

MACROPHAGES FROM C3 DEFICIENT MICE HAVE IMPAIRED POTENCY TO STIMULATE ALLOREACTIVE T CELLS

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Abstract

Impaired T cell reactivity is a feature of C3 deficient mice in several disease models. The mechanism behind the reduced T cell response is however poorly understood. We explored the hypothesis that antigen-presenting cells (APCs) from C3^{-/-} mice have impaired potency to stimulate antigen-specific T cells, in an alloantigen dependent model. Our results show that C3^{-/-} macrophages have reduced ability to elicit alloreactive T cell responses *in vitro* and *in vivo*, affecting both the primary and secondary responses. The C3 status of donor macrophages had a major impact on the CD4 T cell response. The impaired CD4 T cell response was associated with reduced expression of MHC class II on the surface of C3^{-/-} macrophages, without loss of class II gene expression. Furthermore, inhibition of C3 gene expression in C3^{+/+} macrophages reduced their ability to stimulate alloreactive T cells, suggesting that endogenous production of C3 could in part contribute to the potency of APCs. Our data provide compelling evidence that C3 deficiency modulates the potency of APCs to stimulate the T cell response, suggesting a critical role for complement in the maintenance of APC function. This could offer a partial explanation as to why the T cell response is impaired in C3^{-/-} mice.

Introduction

Recent evidence has emphasized the importance of the innate immune system in the establishment of the adaptive immune response¹. The complement system is an important component of innate immunity. In addition to its pivotal role in the regulation of inflammation and host defence, the complement system also participates in regulating the antigen specific immune response^{2,3}. The activation of C3 is the converging point of the three pathways, from where effector functions of complement are generated. Therefore, C3 is vital for participation of the complement system in both the innate and adaptive immune responses.

C3, the most abundant complement protein in serum (1.3 mg/ml in man), is an acute-phase protein and rapidly responds to many noxious stimuli including microbial infection and tissue injury. The liver is the primary source for the synthesis of C3⁴; however, many other specialized cells have the capacity to synthesise C3 including myeloid derived cells and parenchymal cells⁵⁻⁹. These cells synthesize C3 spontaneously or in response to cytokine stimulation. Extrahepatic synthesis of C3 is thought to serve important physiological and pathological functions, in terms of regulation of the inflammatory response, host defence and allograft rejection¹⁰⁻¹³.

Human and animal studies have demonstrated the importance of C3 in the adaptive immune response^{14,15}. C3^{-/-} mice exhibit an impaired B cell response to both T cell-independent and –dependent antigens. The mechanism of complement regulation of the B cell response appears to involve the interaction of complement activation products (C3b, C3d) and complement receptors 1 and 2 (CR1/CD35, CR2/CD21) on cell surfaces, thus increasing the retention of antigen in lymphoid tissue and enhancing the B cell response^{16,17}. In addition to the regulation of B cell responses, participation of complement in T cell immunity has more recently been described. Impaired T cell responses in C3^{-/-} mice were reported in several disease models including viral infection, autoimmune disease and transplantation¹⁸⁻²¹. However the means by which C3 deficiency leads to impaired antigen-specific T cell reactivity in such conditions remains unclear.

Although T cell priming is impaired in C3^{-/-} mice in the influenza virus model, T cell priming is normal in mice deficient of CD35 and CD21¹⁸. This suggests that the complement receptors CD35 and CD21 are not involved in the development of T cell reactivity; therefore, the mechanism for C3 regulating T cell responses may differ from that regulating the B cell response. Previous studies have found that antigen coupled covalently to C3b is protected from excessive proteolytic degradation in the endosomal/lysosomal compartment of the MHC class II pathway in B cells. This enhances peptide loading onto MHC class II molecules and increases stable MHC class II molecules in this compartment²²⁻²⁴. These studies suggest that activated C3 fragment functions as a “chaperone” in the MHC class II pathway of APCs, which is essential for maintaining the normal

function of APCs. It also raises the possibility that in the absence of C3, APC function may be defective.

In this study, we explored the hypothesis that C3 deficiency reduces the potency of APCs to stimulate T cell responses. We examined an alloantigen dependent model of T cell stimulation using macrophages from C57BL/6 mice as stimulators and splenic T cells from BALB/c mice as responders. We assessed the alloreactive T cell response *in vitro* and *in vivo* following stimulation with C3^{+/+} and C3^{-/-} macrophages. We assessed the gene and cell surface protein expression of MHC class II and co-stimulatory molecules. We also investigated the role of endogenous production of C3 by macrophages in alloreactive T cell response and the way that this production might increase the capacity of macrophages to stimulate alloreactive T cells.

Materials and Methods

Mice. Homozygous C3^{-/-}, C4^{-/-} and factor B (FB)^{-/-} mice were derived by homologous recombination in embryonic stem cells²⁵⁻²⁷ and backcrossed on to the C57BL/6 parental strain for 11 generations. WT (C57BL/6^{H-2b}) mice, BALB/c^{H-2d} mice were purchased from Harlan UK. Male mice (6-7 week old) were used in all experiments. All animal procedures were carried out within the Animals (Scientific Procedures) Act, 1986 UK.

Preparation of macrophages. Resident and thioglycollate-elicited peritoneal macrophages and bone marrow (BM) macrophages were prepared using previously described methods²⁸ (see the details in Supplementary Methods). Macrophages were further purified with CD11b MicroBeads (Miltenyi Biotec UK) according to manufacturer's instruction, unless otherwise specified. Following the purification, 85-90% of cells were found to stain positive with anti-mouse F4/80 as determined by flow cytometry (Supplementary Figure 1). In each experiment, macrophages were prepared from 3-5 mice and pooled together for analysis, unless otherwise stated. Thioglycollate-elicited macrophages were used in the majority of our experiments, unless otherwise specified.

Preparation of T cells. Naive alloreactive T cells were derived from spleens of normal BALB/c mice. Primed alloreactive T cells were derived from BALB/c mice that had received a C57BL/6 skin graft 14 days in advance. CD3⁺, CD4⁺ and CD8⁺ T cells were prepared from splenocytes using Spin-SepTM Enrichment Cocktail Kit (StemCell Technology Inc., UK). Following the isolation, the purity of the T cell preparation was routinely >90%, as determined by flow cytometry. T cells were prepared from 3-6 mice depending on the number of purified T cells needed in each experiment.

Skin grafting. Tail skin from the donor animals was grafted onto the left flank of the recipients under enflurane anaesthetic. The graft site was covered with paraffin embedded gauze and a dressing of dry gauze and clear tape. The dressing was removed on day 7, and the graft was assessed visually on a daily basis for signs of rejection. The time to rejection was determined as the day on which >90% of the graft area was necrotic.

Analysis of alloreactive T cell response *in vitro*. Irradiated macrophages (2000 Rad) (2×10^5) and 2×10^5 purified alloreactive T cells ($CD3^+$ or $CD4^+$ or $CD8^+$) were co-cultured in RPMI-1640 containing 10% heat inactivated FCS, 50 μ M 2-mercaptoethanol, 100 U/ml penicillin, and 100 μ g/ml streptomycin for up to 5 days, unless otherwise specified. For measuring the production of IFN- γ and IL-2 by T cells, co-culture supernatants were collected and used to perform ELISA. T cell proliferation was assessed at 96h after culture by measuring the incorporation of 3 H-thymidine (see Supplementary Methods) or using the CellTiter 96[®] AQueous Non-Radioactive Cell Proliferation Assay Kit (Promega, UK) in a 3 day co-culture, according to manufacturer's instructions.

