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IL-6 promotes CD4$^+$ T-cell and B-cell activation during *Plasmodium* infection.

Short Title: IL-6 promotes T & B-cell responses in malaria.

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ABSTRACT

Aims: Humoral immunity develops in the spleen during blood-stage *Plasmodium* infection. This elicits parasite-specific IgM and IgG, which control parasites and protect against malaria. Studies in mice have elucidated cells and molecules driving humoral immunity to *Plasmodium*, including $CD4^+$ T-cells, B-cells, interleukin (IL)-21 and ICOS. IL-6, a cytokine readily detected in *Plasmodium*-infected mice and humans, is recognized in other systems as a driver of humoral immunity. Here, we examined the effect of infection-induced IL-6 on humoral immunity to *Plasmodium*.

Methods and Results: Using *P. chabaudi chabaudi* AS (*Pc*AS) infection of wild-type and *IL-6*$^{-/-}$ mice, we found that IL-6 helped to control parasites during primary infection. IL-6 promoted early production of parasite-specific IgM but not IgG. Notably, splenic $CD138^+$ plasmablast development was more dependent on IL-6 than germinal centre (GC) B-cell differentiation. IL-6 also promoted ICOS expression by $CD4^+$ T-cells, as well as their localisation close to splenic B-cells, but was not required for early Tfh-cell development. Finally, IL-6 promoted parasite control, IgM and IgG production, GC B-cell development and ICOS expression by Tfh cells in a second model, *Py*17XNL infection.

Conclusions: IL-6 promotes $CD4^+$ T cell activation and B-cell responses during blood-stage *Plasmodium* infection, which encourages parasite-specific antibody production.

Keywords: Malaria, Cytokine, Humoral Immunity, Costimulatory Molecules, B lymphocyte, CD4 T lymphocyte, Animal model

INTRODUCTION

Plasmodium-specific antibodies can control blood-stage parasite numbers, both in humans and in experimental animals (1-4). In fact, antibody-mediated parasite control remains a primary mechanism by which natural infection or immunization confers partial immunity to blood-stage malaria (1, 3). Given that long-lived immunity to malaria is not easy to induce in humans, a better understanding of how *Plasmodium*-specific antibodies are generated *in vivo* may offer strategies for improving naturally-acquired and vaccine-mediated immunity.

To model the cellular and molecular processes that govern the onset of humoral immunity, many research laboratories have examined inbred mice infected with non-lethal, rodent-infective *Plasmodium* strains, such as *P. yoelii* 17XNL (*Py*17XNL) and *P. chabaudi chabaudi* AS (*Pc*AS) (2, 4-8). Detailed immune processes have been identified using these *in vivo* systems, which indicate that $CD4^+$ T cells and B-cells interact in the spleen to drive the production of parasite-specific antibodies. For example, during *Pc*AS infection, a rapid and transient blurring of T- and B-cell zones was reported during the first ten days of infection, which suggested that early T-cell and B-cell interactions occurred (9). Furthermore, a strong extra-follicular B-cell response was observed, where $CD138^+$ antibody-secreting plasmablasts were robustly generated (9). More recently, it was also shown in this model that $CD4^+$ T-cell-derived interleukin-21 (IL-21) played a crucial role in the development of the germinal centre (GC) reaction, in which somatic hypermutation of activated B-cells occurs, which drives the development of high-affinity, parasite-specific IgG antibodies (4). Interestingly, in this report there was little evidence of a role for IL-21 in promoting early humoral immune responses such as extra-follicular plasmablast development within the first week of infection, although a requirement for IL-21 for long-term humoral immunity was clear. Another recent study using *Pc*AS infection reported that Inducible T-cell Co-stimulatory molecule (ICOS) played an important role in the development of humoral immunity (10), a finding that we subsequently also observed during *Py*17XNL infection (6). Several recent studies, using a number of different *Plasmodium* strains in mice also identified molecules that suppressed the onset of humoral immunity, including PD1 and Lag3 (2), inflammatory cytokines IFNγ and TNF (5), and Type I Interferon signalling via IFNAR1 (6, 7). Moreover, we showed that IFNAR1-signalling exerted its suppressive effects at least partially by limiting ICOS expression on $CD4^+$ T cells (6). Therefore, over the past few years the literature has begun to reveal the existence of factors that regulate the onset of humoral immunity to *Plasmodium in vivo*.

The development of humoral immunity has been studied in numerous experimental systems in mice, most often during viral infections and immunization (11-15), but less so during parasitic infection. These studies also revealed crucial roles for $CD4^+$ T-cells, B-cells, IL-21 and ICOS in promoting humoral responses, as well as a large number of other molecules including the cytokine, IL-6 (11-17). During viral infection and immunization, IL-6 was reported to play specific roles different to IL-21, for example in driving early plasmablast responses (17, 18). In addition, IL-6 has been reported to support ICOS expression by $Foxp3^+$ Treg cells in aged mice *in vivo* (19), by human $CD4^+$ T-cells during *in vitro* culture (20), and late during infection by LCMV-specific $CD4^+$ T cells (12). However, whether IL-6 plays any such roles during *Plasmodium* infection has yet to be determined.

IL-6 is a pleiotropic cytokine. It is expressed by many different cell types throughout the body, and can exert its functions via different signalling processes, termed classical and trans-signalling (21). Notably, IL-6 is readily detected in the plasma of humans and mice infected with blood-stage *Plasmodium* parasites (22-25). Previous studies demonstrated a role for infection-induced IL-6 in driving pathology during *Plasmodium* infection (26-28). Another interesting study improved IgG antibody responses via exogenous injection of recombinant IL-6 cytokine (22), which suggested that IL-6 can control the development of humoral immune responses during *Plasmodium* infection. Here, we principally employed *Pc*AS infection of C57BL/6J mice to further explore a role for infection-induced IL-6 in the development of humoral immune responses during experimental blood-stage malaria, with a specific focus on B-cell and T-cell responses in the spleen.

MATERIALS AND METHODS

Animal Ethics

All procedures on animals were approved by the QIMR Berghofer Medical Research Institute Animal Ethics Committee (approval numbers A02-633M and A1503-601M), in accordance with the "Australian Code of Practice for the Care and Use of Animals for Scientific Purposes" (Australian National Health and Medical Research Council).

