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An intracellular self protein synthesized in macrophages is presented but fails to induce tolerance

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Abstract

Mice deficient for the fifth component of murine complement (C5), unlike normal mice, do not possess the secreted form of C5 in their body fluids and can be readily immunized to serum-derived normal C5. Although macrophages from C5-deficient mice do not secrete C5, they synthesize the precursor form (pro-C5). Therefore contact of T cells with autologous pro-C5 presented by macrophages is theoretically possible. We show that macrophages from C5-deficient mice can indeed stimulate a class II restricted C5-specific T cell clone without addition of exogenous C5. Immunization of C5-deficient mice with autologous pro-C5 induces vigorous C5-specific T cell proliferation and pro-C5 is recognized by C5-specific T cells *in vitro*, demonstrating that this protein fails to induce tolerance under physiological conditions. Thus, intracellular pro-C5 is processed and presented by C5-deficient macrophages and can activate T cell clones *in vitro*, yet is neither immunogenic nor tolerogenic for T cells *in vivo*.

Introduction

The fifth component of murine complement (C5) is a self antigen that is normally present in plasma at a concentration of $\sim 10^{-7}$ M (1). The widespread occurrence in the mouse population of a spontaneous mutation affecting synthesis and secretion of C5 has generated a natural model for studies of self tolerance (2,3). Mice deficient for C5 do not possess secreted C5 protein and as a consequence are not tolerant of C5 Immunization with normal serum results in generation of C5-specific antibodies (2,3) and $CD4^+$ T cells that recognize C5 in the context of class II MHC (4).

Tolerance induction presumably involves self molecules expressed by cells in the thymus as well as secreted proteins which can reach the thymus to be presented to developing T cells. In C5-sufficient mice which do not generate anti-C5 T cell responses, endogenous secreted C5 is processed and presented by antigen-presenting cells in thymus and periphery (4), allowing induction and maintenance of tolerance. Although C5-deficient mice are devoid of secreted C5, their macrophages (and hepatocytes) synthesize a single-chain precursor molecule (pro-C5) (5). In normal mice pro-C5 is subsequently cleaved and secreted as a disulfide-linked heterodimer (6). A defect at a yet undefined site reduces the rate of synthesis of pro-C5 in C5-deficient macrophages and prevents its modification and secretion (5,7).

The synthesis of pro-C5 in macrophages, which process and present not only exogenous but also endogenous proteins (8,9), raises the question of whether this molecule is presented and recognized by T cells from C5-deficient donors.

In this paper we show that macrophages from C5-deficient mice indeed present their endogenous pro-C5 which can activate T cell clones *in vitro*. However, T cells *in vivo* appear to ignore this self antigen so that, as a consequence, neither tolerance nor autoimmunity is induced

Methods

Mice

B10.D2/oSn (O) and B10.D2/nSn (N) mice were obtained from the Jackson Laboratories, Bar Harbor, ME and maintained in the Basel Institute animal facilities.

T cell proliferation assays

Draining lymph nodes from mice immunized in the base of the tail 8 – 10 days previously were aseptically removed and cell suspensions were prepared. C5-specific proliferation assays were set up as described (4) Briefly, 4×10^5 responding lymph node cells/well of flat-bottom, 96-well culture plates (Costar) were

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cultured in 0.2 ml total volume of Iscove's modified Dulbecco medium supplemented with 5% fetal calf serum (FCS; Gibco), 5×10^{-5} M mercaptoethanol, 2×10^{-3} M L-glutamine, and 100 μg/ml gentamycin. Cultures were incubated for 3 days with affinity-purified C5 antigen prepared as described (10). For the last 20 h 1 μCi/well [^{3}H]thymidine was added Cultures were harvested and counted in a liquid scintillation counter.

