Antigen handling and cross-presentation by dendritic cells

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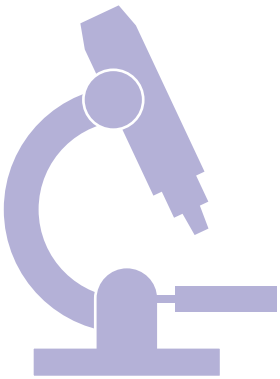


C1q-dependent dendritic cell cross-presentation of in vivo-formed antigen-antibody complexes

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ABSTRACT

Dendritic cells (DCs) are specialized in antigen engulfment via a wide variety of uptake receptors on their cell surface. In the current study we investigated antigen uptake and presentation of *in vivo* formed antigen-antibody complexes by injecting intravenously mice with antigen-specific antibodies followed by the cognate antigen. We show by this natural antibody-mediated antigen targeting system that uptake by splenic APC subsets is severely hampered in mice lacking complement factor C1q ($C1qa^{-/-}$). Moreover, no detectable antigen cross-presentation by $CD8\alpha^{+}$ DCs from $C1qa^{-/-}$ mice was found. On the contrary, antigen uptake was not hampered by APCs in FcγRI/II/III/IV-deficient (FcγR quadruple$^{-/-}$) mice and the cross-presentation ability of $CD8\alpha^{+}$ DCs was not affected.

In conclusion, we show that C1q rather than FcγR controls the antibody- mediated antigen uptake and its presentation by spleen APC subsets to T cells.

INTRODUCTION

Dendritic cells (DCs) are professional antigen presenting cells (APCs) bridging innate and adaptive immunity. DCs are specialized in antigen uptake and internalization via pinocytosis, phagocytosis or receptor mediated endocytosis and subsequently process and present the antigen peptides in MHC molecules to specific T cells (1). DCs express a wide variety of pathogen recognition receptors on their cell surface including C-type lectin receptors, toll-like receptors and Fcγ receptors (FcγR) (2). Several studies revealed that FcγR are important in T cell mediated anti-tumor responses (3, 4). DCs loaded with specific antigen-IgG-immune complexes resulted in induction of DC maturation, priming of specific $CD8^+$ CTL responses and tumor protection *in vivo* (5, 6). We have demonstrated that FcγR mediated uptake of model antigen ovalbumin (OVA) bound to anti-OVA IgG, also called OVA immune complexes (OVA IC), is at least 1000 fold more efficient in antigen cross-presentation than soluble OVA (6). Binding of antigen-IgG complexes triggers crosslinking of the Fcγ receptors resulting in internalization of the IC towards antigen storage and presentation compartments (7, 8). In mice, four FcγR have been described, FcγRI (CD64), FcγRIIB (CD32B), FcγRIII (CD16) and FcγRIV (9). The activating receptors FcγRI, FcγRIII and FcγRIV have an immunoreceptor tyrosine-based activation motif (ITAM), whereas the inhibitory receptor FcγRII has an immunoreceptor tyrosine-based inhibitory motif (ITIM). Both activating and inhibitory FcγR can be co-expressed on the same cell, which can regulate and determine the cellular response (10, 11).

Besides binding to FcγR, most IgG subclasses can also activate complement by binding to C1q, the first recognition subcomponent of the classical pathway (12, 13). C1q is a hexameric glycoprotein, composed of 18 polypeptide chains that are formed by 3 types of chains (A-chain, B-chain and C-chain) (14, 15). Each chain consists of a collagen-like domain (binding site for anti-C1q autoantibody) to which the serine proteases C1r and C1s are localized, and a globular head, which binds to the Fc part of IgG and IgM when bound to the cognate antigen (16). Upon binding of C1q to IC, the serine proteases C1r and C1s are activated, resulting in the activation of the classical complement pathway (17). C1q is mainly produced by macrophages and immature DCs (18).

The complement system plays an important role in the physiological clearance of IC by binding complement coated IC to complement receptor-1 (CR1) on erythrocytes and thereby preventing IC deposition (19). Defects in IC clearance mechanisms, resulting in tissue inflammation and damage, have been described in patients with systemic lupus erythematosus (SLE) (20). Several studies have also shown the importance of C1q in adaptive immunity as the uptake and processing of IC in the spleen and antigen cross-presentation *in vivo* were hampered in C1q deficient mice (21, 22).

In the present study we used *in vivo* formed OVA IC obtained by injecting mice sequentially with anti-OVA IgG and OVA to investigate the contribution of FcγR and C1q in IC uptake in splenic APC subsets. We have previously reported that this natural formation of antigen-IgG complexes *in vivo* leads to efficient antigen cross-presentation to $CD8^+$ T cells (23). We now show that FcγR play minor roles in antibody-mediated antigen uptake *in vivo*, as the antigen uptake and cross-presentation were not hampered in APCs from FcγRI/II/III/IV-deficient (FcγR quadruple$^{-/-}$) mice. On the contrary, our results indicate a dominant role for C1q in controlling antigen targeting and handling by dendritic cells *in vivo*, as mice lacking C1q (C1qa$^{-/-}$) showed no detectable antigen uptake in APCs and strongly reduced antigen presentation to $CD8^+$ or $CD4^+$ T cells.

RESULTS

Efficient antibody-dependent antigen uptake by dendritic cell subsets *in vivo*

We have previously demonstrated by using a classical haptenated protein that circulating hapten-specific antibodies enhanced antigen uptake by DCs *in vivo* which can activate naïve $CD4^+$ and $CD8^+$ T lymphocytes (23). Here, we analyzed the uptake of the model antigen ovalbumin (OVA) by murine splenic APC subsets *in vivo* mediated by circulating OVA specific antibodies. Mice were first i.v. injected with anti-OVA Ab followed by OVA after 30min to allow natural formation of immune complexes (IC) by circulating antibodies *in vivo* (Fig. 1A). Antigen uptake was analyzed in four splenic APC subsets: $CD8\alpha^+$ DCs, $CD8\alpha^-$ DCs, pDCs and $CD11b^+$ Macrophages. Mice with circulating anti-OVA Ab showed OVA uptake in all four APC subsets (Fig. 1B). Although the percentage of antigen positive cells differed between the subsets (~2%- 10%), the overall level of uptake (MFI) was comparable. Mice that were injected with OVA without anti-OVA Ab showed no detectable antigen uptake in any APC subsets, indicating antibody dependence of antigen engulfment.

Antibody-mediated antigen uptake by APC subsets *in vivo* is Fcγ receptor independent

Several studies have shown that FcγR are involved in Ab-mediated uptake of antigen (6, 10, 24). Here, we investigated the role of FcγR in antigen-antibody complex uptake by APC subsets *in vivo* by using FcγRI/II/III/IV-deficient (FcγR quadruple$^{-/-}$) mice. Expression of all four FcγR was analyzed on BL/6 WT and FcγR quadruple$^{-/-}$ murine spleen APC subsets (Supplemental Fig. 1). $CD8\alpha^+$ DCs, $CD8\alpha^-$ DCs and Macrophages from BL/6 mice expressed all four FcγR, whereas pDCs only expressed FcγRII. OVA was injected in mice with circulating anti-OVA Ab as described above (Fig. 1A). Unexpectedly, all four APC subsets in FcγR quadruple$^{-/-}$ mice showed significant uptake of OVA (Fig. 2 and Supplemental Fig. 2A).

