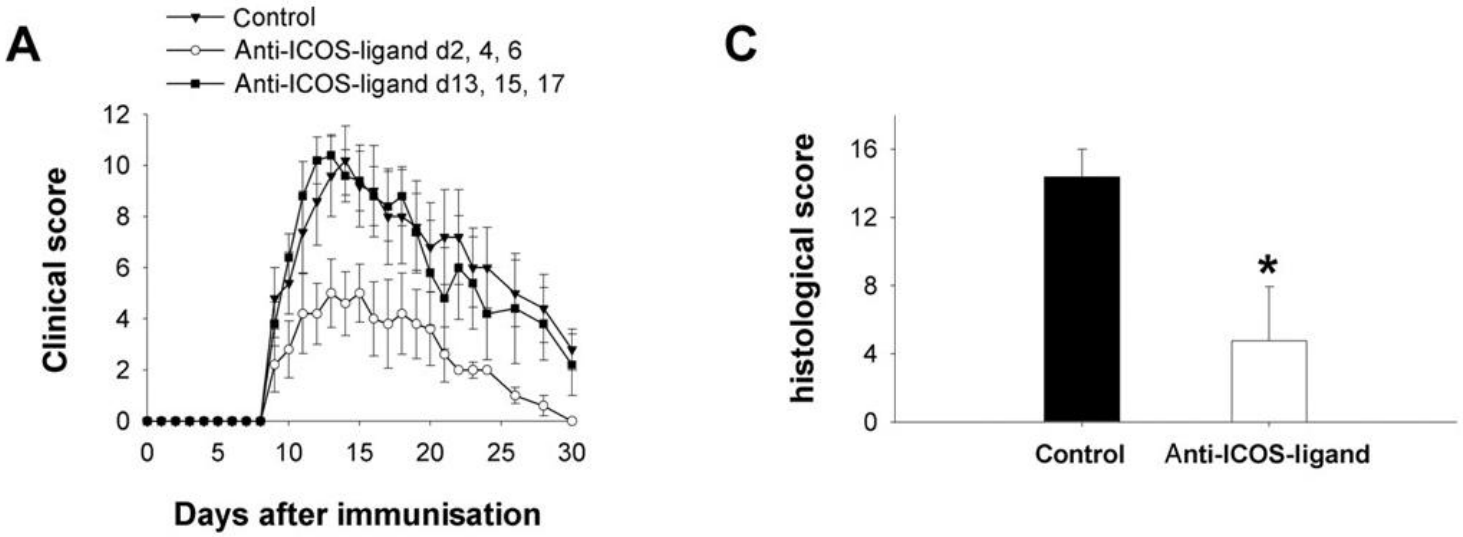


Figure 1 *(continued)*

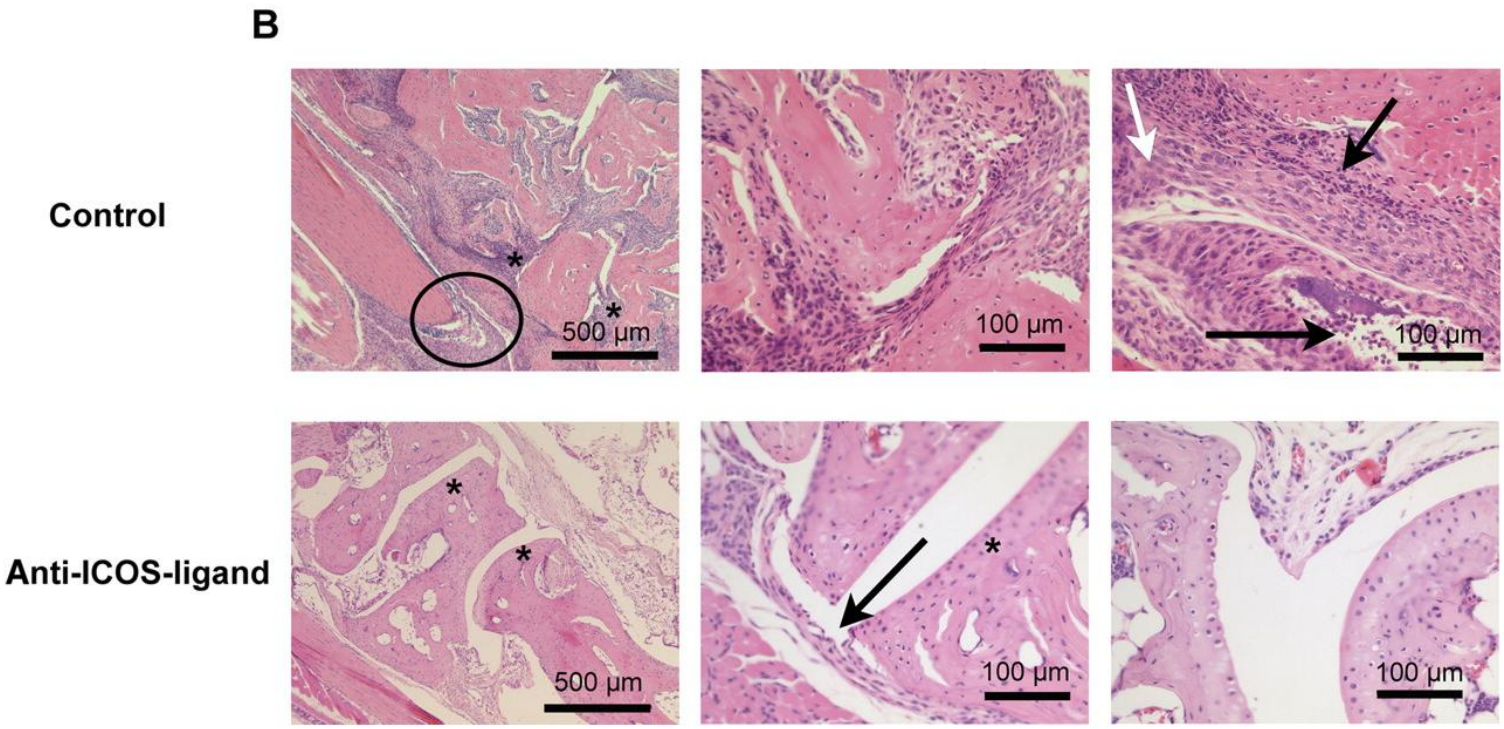


Figure 2 Effects of ICOS blockade on G6PI-specific immunoglobulin production, proliferation and cytokine production. A. DBA/1 mice were immunised with 400 µg G6PI and left untreated (filled bars) or were treated with 100 µg anti-ICOSL monoclonal antibody (mAb) at days 2, 4 and 6 after immunisation (open bars). Sera were obtained at day 9 after immunisation and titres of G6PI-specific immunoglobulins of the indicated isotypes were measured by ELISA. Data shown are from one representative experiment (n=five mice per group). B. Single cell suspensions were prepared from draining lymph nodes from anti-ICOSL mAb-treated mice (open bars) or controls (filled bars) at day 9 after immunisation. Lymph nodes from anti-ICOSL mAb-treated mice and controls yielded equal numbers of cells. C,D. Lymph node cells were restimulated in vitro with G6PI and proliferation was measured using [^{3}H]-thymidine incorporation for the last 16 h of a 72 h restimulation period. Culture supernatants were collected after 48 h and concentrations of interferon γ and interleukin 17 were determined using ELISA. Data are representative for two independent experiments with three to five mice per group. G6PI, glucose-6-phosphate isomerase; ICOS, inducible costimulator; ICOSL, ICOS ligand.