Analysis of alloreactive T cell response *in vivo*. For priming of alloreactive T cells, BALB/c mice, at day 0, were given either $C3^{+/+}$ or $C3^{-/-}$ donor macrophages (5×10^6 , irradiated at 2000 Rad) by i.p. injection; 14 days after immunization the mice received a C57BL/6 skin graft. On day 21, some of the mice were killed; T cell responses in those BALB/c mice were then measured *ex vivo* by mixed lymphocyte reaction (MLR). The remaining mice were kept for monitoring skin graft rejection. For reactivation of alloreactive T cells, BALB/c mice, at day 0, were primed with a C57BL/6 skin graft; 14 days after priming the mice were given either $C3^{+/+}$ or $C3^{-/-}$ donor macrophages (5×10^6 , irradiated at 2000 Rad) by i.p. injection. After a further 7 days, the mice were killed; T cell responses in those BALB/c mice were then measured *ex vivo* by MLR.

Mixed lymphocyte reaction (MLR). Splenocytes (2×10^5) derived from immunized BALB/c mice (H-2^d) and irradiated (2000 Rad) splenocytes (2×10^5) derived from C57BL/6 (H-2^b) were co-cultured in T cell culture medium for up to 5 days. Supernatants were collected for measuring the production of IFN- γ and IL-2 by ELISA.

ELISA. Sandwich ELISA was performed using OptEIA ELISA Set for mouse IL-2 and IFN- γ (BD Biosciences, UK) according to the manufacturer's instruction. Mouse C3 ELISA was performed using sheep anti-mouse C3 (ICN Biomedicals, UK) and HRP-conjugated goat anti-mouse C3 (Nordic, The Netherlands). The standard was pooled normal mouse serum, which has a reported concentration of 0.7mg/ml²⁹.

Conventional RT-PCR. cDNA was synthesized as described³⁰ from macrophages. PCR was carried out with 2 μ l diluted cDNA (reflecting 0.2 μ g of total RNA), 12.5 pmol of each 3' and 5' primer pair for MHC Class II A β chain, CD40, ICAM-1, and B7.2 (CD86) in 25 μ l of reaction buffer (Promega, Southampton, UK). The PCR cycle consisted of 1 min at 94°C, 1 min at 62°C, and 1 min at 72°C. Amplified PCR products were visualized after electrophoresis on 1.2% agarose gel containing ethidium bromide. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers, 12.5 pmol each, were also added in every reaction as an internal control. PCR primer sequences and product sizes are shown in Supplementary Methods.

C3 gene silencing. C3^{+/+} macrophages (5 x 10⁵) were transfected with 100 nM of either C3 siRNA (sense sequence: 5'-GGAAUUCAACUCAGAUAAAGtt-3'; anti-sense sequence: 5'-CUUAUCUGAGUUGAAUUCctt-3') or silencer negative control siRNA (Ambion Europe Ltd.) using siRNA transfection kit (Qiagen, UK). After 48h transfection, the reduction of C3 synthesis by macrophages was measured by quantitative real-time RT-PCR and ELISA. After 48h transfection, macrophages were also co-cultured with primed alloreactive T cells for up to 2 days. Supernatants were collected and used for measuring the production of cytokine by ELISA.

Flow cytometry. Macrophages (2 x 10⁵ cells) and splenocytes (1 x 10⁶ cells) were stained with either Fluorescein isothiocyanate (FITC)- or Phycoerythrin (PE)-conjugated antibody or the appropriate isotype control antibody. Antibody reagents are described in Supplementary Methods.

Statistical analysis. ELISA or T cell proliferation assays were performed in 3-6 replicate wells of the co-cultures. Results were expressed as a mean \pm SEM and subjected to statistical analysis. Student's *t* test or Two-way ANOVA or variance component regression analysis was used where appropriate to determine significant differences between samples. Graft survival was analyzed by Log-rank test. All experiments were repeated at least three times.

Results

1. C3^{-/-} macrophages have reduced potency to stimulate alloreactive T cells

Initially we asked if C3^{-/-} and C3^{+/+} macrophages have different potencies in stimulating alloreactive T cells. We examined both naïve and primed T cell responses. Naïve or primed alloreactive T cells, respectively, were co-cultured with either C3^{+/+} or C3^{-/-} macrophages. After 3-day co-culture, the concentrations of secreted IL-2 and IFN- γ were significantly lower with stimulation by C3^{-/-}

macrophages compared to $C3^{+/+}$ macrophages. Both primed and non-primed T cell responses were affected by the C3 status of donor macrophages, though the effect was more pronounced with antigen experienced T cells. Syngeneic controls, where donor macrophages ($H-2^b$) were co-cultured with donor T cells ($H-2^b$), exhibited a very low level of IL-2 and IFN- γ production (Fig. 1A). Based on the result that the primed T cell response, compared to naïve T cell response, was more sensitive to the C3 status of donor macrophages, we decided to use primed alloreactive T cells in the remaining *in vitro* experiments.

In our initial study, we also assessed the proliferative T cell response after stimulation by allogeneic macrophages. T cell proliferation following stimulation by $C3^{-/-}$ macrophages was reduced compared to $C3^{+/+}$ macrophages (Fig. 1B).

2. C3 gene silenced macrophages have lowered ability to stimulate alloreactive T cells

Since macrophages derived from C3 deficient mice exhibited a low level of T cell stimulation, we wished to verify if this effect was attributable to C3 production by the macrophages. For this purpose, we inhibited C3 gene expression in $C3^{+/+}$ macrophages using small interfering RNA and measured the ability of these macrophages to stimulate alloreactive T cells compared with control-treated macrophages. As shown in Figure 2, treatment with interfering RNA significantly reduced the amount of C3 mRNA and C3 secretion, and co-ordinately lowered the level of T cell activation stimulated by these macrophages. However, treatment with irrelevant interfering RNA did not show significant reduction in the amount of C3 produced or in the level of T cell activation. This is consistent with an effect of endogenous production of C3 on the potency of macrophages to stimulate alloreactive T cells.

3. Dependence of the CD4 T cell response on the C3 status of donor macrophages

To determine which of the two main subpopulations of T cells, CD4 or CD8, was more reactive to the C3 status of the stimulator cells, we performed a 5-day co-culture experiment where the purified CD3⁺, CD4⁺ and CD8⁺ T cells were stimulated respectively by $C3^{+/+}$ or $C3^{-/-}$ macrophages. We found that the production of IL-2 (Fig. 3A) and IFN- γ (Fig. 3B) was lower in all preparations of the T cells stimulated by $C3^{-/-}$ macrophages compared with the T cells stimulated by $C3^{+/+}$ macrophages. However, this complement dependent cytokine production was more marked in CD4⁺ T cells than CD3⁺ and CD8⁺ T cells. These data indicate that both CD4⁺ and CD8⁺ T cell responses were influenced by the C3 status of donor macrophages, but CD4⁺ T cells were the more sensitive, suggesting that the effect of C3 was primarily on the MHC class II dependent pathway of donor antigen presentation.

The macrophages in the above experiments were prepared from the inflamed peritoneum of thioglycollate treated mice. We also prepared macrophages from the normal peritoneal cavity and BM of WT and $C3^{-/-}$ mice without further purification with CD11b beads. Then, we stimulated them with LPS (100ng/ml) for 24 hours and subsequently co-cultured these macrophages with primed alloreactive $CD4^{+}$ T cells and assessed the T cell response. We found that the production of IFN- γ and IL-2 was consistently lower in T cells stimulated by $C3^{-/-}$ macrophages compared with the T cells stimulated by $C3^{+/+}$ macrophages, for macrophages from both of these sources (Fig. 3C). $C3$ -dependent T cell stimulation was thus a feature of macrophages regardless of source or inflammatory status.

4. Reduced capacity of $C3^{-/-}$ macrophages for immune stimulation *in vivo*

In the next set of experiments, we determined whether $C3^{-/-}$ macrophages have a reduced ability to elicit an alloreactive T cell response *in vivo*. We examined two protocols. The first tested whether T cell priming was affected by the $C3$ status of donor macrophages. At day 0, BALB/c mice were given either $C3^{+/+}$ or $C3^{-/-}$ C57BL/6 donor macrophages. As a read out of the T cell response, at day 14 after the injection of macrophages the mice received a skin graft from a C567BL/6 donor. On day 21, some mice were killed and their splenocytes were used for analysis of the alloreactive T cell response *ex vivo* using MLR. T cell activation was assessed by measuring the secretion of IL-2 and IFN- γ using ELISA. Other mice were kept for monitoring skin graft rejection. The T cell response in mice that had received $C3^{-/-}$ macrophages was impaired, with significant lowering of IL-2 (Fig. 4A) and IFN- γ (Fig. 4B) production and prolonged graft survival (Fig. 4C), compared to mice that had received $C3^{+/+}$ macrophages. Thus immunization with $C3^{-/-}$ macrophages resulted in defective T cell priming *in vivo*.

