Complement Activation Regulates the Capacity of Proximal Tubular Epithelial Cell to Stimulate Alloreactive T Cell Response

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Abstract. Tissue expression of C3 is an unexpected regulator of the alloimmune response in mouse kidney transplantation. It is unclear, however, whether a direct or an indirect action of complement on the host immune response is involved. Also unknown is which of the complement effector products, cleaved C3, cleaved C5, or C5b-9, is responsible. Proximal tubular epithelial cells (PTEC) not only constitute a major target of the alloimmune response but also produce substantial amounts of C3. This study investigated the property of mouse PTEC to stimulate alloreactive T cells in a complement-dependent manner. The proliferative and cytokine responses of primed alloreactive T cells were measured after exposure to donor-specific PTEC that had been pretreated with normal mouse serum, heat-inactivated mouse serum, or complement-deficient (C3, C5, or C6) mouse sera to differentially deposit complement components. PTEC were able to stimulate alloreactive T cells in an antigen-specific manner. Complement activation leading to the deposition of cleaved C3 on PTEC enhanced the alloreactive T cell response. This complement-mediated stimulation of the T cell response was dependent on C3 but not on C5 or C6. The primary influence of tissue-bound complement was on CD4$^+$ T cells. Moreover, the effect of complement on alloreactive T cells was B7 dependent, shown by inhibition studies with CTLA4-Ig. These results suggest that donor epithelium-bound C3 can upregulate the alloimmune response. It is postulated that surface-bound C3 interacts with complement receptors on alloreactive T cells or on antigen presenting cells to increase allo-immune stimulation.

Evidence has emerged that the innate immune system regulates the development of the adaptive immune response. Complement is one of the major components of the innate immune system and not only plays an important role in host defense against initial infection but also modulates both B cell and T cell function. Much of the evidence linking the immunoregulatory properties of complement with receptor activation on immune cells is in the context of infection and autoimmunity. The regulation of the alloimmune response by complement is much less clear.

Complement protein C3 is the point of convergence for the three recognized pathways of complement activation and thus plays a critical role in biologic processes mediated by the complement cascade, which includes anaphylatoxic activity, opsonization of pathogens and foreign particles, and lysis of cells. In addition to these effector functions, it has become increasingly evident that C3 participates in the regulation of the antigen-specific immune response. The C3 split product C3b or its metabolite C3d, either conjugated to antigen or bound on antigen-presenting cells (APC), can regulate the specific immune response against exogenous antigens (1–4). Furthermore, studies in C3-deficient (C3-def) mice have demonstrated that C3 has an ability to augment the specific T cell response against viral antigens and autoantibodies (5,6). Recently, two studies in our laboratory provided evidence that complement also has an important role in the regulation of the alloresponse. In a skin graft model, C3- and C4-def recipient mice but not C5-def mice had a profound defect in the primary alloantibody response, suggesting that complement participates in modulating alloantibody production (7). In another study, most allogeneic C3-def mouse kidneys transplanted into complement-sufficient mice survived for at least 100 d, whereas control grafts from C3-sufficient (C3-suf) donors were rejected within 14 d (8). Both studies strongly suggest that tissue C3 is essential for the regulation of transplant rejection and that local production of C3 has a significant effect on the T cell alloresponse.

Alloreactive T cells are critical for mediating acute allograft rejection. Activation of allospecific T cells after transplantation requires two signals: stimulation by donor antigen (alloantigen) and co-stimulation through antigen-independent pathways (9). Alloantigen is a unique antigen, which could be allo-MHC molecules or peptide:MHC complexes; the latter could be any peptide:allo-MHC or allo-peptide:self MHC (10–12). Allo-

reactive T cells recognize all of them by either the direct or indirect antigen presentation pathway. In the kidney, apart from passenger leukocytes, parenchymal cells are also thought to be able to present antigen to T cells (13,14). However, the extent, regulation, and significance of antigen presentation by kidney parenchymal cells are at present unclear. Renal proximal tubular epithelial cells (PTEC), besides expressing MHC class II molecules, are able to produce a number of inflammatory mediators, including C3, and co-stimulatory molecules (13,15,16), whose expression is increased by many inflammatory stimuli. In addition, PTEC reside at the interface between the urinary space, interstitium, and peritubular capillaries. These features allow PTEC to have a potential role in coordinating proinflammatory and immunoregulatory functions in the renal interstitium. PTEC functioning as nonprofessional APC therefore could participate in the alloimmune response during transplant rejection.

Previous work has shown that C3 is markedly upregulated in the tubules of inflamed kidney (*i.e.*, ischemia/reperfusion injured or rejected) and is deposited on the tubule surface adjacent to the T cell infiltrate (17–19). These data suggest that complement, in addition to regulating the specific immune response in infection and autoimmunity, participates in the regulation of the alloimmune response in kidney transplantation. In the present study, we examined the effect of complement on the capacity of PTEC to stimulate primed alloreactive T cells and investigated the precise level of the complement cascade (C3/C5/C6) involved.

Marerials and Methods

Mice

BALB/c (H-2^d), C57BL/6 (H-2^b), C3H/He (C6-suf), DBA/1 (C5-suf) and DBA/2 (C5-def) were purchased from Harlan UK Ltd (Bicester, UK). Homozygous C3-def mice were derived by homologous recombination in embryonic stem cells (20) and backcrossed onto the C57BL/6 parental strain for 11 generations. C6-def mice, which were derived from a Peruvian strain and backcrossed with C3H/He mice for 10 generations, were provided by Professor P. Lachmann (Cambridge,

UK) (21). C3-, C5-, and C6-def mice had no plasma activity of their respective deficient complement components. Only male mice were used in the study to avoid gender-related variations in complement activity that occur in some strains. All procedures were conducted in accordance with the Home Office Animals (Scientific Procedures) Act of 1986.