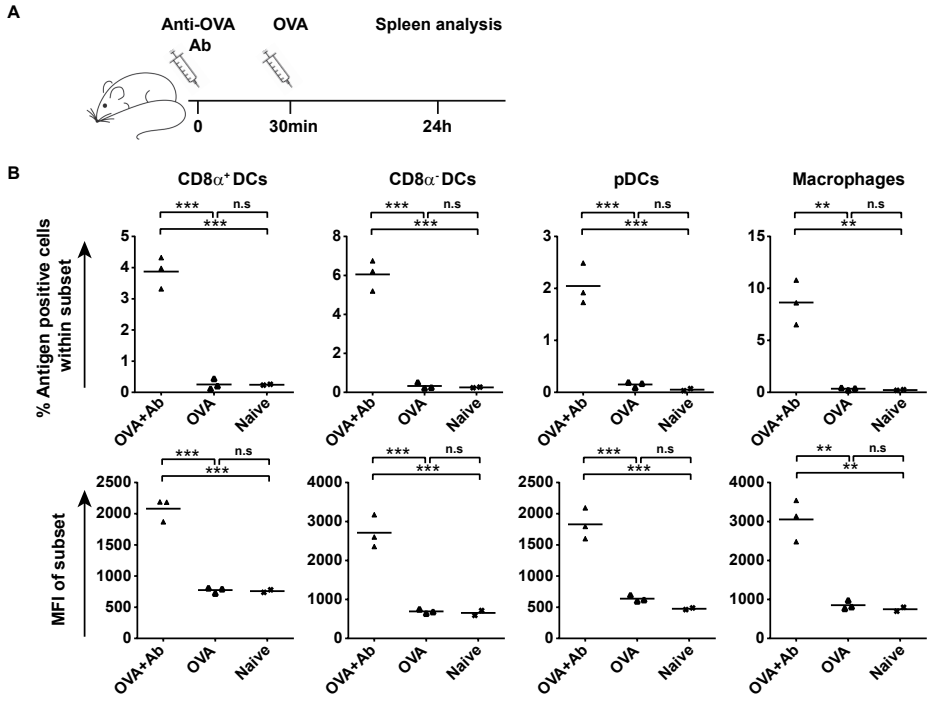

Figure 1. Antibody-mediated antigen uptake by APCs *in vivo*. C57BL/6 (BL/6) mice (each dot represents one mouse) were i.v. injected with 100µg anti-OVA IgG (Ab) and 30min later i.v. injected with 5µg OVA (Alexa Fluor 647 labeled). Antigen presence was measured in spleen APC subsets after 24h **(A)**. Antigen presence in spleen APC subsets measured after 24h, indicated by percentage antigen positive cells and MFI within each subset. Representative experiment is shown for 4 independent experiments **(B)**.

The antigen uptake was slightly higher in CD8α$^+$ DCs and pDCs from FcγR quadruple$^{-/-}$ mice compared to BL/6 mice, indicated by percentage positive cells and MFI. The OVA uptake by CD11b^{high}F4/80$^+$ Macrophages was lower when all four FcγR were lacking. FcγR are also known for their function in antigen-antibody complex clearance from the blood (25). By measuring the OVA amount in serum of mice after anti-OVA Ab and OVA injection in time, we could show that FcγR indeed play a role in rapid antigen clearance (Supplemental Fig. 3). Most of the circulating OVA was already cleared after 30min of injection in BL/6 mice, while the clearance was delayed in FcγR quadruple$^{-/-}$ mice and OVA was still detectable in circulation after 4h. This lower antigen clearance in FcγR quadruple$^{-/-}$ mice, and therefore more availability of OVA antigen, is likely the explanation for the higher uptake found in some of the APC subsets compared to BL/6 WT mice. Importantly, the uptake of OVA in FcγR quadruple$^{-/-}$ mice is antibody dependent, as mice injected with only OVA showed no significant OVA uptake in any APC subset (Supplemental Fig. 2B). These results indicate that antibody-mediated antigen uptake by *in vivo* APC subsets is FcγR independent and is likely facilitated by a different uptake route.

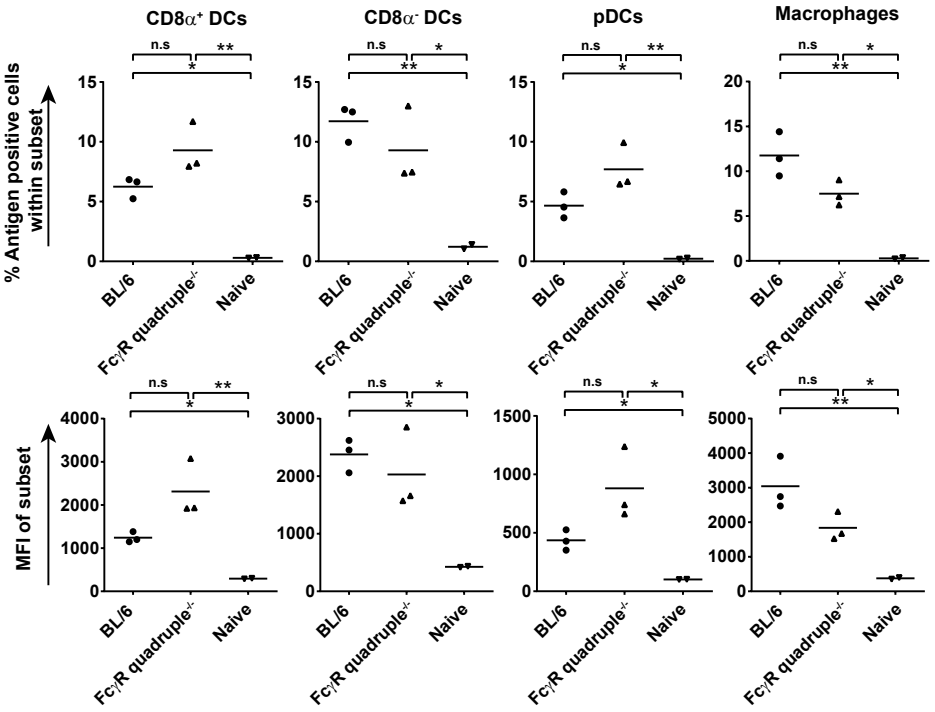

Figure 2. FcγR independent uptake of antigen-antibody complexes by APCs *in vivo*. BL/6 and FcγR quadruple$^{-/-}$ mice (each dot represents one mouse) were injected i.v. with 100µg anti-OVA Ab, followed by 5µg OVA (Alexa Fluor 647 labeled) i.v. injection 30min later. After 24h hours, antigen presence was measured in spleen APC subsets with flow cytometry. The percentage antigen positive cells within each subset is indicated in the upper panel. The mean fluorescence intensity (MFI) of antigen within each APC subset is shown in the bottom panel. Representative FACS data from one representative experiment are shown for 3 independent experiments.