We then tested whether the reactivation of previously primed T cells was affected by the $C3$ status of donor macrophages. At day 0, BALB/c mice received a C57BL/6 donor skin graft, and after 14 days the skin-grafted mice were given either $C3^{+/+}$ or $C3^{-/-}$ macrophages. On day 21, the mice were killed and their splenocytes were used for analysis of the alloreactive T cell response *ex vivo* using MLR. We found that primed alloreactive T cells re-stimulated by $C3^{-/-}$ macrophages *in vivo* produced less IFN- γ , compared to those re-stimulated by $C3^{+/+}$ macrophages *in vivo* (Fig. 4D). However, the production of IL-2 was no significantly different between re-stimulation with $C3^{-/-}$ and $C3^{+/+}$ macrophages (data not shown). Although the reason for this discrepancy between the IFN- γ and IL-2 responses is unclear, IFN- γ is the hallmark of Th1 lymphocytes, which are thought to be the predominant T cell population involved in the alloimmune response. On the basis of these results, both T cell priming and reactivation were impaired when stimulated by $C3^{-/-}$ macrophages.

5. Reduced cell surface expression of MHC class II in $C3^{-/-}$ macrophages

Full activation of T cells requires stimulating signals from both antigen (allo-MHC) and co-stimulatory molecules present on APCs. In order to assess the expression of MHC and co-stimulatory molecules, we first examined thioglycollate-elicited peritoneal macrophages. Using flow cytometry we found that the surface expression of MHC class II was reduced in $C3^{-/-}$ cells compared with $C3^{+/+}$ cells (Fig. 5A and 5B). However, cell surface staining for co-stimulatory molecules showed no reduction of expression of B7.2, ICAM-1 or CD40 on the $C3^{-/-}$ cells (data not shown). To confirm our observations in a second macrophage population, we examined BM-derived macrophages, both before and after activation with LPS. Results showed that activated BM-derived macrophages exhibited higher expression of MHC class II than resting BM-derived macrophages and the surface expression of MHC class II was lower in $C3^{-/-}$ cells compared with $C3^{+/+}$ cells by flow cytometry (Supplementary Figure 4), confirming our observations with peritoneal macrophages. Given the importance of the MHC/peptide complex on the surface of APCs in providing a cognate interaction with T cells, reduced MHC class II surface expression may contribute to the weak property of $C3^{-/-}$ APCs in allo-stimulation.

In addition we examined mRNA expression for MHC and co-stimulatory molecules, to ensure that the recombinant DNA procedures used to generate the $C3^{-/-}$ mice had not interfered with the expression of non-targeted genes that were essential for immune stimulation. Using semiquantitative PCR we found that all the genes examined (MHC class II, B7.2, ICAM-1 and CD40) were transcribed, and that the level of gene expression was not reduced in the $C3^{-/-}$ macrophages. The RT-PCR data for MHC class II are shown in Figure 5C. Real-time PCR results confirmed that there was no loss of mRNA expression for MHC class II, B7.2, ICAM-1 and CD40 (data not shown). Thus, defective immune stimulation by $C3^{-/-}$ macrophages was not due to loss of essential co-stimulatory molecules. Moreover, the lower frequency of MHC class II expressing cells in $C3^{-/-}$ macrophages was not caused by a decrease in gene transcription. This raises the possibility that reduced surface expression of MHC class II in the $C3^{-/-}$ mouse cells could be due to a post-transcriptional defect of MHC class II expression.

6. Extracellular C3 does not explain the different ability of $C3^{-/-}$ and $C3^{+/+}$ macrophages to stimulate alloreactive T cells *in vitro*

Next, we explored how endogenous production of C3 might increase the capacity of macrophages for stimulation of alloreactive T cells. Previous studies have shown that C3 attached covalently to macrophages can result in stronger T cell stimulation, presumably via interaction of macrophage-bound C3 with complement receptors on T cells^{31,32}. To assess the potential for this interaction in our experiments, we prepared thioglycollate-elicited macrophages and performed staining for

surface-bound C3 fragment by flow cytometry. There were no detectable differences between C3^{+/+} and C3^{-/-} macrophages (Fig. 6A). Therefore the differences in T cell stimulation by C3^{+/+} and C3^{-/-} macrophages *in vitro* could not be explained by macrophage-adherent C3.

Another possibility is that soluble fragments released after the cleavage of secreted C3 could interact with C3a and C5a receptors on APCs or T cells^{33,34}. We measured the amount of C3 secreted during co-culture in our experiments with irradiated C3^{+/+} macrophages, and found only small quantities of C3 were produced (4-14ng/ml). To determine if this small amount of secreted C3 could explain the functional difference between C3^{+/+} and C3^{-/-} macrophages, we performed a reconstitution study. We co-cultured C3^{-/-} macrophages with primed T cells in the presence of either 0.005% or 0.05% fresh WT mouse serum (which contains at least 10x the amount of C3 as secreted by our irradiated macrophages) or C3^{-/-} serum at the same concentration, and measured the T cell response. As shown in Figure 6B, the production of IFN- γ was not significantly increased by the addition of C3 in the co-culture medium. The response with C3^{-/-} stimulators remained far below that with C3^{+/+} stimulators.

Additionally, we performed co-culture of WT peritoneal macrophages with primed T cells in the presence of C3 inhibitor (anti-C3 antibody or soluble complement regulator, Crry-Ig³⁵). Compared with isotype-matched control Ig, neither of these complement inhibitors led to a reduction in T cell stimulation, as measured by IL-2 or IFN- γ release at day 3 of incubation (data not shown).

In all of the aforementioned experiments the difference between C3^{-/-} and C3^{+/+} macrophages in their ability to stimulate alloreactive T cells is not explained either by C3 fragment deposited on the macrophage surface or by the small amount of C3 released by irradiated cells *in vitro*. It is important to note that our data do not exclude the possibility of such effects occurring *in vivo*, thus promoting the interaction of macrophages and T cells.

7. Factor B^{-/-} macrophages have impaired ability to elicit a T cell response

Since C3^{-/-} macrophages originate from an environment in which complement activation is lacking, we postulated that other factors required for complement activation might produce a similar effect on macrophage function. We therefore assessed the influence of Factor B (alternative pathway) and C4 (classical and lectin pathways) on macrophage induced T cell stimulation. Macrophages derived from C3^{-/-}, Factor B^{-/-} and C4^{-/-} mice were evaluated in T cell cytokine and proliferation assays. The results are presented in Figures 7A and 7B, respectively. These confirm that the macrophage-effect on the stimulation of primed T cells is dependent on C3. Additionally, the data show that macrophages from Factor B^{-/-} mice elicit weaker T cell responses compared to wild type macrophages, although the effect of Factor B is not as pronounced as for C3. In contrast, the T cell response with C4^{-/-} macrophages was unimpaired. This suggests that the effect of C3 in augmenting

the T cell response may partly depend on activation through the alternative pathway, but not through the classical and lectin pathways.

Discussion

While the effect of complement on B cell regulation has been comprehensively studied, the way in which C3 stimulates the T cell response is poorly understood. Our results show that macrophages from C3 deficient mice have reduced ability to stimulate alloreactive T cells. These findings, shown both for T cell priming and T cell reactivation, *in vitro* and *in vivo*, suggest a critical role for complement in the maintenance of APC function.