Preparation of liver extracts

Liver extracts from O and N were prepared as described (11). Briefly, livers were homogenized in 1 1 (w/v) PBS containing 1 mM PMSF. They were then spun at 11,000 *g* for 30 min The supernatants were extracted with chloroform and spun at 10,000 *g* for 1 h. The resulting supernatants were once more spun at 35,000 *g* for 40 min

Precipitation of pro-C5

Liver extracts were precleaned by addition of protein A–Sepharose. Pro-C5 in liver extracts was precipitated by addition of 50 μg of the IgG fraction of a polyclonal anti-C5 antiserum per 2 g original liver input Precipitates were bound by addition of protein A–Sepharose, washed extensively with PBS containing 1 mM EDTA, and then emulsified in complete Freund's adjuvant (CFA) For use in culture, pro-C5 from 5 g of C5-deficient liver was immunoprecipitated and subsequently eluted with 1 ml of 1 M KBr and then dialyzed against PBS.

Immunization

Mice were immunized in the base of the tail with 100 μl antigen preparation emulsified in CFA Serum-derived C5 was prepared by precipitating the euglobulin fraction of serum containing C5 as described (2). Mice received ~25 μg C5, assuming a concentration of 50 μg/ml serum.

Establishment of C5-specific T cell clones and hybrids

The generation of C5-specific T cell clones and hybrids has been described in detail (4).

Assay for evaluation of IL-2 production

The IL-2-dependent T cell line CTLL was cultured at 5×10^3 cells/well with supernatants to be tested for their IL-2 content. Culture medium provided the negative control. After 24 h, 1 μCi [^{3}H]thymidine was added per well and cultures were harvested 6 h later

Enrichment of antigen-presenting cells (APC)

Because of their low buoyant density, APC can be isolated on discontinuous Percoll gradients. Thymus or spleen cell suspensions were prepared and 2×10^7 cells were layered on a gradient consisting of Percoll density 1.074 followed by densities 1 062 and 1.05 in 11 ml polycarbonate tubes (Nunc) These tubes were spun at 5000 *g* for 20 min and APC were isolated from the low-density fraction between densities 1.05 and 1 062. The recoveries were ~7% from spleen and 0.4% from thymus.

Preparation of different feeder cell populations

O adherent cells (enriched for macrophages) were prepared by adsorption of spleen cells on 35 mm tissue culture dishes (Nunc) for 1.5 h Non-adherent cells were removed by vigorous washing and adherent cells were subsequently removed by addition of PBS containing 1 mM EDTA

O B cells were prepared by panning spleen cells from C5-immune O mice on Petri dishes (Optilux, Falcon) coated with a 1/1000 dilution of ascites from monoclonal anti-Thy 1.2 antibody 6-68. Non-adherent cells were subsequently subjected to two sequential rounds of adsorption on 35 mm tissue culture dishes to remove macrophages. B cells were activated with 30 μg/ml LPS for 24 h

O T cells were isolated by nylon wool passage of lymph node cells.