PTEC Cultures

Primary PTEC cultures were prepared from kidneys of male C57BL/6 (H-2^b) or BALB/c (H-2^d) mice as described previously (15). Minced cortex was digested with 0.1% collagenase II and passed through a 40-μm nylon sieve. The cells and tubules were collected and cultured in a DMEM-12 medium that contained 2% FCS, insulin (5 μg/ml), transferrin (5 μg/ml), selenium (5 ng/ml), hydrocortisone (40 ng/ml), and tri-iodothyronine (10^{-2} M). All cells used in this study were nonpassaged 5- to 6-d cultures. They displayed epithelial morphology, expressed the brush border enzyme alkaline phosphatase, and stained positively for cytokeratin. Examination by electron microscopy demonstrated the presence of numerous apical microvilli of a rudimentary brush border, with reassembly of tight junctions.

Reverse Transcription of RNA and Subsequent PCR

Total RNA was extracted from PTEC and followed by cDNA synthesis as we described previously (16). PCR was carried out with 2 μl of diluted cDNA (reflecting 0.2 μg of total RNA), 12.5 pmol of each 3' and 5' primer pair for MHC class II Aβ chain, CD40, intracellular adhesion molecule-1 (ICAM-1), and B7.2 (CD86) (Table 1) in 25 μl 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 that contained ethidium bromide. Glyceraldehyde-3-phosphate dehydrogenase primers, 12.5 pmol each, were also added in every reaction as an internal control.

Serum Preparation

Mouse sera were freshly prepared on the day of use. Normal serum (NS) was obtained from C57BL/6 mice; heat-inactivated serum (HIS) was prepared by incubating this serum at 56°C for 30 min. Complement component-sufficient sera (C3, C5, and C6) were obtained from C57BL/6, DBA/1, and C3H/He mice, respectively. Sera deficient in a

Table 1. PCR primer sequences and product sizes^a

Primer^b	Oligonucleotide Sequence	Product Size (bp)	Gene Bank Code
Class II-1	5′-CTCAACCACCACAACACTCTGG-3′	344	V01527
Class II-2	5′-GCCTTTTCATCCGTCACAGGAG-3′		
CD40-1	5′-TGTGATTTGTGCCAGCCAGG-3′	620	M83312
CD40-2	5′-CCCAGGAGATGGAAGATTATCC-3′		
ICAM-1-1	5′-TCTGTTCTTGCTGCTGTTGAGC-3′	728	AH001921
ICAM-1-2	5′-CTCTACGCAGCACAGAACATC-3′		
B7.2-1	5′-CGGACTTGAACAACCAGACTCC-3′	563	L25606
B7.2-2	5′-GGTCACCCGAAACCTAAGAAGA-3′		
GAPDH-1	5′-ACCACAGTCCATGCCATCAC-3′	453	XM_033260
GAPDH-2	5′-TCCACCACCCTGTTGCTGTA-3′		

^{a} ICAM, intracellular adhesion molecule; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
^{b} Primer 1 is identical to the coding strand; primer 2 is complementary to the coding strand. All primers were designed such that there are intronic sequences between primer 1 and primer 2.

specific complement component were obtained from C3-, C5-, and C6-def mice.

Preparation of Stimulators (PTEC)

PTEC used in this study were incubated with IFN-γ for 24 h to maximize expression of MHC and co-stimulatory molecules. Single-cell suspensions were prepared by trypsin/EDTA treatment and then incubated with 10% mouse serum for 30 min, unless otherwise stated. PTEC were then washed three times with endotoxin-free PBS to remove unbound complement proteins and resuspended in T cell culture medium (RPMI-1640 containing 10% heat-inactivated FCS, 50 μM 2-mecaptoehtonal, 100 U/ml penicillin, and 100 μg/ml streptomycin) and irradiated at 1000 RADS. Cell viability was assessed before and after serum treatment, by trypan blue dye exclusion, which showed no increase in the number of dead cells after treatment with serum. Viable cells were >90% of the total. Allogeneic H-2^b PTEC monolayers were used in all experiments, except the syngeneic control experiment in which H-2^d PTEC were used.

Preparation of Responders (Alloreactive T Cells)

Primed alloreactive T cells were isolated from spleens of BALB/c (H-2^d) mice 14 d after they received a C57BL/6 skin graft. Minced spleen was forced through a 40-μm nylon sieve using a 5-ml syringe plunger. The filtrates were collected and washed once with endotoxin-free PBS. Enriched T cells were prepared using anti-mouse IgG beads (Dynal, Oslo, Norway) to remove IgG$^+$ cells. After enrichment, the T cell preparation routinely consisted of >80% CD3$^+$ T cells. Purified CD4$^+$ and CD8$^+$ T cells were prepared using Spin-Sep Enrichment Cocktail Kit (StemCell Technologies, London, UK). After the purification, the purity of those T cell preparations is >90% as determined by flow cytometry.