The C3 knockout mouse used in our study was an 11th generation backcross that exhibited indefinite skin graft survival with the wild type strain (C57BL/6). Thus it is unlikely that the C3 deficient and wild type macrophages had any significant differences at histocompatibility loci. Moreover we were unable to demonstrate defects in macrophage gene expression of several vital pathways involved in immune stimulation. Therefore the differences in T cell stimulation obtained with wild type and C3 deficient macrophages were not obviously explained by defects of other genes involved in antigen presentation. Although a mutant strain of mouse with spontaneous deficiency of C3 was not available, an inbred guinea pig strain with partial functional deficiency of C3 exists, and peritoneal macrophages from these guinea pigs were shown to elicit reduced T cell responses, against conventional antigens³². These observations offer strong support that the differences between deficient and wild type macrophages detected in our study are due to lack of C3.

The mechanism by which C3 enhances the T cell stimulatory capacity of macrophages has a number of possible explanations. The simplest of these is that macrophages derived from C3 deficient mice have an immature phenotype and are thus less competent to function as APC. The reduced cell surface MHC class II expression and associated defect of CD4 T cell stimulation found in our study could be an example of this. *In vivo*, complement activation can occur spontaneously under physiological conditions and increase rapidly in response to inflammatory stimuli^{36,37}. Complement effector products may directly activate macrophages through engagement of C3a or C5a receptors on the macrophage surface or through membrane insertion with a sublethal dose of C5b-9^{38,39}. In addition, complement mediated local inflammation could indirectly activate macrophages through cytokine, autocrine and paracrine regulation³⁸. Thus, deficiency of C3 or other activating factors required for the cleavage of C3, such as Factor B, would result in a less mature macrophage phenotype with reduced capacity for stimulating alloreactive T cells. It is also apparent from our results that Factor B had a weaker effect than C3 on the capacity of APCs for T

cell stimulation, suggesting that the alternative pathway (of which Factor B is an essential component) is not the only pathway leading to C3 cleavage in our model. On the other hand, our results identified no significant role for the classical and lectin pathway component C4. This apparent discrepancy requires further investigation.

Another possibility to explain the effect of complement is that membrane-bound fragments of C3 behave as adhesion molecules increasing the contact between macrophages and T cells, which are known to express receptors for such fragments^{40,41}. A similar model has been proposed in the study of conventional antigens^{31,32}. Thus, C3 receptor-ligand interaction may provide a bridge between these two types of cells, facilitating immune adherence and T cell activation. Our study differed from those with conventional antigen, in that alloantigen is synthesized by the donor APC, though may also be taken up by recipient APCs and presented to the immune system. Moreover, we were unable to show that the addition of complement could restore allo-stimulation by C3 deficient mouse macrophages or show an effect of C3 blockade *in vitro*. However, our findings do not preclude a role of C3 for opsonisation of APCs *in vivo*.

A third potential mechanism suggested by our data is that local production of C3 by donor APCs could be crucial to their relationship with T cells. Macrophages are able to synthesise C3 and Factor B, as well as other components of the complement activation pathways in a cytokine-dependent manner^{5,42}. Local production of C3 and other activating factors thus has the ability to alter the microenvironment in the region of the APC *in vivo* and therefore to contribute to their innate and adaptive immune functions. Our findings in C3 gene silencing studies showing a causal relationship between macrophage expression of C3 and their immunoregulatory capacity support the notion that local production contributes to normal macrophage function.

Finally, it has been proposed that C3 taken up with antigen via a complement-receptor dependent mechanism can modulate the presentation of antigen to T cells⁴³. Using B cells as APCs, research has shown that C3-coupled antigen is protected from excessive proteolytic degradation in the endosomal/lysosomal compartment of the MHC class II pathway, suggesting that activated C3 fragment acts as a chaperone in this antigen presenting pathway²²⁻²⁴. It is therefore possible that C3, either synthesised or taken up by macrophages, could act as an adjuvant for alloantigen. Thus it could be speculated that the lowered expression of MHC class II on the surface of C3 deficient APC, presumably as a result of a post transcriptional mechanism, arises from defective antigen processing.

In conclusion our data show that macrophages derived from C3 deficient mice have impaired ability to generate alloreactive T cell responses *in vitro* and *in vivo*. Although it is possible that APCs arising in a complement-deprived environment have defective maturation, the results of C3 gene silencing point to an additional mechanism that is dependent on the local synthesis of C3. This is

the subject of ongoing studies. Thus, defective APC function offers an explanation, at least in part, for C3 deficient mice having impaired T cell responses. Additionally, our data that APC function could depend on the local synthesis of C3 may inform new approaches for vaccine development, cancer therapy and prevention of organ graft rejection.

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Figure legends

Figure 1: C3^{-/-} macrophages have reduced potency to stimulate alloreactive T cells.

Thioglycollate-elicited macrophages were prepared from C3^{+/+} and C3^{-/-} mice. Alloreactive CD3⁺ T cells were prepared from either naïve BALB/c mice or BALB/c mice that had been primed by grafting with C57BL/6 donor skin. Syngeneic CD3⁺ T cells were prepared from C57BL/6 mice. Figure 1A, 2 x 10⁵ macrophages and 2 x 10⁵ T cells were co-cultured for 3 days; supernatants were collected for measuring the production of IL-2 and IFN- γ by ELISA. Figure 1B, 2 x 10⁵ macrophages and 1 or 2 x 10⁵ T cells were co-cultured for 3 days, after which T cell proliferation and cytokine assays were performed. T cell proliferation was assessed using Non-Radioactive Cell Proliferation Assay Kit. The absorbance at 490nm reflecting the amount of formazan in cell culture medium was measured. All data were generated from 3 independent experiments with three mice/group in each experiment, and analyzed by Student's *t* test. *P* values are for comparisons between C3^{+/+} and C3^{-/-} macrophages. *, *P* <0.05; **, *P* <0.005; ***, *P* <0.0001; ns, no significant difference.

Figure 2: C3 gene silenced macrophages have lowered ability to stimulate alloreactive T cells.

Thioglycollate-elicited C3^{+/+} macrophages were transfected with 100 nM of either C3 siRNA or control siRNA using a siRNA transfection kit (Qiagen). After 48h transfection, C3 expression was measured by quantitative real-time RT-PCR (Fig. 2A) and ELISA (Fig. 2B). After 48h transfection, 1x10⁵ macrophages were co-cultured with 1x10⁵ primed alloreactive CD3⁺ T cells for 2 days. Supernatants were collected and used for measuring the production of IL-2 by ELISA (Fig. 2C). All data were generated from 3 independent experiments, with macrophages pooled from 5 mice in each experiment, and analyzed by Student's *t* test. *P* values are for comparisons between untreated C3^{+/+} macrophages and other groups of macrophages, ***, *P* <0.0001; ns, no significant difference.

Figure 3: Dependence of CD4 T cell response on the C3 status of donor macrophages.

In Figure 3A and 3B, thioglycollate-elicited macrophages were prepared from C3^{+/+} and C3^{-/-} mice. Primed alloreactive CD3⁺, CD4⁺ and CD8⁺ T cells were prepared from BALB/c mice. 2 x 10⁵ pooled macrophages from each group were co-cultured with 2 x 10⁵ T cells for up to 5 days. Supernatants were collected for measuring the production of IL-2 (Fig 3A) and IFN- γ (Fig. 3B) by ELISA. In Figure 3C, normal peritoneum or bone marrow (BM) macrophages were prepared from C3^{+/+} and C3^{-/-} mice. The pooled macrophages without further purification with CD11b beads were stimulated with LPS (100ng/ml) for 24 hours, and then co-cultured with primed alloreactive CD4⁺ T cells for 3 days. Supernatants were collected for measuring the production of IL-2 and IFN- γ by ELISA. All data in Figure 3A and 3B were generated from 3 independent experiments, with 5

mice/group in each experiment. Data were analyzed by variance component regression analysis. In Figure 3C, the data were generated from 3 experiments, with 3 mice/group in each experiment, and analyzed by Two-way ANOVA. *P* values are for comparisons between $C3^{+/+}$ and $C3^{-/-}$ macrophages. Six additional T cell stimulation experiments were performed, where CD3 or CD4 T cells were incubated for only 3 days with thioglycollate-elicited macrophages. The data are shown in Supplementary Figure 2.