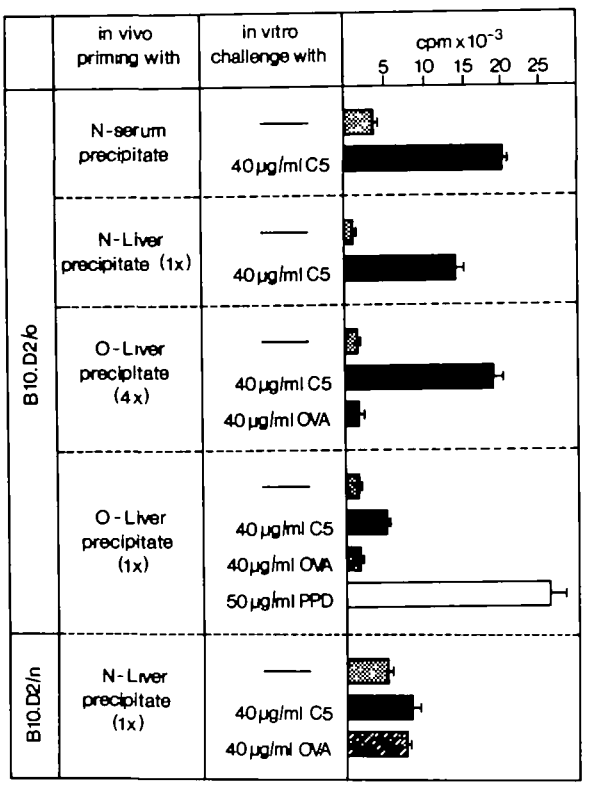

Fig. 1. C5-specific T cell proliferation after immunization with precipitates from liver or serum Three O mice per group were immunized in the base of the tail with immunoprecipitates emulsified in CFA of either N serum equalling ~10 μg C5/mouse, N liver equalling one liver equivalent and O liver equalling four or one liver equivalents, respectively A control group of N mice was injected with N liver precipitate Nine days later cells from draining lymph nodes were pooled and assayed for C5-specific proliferation in the presence of 40 μg/ml affinity-purified C5. Control wells received medium or an irrelevant antigen, ovalbumin Tuberculin PPD (50 μg/ml, Statens Institute) provided a positive control [^{3}H]Thymidine uptake (cpm) after 72 h of culture is shown.

Treatment with γ-interferon (γ-IFN)

APC from thymus and spleen were incubated in 96-well, round-bottom tissue culture plates with 250 U/ml recombinant rat γ-IFN for 24 h. The medium was then discarded and the plates were washed once with prewarmed culture medium.

Activation of T cell clones with fibroblast APC

Fibroblast APC (1×10^5/well; ref. 12, CA36.2.1 Ii) were pulsed with antigen for 6 h at 37°C, subsequently washed and irradiated

Table 1. T cell reactivity to pro-C5 *in vitro*

Responder	Medium[a]	pro-C5	C5	PR8 virus
C5 hybrid HA2	143 ± 5	45,545 ± 3218	45,781 ± 2226	285 ± 112
C5 hybrid C8-15	11,622 ± 403	47,386 ± 1209	42,628 ± 1083	9432 ± 1543
Influenza hybrid BV2-15	167 ± 23	193 ± 57	225 ± 167	59,252 ± 627

1×10^5 cells/well from hybrid HA2, C8-15, or BV2-15 were cultured with 1×10^5/well CA36.2.1 Ii fibroblast APC. APC were pulsed for 6 h at 37°C with either medium or affinity-purified C5 (5 μg/ml) or pro-C5 immunoprecipitated from extracts of 5 g B10.D2/o livers Immunoprecipitated material was eluted from Sepharose beads with 1 M KBr and dialyzed against PBS before addition to the APC After the 6 h pulse, APC were washed three times and irradiated 10,000 rad PR8 virus antigen (10 U/ml) was added to irradiated APC together with responder cells rather than pre-pulsed on APC. After 24 h of culture, 100 μl of supernatant were transferred to a fresh 96-well culture plate containing 5000/well IL-2-dependent CTLL cells for another 24 h culture period For the last 6 h 1 μCi/well [^{3}H]thymidine was present Results are expressed in mean cpm of thymidine upake ± SD.
[a]Values represent cpm $\times 10^{-3}$

with 10,000 rad. They were cultured with 2×10^4/well cloned T cells for 24 h. One hundred microliters of supernatants were then transferred to wells containing 5×10^3 CTLL cells for assessment of IL-2 production

Immunization with Listeria monocytogenes

Mice were immunized i v with 5×10^4 live *Listeria* (a gift from Dr Stefan Kaufmann, Ulm)

Results

T cell reactivity of C5-deficient mice to autologous C5

To test T cell reactivity to autologous pro-C5, liver extracts from C5-deficient B10.D2/o (O) mice and the congenic C5-sufficient strain B10.D2/n (N) were prepared and pro-C5 was precipitated with polyclonal anti-C5 antibody Precipitate from N and O liver extracts showed a single band of mol. wt ~180,000 corresponding to pro-C5 on SDS gels run under reducing conditions. Precipitates from N serum showed the two chains of secreted C5. The concentration of O pro-C5 was estimated to be ~8-fold lower than N pro-C5 (data not shown)

C5-deficient O mice and one group of C5-sufficient N mice were immunized in the base of the tail with precipitates in CFA They either received one liver equivalent of N or O or four liver equivalents of O, and N serum precipitate containing ~10 μg C5

The results presented in Fig. 1 clearly show that immunization with N serum precipitate, N liver precipitate, and O liver precipitate equalling four liver equivalents induced comparable C5-specific proliferation. Priming with one liver equivalent of O, which was estimated to contain ~8-fold less precipitated pro-C5, induced low but detectable proliferation. Absence of proliferation to the control antigen ovalbumin, as well as the failure of N mice immunized with N liver precipitate to respond to C5 in culture, excluded potential non-specific activation by protein A or antibody in the priming inoculum.