MHCI cross-presentation by CD8α$^+$ DCs of antibody bound antigen is Fc receptor independent

Since APC subsets lacking all four FcγR were still able to take up antigen-antibody complexes, we further investigate the possible role of FcγR in antigen presentation to CD4$^+$ and CD8$^+$ T cells. APC subsets were freshly sorted from spleens of mice that were 24h before injected with anti-OVA Ab and OVA. Each *ex vivo* isolated subset was incubated with either CD8$^+$ (OTI) or CD4$^+$ (OTII) T cells. CD8α$^+$ DCs from BL/6 WT mice showed efficient antigen cross-presentation (~87% dividing cells) to OTI cells (Fig. 3A). CD8α$^-$ DCs were also able to cross-present antigen, although in a lower extent (~57% division) compared to CD8α$^+$ DCs. The antigen cross-presentation ability of CD8α$^+$ DCs from FcγR quadruple$^{-/-}$ was comparable to BL/6 WT mice (~85%), but antigen cross-presentation by CD8α$^-$ DCs lacking FcγR was drastically hampered. pDCs and Macrophages from both BL/6 WT and FcγR quadruple$^{-/-}$ showed no detectable antigen cross-presentation to OTI cells. CD8α$^-$ DCs from BL/6 WT was the only subset capable of presenting antigen to OTII cells, but again hampered when

lacking FcγR (Fig. 3B). In conclusion, in this circulating antigen-antibody uptake model, *ex vivo* isolated CD8α⁺ DCs can cross-present antigen regardless of the presence of FcγR. On the other hand, FcγR play a role in CD8α⁻ DC mediated cross-presentation.

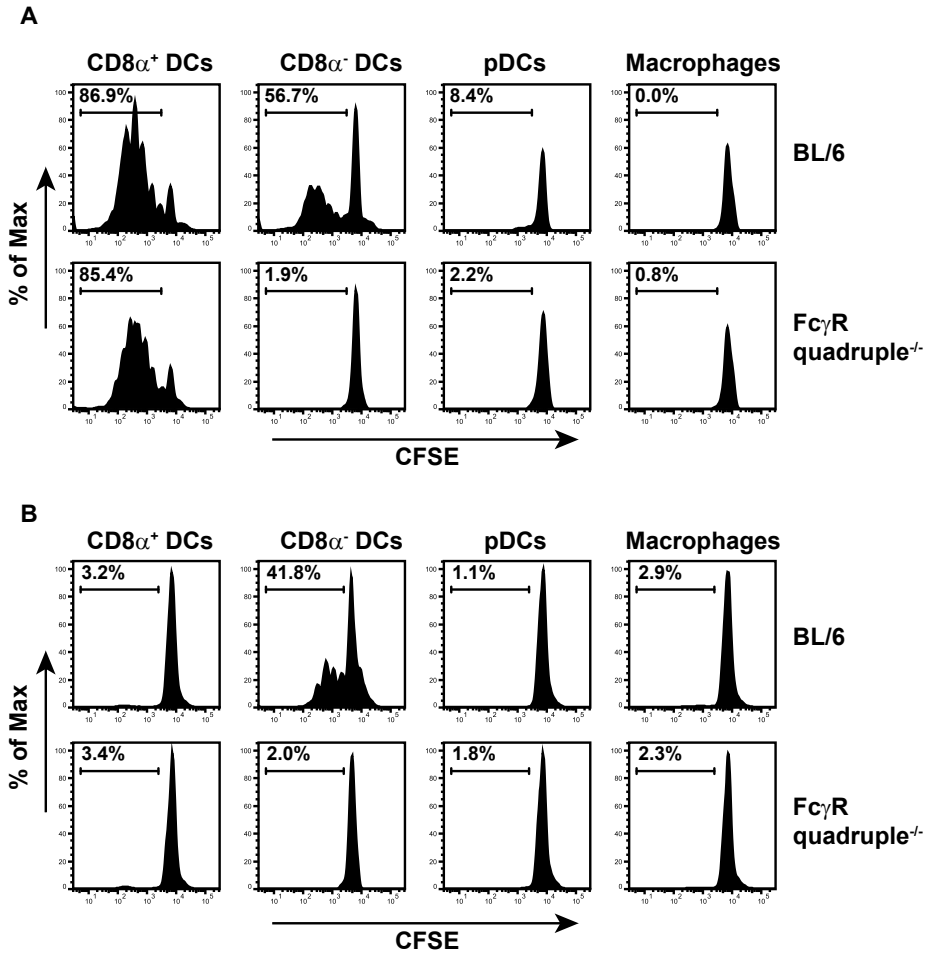

Figure 3. Antibody-mediated antigen cross-presentation by CD8α⁺ DCs is FcγR independent. BL/6 and FcγR quadruple$^{-/-}$ mice (2 mice per group) were injected i.v. with 100µg anti-OVA Ab, 30min later 5µg OVA was i.v. injected. 24h later, spleen APC subsets were sorted and incubated with CFSE labeled OTI (CD8⁺) T cells **(A)** or OTII (CD4⁺) T cells **(B)**. T cell proliferation was measured after 4 days by flow cytometry. Representative FACS data are shown for 3 experiments.

Antigen uptake mediated by circulating antibodies *in vivo* is C1q dependent

Several studies suggest that the complement system is involved in IC clearance which is mainly mediated by C1q, the activating molecule of the classical complement pathway (21, 22, 26). To investigate the possible role of C1q in *in vivo* formed IC, we analyzed antigen uptake

in splenic APC subsets of BL/6 WT in comparison with FcγR quadruple$^{-/-}$ and C1qa$^{-/-}$ mice. As shown previously, antigen uptake by all APC subsets in FcγR quadruple$^{-/-}$ mice is comparable to BL/6 WT mice (Fig. 4). However, mice lacking C1q showed no uptake of antigen in CD8α^{+} DCs, pDCs and macrophages, and a strongly reduced uptake in CD8α^{-} DCs. The antigen clearance rate is not affected in mice lacking C1q (Supplemental Fig. 3). These results indicate that C1q plays a crucial role in the uptake of antigen bound to circulating antibodies *in vivo*.

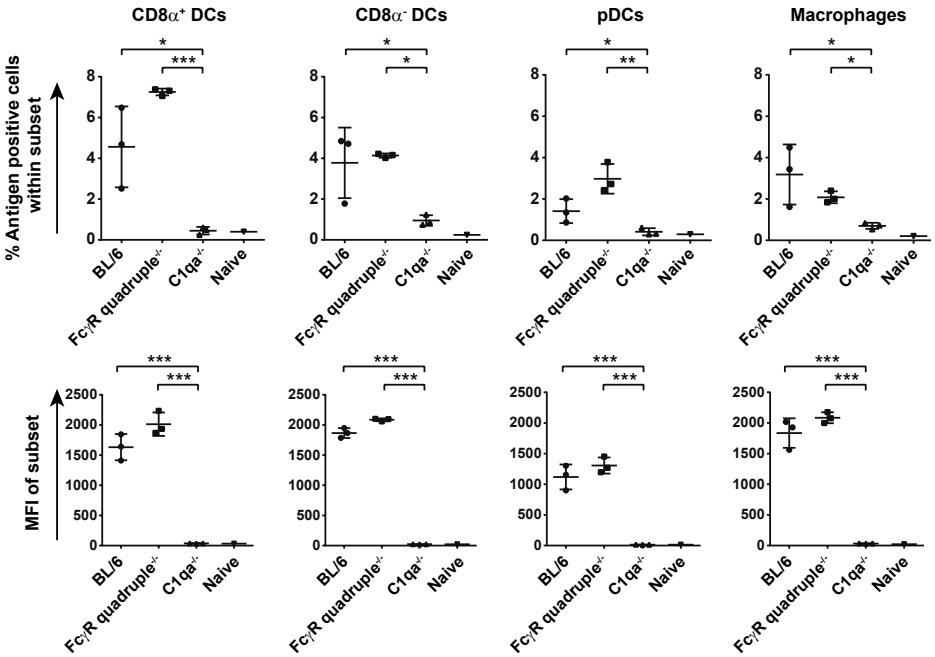

Figure 4. C1q dependent uptake of antibody-mediated antigen by APCs *in vivo*. BL/6, FcγR quadruple$^{-/-}$ and C1qa$^{-/-}$ mice (each dot represents one mouse) were i.v. injected with 100μg anti-OVA Ab and 30min later i.v. with 5μg OVA (Alexa Fluor 647 labeled). Antigen presence in spleen APC subsets were measured after 24h by flow cytometry, indicated by percentage antigen positive cells (upper panel) and MFI (bottom panel) within each subset. Representative FACS data are shown for 3 experiments.