Figure 4: Reduced capacity of $C3^{-/-}$ macrophages for immune stimulation *in vivo*.

BALB/c mice (n=12/group) were given either $C3^{+/+}$ or $C3^{-/-}$ C57BL/6 thioglycollate-elicited macrophages by i.p. injection. The primed mice then received a C57BL/6 donor skin graft. T cell responses in those BALB/c mice were then measured *ex vivo* by MLR at day 3 of co-culture. The production of IL-2 (Fig. 4A) and IFN- γ (Fig. 4B) were measured by ELISA. Skin graft survival is shown in Figure 4C. Data were analyzed by Log-rank test. An additional experiment is shown in Figure 4D, in which BALB/c mice (n=10/group) were primed by skin grafting from C57BL/6 donors and re-challenged with $C3^{+/+}$ or $C3^{-/-}$ C57BL/6 macrophages. T cell responses in those BALB/c mice were then measured *ex vivo* by MLR. The production of IFN- γ was measured by ELISA (Fig. 4D). In Fig 4A, 4B and 4D, results from each animal are shown as separate data points, and all data points were analyzed by Student's *t* test. *P* values are for comparisons between the two mouse groups. Additional results for MLR at day 1-5 of co-culture are presented in Supplementary Figure 3.

Figure 5: Reduced cell surface expression of MHC class II in $C3^{-/-}$ macrophages

In Figure 5A and 5B, thioglycollate-elicited macrophages were prepared from $C3^{+/+}$ (n=6) and $C3^{-/-}$ (n=6) mice. Cells from each mouse were then stained for surface molecule MHC class II using PE-conjugated anti-mouse Ig or isotype control Ig. Figure 5A, representative histogram plots for $C3^{+/+}$ and $C3^{-/-}$ peritoneal macrophages. Figure 5B, expression levels (mean fluorescence intensities, MFI) of MHC class II on macrophages. Data were analysed by Student's *t* test. Figure 5C, $C3^{+/+}$ and $C3^{-/-}$ peritoneal macrophages prepared from 4 mice in each group were pooled and used for RNA extraction and cDNA synthesis, and subsequently for semi-quantitative PCR. A typical agarose gel shows PCR products for MHC class II, CD40, ICAM-1, B7.2 and GAPDH (internal control). Results are representative of three independent experiments.

Figure 6: Extracellular C3 does not account for the different ability of C3^{-/-} and C3^{+/+} macrophages to stimulate alloreactive T cells

Thioglycollate-elicited macrophages were prepared from C3^{+/+} and C3^{-/-} mice (n=5/group). Figure 6A, C3^{+/+} or C3^{-/-} macrophages and C3^{+/+} macrophages that were pre-incubated with 25% mouse serum at 37°C for 30 min and washed 3 times were stained for deposition of C3 using FITC-conjugated goat anti-mouse C3 IgG F(ab')₂ fragment. In all histogram plots, the control peak (dashed line) corresponds to unstained cells. The detection peak (intact line) shows the binding of C3 antibody. Figure 6B, C3^{+/+} or C3^{-/-} macrophages were co-cultured with primed alloreactive CD4⁺ T cells in the absence or presence of either WT normal serum or C3^{-/-} serum for 5 days. Supernatants were collected and used for measuring the production of IFN-γ by ELISA. Data were analyzed by *t* test and showed no significant difference between untreated C3^{-/-} macrophages and serum treated C3^{-/-} macrophages. Results presented in Figure 6A and 6B are representative of three independent experiments.

Figure 7: Effect of FB^{-/-} and C4^{-/-} macrophages on immune stimulation

Primed alloreactive CD4⁺ T cells were prepared from four BALB/c mice. Thioglycollate-elicited macrophages were prepared from WT (C57BL/6), C3^{-/-}, C4^{-/-} and FB^{-/-} mice (6 mice in each group). 2x10⁵ macrophages from each mouse were co-cultured with 2x10⁵ T cells. Figure 7A, T cell activation was assessed at 3 days after culture by measuring the production of IL-2 and IFN-γ by ELISA. Figure 7B, T cell proliferation was assessed at 96h after culture by measuring the incorporation of ³H-thymidine. Data were analyzed by Student's *t* test. *P* values are for comparisons between complement deficient and complement sufficient macrophages. ns, no significant difference. Two further experiments were undertaken and the results of these confirm the data shown in Figure 7A (see Supplementary Figure 5).

Figure 1A

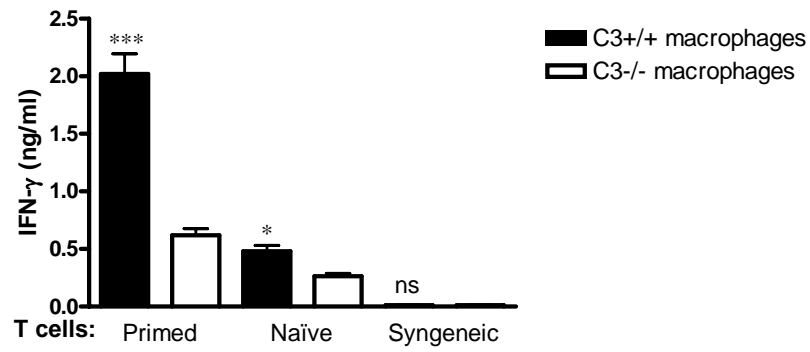
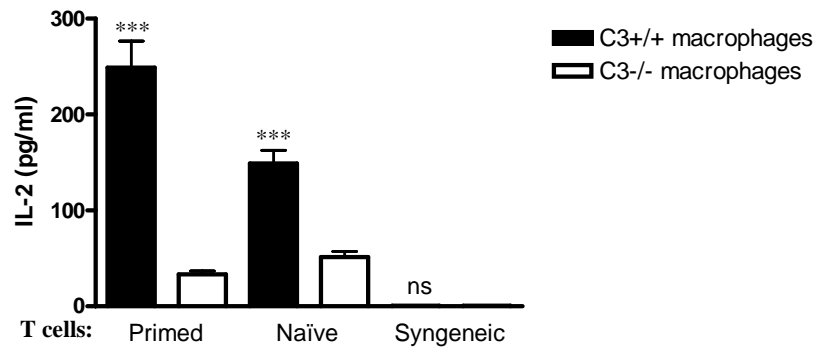