All O mice, with the exception of those immunized with one liver equivalent of O, generated C5-specific antibodies which were predominantly IgG_1 (data not shown). No antibody responses to C5 were detected in N mice regardless of the mode of immunization. These data provide evidence that T cells from C5-deficient mice are not tolerized to autologous pro-C5.

Pro-C5 from C5-deficient mice is immunogenic in vitro

Pro-C5 immunoprecipitated from C5-deficient livers can prime mice for C5-specific responses as shown in the previous

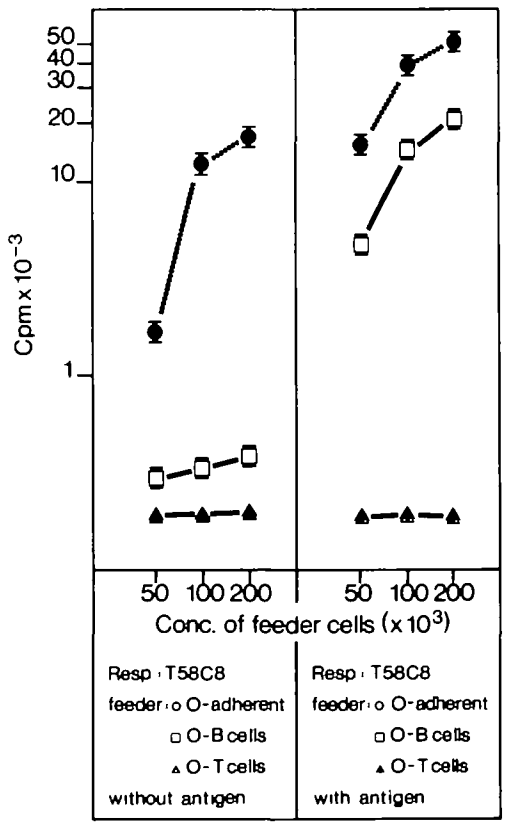

Fig. 2. Proliferative response of clone T58C8 in the presence of different O feeder cells. 2×10^4 cells/well of clone T58C8 were cultured in the presence of various doses of 3000 rad irradiated spleen adherent cells (●), LPS-activated B cells (□), or T cells (▲) without (left panel) or with (right panel) 5 μg/ml C5 for 72 h; 1 μCi/well [^{3}H]thymidine was added for the last 20 h

experiment. Its immunogenicity *in vitro* could not be tested in T cell proliferation assays since pro-C5 could not be further purified because the monoclonal antibody used for affinity purification of C5 does not cross-react with intracellular pro-C5.

To circumvent this problem we took advantage of C5-specific T cell hybrids which secrete IL-2 upon culture with I-E^d-bearing APC and C5 (4). For the following experiment we used I-E^d-bearing fibroblasts (13) which had been supertransfected with the invariant chain gene (CA36.2.1 Ii) and were extremely potent