MHCI cross-presentation of antibody bound antigen is C1q dependent

To determine whether C1q is important in antigen presentation to T cells, C1qa$^{-/-}$ mice were injected with anti-OVA Ab and subsequently with OVA. Splenic APC subsets were sorted after 24h and incubated with either CD8^{+} (OTI) or CD4^{+} (OTII) T cells. CD8α^{+} DCs, pDCs and macrophages from mice lacking C1q showed no detectable antigen presentation to CD8^{+} T cells (Fig. 5A). However, CD8α^{-} DCs from C1qa$^{-/-}$ mice were still able to present antigen to CD8^{+} T cells (~54% proliferating T cells) although in a lower extent than BL/6 WT mice (~69% proliferating T cells). All four APC subsets from C1qa$^{-/-}$ mice showed no efficient antigen

presentation to CD4+ T cells (Fig. 5B). Taken together, C1q drastically affects *in vivo* circulating antibody-mediated antigen uptake and thereby controlling antigen presentation efficiency by spleen APC subsets to T cells.

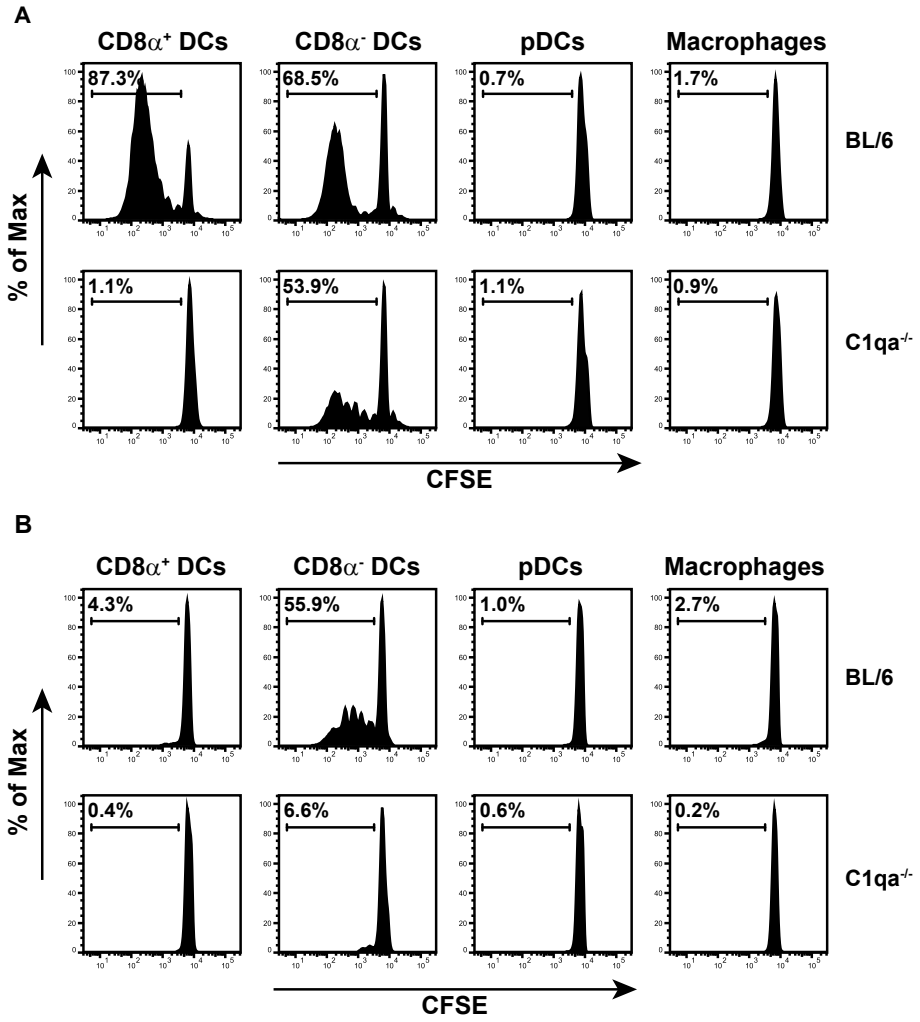

Figure 5. C1q dependent antigen presentation by APCs *in vivo*. BL/6 and C1qa$^{-/-}$ mice (2 mice per group) were i.v. injected with 100µg anti-OVA Ab, followed by 5µg OVA i.v. injection 30min later. Spleen APC subsets were sorted after 24h and incubated with CFSE labeled OTI **(A)** or OTII **(B)** cells. T cell proliferation was measured after 4 days by flow cytometry. Representative data are shown for 3 experiments.

In vivo antigen uptake is reduced by C1q depleting antibodies

To further analyze the crucial role of serum C1q in circulating antibody-mediated antigen uptake *in vivo*, BL/6 WT mice were injected with C1q depleting antibody (27) prior to anti-OVA Ab and OVA injection. All four splenic APC subsets showed reduced uptake of antigen when C1q was depleted from the serum (Fig. 6A). The C1q levels in mice that received C1q depletion treatment were monitored, showing reduced C1q in circulation even up to 24h (Fig. 6B). The antigen clearance rate from the system was not affected in the presence of C1q depleting antibodies (Fig. 6C). These results show that C1q in circulation plays an important role in antibody-mediated antigen uptake by splenic APC subsets *in vivo*.