T Cell Proliferation Assay

After cytokine $\pm$ serum treatment, 2×10^5 washed, irradiated PTEC (H-2^b) were added into cultures of 2×10^5 enriched T cells or purified CD4$^+$ and CD8$^+$ T cells (H-2^d) in 96-well plates and cultured for 96 h. ^{3}H-thymidine (1 μCi/well) was added during the last 24 h. The amount of [^{3}H] TdR incorporation was counted. Control cultures included wells of irradiated PTEC with irradiated T cells. Data were expressed as the difference in cpm of experimental and control cultures and referred to as "Δ cpm." T cells alone and PTEC alone controls were also included in each experiment and gave consistently low backgrounds.

T Cell Function Assay

The amount of IFN-γ and IL-2 secreted into cell culture supernatants was measured by ELISA. Sandwich ELISA was performed using ELISA Kit for mouse IFN-γ or IL-2 (R&D Systems, Oxford, UK) according to the manufacturer's instructions.

Flow Cytometry

Single-cell suspensions of PTEC were stained with either FITC- or phycoerythrin (PE)-conjugated antibody or the appropriate isotype control antibody. The stained cells were analyzed by flow cytometry (FACScan; Becton Dickinson, Oxford, UK). Ab reagents used were FITC-conjugated cross-reactive rabbit anti-human C3d antibody (Dako, High Wycombe, UK), PE-conjugated rat anti-mouse MHC class II (I-A/I-E), PE-conjugated rat anti-mouse CD40, PE-conjugated Armenian hamster anti-mouse ICAM-1 (CD54), and PE-conjugated rat anti-mouse B7.2 (CD86; BD Biosciences, Cowley, UK).

Statistical Analyses

All T cell proliferation data shown are representative of at least three independent experiments. ELISA data shown are representative of three independent experiments. Statistical analysis was performed on a complete data set of one experiment, with T cell proliferation and ELISA assessed in triplicate. Data are expressed as mean $\pm$ SEM and subjected to t test.

Results

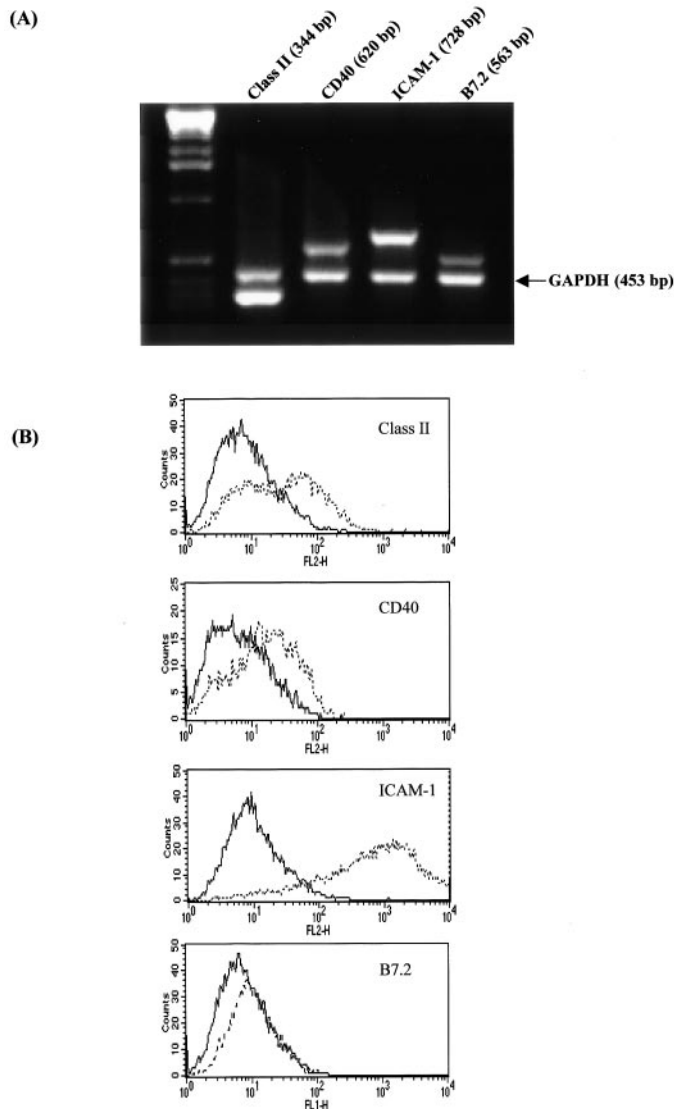## MHC Class II and Co-stimulatory Molecule Expression

To examine whether primary cultured murine PTEC are able to express MHC class II and a number of co-stimulatory molecules and thereby can potentially function as APC, we performed reverse transcription–PCR (RT-PCR) and flow cytometry on PTEC treated with IFN-γ (1000 U/ml) for 24 h. Gene expression of MHC class II, CD40, ICAM-1, and B7.2 was clearly detected by RT-PCR. Protein expression of MHC class II, CD40, and ICAM-1 was correspondingly detected by flow cytometry, with the exception of B7.2, which is only weakly detected (Figure 1). In addition, gene expression of MHC class II, CD40, and ICAM-1 was detected in unstimulated PTEC by RT-PCR (data not shown).