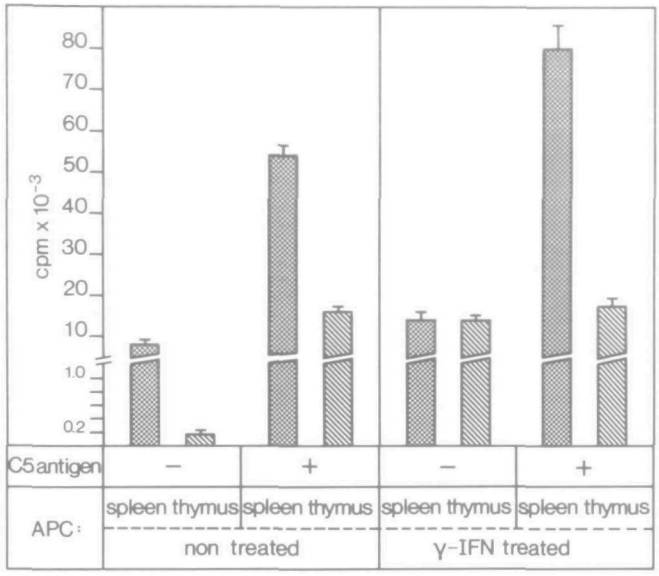

Fig. 3. C5-presenting capacity of spleen and thymus APC before and after treatment with γ-IFN. APC from thymus and spleen were isolated on discontinuous Percoll gradients. 2 × 10^5 APC/well were treated with either medium or 250 U/ml recombinant rat γ-IFN in 96-well, round-bottom tissue culture plates for 24 h. 2 × 10^4 cells/well of clone T58C8 were added to either non-treated APC or γ-IFN-treated APC in the absence or presence of 5 μg/ml C5 and cultured for 72 h. For the last 20 h, 1 μCi/well [^{3}H]thymidine was added to the wells.

APC (12). They were pulsed with either affinity-purified C5 or pro-C5 immunoprecipitated from C5-deficient livers, or C5 precipitated from normal serum and subsequently cultured with the C5-specific T cell hybrids C8-15 and HA2 for 24 h. Table 1 shows that not only normal C5 but also pro-C5 from C5-deficient mice was capable of activating these hybrids for IL-2 secretion. A T cell hybrid specific for influenza hemagglutinin was not stimulated by fibroblasts pulsed with C5 or pro-C5. We can conclude that C5-specific T cells are not tolerized to pro-C5 but recognize 'foreign' secreted C5 just as well as autologous pro-C5 which is still synthesized in C5-deficient mice.

Reactivity of T cells to pro-C5 presented by autologous macrophages

In vivo autologous pro-C5 does not appear in the circulation of C5-deficient mice and so cannot be processed and presented by every class II-bearing APC. The only cells that might present pro-C5 in C5-deficient mice are macrophages which synthesize this molecule themselves. Therefore it was of interest to test if T cells from C5-deficient mice could perceive autologous pro-C5 presented by macrophages in the absence of any exogenously added antigen. For these experiments, spleen cells from C5-deficient O mice were either enriched or depleted of Mϕ and tested for their antigen-presenting capacity to the C5-specific T cell clone T58C8 with or without exogenously added C5.

As shown in Fig. 2, T58C8 proliferated vigorously after culture with O adherent spleen cells that were enriched for macrophages in the presence or absence of C5. In contrast, feeder cells enriched for activated B cells and depleted for adherent cells were excellent APC for exogenously added C5 but they did not stimulate the clone in the absence of C5. Nylon wool-purified T cells could not stimulate the clone whether C5 was added or not.

Table 2. Absence of autoimmune reactivity in *Listeria* primed mice

	Antigen	B10.D2/N[a]	B10.D2/o
Exp. 1	–	13,252	7393
	C5	11,824	6350
	Listeria	109,604	76,571
Exp. 2	–	4769	3627
	C5	4934	4287
	Listeria	56,544	69,967

O and N mice were immunized twice with an interval of 1 week with 5 × 10^4 live *Listeria* i.v. For experiment 1, spleen and lymph node cells from three mice were pooled 8 days after the second immunization. 5 × 10^5 responder cells/well of a 96-well culture plate were cultured with 20 μg/ml C5 or 10^7 heat-killed *Listeria* for 72 h. For the last 20 h, 1 μCi/well thymidine was present. For experiment 2, spleen and lymph node cells from three mice 8 days after the second immunization were passed over nylon wool columns. 5 × 10^5 responder cells/well were cultured with 3 × 10^5/well 3000 rad irradiated spleen cells from untreated B10.D2/o mice in the presence or absence of C5 or *Listeria* as stated above.