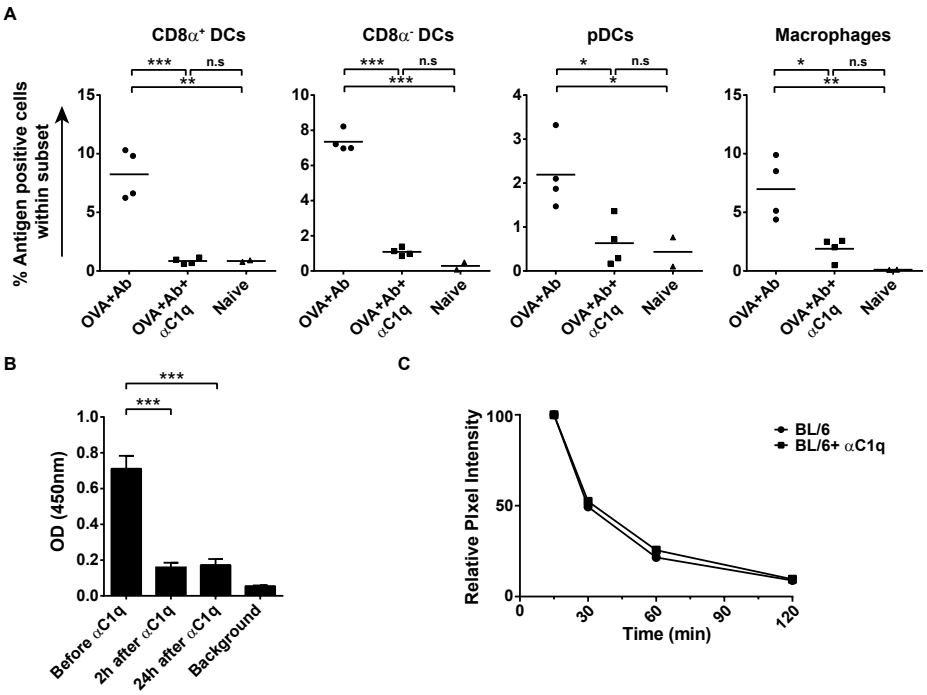

Figure 6. Antibody-mediated antigen uptake after C1q depletion. BL/6 mice (each dot represents one mouse) were i.p. injected with 1mg anti-C1q mAb (αC1q). Two hours later, mice were injected i.v. with 100µg anti-OVA Ab and after 30min i.v. with 5µg OVA (Alexa Fluor 647 labeled). Antigen presence in APC subsets were measured after 24h with flow cytometry **(A)**. C1q levels in serum were measured by C1q ELISA at indicated time points before or after C1q depletion **(B)**. OVA antigen clearance from mice serum was analyzed by withdrawing blood at the indicated time points. Serum was loaded on SDS/PAGE and the amount of fluorescent OVA was quantified, indicated by relative pixel intensity **(C)**. Representative data are shown for at least 2 independent experiments.

Antigen-antibody complex uptake *in vitro* is improved by C1q

In apparent contrast to the *in vivo* results from the current study, we have previously shown by using FcR gamma chain deficient mice (lacking FcγR I/III/IV) that the uptake of antibody-bound antigen by bone marrow derived DCs (BMDCs) is FcγR dependent *in vitro* (7). We confirmed these results with the use of BMDCs from FcγR quadruple$^{-/-}$ mice, showing no antigen uptake and antigen cross-presentation to CD8^{+} T cells *in vitro* (Supplemental Fig. 4A). Together, these studies show that FcγR are crucial in the uptake of antigen-antibody complexes by DCs *in vitro*. To determine whether this uptake can be improved by adding additional C1q, spleen cells from untreated BL/6 or FcγR quadruple$^{-/-}$ mice were incubated with different combinations of anti-OVA Ab, OVA and C1q *ex vivo*. Splenic APC subsets from BL/6 WT mice showed an increased antigen uptake when C1q was added to anti-OVA Ab and OVA (Fig. 7). As expected, the uptake of antigen-antibody complexes in splenic APC subsets in absence of all four FcγR is much lower compared to BL/6 WT, however, by adding C1q the uptake increased significantly. The uptake in FcγR quadruple$^{-/-}$ subsets could be increased in the presence of C1q, however the levels are only comparable with those in BL/6 WT without adding additional C1q. Adding additional C1q to OVA without anti-OVA Ab did not enhance antigen uptake by FcγR quadruple$^{-/-}$ APC subsets, indicating the antibody dependence for antigen uptake *in vitro* (Supplemental Fig. 4B). These results indicate that although C1q can facilitate the uptake of antigen-antibody complexes, FcγR are the more dominant uptake receptors under *in vitro* conditions.

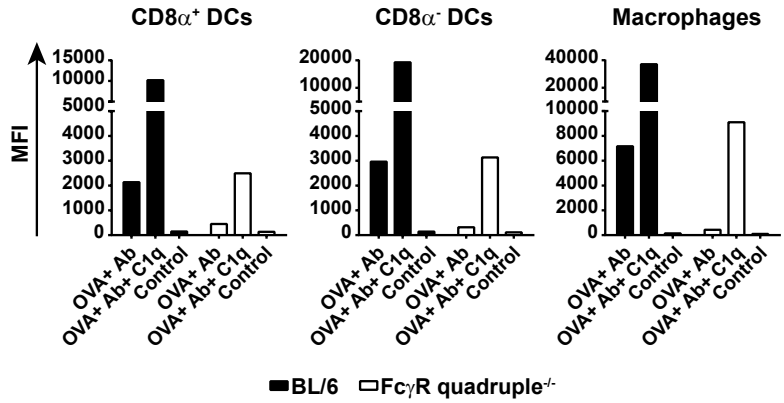

Figure 7. C1q induced antigen-antibody complex uptake by APCs *in vitro*. Naïve spleens from BL/6 or FcγR quadruple$^{-/-}$ mice (2 mice per group) were depleted for B cells and incubated with anti-OVA Ab and OVA (Alexa Fluor 488 labeled) with or without C1q for 1h. Antigen uptake in APC subsets was measured by flow cytometry, indicated by MFI. Representative FACS data are shown for 3 independent experiments showing similar results.

DISCUSSION

It is well established that targeting antigens to dendritic cells (DCs) via FcγR results in high efficient antigen uptake, DC maturation and antigen processing and presentation to T cells (5, 8, 9). Antibody-bound soluble antigen is much more efficient than free antigen since binding of immune complexes triggers crosslinking of FcγR, resulting in DC activation, prolonged antigen presentation, priming of specific $CD8^+$ CTL responses and tumor protection *in vivo* (6, 7, 28). In contrast to most studies that used *in vitro* preformed antigen-antibody complexes before injecting in mice, we injected mice sequentially with antibody followed by antigen (i.v.) to allow natural *in vivo* binding of IgG with cognate protein in circulation. As we have previously shown this leads to functional cross-presentation *in vivo* (23). In the present study we show a crucial role for complement factor C1q in antibody-mediated antigen uptake in DCs *in vivo* (illustrated in Fig. 8). Antibody-mediated antigen uptake in APC subsets of $C1qa^{-/-}$ mice was severely hampered in $CD8\alpha^+$ DCs, pDCs and macrophages, and strongly reduced in $CD8\alpha^-$ DCs. Moreover, $CD8\alpha^+$ DCs, pDCs and macrophages isolated from $C1qa^{-/-}$ mice that were injected with circulating antibody and antigen, showed no detectable MHCI and MHCII antigen presentation.