Mice and Plasmodium infections

Female wild-type (WT) C57BL/6J mice (6-12 weeks old) purchased from Australian Resource Centre (Canning Vale, Western Australia) and were maintained under conventional conditions. C57BL/6J *IL-6*$^{-/-}$ mice were maintained in-house. *Plasmodium chaubadi chaubadi* AS (*Pc*AS) or *P. yoelii* 17XNL (*Py*17XNL) parasites were prepared after one *in vivo* passage in WT C57BL/6J mice, and were injected in 200µl volumes via intravenous tail-vein injection such that mice received either 10^5 pRBCs (*Pc*AS) or 10^4 pRBCs (*Py*17XNL). Peripheral blood from tail bleeds were assessed for parasitemia by preparing thin blood smears and using Diff-Quick stains (Lab Aids, Narrabeen, NSW, Australia). More usually, we employed an established flow cytometric method to measure parasitemia (29). One drop of blood (~20µl) was diluted in 250µl RPMI + 5U/ml heparin sulphate, and then co-stained with Syto84 (5µM; Life Technologies) and Hoechst33342 (10µg/ml; Sigma) for 30 minutes (room temperature, in the dark). Reactions were stopped with ice-cold RPMI (10x volumes), immediately analysed by flow cytometry (BD FACS CantoII or LSR Fortessa analyser (BD Biosciences)) and FlowJo software (Treestar, CA, USA). pRBC were detected as Hoechst33342$^+$ Syto84$^+$. PBMC were easily excluded on the basis of size, granularity and much higher Hoechst33342/Syto84 staining.

Detection of parasite-specific serum antibodies by ELISA

Plasmodium antigen extract was prepared and ELISAs conducted as previously described (6, 30, 31). 96-well flat-bottomed plates (Costar EIA/RIA) were coated overnight with *Plasmodium* extract (2.5µg/ml) in bicarbonate buffer (pH 9.6), overnight at 4ºC. The following day, plates were washed three times (0.005% Tween-20 in PBS) and blocked with 1% BSA/PBS, for 1hr at 37°C. After washing three times, 100ul of diluted sera (1/400) was added. Plates were incubated for 1hr at 37°C. Plates were then washed six times, and then biotinylated anti-IgM or anti-total IgG (Jackson ImmunoResearch) was added for 1hr at room temperature. Plates were again washed six times, after which streptavidin HRP (BD Biosciences) was added for 30 minutes at room temperature in the dark. Wells were washed six times prior to developing with 100µl of OPD (Sigma-Aldrich) for five minutes in the dark. Colour changes were fixed with 100µl of 1M HCl. Absorbance was determined at 492nm on a Biotek synergy H4 ELISA plate reader (Biotek, USA). Data were analysed using Gen5 software (version 2) and GraphPad Prism (version 6).

Flow Cytometry and Antibodies.

Splenocytes were prepared as previously described (32). Monoclonal antibodies, anti-mouse B220-Alexa Fluor 700 (RA3-6B2), B220-Pacific blue (RA3-6B2), CD19-FiTC (6D5), CD138-BV605 (281-2), IgD-APCCy7 (11-26c.2a), IgM-PECy7 (RMM-1), TCRβ-Alexa Fluor 700 (H57-597), TCRβ-APC/Cy7 (H57-597), CD4-BV605 (RM4-5), ICOS-PE (7E.17G9), CD23-APC (B3B4), CD21/CD35 (7E9), Streptavidin-PE/Cy7 and Zombie Aqua™ fixable viability dye were purchased from Biolegend (San Diego, CA). Anti-mouse CD95/Fas-BV421 (Jo2), CXCR5-biotin (2G8), and Bcl6-PerCP/Cy5.5 (K112-91) were sourced from BD Biosciences (Franklin Lakes, NJ). PD1-APC/Cy7 (J43) purchased from eBioscience. FACS staining was performed as previously described (32, 33).

In vivo IL-6R blockade.

Anti-IL-6R blocking monoclonal antibody (clone MR-16, Chugai, Japan) and its isotype control mAb were administered in 0.5mg doses, via *i.p* injection in 200 µl 0.9% NaCl (Baxter) on the day of infection, and subsequently on days 3, 6 and 9 post infection.

Confocal microscopy analysis

10–20 µm frozen spleen sections were employed as previously described (34, 35). Tissues were snap-frozen in optimal cutting temperature (OCT) medium (Sakura) and stored at -80°C. Sections were fixed (10 minutes) in ice-cold acetone before adding anti-CD3-Biotin (clone-17A2), anti-

B220-PE (clone-RA3-6B2) and anti-ICOS-APC (clone-C398.4A). Anti-CD3 was detected by streptavidin -Alexa Fluor 594. All antibodies were sourced from Biolegend, San Diego, CA. In addition, we used DAPI was used to help visualization of white pulp. We imaged samples using a Zeiss 780-NLO laser-scanning confocal microscope (Carl Zeiss). Resulting image data was analysed using Imaris image analysis software, version 8.1.2 (Bitplane). Cells were identified using the "spots function" in Imaris, with thresholds set at <10μM and intensities set at <150. The border between T and B-cell zones were defined by the region between $CD3^+$ cells closest to the B cell follicle and $B220^+$ cells furthest into the T-cell zone. All objects were manually inspected for accuracy before data were plotted and analyzed in GraphPad Prism (version 6).

Statistical analysis

Comparison between two groups was performed using non-parametric Mann-Whitney tests. P< 0.05 was considered significant (P<0.05 = *; P<0.01=**; P<0.001 =***; P<0.0001=****). Graphs depict mean values ± SEM, except where individual mouse data points are depicted, in which case median values are shown. All statistical analyses were performed using GraphPad Prism v6 or v7 software.

RESULTS

IL-6 promotes parasite control during PcAS infection.

To begin exploring a role for IL-6 during experimental blood-stage malaria, C57BL/6J wild-type (WT) and *IL-6*$^{-/-}$ mice were infected with *Pc*AS and examined over a time course for peripheral blood parasitemia. WT and *IL-6*$^{-/-}$ mice displayed similar parasitemias over the first ten days of infection (Figure 1A). However, for approximately one week thereafter, *IL-6*$^{-/-}$ mice exhibited higher parasitemias than WT controls (Figure 1A). Ultimately, *IL-6*$^{-/-}$ mice resolved primary *Pc*AS infection (Figure 1A), indicating a modest role for IL-6 in controlling parasite numbers. To substantiate these findings, WT mice were also treated with anti-IL-6R (α-IL-6R) blocking antibody or an isotype control during *Pc*AS infection, and parasitemias similarly assessed. IL-6R blockade transiently impaired parasite control from day 12-17 *p.i.* with *Pc*AS infection (Figure 1B), once again suggesting that IL-6 played a non-redundant role in optimizing parasite control during *Pc*AS infection.

IL-6 promotes early humoral immune responses during PcAS infection.

Given that IL-6 supported parasite control after peak parasitemia had been reached, when humoral immune responses are considered to act (36, 37), we next examined the impact of IL-6 on the development of humoral immune responses. Firstly, parasite-specific IgM levels, but not total IgG levels were significantly lower in *IL-6*$^{-/-}$ mice compared to infected WT controls, at the end of the second week of infection (Figure 2), suggesting a role for IL-6 in supporting early production of parasite-specific IgM but not IgG.