[a]Values represent cpm × 10^{-3}.

This indicates that autologous intracellular pro-C5 is immunogenic for class II MHC-restricted T cells of C5-deficient mice, not only after exogenous application in CFA but also in its native form presented by macrophages from C5-deficient donors.

These results raise a puzzling paradox: given that pro-C5 presented by autologous macrophages is immunogenic for T cells, why are they not tolerized during thymic differentiation?

C5-presenting capacity of spleen and thymus APC

Lack of tolerance to pro-C5 might be the consequence of a combination of quantitative effects. Obviously the concentration of pro-C5 is lower in macrophages from C5-deficient mice than in C5-sufficient mice both at the level of mRNA (7) and at the protein level (our experiments with liver extracts).

In addition, class II MHC expression on macrophages is not constitutive (14) and, although macrophages in thymus have been reported to express class II (15), the levels of expression are low (16) so that peptides from pro-C5 might not be appropriately presented to class II MHC-restricted T cells.

Indeed, we found that thymus APC isolated by density gradient centrifugation could not present endogenous pro-C5 to clone T58C8 in contrast to spleen APC. Addition of exogenous C5 induced vigorous proliferation both with spleen and thymus APC, indicating that thymus APC contain dendritic cells that are constitutively class II MHC positive and can present exogenous C5. However, after treatment of thymus and spleen APC with recombinant rat γ-IFN, which induces MHC class II expression on macrophages (17) prior to addition of the T cell clone, thymus APC were capable of presenting endogenous pro-C5 (Fig. 3). These data support the assumption that the apparent deficiency of class II expression on thymic macrophages under physiological conditions may be one of the reasons that prevent induction of tolerance to pro-C5 during thymic ontogeny of T cells.

Absence of autoimmune anti-C5 reactivity in C5-deficient mice

Given the finding that T cells in C5-deficient mice are not tolerized

to pro-C5, one might expect development of autoimmune T cell responses triggered by recognition of class II positive, pro-C5-presenting macrophages in the periphery. However, we have failed to detect any C5-specific T cell reactivity in the absence of prior *in vivo* priming with C5.

It has been stated previously that the magnitude of a T cell response is determined by the product of concentration of antigen and MHC (18). Increasing one of the two parameters might be a means to stimulate an otherwise non-detectable T cell response. Likewise, we reasoned that up-regulation of MHC class II expression on macrophages might tip the physiological balance in C5-deficient mice in favor of triggering an autoimmune C5-specific response.

It is well documented that macrophage class II expression is up-regulated in the course of bacterial infection, most notably with intracellular pathogens like *Listeria* monocytogenes (19), mycobacterium tuberculosis (20), or *Trypanosoma cruzi* (21). In order to test if increasing the amount of macrophage class II would allow priming of C5-specific T cells, we immunized O or N mice with *Listeria* monocytogenes and tested for C5-specific T cell proliferation at various time points after secondary immunization. In no case could we detect C5-specific reactivity. Two representative experiments are shown in Table 2. For experiment 1 we used spleen and lymphocyte cells from *Listeria*-primed mice 8 days after the second immunization. For experiment 2 we enriched for T cells and depleted macrophages by passage of spleen and lymph node cells over nylon wool. In the latter case, fresh syngenic spleen cells were added to the cultures to provide purified responder T cells with APC. In both types of experiments we consistently obtained good *Listeria*-specific T cell responses but no C5-specific response.

So it appears that recognition of this intracellular self antigen *in vivo* does not occur even under conditions that induce strong MHC class II expression on pro-C5 synthesizing macrophages.

Discussion

C5-deficient mice are known not to be tolerant of C5 protein present in serum of normal mice. In this paper we present evidence that lack of tolerance extends to the intracellular precursor molecule pro-C5 which is synthesized but not secreted by C5-deficient macrophages (and hepatocytes).

Immunization with pro-C5-primed C5-deficient mice for C5-specific T cell responses and C5-specific T cells were capable of recognizing pro-C5 *in vitro*.