C3 Deposition on PTEC

To test whether complement can be activated and deposited on murine PTEC, the suspension cells were incubated with 10% mouse sera (NS, HIS, C3-def, C5-def, or C6-def sera). C3 deposition was examined by flow cytometry. The result of C3d staining shows that membrane-bound C3 was clearly detected in NS or C5-def serum-treated cells but barely detected in HIS or C3-def serum-treated cells (Figure 2). These results indicate that after complement activation, C3 split product was deposited on PTEC. The very weak staining of C3d observed in HIS- or C3-def serum-treated cells could be explained by the C3 produced by PTEC themselves (15,16). The detection of C3d in C5-def (or C6-def; data not shown) serum-treated cells indicates that without C5 or C6, complement activation of C3 occurs despite C5b-9 being unable to form. Our data suggests that mouse PTEC can spontaneously activate complement, confirming observations with human PTEC (22,23).

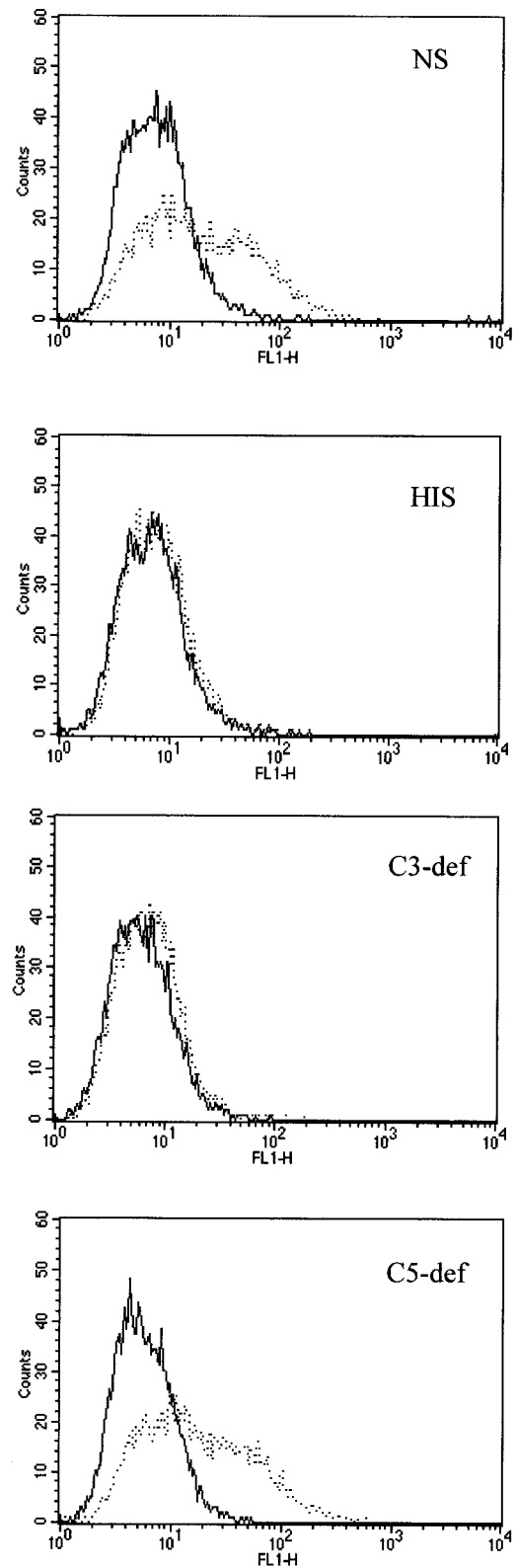## C3 Deposition Enhances the Ability of PTEC to Stimulate Alloreactive T Cell Proliferation

To determine whether PTEC are able to stimulate alloreactive T cells, nonstimulated or IFN-γ–stimulated PTEC were subsequently co-cultured with alloreactive T cells. As shown in Figure 3A, T cell proliferation was clearly detected and was increased by pretreating the stimulator cells with IFN-γ compared with untreated cells. To determine the effect of complement deposition on the activity of PTEC to stimulate T cells, we treated IFN-γ–stimulated PTEC with NS or HIS before their co-culture with responder T cells. The results showed that treatment of PTEC with NS significantly increased T cell proliferation compared with HIS treatment. Syngeneic control experiments with H-2^d stimulator PTEC elicited a low level of T cell proliferation (Figure 3B). These data demonstrate that PTEC are able to stimulate alloreactive T cells and that complement activation on PTEC enhances their APC function.

Figure 1. Detection of MHC class II and co-stimulatory molecules. Monolayers of proximal tubular epithelial cells (PTEC) were stimulated with IFN-γ (1000 U/ml), followed by reverse transcription–PCR (RT-PCR) and flow cytometry analysis. (A) RT-PCR. An agarose gel showing the size of the detected fragments and the 453-bp glyceraldehyde-3-phosphate dehydrogenase (internal control) band. The 1-kb DNA markers are shown along side the gel. (B) Flow cytometry. In all histogram plots, the control peak (heavy line) corresponds to staining cells with the appropriate isotype control antibody. The detection peak (dotted line) shows surface binding of specific antibody.

Enhancement of the Alloreactive T Cell Response Is Dependent on PTEC-Bound C3 but not C5 or C6

Complement activation produces a number of biologically active products, including C3b, C5a, and C5b-9, whose presence on the PTEC may participate in the regulation of the immune response. To investigate further which components might contribute to PTEC stimulation of the alloreactive T cell response, we treated PTEC with different pairs of sera, including C3-suf/C3-def, C5-suf/C5-def, and C6-suft/C6-def sera. The treated PTEC were then used to stimulate alloreactive T

Figure 2. Detection of complement C3 deposition on PTEC. Single-cell suspension PTEC were treated with 10% various mouse sera (normal serum [NS], heat-inactivated serum [HIS], C3-deficient [C3-def], and C5-def) for 30 min. Washed serum-treated cells were subsequently stained for C3d by FACS. PTEC that were treated with serum are represented by the dotted line, and control cells (no serum added) are represented by the heavy line.