A previous single study reported robust differentiation of CD138^{+} antibody-secreting cells in the spleen during the second week of *Pc*AS infection, particularly in areas outside B-cell follicles (9). Here, we first confirmed at day 8 *p.i.* that *Pc*AS infection had triggered potent development of CD138^{+} plasmablasts in the spleen that were almost exclusively CD21^{lo} CD23^{lo}, consistent with extra-follicular localisation (Figure 3). Next, *IL-6*$^{-/-}$ mice displayed significantly reduced proportions and absolute numbers of splenic IgD^{lo}CD138^{+} plasmablasts compared to infected WT controls (Figure 4A), although early GC B-cell development was less affected (Figure 4B). Together, these data suggested that IL-6 supported splenic plasmablast development and parasite-specific IgM production during *Pc*AS infection.

IL-6 supports ICOS expression by splenic CD4^{+} T-cells

Previous reports showed that during *Pc*AS infection splenic CD138^{+} cells developed in extra-follicular areas, close to T-cell zones in the spleen (9). More recently we showed during *Py*17XNL infection, that splenic CD138^{+} plasmablast development was almost entirely dependent on CD4^{+} T-cells (6). Therefore, we hypothesized that reduced plasmablast development in *IL-6*$^{-/-}$ mice compared to WT controls was linked to effects on CD4^{+} T cell activation. Given reports in other experimental systems that IL-6 can support ICOS expression (12, 19, 20), we assessed ICOS up-regulation on splenic CD4^{+} T-cells, 8 days after *Pc*AS infection (Figure 5A). We noted an impairment in ICOS upregulation, in terms of the proportion and absolute numbers of T-cells expressing ICOS (Figure 5A), as well as the magnitude of ICOS expression by those cells which had upregulated this marker. Thus, ICOS up-regulation by splenic CD4^{+} T cells during *Pc*AS infection was partially dependent upon IL-6.

Next, given that ICOS expression by CD4^{+} T cells has been implicated in facilitating interaction with ICOS-ligand-expressing B-cells at the periphery of B-cell zones (14, 38, 39), we examined the impact of IL-6 on CD4^{+} T-cell localization within the spleen (Figure 5B). Densities of ICOS^{+} T-cells at the T/B border and within B-cell follicles were significantly reduced in spleens of *IL-6*$^{-/-}$ mice compared to WT controls (Figure 5B) by 8 days *pi*, consistent with the idea that IL-6-supported interactions between ICOS^{+} CD4^{+} T-cells and B-cells. Taken together, our data suggested

that IL-6 supported early expression of ICOS by $CD4^+$ T-cells, and their subsequent interaction with B-cells located outside or at the periphery of B-cell follicles.

IL-6 is not required for early development of Tfh-like cells during PcAS infection.

IL-6 has previously been reported to support $CD4^+$ T follicular helper (Tfh) cell differentiation in viral infection models (12, 40). Tfh cells have been reported to develop in several *Plasmodium* infection models including *Pc*AS infection (2, 4, 5). Therefore, we next explored whether IL-6 influenced the differentiation of Tfh cells. WT and *IL-6*$^{-/-}$ mice were infected with *Pc*AS, and splenic $CD4^+$ T-cells examined eight days *p.i.* for the expression of Tfh markers. Similar proportions and absolute numbers of splenic $CD4^+$ T cells up-regulated PD1 and CXCR5, or Bcl-6 and CXCR5 in both WT and *IL-6*$^{-/-}$ mice, suggesting that IL-6 played no essential role in early Tfh differentiation during *Pc*AS infection (Figure 6A). However, ICOS expression was significantly reduced on these emerging Tfh cells in *IL-6*$^{-/-}$ mice compared to WT controls (Figure 6B). Together, these data suggested that IL-6 was not required for early Tfh differentiation during *Pc*AS infection, but did influence their expression of ICOS.

Effect of IL-6 on humoral immune responses in a second model, P. yoelii 17XNL infection.

Finally, we sought to determine whether IL-6 promoted the development of humoral immune responses in a second model, *Py*17XNL infection. Firstly, we noted that IL-6 was required for optimal control of primary parasitemia, particularly during the second and third week of infection (Figure 7A), and similar to observations made during *Pc*AS infection, was ultimately resolved. Next, we found that IL-6 supported not only parasite-specific IgM production, as seen during *Pc*AS infection, but also IgG production, which had not been observed during *Pc*AS infection (Figure 7B). Consistent with this, IL-6 promoted GC B-cell development by day 14 of *Py*17XNL infection (Figure 7C). Finally, as with *Pc*AS infection, ICOS expression by Tfh cells, but not in their development *per se*, was partly reduced in the absence of IL-6 (Figure 7D). Therefore, taken together our data indicated that IL-6 supported the development of humoral immune responses during *Py*17XNL infection, including parasite-specific IgM and IgG production, ICOS upregulation on $CD4^+$ T-cells and GC B-cell development.

DISCUSSION

In this study we report *in vivo* roles for endogenous IL-6 in supporting $CD4^+$ T-cell expression of ICOS and localization near B-cells, differentiation of splenic B-cells into $CD138^+$ plasmablasts or GC B-cells, production of parasite-specific antibody, and optimal control of blood-stage *Plasmodium* parasites. Although previous reports have studied IL-6 in mouse models of malaria, these have tended to be in relation to immune-pathology and the effects of exogenous

recombinant IL-6 (22, 26-28). Therefore, to our knowledge, this is the first study to suggest a positive role for infection-induced IL-6 in supporting the early development of humoral immune responses during blood-stage *Plasmodium* infection.

Although it is generally accepted that *Plasmodium*-specific antibodies can provide excellent protection against malaria (2, 3), our understanding of immunological factors that control the development of these humoral immune responses during infection is limited. Current understanding of cytokine-mediated control of humoral immunity derives mostly from viral infection or experimental immunization in mice, in which roles for IL-21 and IL-6 have been described (11, 12, 40-42). More recently, we and others have reported suppressive roles for IFNγ, TNF and Type I IFN-signalling, and positive roles for IL-21 in controlling humoral immunity during *Plasmodium* infection (4-8, 43). In particular, IL-21 mediated GC B-cell responses and IgG class switching but not Tfh cell development or plasmablast responses (4). This study found that IL-6 also played no essential role in early Tfh cell development, except in terms of supporting ICOS expression, but was important in supporting B-cell responses and antibody production. Taken together, these observations suggest that during *Plasmodium* infection, neither IL-21 nor IL-6 are crucial for the initial development of Tfh cells. Instead these cytokines appear to play other distinct roles: IL-6 optimizes ICOS-dependent positioning of $CD4^+$ T cells in B-cell areas of the spleen, and influences the development of splenic B-cell responses and antibody production, while IL-21, produced predominantly by Tfh cells within the GC, influences IgG production. These observations suggest differing roles for IL-6 and IL-21, where IL-6 may tend to act earlier to drive extra-follicular plasmablast development, while IL-21 is produced later in the GC and acts to drive affinity maturation of IgG antibodies.