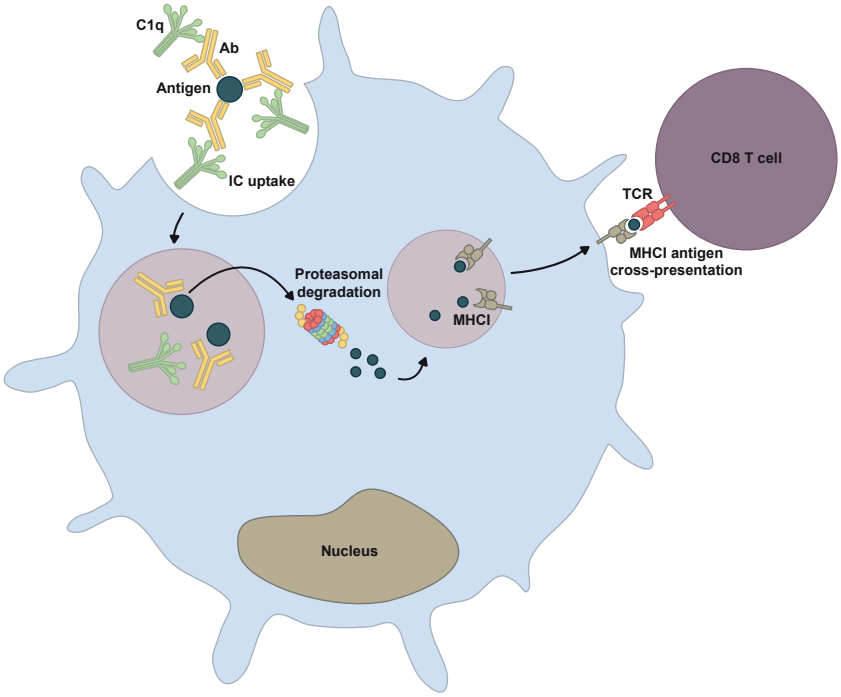

Figure 8. C1q-dependent dendritic cell cross-presentation of antigen-antibody complexes. Soluble protein antigen binds to antibodies in circulation to form antigen-antibody complexes. C1q facilitates the uptake of these complexes by dendritic cells via an as yet undefined uptake route *in vivo*. After antigen uptake, cross-presentation takes place and MHCI molecules are loaded with processed peptides and presented on the cell surface to $CD8^+$ T cells.

By using FcγRI/II/III/IV-deficient (FcγR quadruple$^{-/-}$) mice, we show that antibody-mediated antigen uptake was not hampered but slightly increased in all APC subsets compared to wildtype mice *in vivo*. Detailed characterization study of the FcγR quadruple$^{-/-}$ mice has been done by Fransen *et al.* (29) and no significant differences in circulating levels of C1q or other complement factors were observed. A likely explanation for the enhanced uptake in FcγR quadruple$^{-/-}$ mice is the prolonged presence of antigen in circulation since the clearance of antigen was lower in mice lacking all FcγR. Elimination of circulating antigen-antibody IC is generally assumed to be mediated by Kupffer cells and endothelial cells expressing FcγR in the liver. Although antigen uptake by APC subsets is FcγR independent, we showed that CD8α$^-$ DC antigen cross-presentation was hampered in the absence of FcγR. On the other hand, CD8α$^+$ DCs could still cross-present antigen regardless of the presence of FcγR. These results are in line with earlier work using FcRychain$^{-/-}$ mice where it was shown that FcR γ-chain activation is necessary for CD8α$^-$ DCs to enable MHCI cross-presentation (30). Taken together, we show that C1q drastically affects *in vivo* circulating antibody-mediated antigen uptake and thereby controlling antigen presentation efficiency by splenic APC subsets to T cells, a process which is mostly independent of FcγR targeting.

In contrast to the *in vivo* results in the present study, we have previously shown in FcRychain$^{-/-}$ BMDCs that IC uptake *in vitro* is FcγR dependent (7). Here, we show that *in vitro* uptake of IC in FcγR quadruple$^{-/-}$ BMDCs is also hampered compared to wildtype BMDCs, suggesting a distinct contribution of FcγR in antibody-mediated antigen uptake *in vitro* compared to *in vivo*. By adding additional C1q, antigen uptake was highly improved in wildtype APC subsets *in vitro*. The uptake in FcγR quadruple$^{-/-}$ APC subsets could be increased by the presence of C1q, however the levels are only comparable with those in wildtype cells without adding additional C1q. These results indicate that although C1q can facilitate the uptake of antigen-antibody complexes, FcγR are the more dominant uptake receptors *in vitro* compared to C1q mediated uptake, in contrast to their role *in vivo*. One possible explanation is that FcγR *in vivo* are more important in IC clearance from the circulation by FcγR expressing cells in the liver than in IC uptake by DCs in the spleen. Another possible explanation could be the fact that DCs *in vivo* are strategically positioned for efficient take up of antigen to initiate adaptive immunity. Studies on the anatomy of mouse spleen show high organization of immune cells in different zones within the spleen. DCs in the spleen are a heterogeneous population that can be distinguished into numerous smaller subsets according to specific markers. It has been shown that CD8α$^+$ DCs that express higher levels of DEC205/CD205 are more restricted to periarterial lymphoid sheaths (PALS) in the spleen, whereas CD8α$^-$ DCs that express DCIR2 are restricted to the bridging region of the marginal zone (MZ) (31). Moreover, it has been shown that Langerin/CD207$^+$CD103$^+$CD8α$^+$ DCs were mainly localized to the MZ, but migrated into the T cell zone for cross-presentation after phagocytosis of apoptotic cells (32). Since distinct DC subsets are distributed differently

in the spleen, there is a possibility that DCs expressing a receptor for C1q-mediated IC uptake is better positioned in the spleen and therefore having better access to circulating IC compared to DCs that lack C1q receptor. Considering the fact that we only found a small percentage of antigen positive cells within each APC subset after i.v. injection, it is possible that this is a subpopulation that is either expressing high levels of C1q receptor and/or better positioned in the spleen. Further anatomic studies on the spleen are needed to determine the distribution of subpopulations expressing FcγR and C1q receptors.

Many C1q receptors have been described over the years, however it is still unclear which C1q receptor is expressed on DCs to mediate IC uptake (14, 33, 34). Both the collagen-like region and the globular head are suggested as binding sites for C1q receptors. Several studies demonstrated that a monomeric IgG binding to one C1q head domains is of low affinity and gives poor complement activation (35, 36). More recent studies showed that IgG molecules form ordered hexamerix structures after binding to antigen that bind C1q with high avidity and promote efficient complement activation (16, 37). By using different Ab mutants that either promote or inhibit hexamerization in solution, they showed that IgG hexamerization is prerequisite to C1q binding and C1 activation. These findings present possibilities to further fine-tune C1q-mediated IC uptake by DCs and thereby enhancing immune responses.

In conclusion, we have shown that antibody-mediated antigen uptake from blood circulation and presentation by APCs *in vivo* is mainly controlled by C1q, independent of FcγR. Further studies on C1q-mediated IC uptake mechanisms by DCs may be relevant for the design of vaccination strategies for optimal induction of T cell immunity against cancer and infectious diseases.

MATERIALS AND METHODS

Mice

All animal experiments in this paper have been approved by the review board of Leiden University Medical Center. C57BL/6 (BL/6) mice were purchased from Charles River laboratories. FcγRI/II/III/IV-deficient (FcγR quadruple$^{-/-}$) (29) and C1q-deficient (C1qa$^{-/-}$) mice were on C57BL/6 genetic background (38). OTI mice (CD8^{+} T cell transgenic mice expressing a TCR recognizing the OVA derived K^{b} associated epitope SIINFEKL) and OTII mice (CD4^{+} T cell transgenic mice expressing a TCR recognizing the OVA derived Th epitope ISQAVHAAHAEINEAGR in association with IA^{b}) were bred and kept at the LUMC animal facility under SPF conditions. All mice were used at 8-12 weeks of age.

In vivo formed OVA-IgG complexes

Mice were intravenously (i.v.) injected with 100µg polyclonal rabbit anti-OVA IgG (Ab, ICN Biomedicals). After 30 min of antibody circulation, mice were injected i.v. with 5µg Ovalbumin (OVA, Worthington Biochemical Corporation) or OVA conjugated with Alexa Fluor 647 (Life Technologies).