(A)

(B)
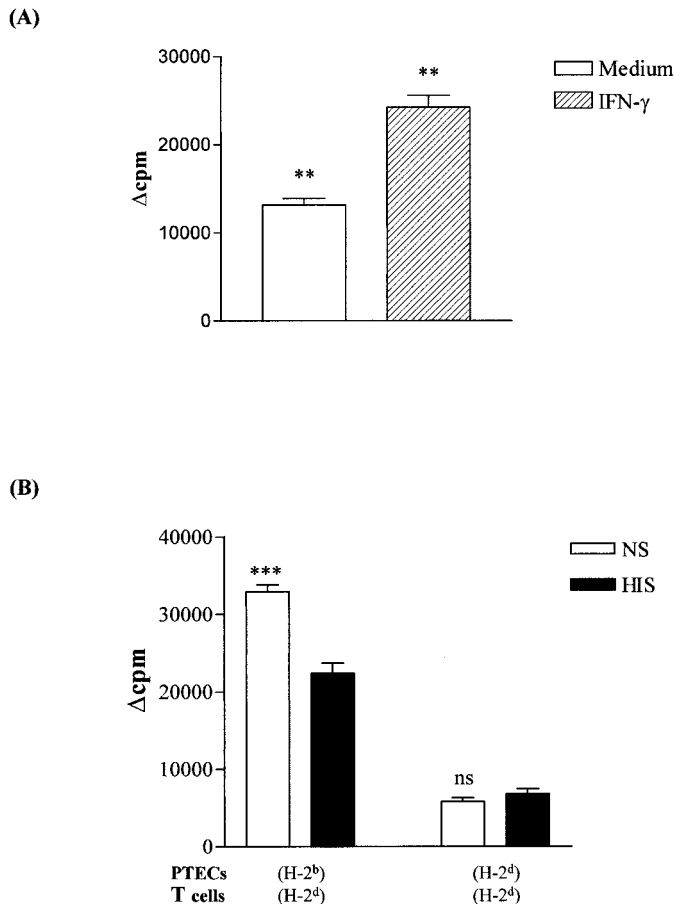
Figure 3. Complement deposition enhances the activity of PTEC to stimulate alloreactive T cell proliferation. (A) PTEC were incubated with IFN-γ (1000 U/ml) or medium alone for 24 h and then irradiated and assessed as stimulators in a 4-d T cell proliferation assay. (B) PTEC (derived from either H-2^b or H-2^d mice) were treated with 10% NS or HIS for 30 min, followed by T cell proliferation assay. The incorporation of ³H-thymidine was measured in the last 24 h. *P* values are for comparisons between IFN-γ and medium alone in A and for comparisons between NS and HIS treatment in B. ***P < 0.0001; **P < 0.005; ns, no significant difference.

cells. Treatment of PTEC with C3-def serum induced a weaker T cell response compared with treatment with C3-suf serum. However, the effect of treatment of PTEC with either C5-def or C6-def serum on T cell proliferation was not significantly different from that with their normal wild-type counterparts (Figure 4). These data suggest that the property of complement to enhance the alloreactive T cell response is dependent on C3 but not on C5 or C6.

Complement C3 Dose-Effect on the Alloreactive T Cell Response

The dose-effect of C3 bound to the stimulator PTEC on the magnitude of the T cell alloresponse was examined next. These experiments were performed with C5-def serum to avoid C5a and C5b-9 formation but to allow deposition of C3. After incubation of PTEC with various concentrations of C5-def serum or C5-def HIS, cell-bound C3 was analyzed by FACS and the serum-treated
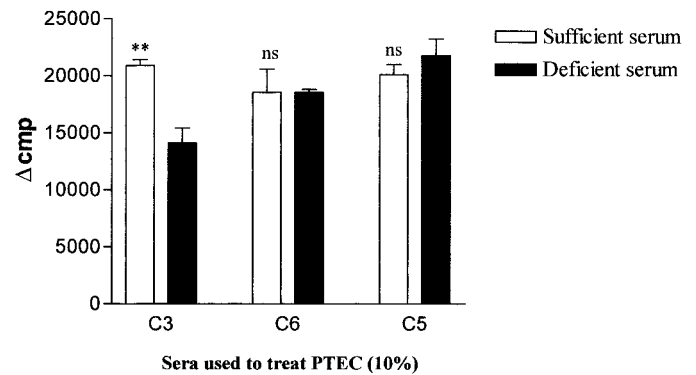
Figure 4. Enhancement of the alloreactive T cell response is dependent on PTEC-bound C3 but not C5 and C6. IFN-γ–stimulated PTEC were treated with different mouse sera, including C3-suffficient (C3-suf) and C3-def, C5-suf and C5-def, and C6-suf and C6-def sera, and subsequently co-cultured with alloreactive T cells for 96 h. The incorporation of ³H-thymidine was measured in the last 24 h. *P* value is for comparisons between C3-suf and C3-def serum. **P < 0.005.