One important question remaining from our study is whether IL-6 influenced splenic plasmablast development indirectly by driving ICOS expression on $CD4^+$ T-cells, or directly via signaling to activated B-cells. Given that IL-6 was previously identified as B-cell stimulatory factor-2 and B-cell Growth Factor, it is important to recognize its capacity for signalling directly to B-cells (44-46). Therefore, while IL-6 may have acted directly on B-cells, the relative contribution of IL-6-signalling to T-cells and B-cells in driving plasmablast development remains to be determined.

Another important question that remains is whether cytokines other than IL-21 and IL-6 are crucial for the early development of Tfh cells during *Pc*AS infection. Our recent single-cell transcriptomic study showed that $CD4^+$ T-cells passed through an intermediate state prior to becoming an early Tfh cell (47), but that no cytokine or chemokine-signalling pathways (other than CXCR5 expression) clearly associated with early Tfh cell development. These data suggest that $CD4^+$ T cells pass through a Tfh-like intermediate state before final commitment towards a Tfh fate

without a requirement for any cytokine-signalling. Instead, capture of $ICOS^+$ $CXCR5^+$ $CD4^+$ T-cells by B-cells may play the major role in driving final Tfh-cell commitment.

During *Pc*AS infection we noted a modest role for IL-6 in promoting GC B-cell development, which held for percentages but not absolute numbers of B-cells in the spleen. The reason for this apparent discrepancy was unclear, but suggests possible differences in lymphocyte infiltration or retention in the spleen in WT compared to *IL-6*$^{-/-}$ mice. The possibly subtle effect of IL-6 on the GC reaction during *Pc*AS infection requires further study at later timepoints. In contrast, our experiments using *Py*17XNL parasites revealed a clearer role for IL-6 in promoting IgG production and GC B-cell development by the end of the second week of infection. Thus, our data suggest that depending on the *Plasmodium* infection model employed, IL-6 plays a mild to moderate role in promoting the GC reaction and IgG production, but a stronger role in promoting IgM production. However, further studies are required to study in more detail the role of IL-6 in maintaining IgG production and GC B-cell responses during *Plasmodium* infection.

In this study we found that in the absence of IL-6, parasite control and antibody production was impaired in two model infections. These observations suggest, but do not prove that impaired parasite control in *IL-6*$^{-/-}$ mice was due to reduced antibody production. It is also possible that other immune responses, for example Th1 differentiation, may have been affected by IL-6 deficiency. We believe studies are warranted to examine further the role of IL-6 in cellular and humoral immunity to malaria.

Given that IL-6 played early roles during infection but was not essential for ultimate control of parasites, we speculate that in contrast to IL-21, IL-6 mediates an early attempt by the host to control parasite numbers. Although, such a response provided some protection against parasites, it is not immediately obvious whether this benefits the host or parasite. For instance, early control of parasite numbers could reduce disease severity, keeping the host alive but also facilitating transmission. Moreover, a greater focus of the humoral response on short-lived plasmablast responses and IgM production could theoretically distract from longer-lived and high affinity IgG production, which again could encourage the establishment of chronic infection and facilitate transmission. Therefore although IL-6 promoted early humoral immune responses, and appeared non-essential for resolution of infection, the effect of IL-6 on longer-term immunity, chronic infection and/or transmission remains to be studied. In summary, this study has demonstrated that IL-6 can promote early humoral immune responses during *Pc*AS infection, which further cements the concept that innate and pro-inflammatory cytokines are important potential targets for modulating anti-parasitic antibody responses during blood-stage *Plasmodium* infection.

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FIGURE LEGENDS

Figure 1. IL-6-signalling contributes to parasite control during *Pc*AS infection. WT and *IL-6*$^{-/-}$ mice (n=6) were infected with *Pc*AS. (A) Shows a time-course analysis of parasitemia in WT and *IL-6*$^{-/-}$ mice. (B) Shows a time-course analysis of parasitemia in WT mice treated with anti-IL-6R blocking monoclonal antibody or control IgG. Data representative of three independent experiments. Statistics: Mann-Whitney U test, *P<0.05; **P<0.01

Figure 2. IL-6 supports IgM production during *Pc*AS infection. WT and *IL-6*$^{-/-}$ mice (n=5-6) were infected with *Pc*AS. Graphs show *Pc*AS-specific IgM and total IgG (serum diluted at 1/400) antibody levels in serum of naïve and infected WT and *IL-6*$^{-/-}$ mice, 14 days post-infection (*p.i.*). Data is pooled from 2 independent experiments each showing similar results. Statistics: Mann-Whitney U test, ****P<0.0001.

Figure 3. Early plasmablast formation during *Pc*AS infection is extra-follicular. C57BL/6J mice (n=5) were infected with *Pc*AS and splenic plasmablast formation assessed, 8 days p.i.. Data shows FACS plots and proportions of CD21^{lo}CD23^{lo} plasmablasts (B220^{+}CD19^{+}IgD^{lo}CD138^{hi}, denoted *Pc*AS CD138^{+}) or non-plasmablasts (B220^{+}CD19^{+}CD138^{lo}, denoted PcAS CD138^{-}) in the spleens of naïve and infected mice. Data representative of 2 independent experiments.

Figure 4. IL-6 promotes splenic plasmablast development during *Pc*AS infection. WT and *IL-6*$^{-/-}$ mice (n=5-6) were infected with *Pc*AS. (A) Shows representative FACS plots, proportions and numbers of splenic plasmablasts (B220^{+}CD19^{+}IgD^{lo}CD138^{hi}) in naïve and infected spleens, 8 days p.i.. (B) Shows representative FACS plots, proportions and numbers of splenic GC B-cells (gated as B220^{+}CD19^{+}GL-7^{+}Fas^{+}) in WT and *IL-6*$^{-/-}$ mice, 8 days p.i.. Data representative of 3 independent experiments. Statistics: Mann-Whitney U test, *P<0.05; **P<0.01.