Antigen presence in splenic APC subsets

Mice with *in vivo* formed OVA-Ab complexes or only OVA (Alexa Fluor 647 labeled) were sacrificed at different time points. Spleens were isolated and dissociated with Liberase (Thermolysin Low, research grade, Roche) for 20 min at 37°C. The antigen presence was measured by the percentage of Alexa Fluor 647 positive cells and the mean fluorescence intensity (MFI) within each APC subset. Background fluorescence levels were determined by naïve mice without any injections. APC subsets were gated according to the following markers: $CD8\alpha^+$ DCs ($CD11c^{high}CD11b^{low}CD8^+$), $CD8\alpha^-$ DCs ($CD11c^{high}CD11b^{high}CD8^-$), pDCs ($CD11c^{int}CD11b^{low}CD45R^+Ly6C^+$) and macrophages ($CD11c^{low}CD11b^{high}F4/80^+$).

Ex vivo antigen presentation

Spleens from mice with *in vivo* formed OVA-Ab complexes or only OVA were isolated at different time points. Sorting of APC subsets was performed by BD FACSAria II SORP (BD Biosciences). Each APC subset (50.000 cells) was incubated with CFSE labeled and purified OTI (50.000) or OTII cells (50.000) in a 96 well round bottom plate. $CD8^+$ and $CD4^+$ T cell proliferation was measured after 4 days by flow cytometry.

Quantification of ovalbumin in mouse serum

Age- and weight-matched naïve mice received 100µg polyclonal rabbit anti-OVA IgG i.v. followed 30 minutes later by 5µg Alexa Fluor 647 conjugated Ovalbumin (Life Technologies) i.v.. At indicated time points 50µl blood was withdrawn from the lateral tail vein and serum was collected. 5µl serum was mixed with sample buffer, heated at 95°C for 5 minutes and loaded on SDS/PAGE. Fluorescent Ovalbumin was quantified directly from the SDS/PAGE gels by using a Typhoon 9410 Variable mode imager (GE Healthcare Bio-Sciences) and ImageQuant TL v8.1 software (GE Healthcare Life Sciences), indicated by relative pixel intensity.

C1q depletion *in vivo*

C57BL/6 mice were intraperitoneal injected with 1mg anti-C1q mAb JL-1, which recognizes the collagen-like region of mouse C1q (27). Two hours later, mice were injected i.v. with 100µg polyclonal rabbit anti-OVA IgG followed by i.v. injection of OVA conjugated with Alexa Fluor 647 30min later. Antigen presence in spleens was analyzed after 24h. C1q in mouse serum was measured 2h and 24h after anti-C1q mAb injection with the use of C1q ELISA (39).

Antigen uptake and presentation by DCs *in vitro*

Bone marrow cells from C57BL/6 or FcγR quadruple$^{-/-}$ mice were cultured in the presence of 30% R1 supernatant from NIH3T3 fibroblasts transfected with GM-CSF for 10 days. The generated bone marrow dendritic cells (BMDCs) were incubated with titrated amounts of Alexa 488 labeled or unlabeled OVA IC for 1h. Antigen uptake (OVA IC Alexa 488) was measured by flow cytometry indicated by MFI. BMDCs that were incubated with unlabeled OVA IC for 1h were washed and incubated with CFSE labeled OTI cells. CD8^{+} T cell proliferation was measured after 3 days by flow cytometry.

Spleen cell suspensions from naïve C57BL/6 or FcγR quadruple$^{-/-}$ mice were depleted from B cells (Anti-mouse CD45R/B220 magnetic particles, BD Biosciences) and incubated with 1μg/ml Alexa 488 labeled OVA and 300μg/ml rabbit anti-OVA IgG (LSBio) with or without 10μg/ml hC1q (kindly provided by Cees van Kooten, LUMC). Antigen uptake in APC subsets was measured after 1h with flow cytometry indicated by MFI.

Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) test. Tukey's *post hoc* test was performed to correct for multiple comparisons. The following indications are used in all figures: n.s non-significant, * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

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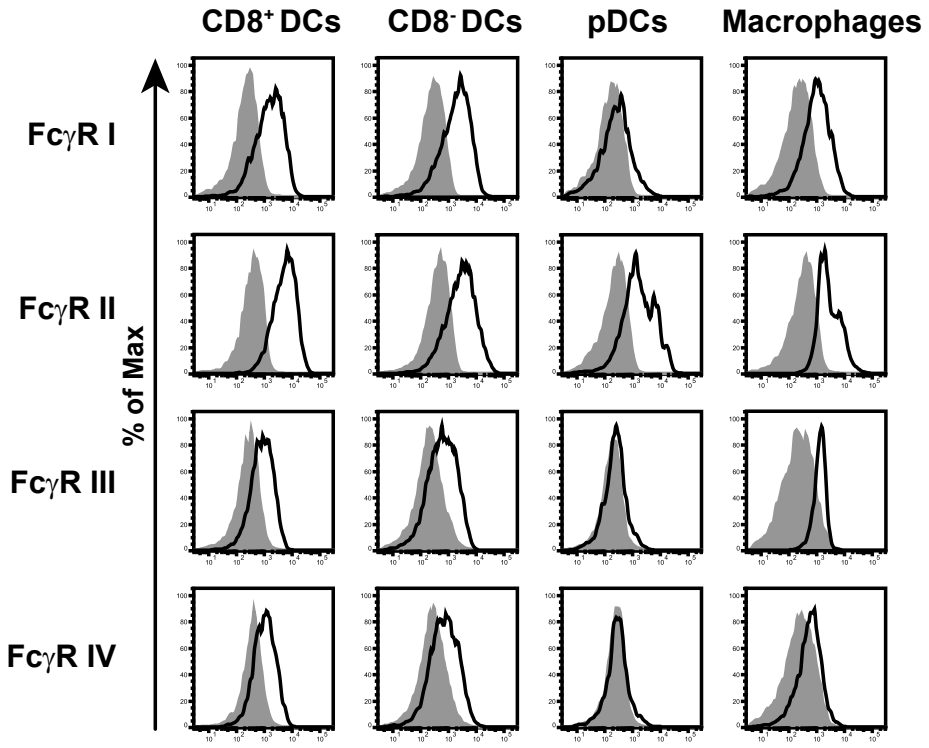
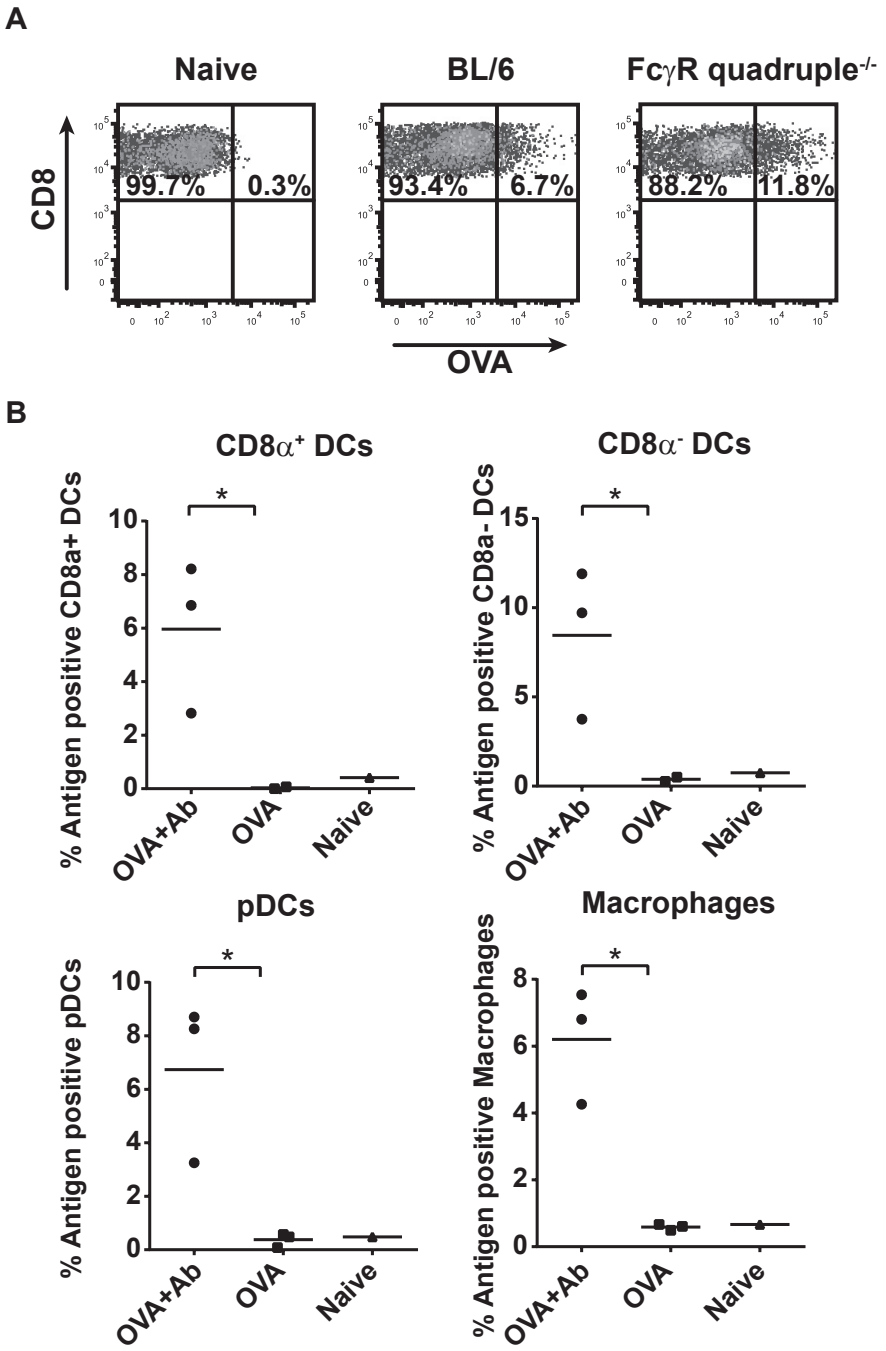
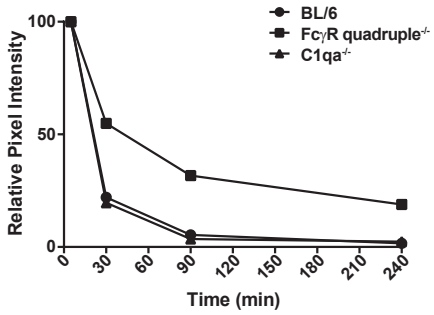