Figure 1B

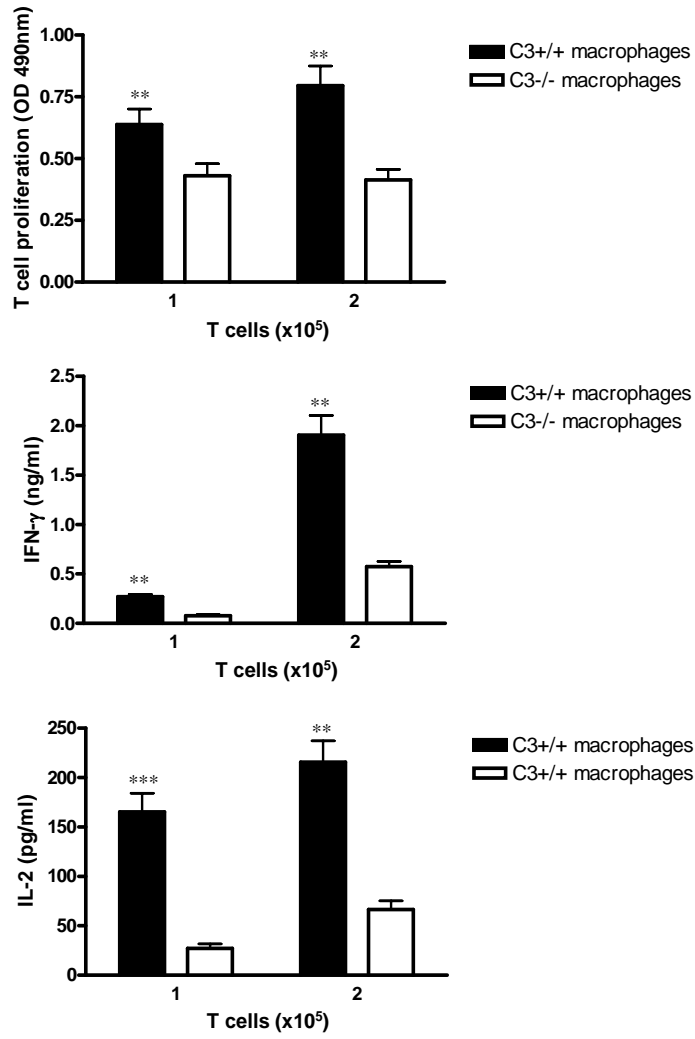


Figure 2

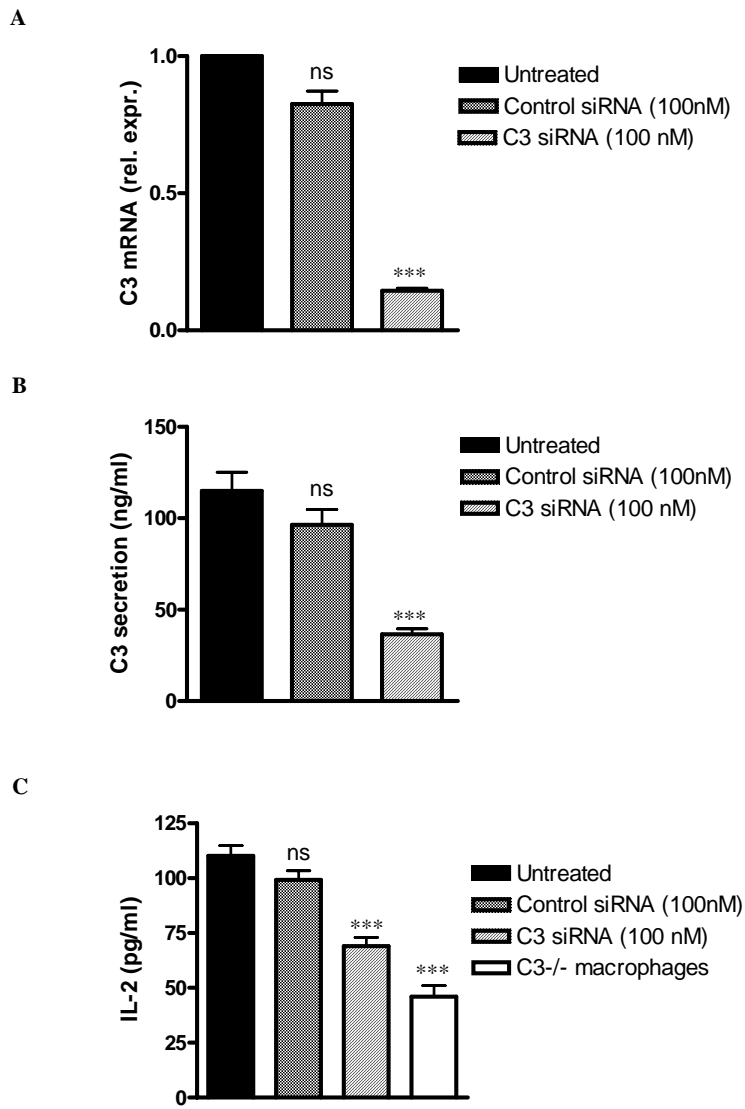


Figure 3A

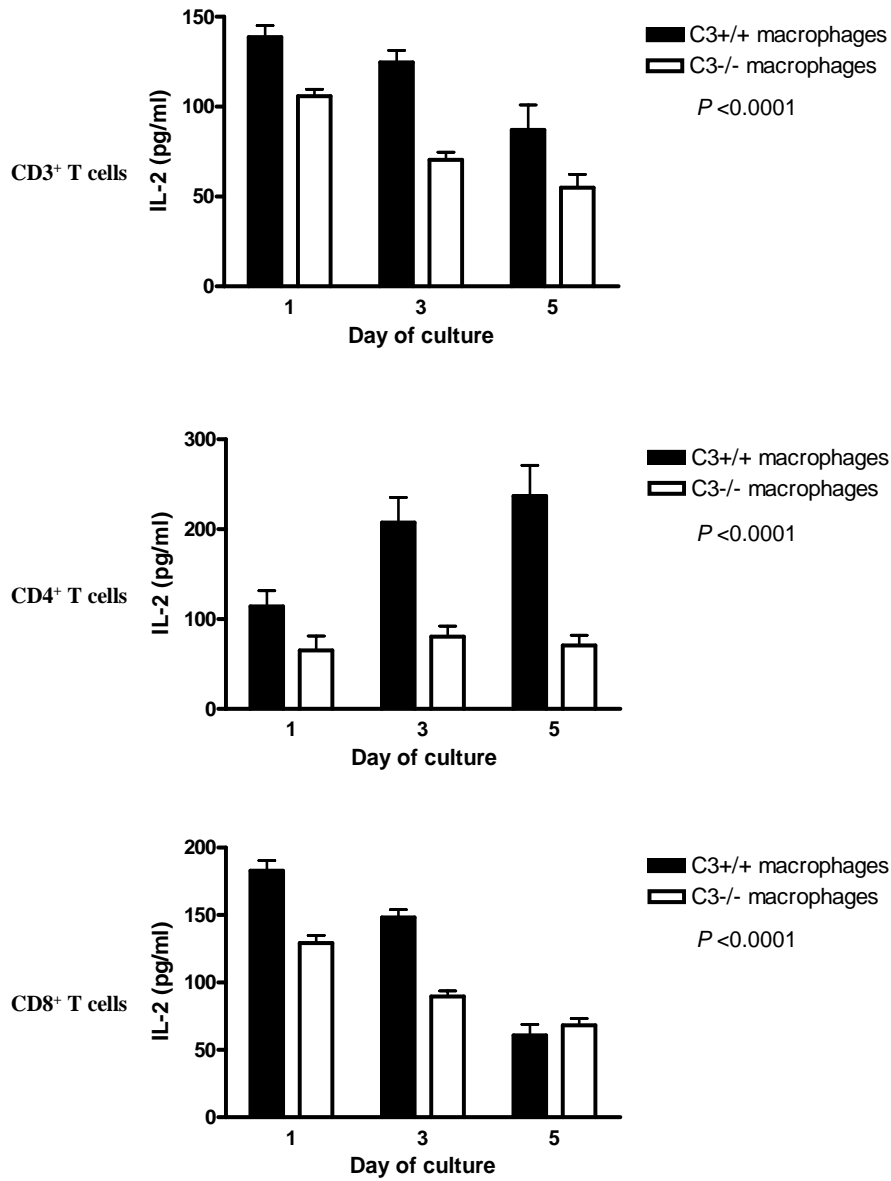


Figure 3B

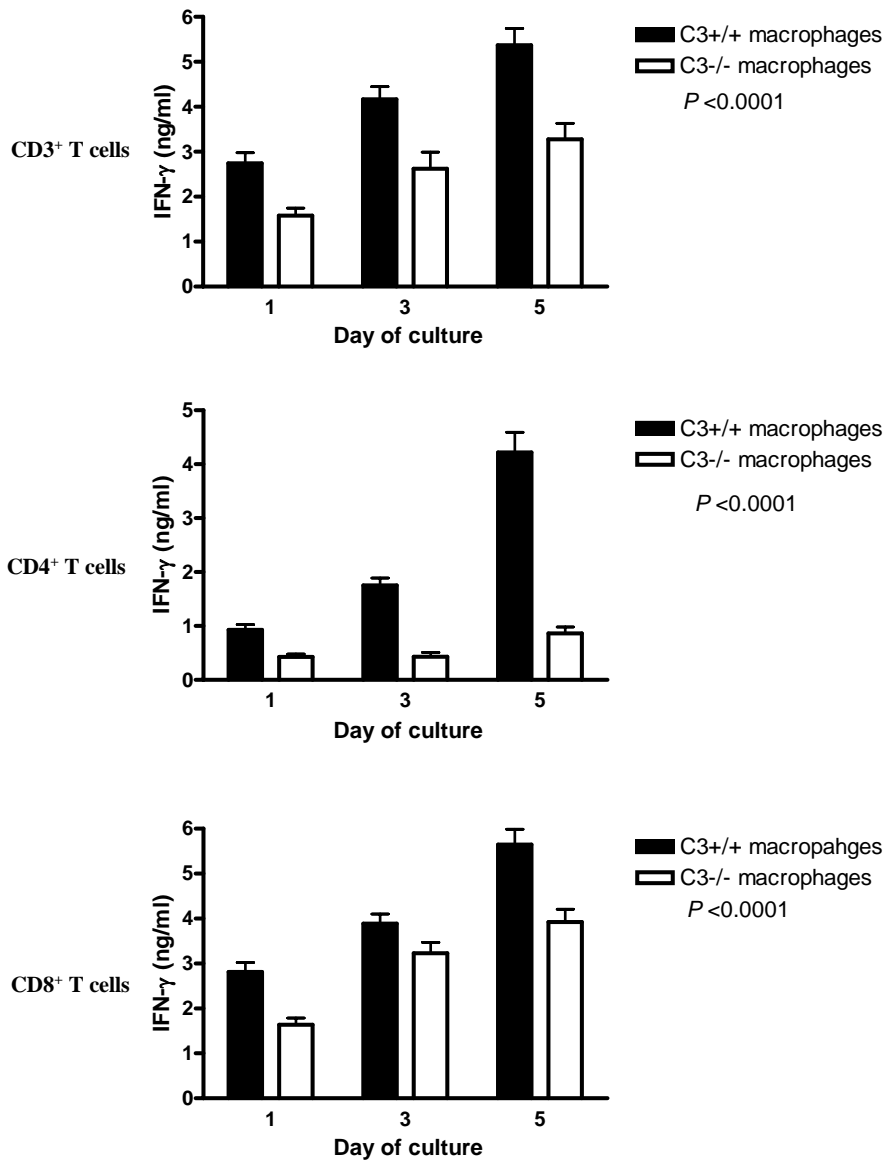


Figure 3C