Figure 5. IL-6 supports ICOS expression and positioning of CD4^{+} T-cells within splenic B-cell areas. WT and *IL-6*$^{-/-}$ mice (n=5-6) were infected with *Pc*AS. (A) Shows representative FACS plots, proportions, absolute numbers and ICOS levels of splenic ICOS^{+} CD4^{+} T-cells (gated on CD4^{+} TCRβ^{+} live singlets) in naïve, and *Pc*AS-infected WT and *IL-6*$^{-/-}$ mice, 8 days p.i.. Data

representative of 3 independent experiments. (B) Shows representative distribution pattern and densities of ICOS expressing T-cells in splenic T-B borders and B-cell follicles of naïve, WT and *IL-6*$^{-/-}$ mice, 8 days *p.i.*. Each symbol represents one T-B border or follicle in the spleen. Data pooled from 3-4 T-B borders or follicles per mouse with n=2 for naïve and n=4 for WT and n=5 for IL-6$^{-/-}$ mice. T-B border defined by region between CD3^{+} cells farthest into follicle (dotted white line, bottom) and B220^{+} cells farthest into the T-cell zone (dotted white line, top). Scale bar, 100μM. Statistics: Mann-Whitney U test, *P<0.05; **P<0.01; ****P<0.0001.

Figure 6. IL-6-signalling is not essential for early Tfh cell development during *Pc*AS infection. WT and *IL-6*$^{-/-}$ mice (n=5-6) were infected with *Pc*AS. (A) Shows representative FACS plots, proportions and absolute numbers of splenic PD1^{+} CXCR5^{+} or Bcl-6^{+} CXCR5^{+} cells (gated on CD4^{+} TCRβ^{+} live singlets) at day 8 p. i.. (B) Shows ICOS expression levels on Bcl-6^{+} CXCR5^{+} CD4^{+} T-cells at day 8 *p.i.*. Data representative of 2-3 independent experiments; Mann-Whitney U test **P<0.01.

Figure 7. IL-6-signalling promotes humoral immune responses in a second model, *Py*17XNL infection. WT and *IL-6*$^{-/-}$ mice (n=5-6) were infected with *Py*17XNL. (A) Time-course analysis of parasitemia in WT and *IL-6*$^{-/-}$ mice infected with *Py*17XNL. (B) *Py*17XNL-specific serum IgM and total IgG (serum diluted at 1/400, 1/800, 1/1600 & 1/3200) in naïve and infected WT and *IL-6*$^{-/-}$ mice, 14 days *p.i.* (C) Representative FACS plots, proportions and numbers of splenic GC B-cells (gated as B220^{+}CD19^{+}GL-7^{+}Fas^{+}) in naïve and infected spleens, 14 days *p.i.* (D) Representative FACS plots, proportions and numbers of splenic CD4^{+} TCRβ^{+} PD1^{+}CXCR5^{+} cells in naive and infected WT and *IL-6*$^{-/-}$ mice, 14 days *p.i.*, and ICOS expression on CD4^{+}TCRβ^{+}PD1^{+}CXCR5^{+} cells. Statistics: Mann-Whitney U test *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001. Data representative of three independent experiments for (A), and two independent experiments showing similar results for (B-D).

Figure 1

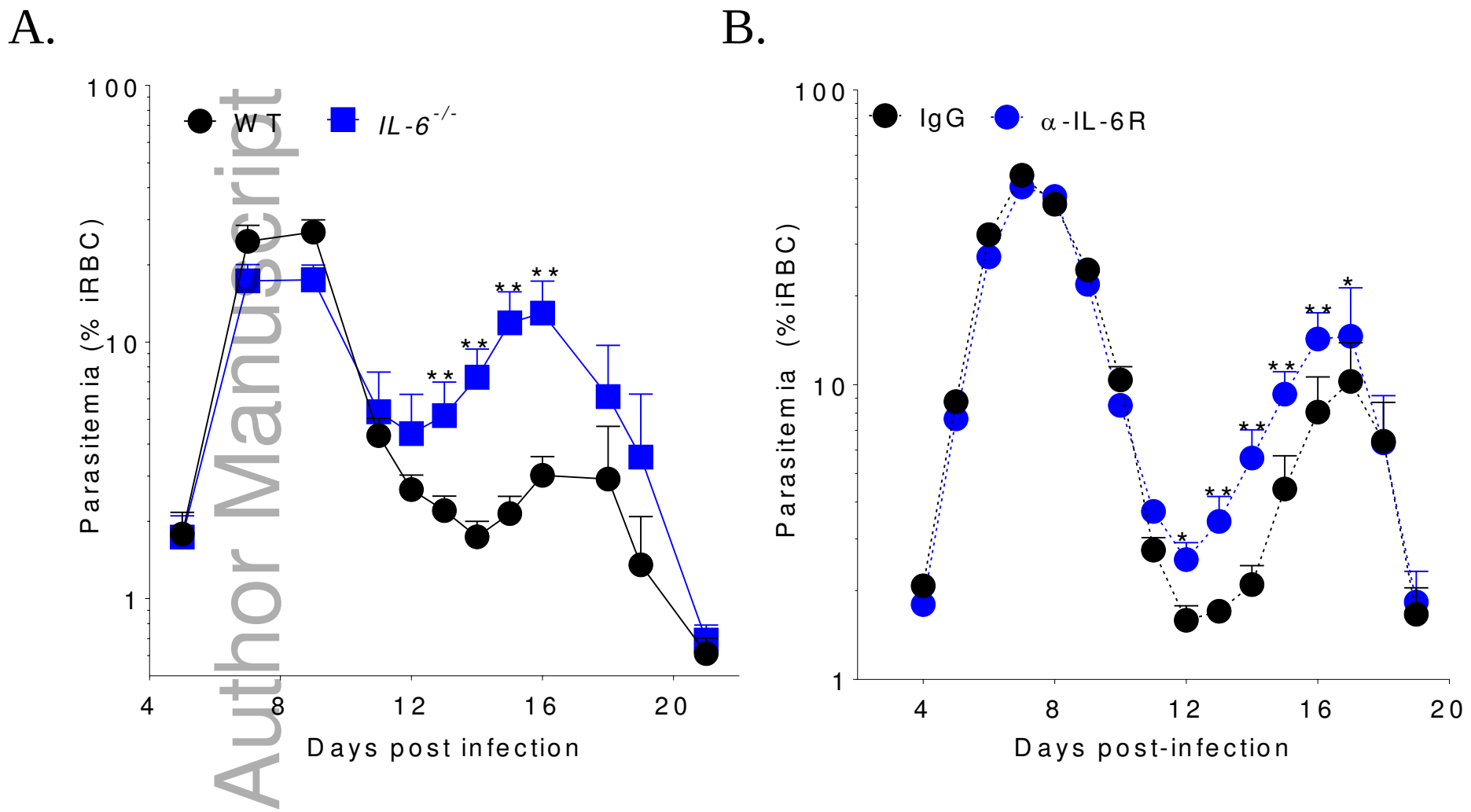
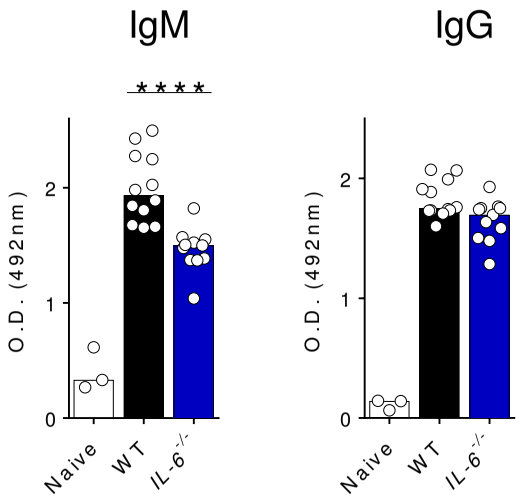