It is worth emphasizing that from the point of T cell recognition pro-C5 and C5 probably do not differ since conversion of the single-chain precursor to the functional heterodimer only removes four amino acids and retains the rest of the sequence (22). Deficient pro-C5 may have additional residues recognizable by T cells since it is slightly larger than normal pro-C5 (7). Nevertheless, our data clearly show that T cell responses to C5 and deficient pro-C5 overlap

Lack of tolerance to pro-C5 could indicate that this intracellular self protein is normally secluded from recognition by the immune system so that T cells encounter it like a foreign antigen upon immunization.

However, the finding that some C5-specific T cell clones (represented by T58C8) can recognize autologous pro-C5 in its native form presented by C5-deficient macrophages supports the assumption that this intracellular self antigen can be perceived by class II-restricted T cells. These clones appear to have a high affinity for antigen because they can be activated by 5- to 10-fold lower doses of antigen presented by fibroblast APC, compared with other clones that do not show stimulation by C5-deficient macrophages in the absence of exogenous antigen (data not shown).

Investigations of the antigenic requirements for activation of MHC-restricted T cell responses have provided evidence for the existence of distinct presentation pathways (reviewed in 23) Class I MHC-restricted T cells seem to be preferentially activated by peptides generated from endogenous proteins which associate with class I MHC (24) while exogenous antigen processed in the low pH milieu of endosomes seems to associate preferentially with class II molecules to trigger class II-restricted T cells (25). In contradiction to an absolute separation of these pathways is the recent description by Weiss and Bogen (26) of presentation of an endogenous lambda light chain determinant to class II-restricted T cells. Our data describing recognition of an endogenous, non-secreted protein by class II MHC-restricted cells indicate that at least some pro-C5 peptides have access to the class II presentation pathway.

However, presentation of pro-C5 peptides with class II MHC does not appear to be efficient enough *in vivo* to ensure induction of tolerance to this self antigen

Furthermore, there are no obvious signs of autoimmunity in C5-deficient mice despite the fact that C5-deficient macrophages from spleen were shown to activate efficiently some C5-specific T cell clones. There are several quantitative factors influencing presentation of this self antigen that probably contribute to the finding that macrophage pro-C5 is ignored by the immune system. Firstly, the amount of antigen available to stimulate T cells is low since C5-deficient macrophages synthesize ~ 10-fold less pro-C5 than normal macrophages. Secondly, the generally low and regulated expression of class II on macrophages might normally be insufficient to reach the triggering threshold for activation of C5-specific T cells *in vivo* In support of this assumption, we have shown that thymus APC could not activate a T cell clone in the absence of exogenous antigen unless they were previously treated with γ-IFN. This effect is most likely due to an increase in class II MHC expression since C5 synthesis in contrast to synthesis of other complement components, like factor B and C2, is not induced by γ-IFN (27).

On the other hand, stimulation of class II expression on macrophages in the periphery following *Listeria* infections did not result in activation of C5-specific T cells. This would indicate that a mere increase in class II on macrophages, which to some degree would also occur as a consequence of any ongoing immune response, does not suffice for recognition of pro-C5 by T cells. However, in view of the recently described interference of *Listeria* infection with macrophage presentation of other antigens, we cannot exclude the possibility that presentation of pro-C5 was prevented by *Listeria* infection (28).

A third quantitative factor influencing presentation of pro-C5 by C5-deficient macrophages could concern the biosynthetic pathway of this defective protein. It is conceivable that the majority of peptides resulting from the degradation of endogenous deficient pro-C5 appear in a cell compartment where they would preferentially associate with class I MHC and only a few peptides

would have access to the class II presentation route.

The unusual restriction in expression of a self antigen to macrophages that are potential, but not constitutive, APC for class II-restricted T cells offers the possibility to study qualitative and quantitative factors of antigen presentation. Regulation of this function could have crucial implications for development of autoimmunity.

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Abbreviations

APC	antigen-presenting cells
CFA	complete Freund's adjuvant
ConA	Concanavalin A
C5	fifth component of murine complement
PMSF	phenylmethylsulfonyl fluoride

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