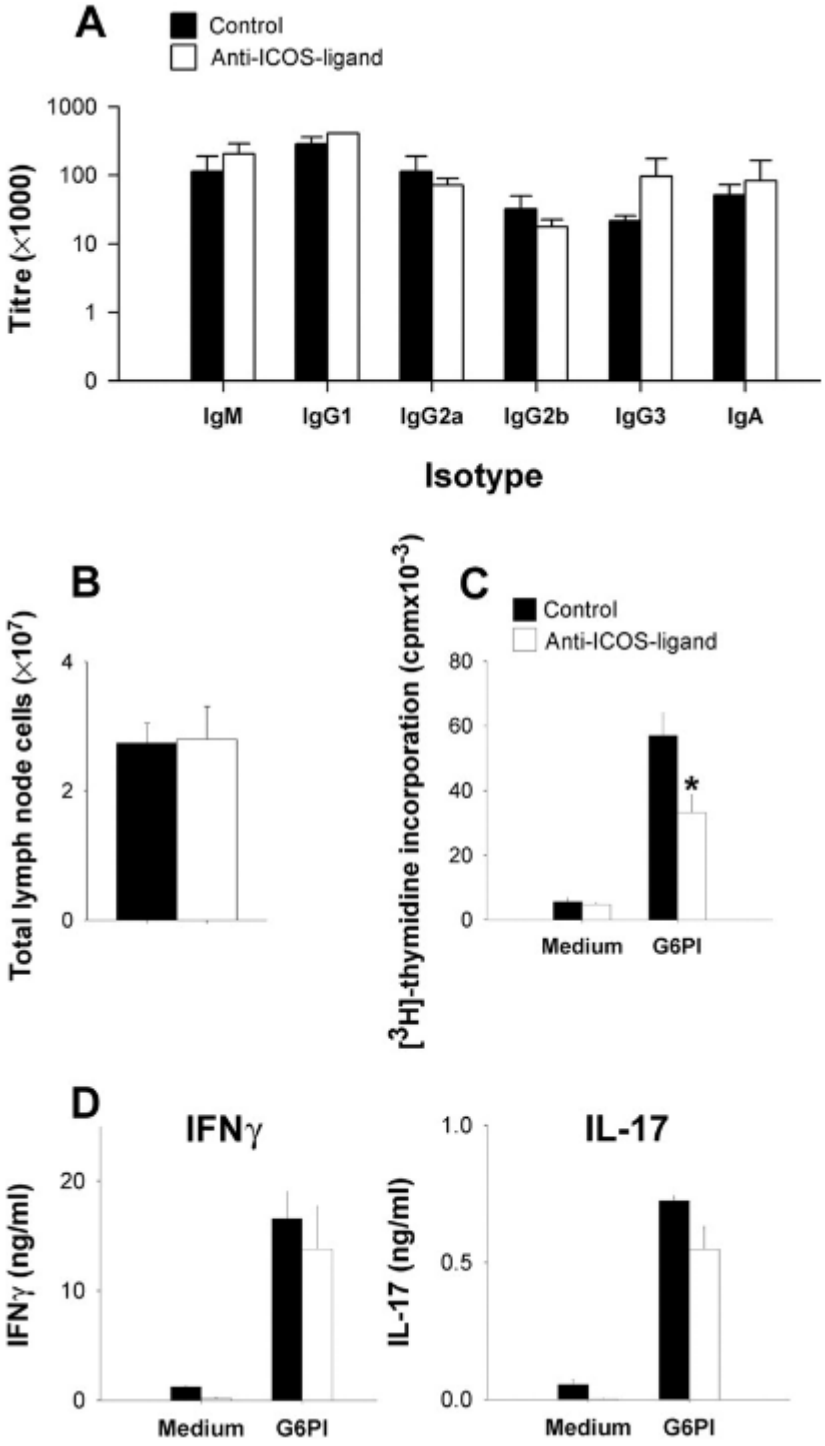
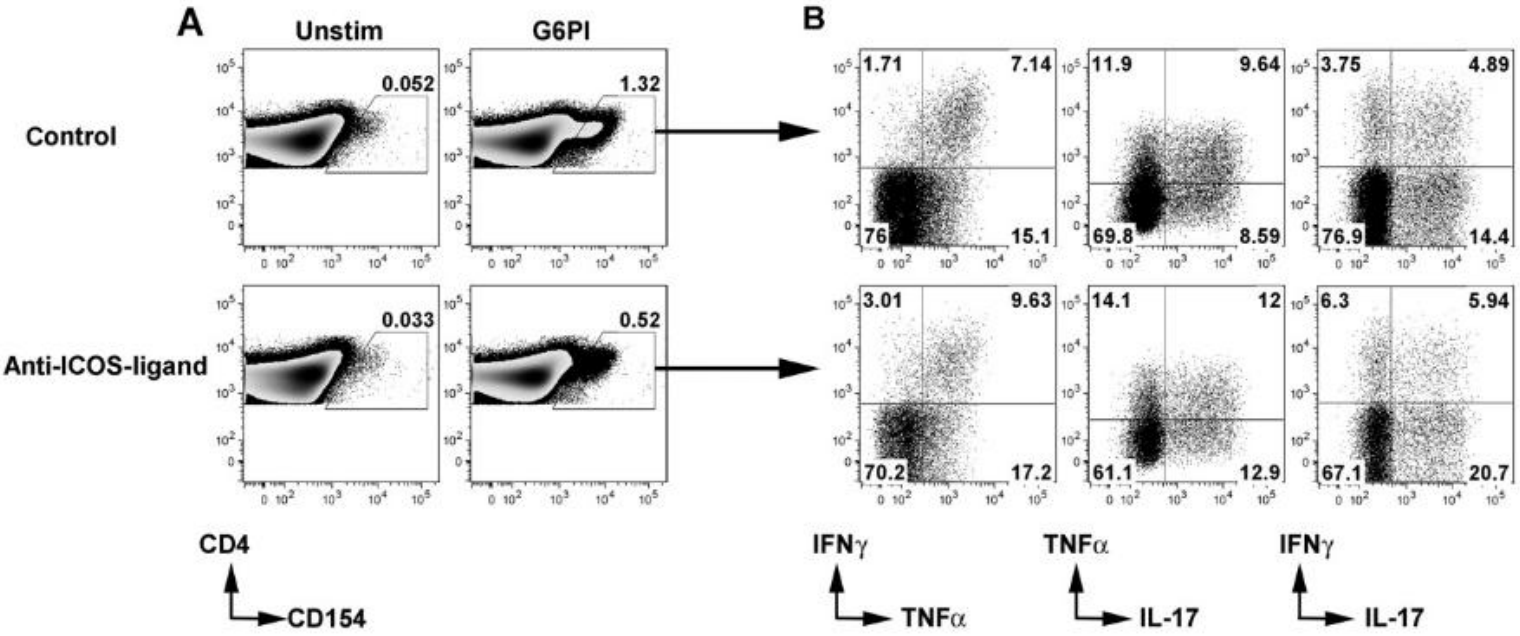