Figure 2



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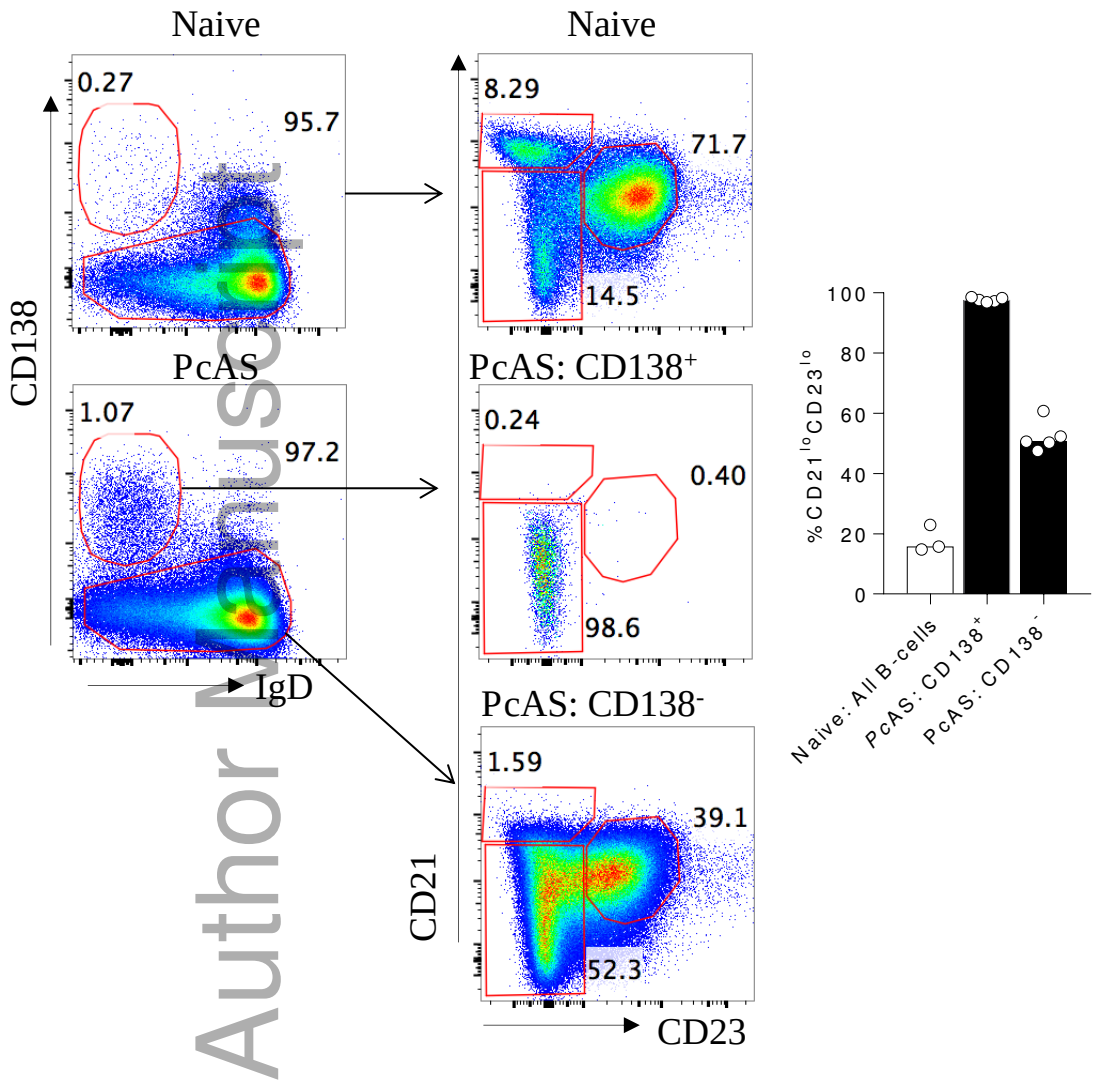

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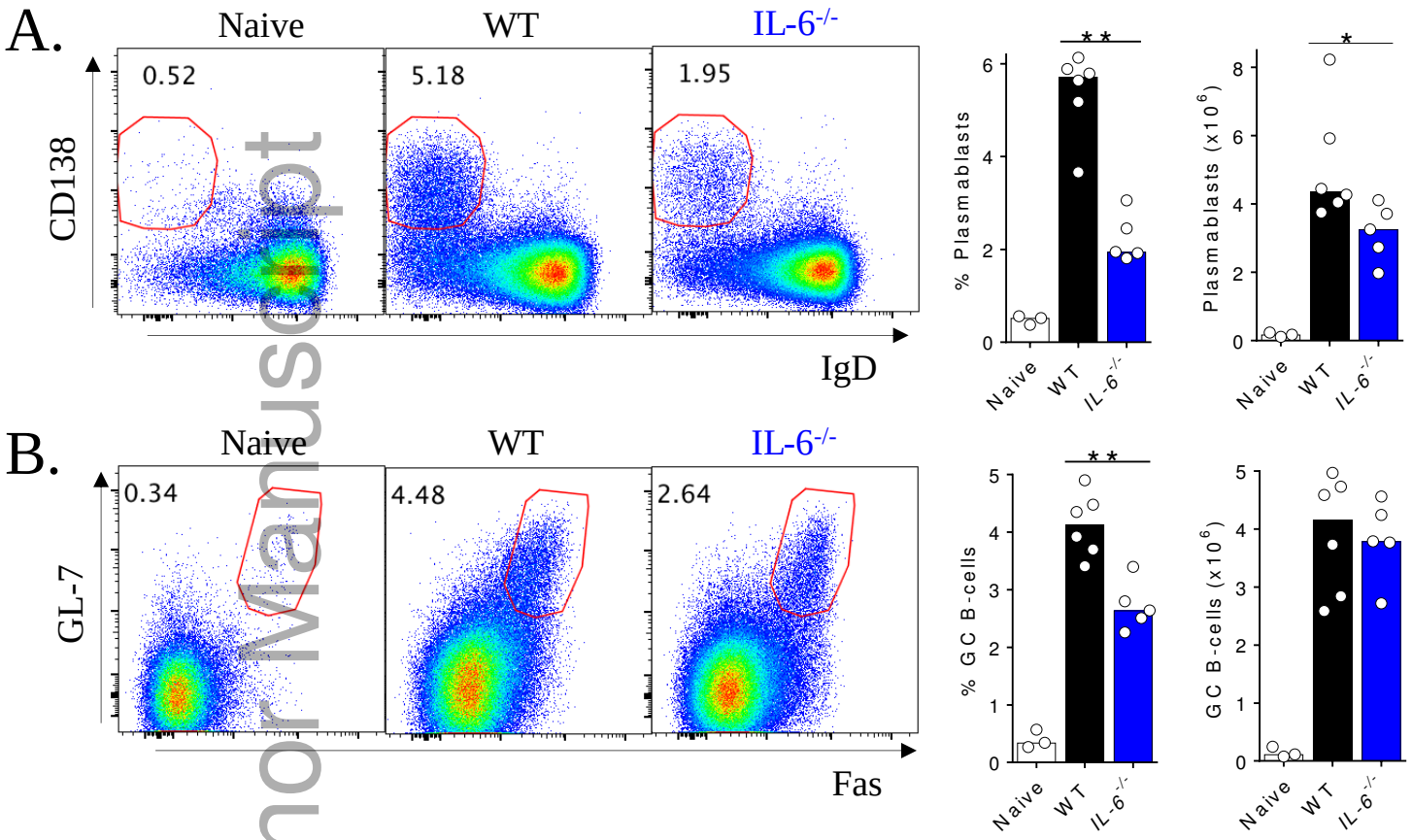