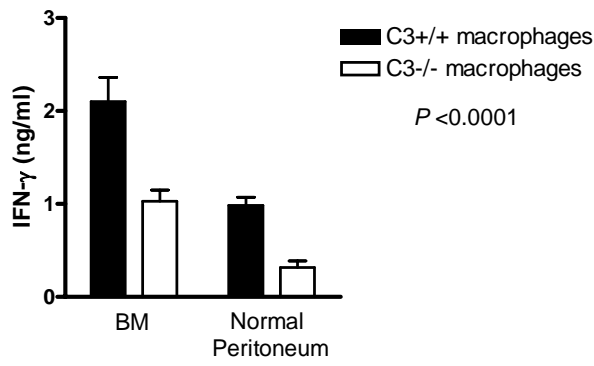
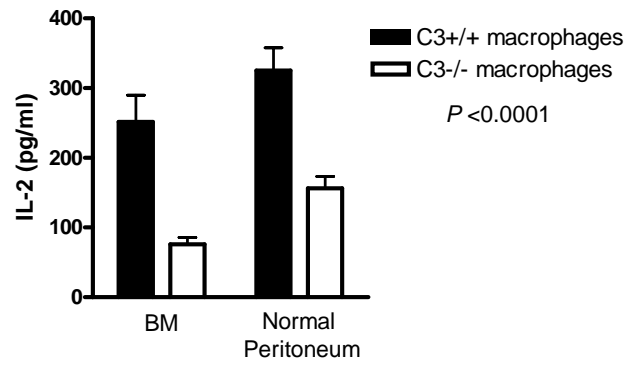


Figure 4

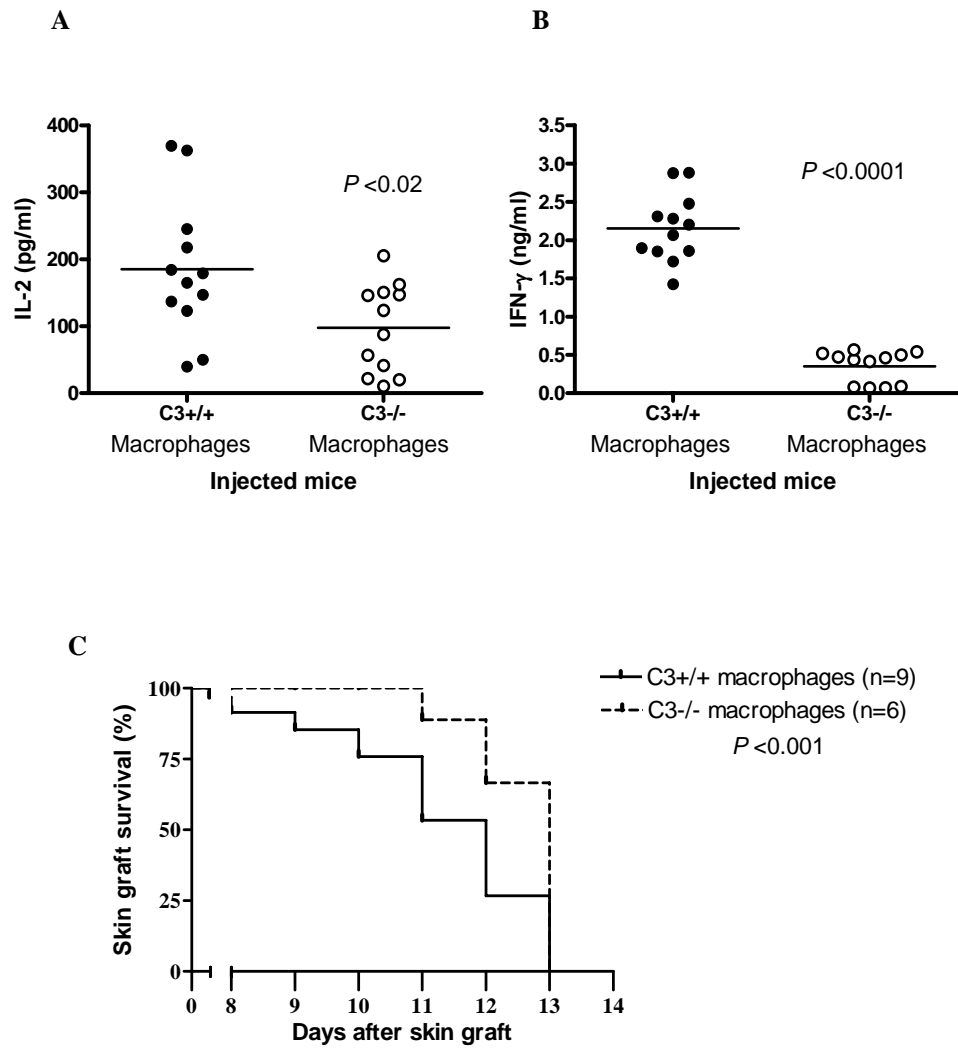


Figure 4

D

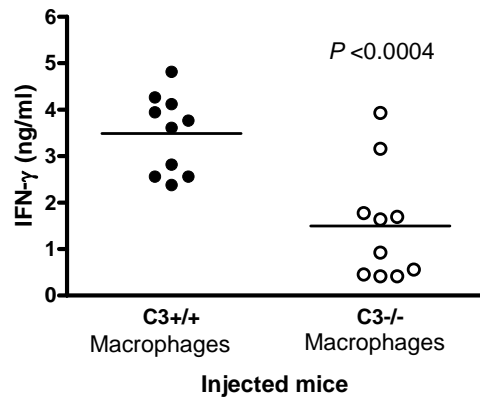
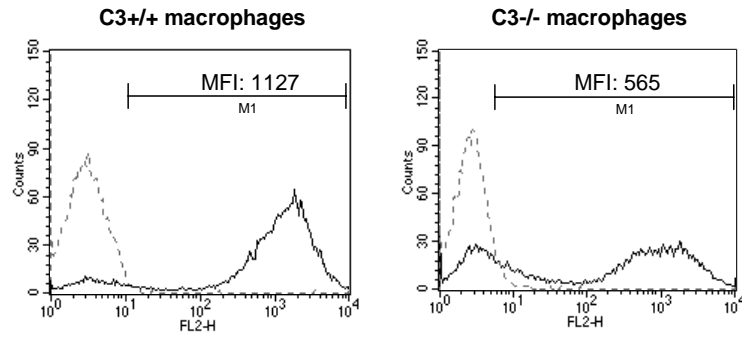