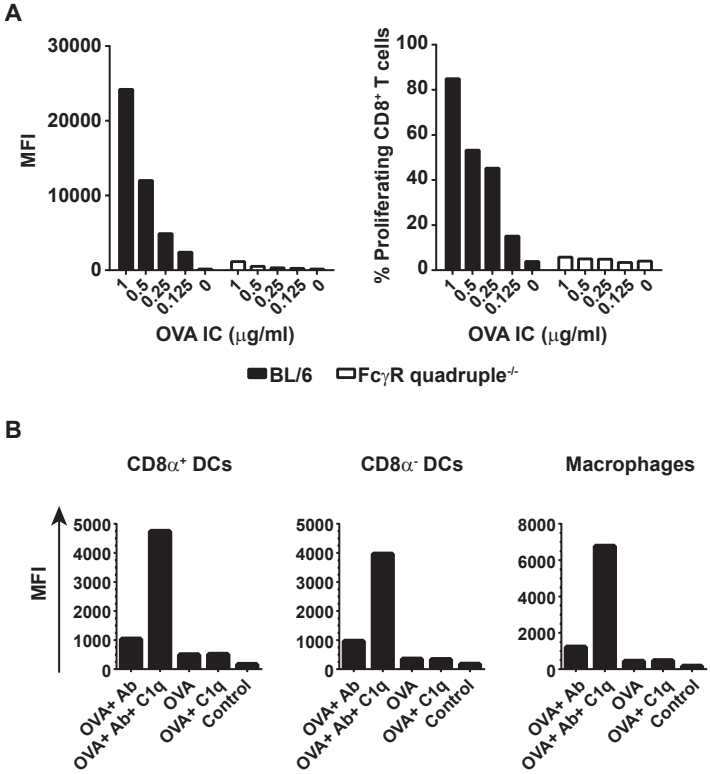
Supplemental Figure 1. FcγR expression on spleen APC subsets. Spleen cells from naïve BL/6 and FcγR quadruple$^{-/-}$ mice were stained with FcγRI-IV antibodies. FcγR expression was measured on each APC subset from BL/6 mice with flow cytometry (black histograms) compared to FcγR quadruple$^{-/-}$ mice (grey histograms). Representative FACS data are shown here for 3 experiments.



Supplemental Figure 2. Antigen-antibody IC uptake in APC subsets. Mice we injected as described in figure 2. Representative FACS plots of antigen uptake in CD8α⁺ DCs from BL/6 and FcγR quadruple$^{-/-}$ mice **(A)**. FcγR quadruple$^{-/-}$ mice were injected i.v. with 100µg anti-OVA Ab and 30min later i.v. with 5µg OVA (Alexa Fluor 647 labeled) or with OVA (Alexa Fluor 647 labeled) only without Ab. Antigen presence in spleen APCs was measured after 24h by flow cytometry **(B)**. Representative data for 4 experiments.



Supplemental Figure 3. Antigen clearance in mouse serum. BL/6, FcγR quadruple$^{-/-}$ and C1qa$^{-/-}$ mice (each dot represents one mouse) were injected with 100µg anti-OVA Ab i.v. followed by 5µg OVA (Alexa Fluor 647 labeled) i.v. after 30min. OVA antigen clearance from mice serum was analyzed by withdrawing blood at the indicated time points. Serum was loaded on SDS/PAGE and the amount of fluorescent OVA was quantified, indicated by relative pixel intensity. Representative data are shown for 4 independent experiments.



Supplemental Figure 4. Antigen-antibody IC uptake by DCs *in vitro*. BMDCs from BL/6 and FcγR quadruple$^{-/-}$ mice were incubated with titrated amounts of Alexa 488 labeled or unlabeled OVA IC for 1h. Antigen uptake (OVA IC Alexa 488) was measured by flow cytometry indicated by MFI. BMDCs that were incubated with unlabeled OVA IC for 1h were washed and incubated with CFSE labeled OTI cells. CD8$^+$ T cell proliferation was measured after 3 days by flow cytometry. Representative data are shown for 4 experiments **(A)**. Spleen cells from FcγR quadruple$^{-/-}$ mice were depleted for B cells and incubated with different combinations of anti-OVA Ab, OVA (Alexa Fluor 488 labeled) and C1q for 1h. Antigen uptake in APC subsets was measured by flow cytometry, indicated by MFI. Representative FACS data are shown for 3 experiments **(B)**.