Figure 5

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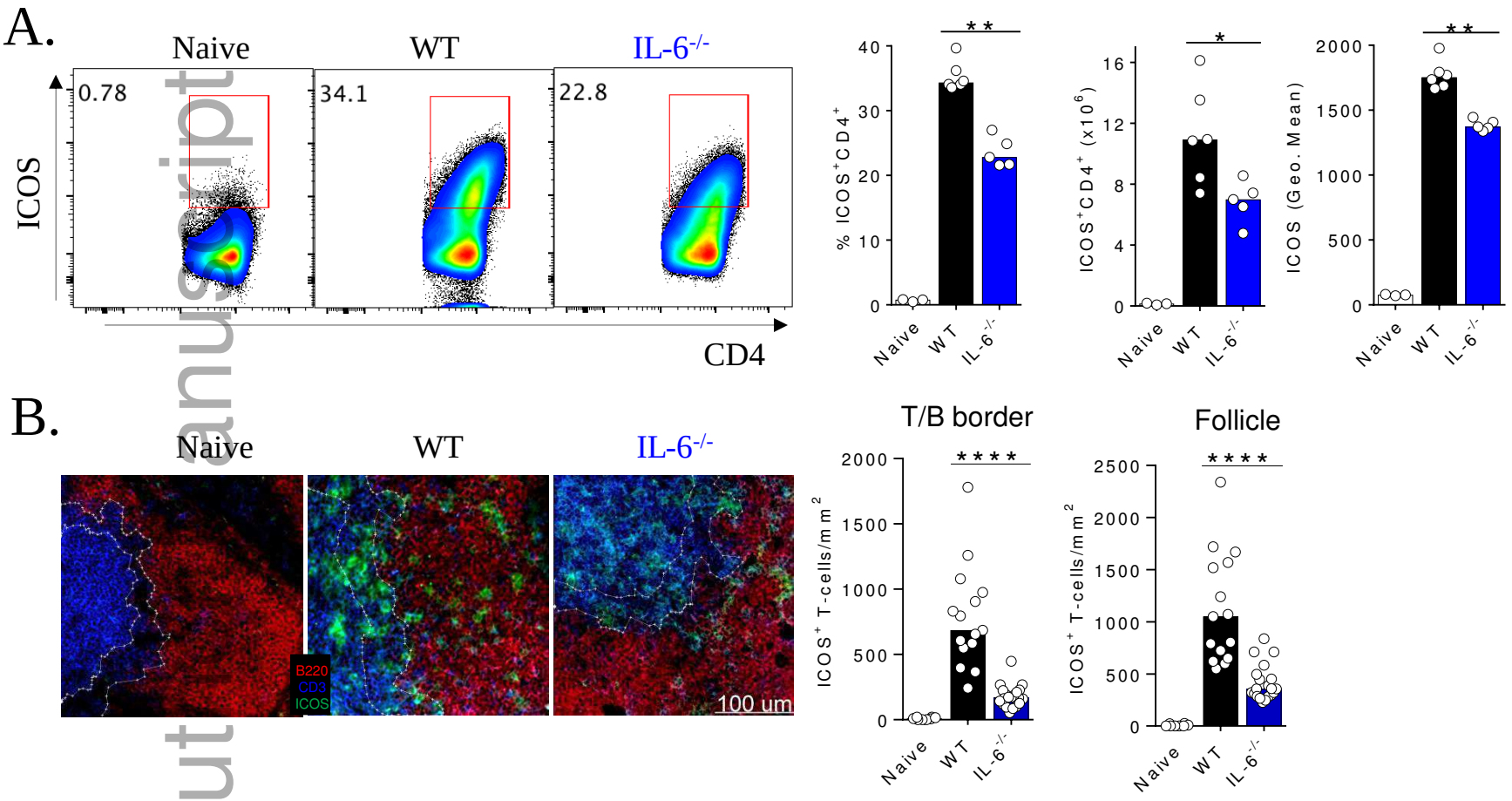