PTEC used in T cell proliferation assays. The absence of PTEC damage after treatment with all serum concentrations was confirmed by their trypan blue dye exclusion. FACS analysis showed that the deposition of C3 on the cell surface increased as the concentration of C5-def serum used (between 2.5 and 50%). This increase was not observed in cells that were treated with C5-def HIS (Figure 5A). The extent of T cell proliferation was significantly elevated by treatment of PTEC with freshly prepared C5-def serum but not the C5-def HIS. There was a significant difference in T cell proliferation at serum concentrations of 2.5 and 10%, suggesting a dose-dependent effect. However, at higher serum concentrations (25 and 50%, the latter not shown), a plateau was reached, suggesting saturation of the effect on T cell proliferation (Figure 5B).

IL-2 and IFN-γ Production by Alloreactive T Cells

Because the T cell proliferation assay detects dividing cells rather than measures T cell effector function and the majority of activated T cells produce IL-2 and/or IFN-γ, we examined IFN-γ and IL-2 production by ELISA. The co-culture, as before, consisted of PTEC incubated with T cells for up to 96 h. Alloreactive T cells produced both IL-2 and IFN-γ, but with different kinetics. The production of IL-2 peaked at 48 h, whereas IFN-γ peaked at 72 h. The effect of NS-treated PTEC was significant at 24 and 48 h for IL-2 and at 48, 72, and 96 h for IFN-γ compared with HIS-treated PTEC (Figure 6). These data correspond with the T cell proliferation results and confirm that T cell responders are functionally activated as well as being stimulated to divide in a complement-dependent manner, after exposure to allogeneic PTEC.

CD4⁺ T Cells Are Responsive to Complement-Mediated PTEC Stimulation of Alloreactive T Cell Response

To evaluate the relative involvement of CD4⁺ and CD8⁺ T cells in the complement-enhanced alloreactive T cell response, we prepared purified alloreactive CD4⁺ and CD8⁺ T cells and

(A)

(B)
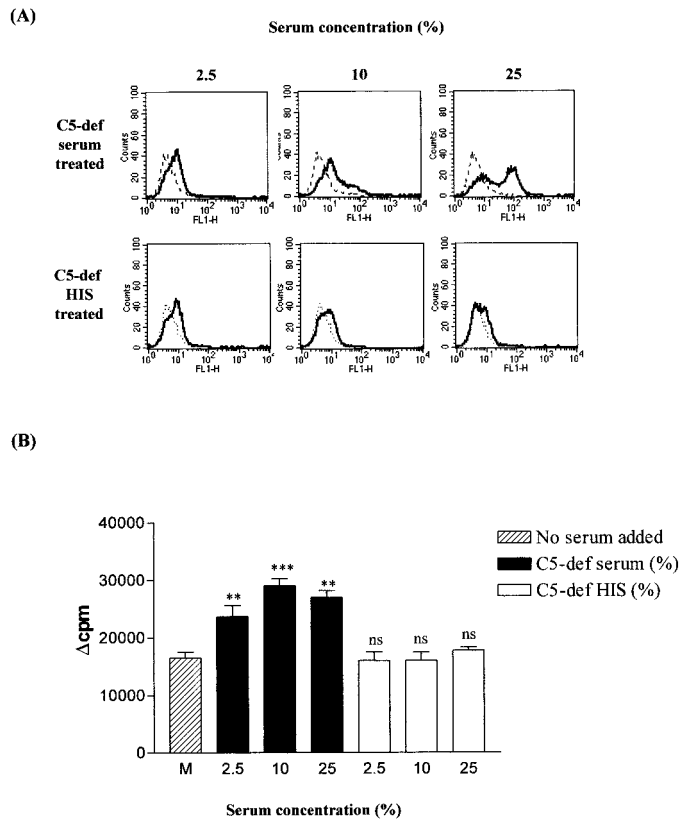
Figure 5. Complement dose-effect on the alloreactive T cell response. Single-cell suspensions of IFN-γ–stimulated PTEC were treated with C5-def or HIS C5-def at various concentrations for 30 min. Washed serum-treated cells were subsequently stained for C3d by FACS or co-cultured with alloreactive T cells. (A) FACS analysis. PTEC that were treated with C5-def serum or C5-def HIS are represented by the heavy line, and control cells (no serum added) are represented by the dotted line. (B) T cell proliferation. Serum-treated PTEC were co-cultured with alloreactive T cells for 96 h. The incorporation of ^{3}H-thymidine was measured in the last 24 h. P values are for comparisons between C5-def serum treatment and control (no serum added). $*P < 0.05$; $**P < 0.005$; $***P < 0.0003$.

co-cultured these cells with NS- or HIS-treated PTEC. Supernatants were collected at 72 h for ELISA analysis of IFN-γ (Figure 7). The production of IFN-γ was much higher in CD4$^+$ T cell preparation compared with CD8$^+$ T cell preparation, regardless of the serum treatment of PTEC. For CD4$^+$ T cell preparation, the production of IFN-γ was significantly higher in NS-treated PTEC compared with either untreated or HIS-treated PTEC. However, for CD8$^+$ T cell preparation, we did not observe a difference of IFN-γ production between NS-treated PTEC and HIS-treated PTEC. These data indicate that the primary influence of tissue-bound complement was on CD4$^+$ T cells.