Figure 3 Multiparameter flow cytometry reveals differences in the number and differentiation of G6PI-specific T cells. A. Single cell suspensions were prepared from draining lymph nodes from anti-ICOSL monoclonal antibody (mAb)-treated mice or controls at day 9 after immunisation. Cells were cultured in vitro with G6PI for 6 h and stained for with mAbs against CD4, CD154 and cytokines as described in the Materials and methods section. Gates were set as exemplified in supplementary figure 2A. Upregulation of CD154 in G6PI-stimulated (right) vs unstimulated samples (left) in control (upper row) and anti-ICOSL-treated mice (lower row). Numbers indicate the frequencies of G6PI-specific CD154 T cells among all CD4 cells. B. Dot plots show the expression of IFNγ vs TNFα (left), IFNγ vs IL17 (middle) and TNFα vs IL17 in control (upper row) and anti-ICOSL-treated mice (lower row). Events in dot plots contain only G6PI-specific (CD154 cells; indicated by arrows). The frequency of cytokine expressing cells among CD154 cells is given in each quadrant. Data depicted in A and B are concatenated data files from five mice per group. C. G6PI-specific T cells producing one, a combination of two or all three cytokines examined were identified using boolean gating (supplementary figure 2B). Total numbers of G6PI-specific cytokine producing T cells per mouse in control (inverted triangles) and anti-ICOSL-treated mice (circles). Data in A–C are from five mice per group, *p<0.05, Wilcoxon rank sum test. G6PI, glucose-6-phosphate isomerase; ICOS, inducible costimulator; ICOSL, ICOS ligand; IFN, interferon; IL, interleukin; TNF, tumour necrosis factor.



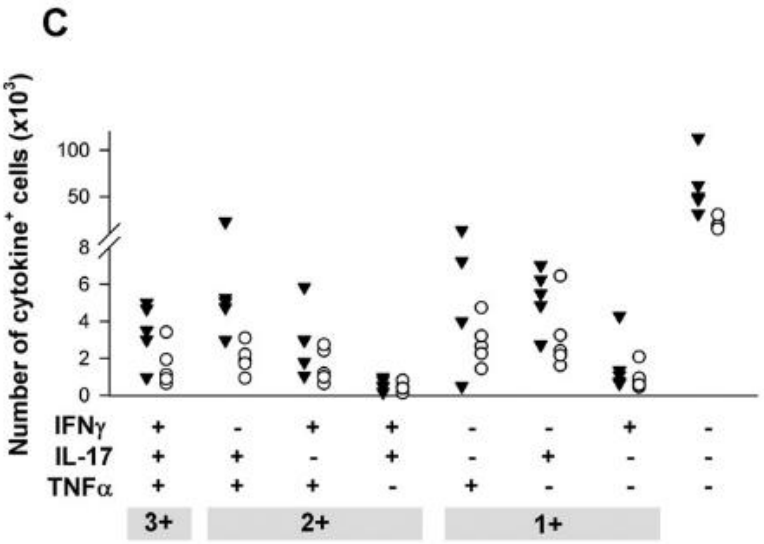


Figure 4 ICOS blockade has no impact on early proliferation and effector cell differentiation in an adoptive transfer model. A. Carboxyfluorescein succinimidyl ester (CFSE)-labelled CD4 T cells (2×10^6) from OT-II mice were adoptively transferred into C57BL/6 mice. Then, 1 day later, recipients were immunised with ovalbumin (OVA)323–339. Mice were treated with anti-ICOSL monoclonal antibody (mAb) at day 0, 2 and 4 relative to immunisation. Numbers of transferred transgenic T cells (TCR Vα2 and Vβ5.1/5.2 positive CD4 cells) among all CD4 T cells in draining lymph nodes 3 (left panel) and 7 (right panel) days after immunisation. B. Analysis of proliferation by CFSE dilution. Frequency of transgenic T cells among total CD4 cells in each division generation is shown for day 3 and day 7. (n=4 per group, *p<0.05). C. Production of interferon γ, tumour necrosis factor α and interleukin17 by TCR transgenic T cells adoptively transferred into anti-ICOSL mAb-treated or control mice at day 3 (upper panel) or day 7 (lower panel). ICOS, inducible costimulator; ICOSL, ICOS ligand, TCR, T cell receptor.