Figure 5

A



B

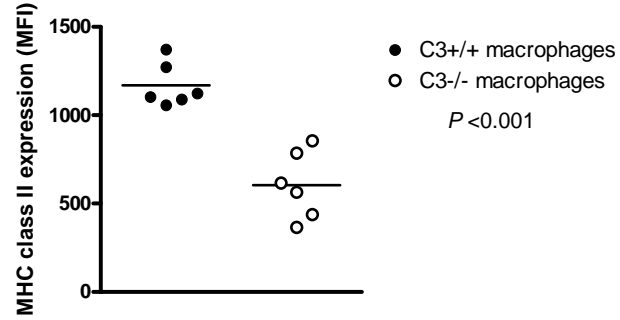


Figure 5

C

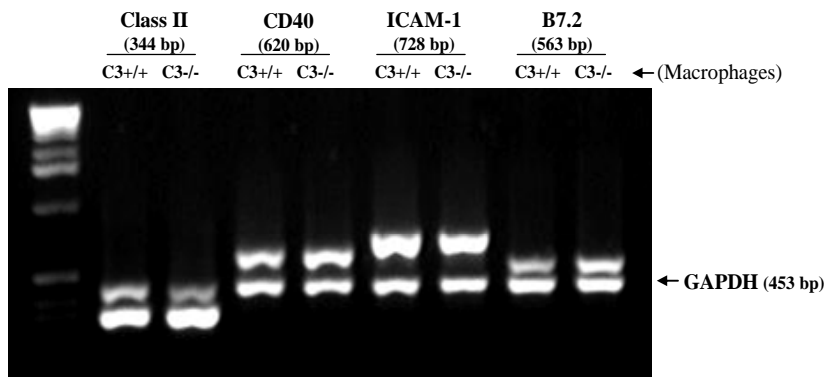


Figure 6

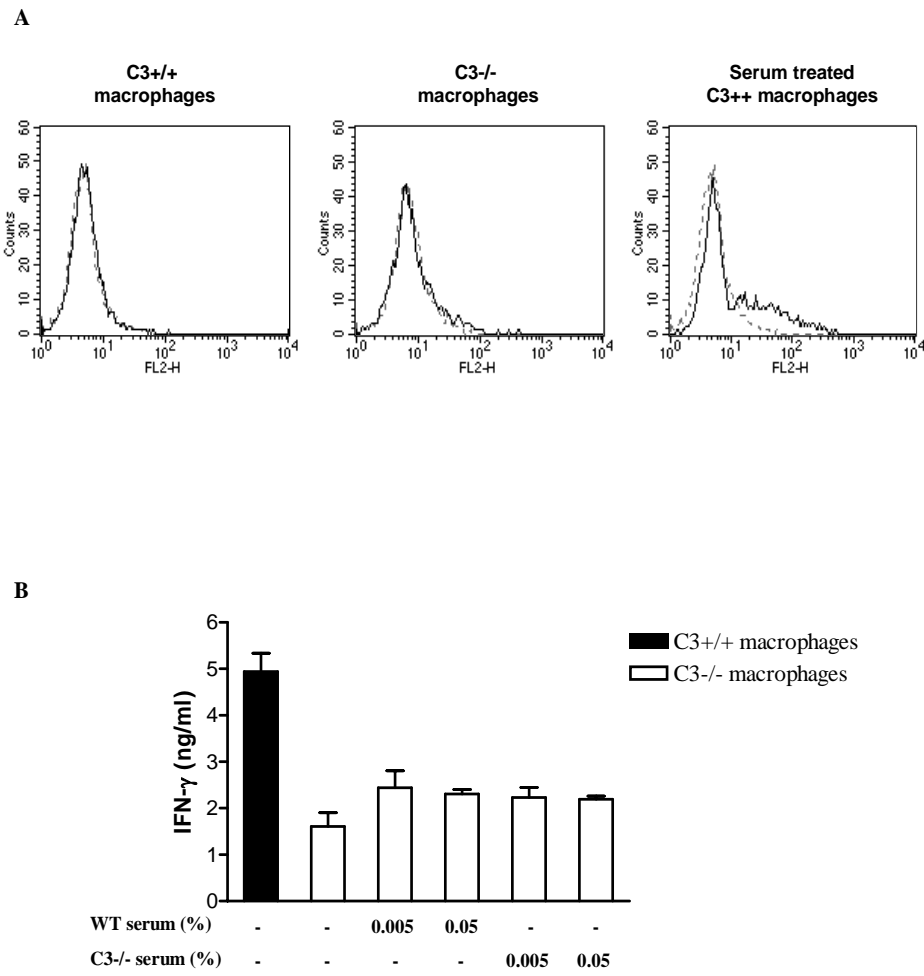


Figure 7

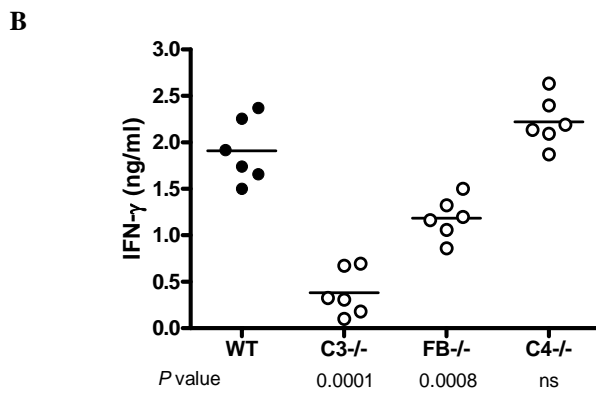
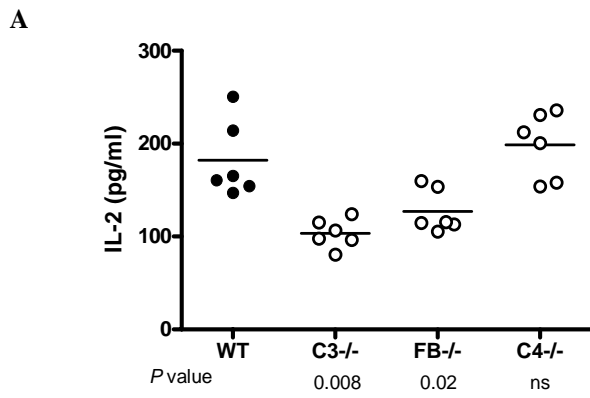
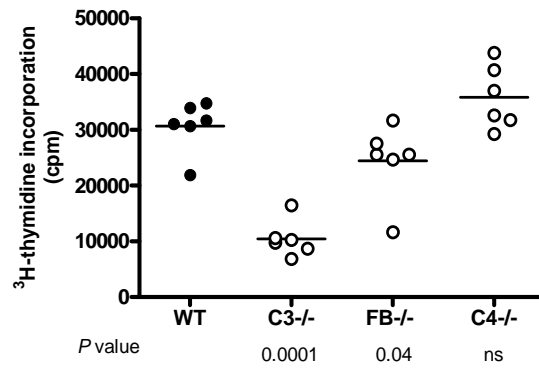


Figure 7

C





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Macrophages from C3 deficient mice have impaired potency to stimulate alloreactive T cells

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