Complement-Mediated PTEC Stimulation of Alloreactive T Cell Response Can Be Inhibited with CTLA4-Ig

To investigate further how complement activation and deposition on PTEC influence T cell co-stimulation, we performed

(A)

(B)
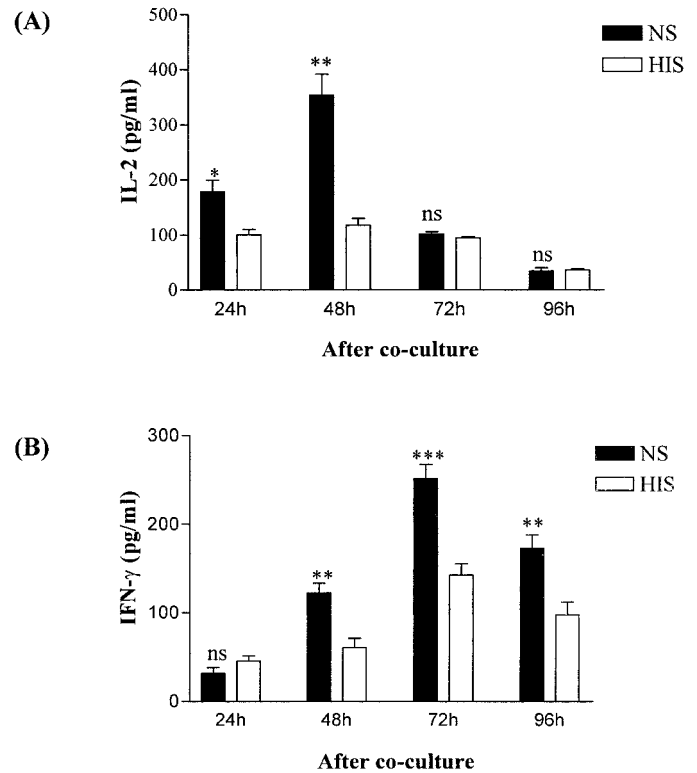
Figure 6. Detection of IL-2 and IFN-γ by ELISA. IFN-γ–stimulated PTEC were treated with NS or HIS for 30 min and subsequently co-cultured with T cells. (A and B) Supernatants were collected from co-culture of 2×10^5 PTEC with 2×10^5 T cells at various time points and assayed for IL-2 and IFN-γ by sandwich ELISA. P values are for comparisons between NS and HIS treatment. $*P < 0.05$; $**P < 0.005$; $***P < 0.0003$.

a CD80 and CD86 blockade experiment. We co-cultured purified alloreactive CD4$^+$ T cells with NS- or HIS-treated PTEC in the presence of CTLA4-Ig (5 μg/ml) or control Ig (human IgG1, 5 μg/ml) in the culture medium. Supernatants were collected at 72 h for ELISA analysis of IFN-γ (Figure 8). The production of IFN-γ was significantly decreased in the presence of CTLA4-Ig compared with either medium alone or control Ig (human IgG1), where 70% reduction in NS-treated PTEC and 57% reduction in HIS-treated PTEC were observed. These data indicate that the co-stimulation of B7 plays an important role in PTEC stimulation of alloreactive T cell response and that the effect of complement on this response is dependent on the B7 co-stimulation.

Discussion

The present investigation is a sequel to mouse kidney transplantation studies, in which local tissue expression of C3 was found to increase the antidonor T cell response and lead to more rapid allograft rejection (8). There are several possible explanations for the effect of immunoregulatory C3 in this animal model, which include the nonspecific effects of complement-mediated inflammation on antigen presentation and a direct effect of complement on T cell stimulation. The present
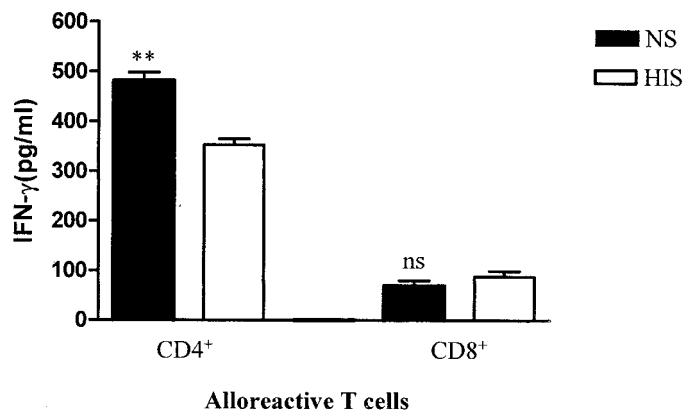
Figure 7. Relative involvement of CD4$^+$ and CD8$^+$ T cells in complement-enhanced alloreactive T cell response. IFN-γ–stimulated and NS- or HIS-treated PTEC were co-cultured with purified alloreactive CD4$^+$ or CD8$^+$ T cells. Supernatants were collected from co-culture of PTEC with T cells after 72 h of co-culturing and assayed for IFN-γ by sandwich ELISA. *P* values are for comparisons between NS and HIS treatment. **P < 0.005.
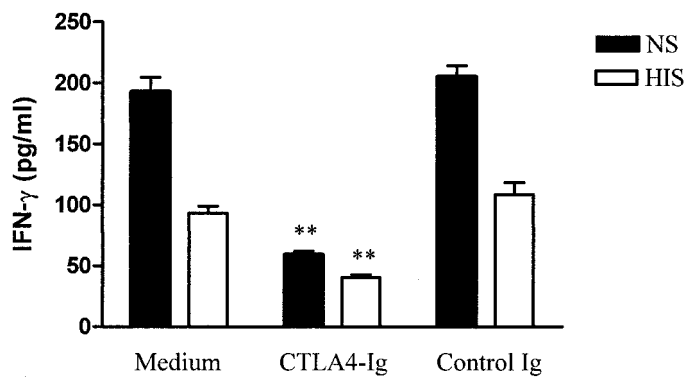
Figure 8. Complement-mediated PTEC stimulation of alloreactive T cell response can be inhibited with CTLA4-Ig. IFN-γ–stimulated and NS- or HIS-treated PTEC were co-cultured with purified alloreactive CD4$^+$ T cells in medium alone or in the presence of CTLA4-Ig (5 μg/ml) or control Ig (human IgG1, 5 μg/ml). Supernatants were collected from co-culture of PTEC with T cells after 72 h of co-culturing and assayed for IFN-γ by sandwich ELISA. *P* values are for comparisons between CTLA4-Ig treatment and medium alone or control Ig treatment. **P < 0.005.