Figure 6

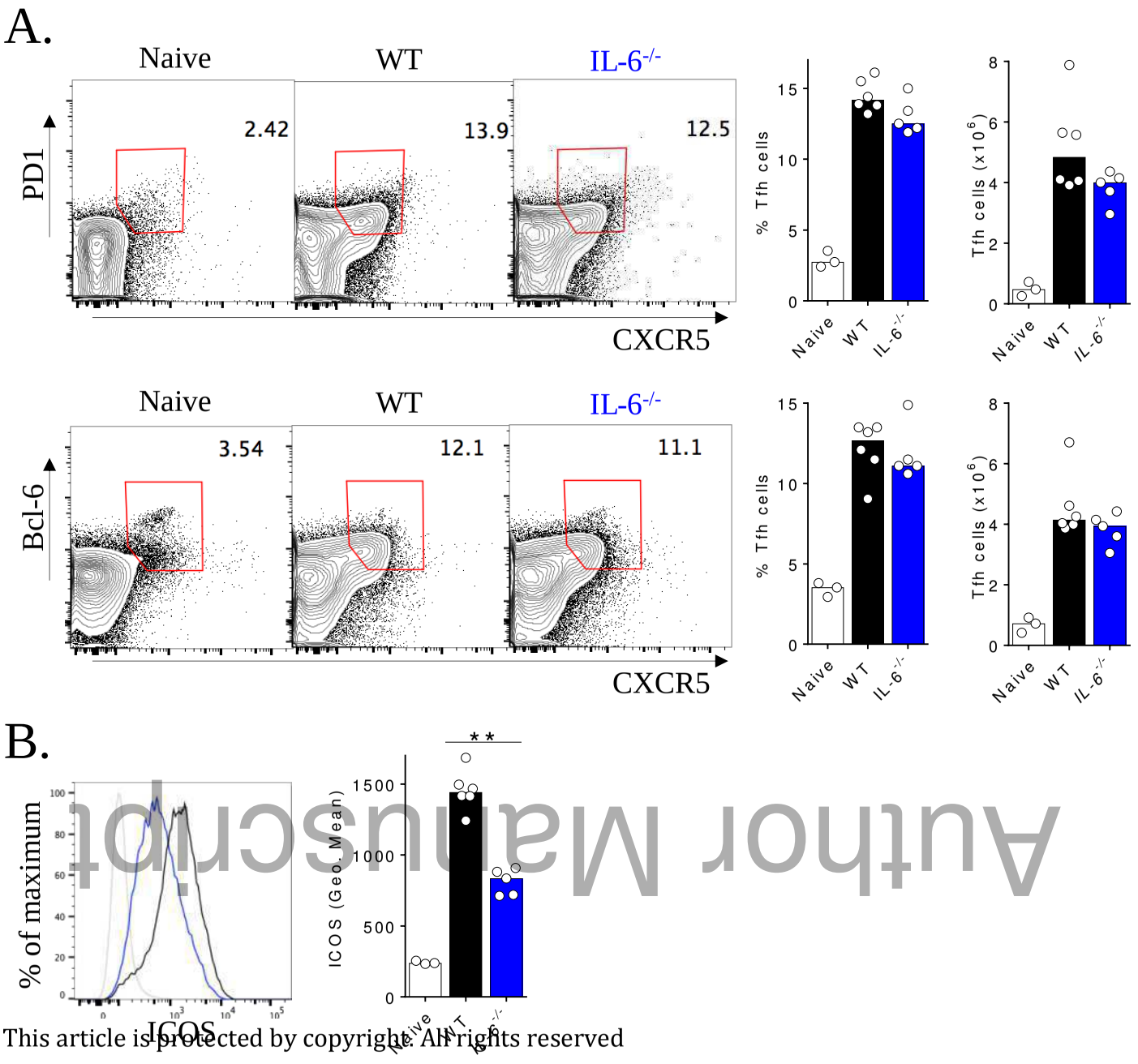
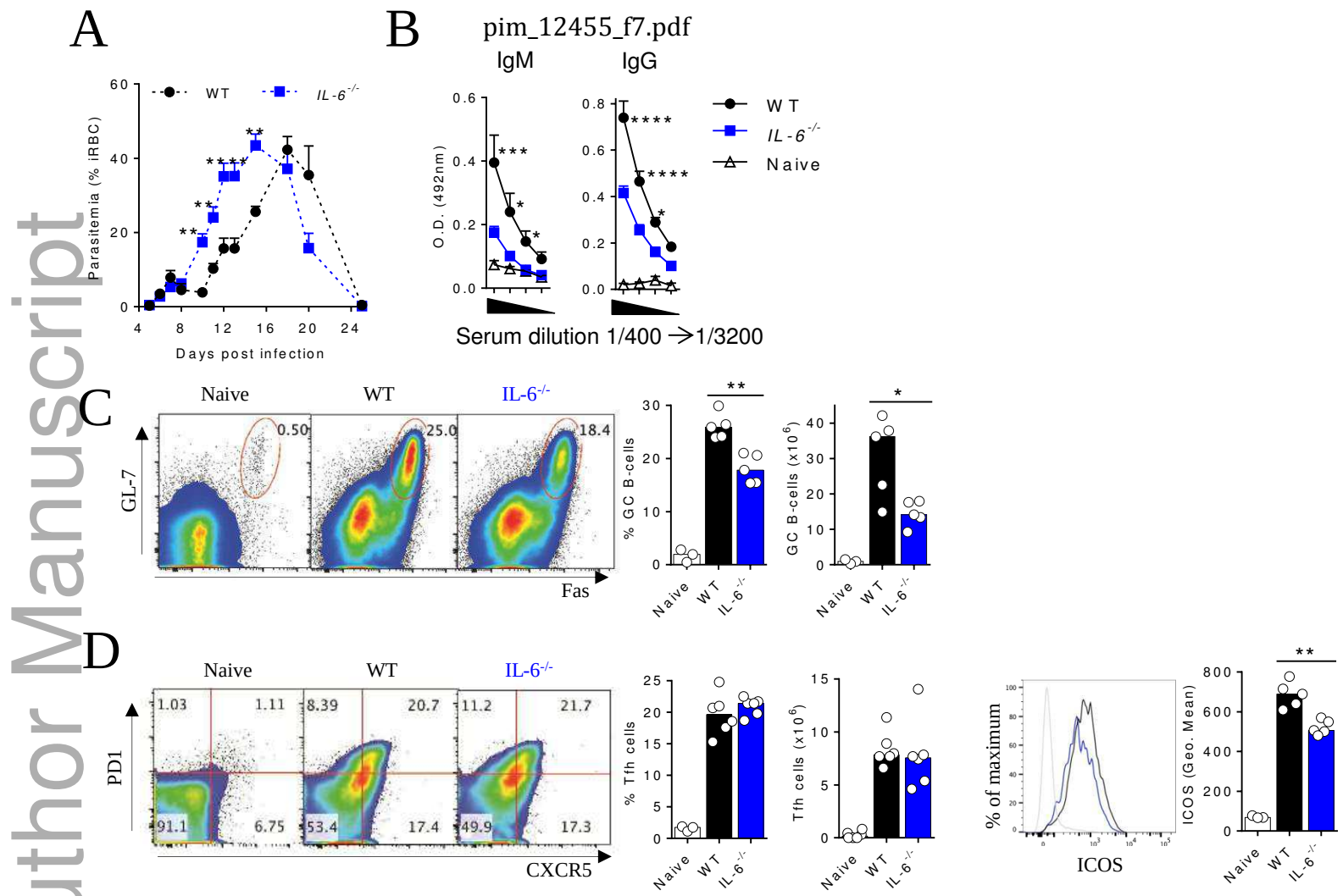


Figure 7



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