results provide a strong case for a direct action of complement on the antigen-presenting property of PTEC *in vitro*. Moreover, the results suggest that because the effect of complement on the tubular cells is independent of C5 and C6, this effect is unrelated to C5b-9 but rather is due to C3 fragment covalently attached to the epithelial cell surface. Our results suggest that direct interaction between tissue-bound C3 fragment and recipient immune cells provides at least one mechanism by which local complement augments the allospecific immune response.

T lymphocytes express receptors that detect a variety of complement activation products, resulting in T cell activation. For example, CR1/2 expression on primed CD4$^+$ T cells

potentially allows T cells to detect bound C3b or its proteolytic cleavage products iC3b and C3d, which remain attached to the activating surface (24). Mouse lymphocytes also express complement control proteins such as CD46, CD55, and Crry that bind C3 and participate in signal transduction, as well as act as co-factors for the proteolytic cleavage of C3 (25–27). Thus, C3 receptor ligand engagement leading to improved contact with tissue APC, with or without ensuing signal transduction, might explain the effect of complement in our T cell stimulation assays. The close physical relationship between the tubular deposition of C3 and infiltrating leukocytes with complement receptor expression, seen in stained histologic sections of rejecting mouse grafts, provides important circumstantial evidence for the interaction suggested by our experiments (8). Moreover, the same tubule cells are a prominent site of C3 synthesis in rejecting grafts (28). Taken together, these data provide evidence for a cooperative effect of C3 on the interaction between donor PTEC and recipient T cells, which may in part explain the effect of complement on the immune response.

Complement receptors that are capable of interacting with attached C3 fragments have also been reported to occur on professional APC, such as macrophages and dendritic cells (29). Previous studies have shown that the immunostimulatory capacity of APC primed by tetanus toxoid antigen is increased with C3-opsonized antigen, thought to be due to more efficient antigen uptake and processing in the presence of C3 "chaperone" activity (30). Possibly, therefore, an alternative or additional explanation for the effect of C3 observed in our T cell stimulation studies is that C3b-opsonized alloantigen, either stripped from or maintained on the cell surface, is taken up or processed more effectively by APC, increasing their capacity as stimulators of T cells.

Indeed, in our B7-CD28 co-stimulation pathway blockade study, CTLA4-Ig was effective at inhibiting the stimulatory effect of complement. Thus, allogeneic T cell stimulation was dependent on B7 pathway signaling as well as complement activation. Because the expression of B7 on PTEC is weak, this suggests that the main effect of complement is mediated *via* another cell type. We suggest that this cell type could be a professional APC in the co-culture, because such cells normally express higher levels of B7 and stimulate T cells in a B7-dependent manner. Possibilities include contaminating donor passenger cells in the PTEC preparation or recipient APC (*e.g.*, B cells or macrophages) in the T cell preparation. Given that our purified T cell preparation responds to mitogen stimulation (data not shown), it seems likely that the T cell preparation contained recipient APC. Therefore, the mechanism of action of complement on T cell stimulation could involve indirect antigen presentation in a manner already described for exogenous antigen (30).

In addition to the ability to detect surface bound C3, leukocytes express receptors that are capable of interacting with the soluble fragments C3a and C5a, leading to cell activation (31,32). Our experimental protocol to activate complement on PTEC included the removal of unbound soluble complement fragments in the wash, making it unlikely for T cells to en-

counter C3a and C5a. Therefore, C3aR- and C5aR-mediated stimulation of the T cell response is unlikely to have played a significant role in our study. It should be noted, however, that the absence of an effect of C3a- or C5a-mediated T cell activation in our co-culture studies does not preclude an effect *in vivo*.

Complement activation through C3 leads to cleavage of C5 and subsequently to the insertion of C5b-9 in the cell membrane. C5b-9–induced cell activation can lead to upregulation of molecules concerned with immunologic function and therefore potentially enhance the immune response (22,23). In our study, however, we found no evidence of increased expression of MHC, co-stimulatory, or adhesion molecules on PTEC after serum treatment. Moreover, in the absence of C5 or C6, complement had an equal effect on the T cell stimulatory function of PTEC, indicating that the action of complement *in vitro* was independent of C5b-9–mediated membrane injury.

Overall, our data provide evidence for complement-mediated regulation of the alloresponse at the level of graft tissue-bound C3 interacting recipient immune cells. Although the precise details of this putative mechanism remain to be worked out, our experiments show an effect independent of C5a and C5b-9 that is likely to involve direct interaction of tissue-bound C3 with complement receptor–bearing alloreactive T cells that lead to CD4$^+$ T cell stimulation.

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