IgM antibodies enhance the phagocytosis of apoptotic cells by immature dendritic cells

A Thesis submitted in partial satisfaction of the Requirements for the degree Master of Science in Biology

by

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<tr>
<td>4-HNE</td>
<td>4-hydroxynonenal</td>
</tr>
<tr>
<td>AC</td>
<td>Apoptotic cell</td>
</tr>
<tr>
<td>AS</td>
<td>Ankylosing Spondylitis arthritis</td>
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<tr>
<td>APC</td>
<td>Allophycocyanin</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CFSE</td>
<td>Carboxyfluorescein succinimidyl ester</td>
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<tr>
<td>C-PS</td>
<td>Pneumococcal cell wall Polysaccharide</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CTCL</td>
<td>Cutaneous T-cell lymphoma</td>
</tr>
<tr>
<td>ECP</td>
<td>Extracorporeal Photopheresis</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunoabsorbent assay</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>GVHD</td>
<td>Graft-versus-host Disease</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>iDC</td>
<td>Immature dendritic cell</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IV</td>
<td>Intravenous</td>
</tr>
<tr>
<td>MBL</td>
<td>Mannose-binding lectin</td>
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<tr>
<td>MDA</td>
<td>Malondialdehyde</td>
</tr>
<tr>
<td>NAb</td>
<td>Natural antibody</td>
</tr>
<tr>
<td>OA</td>
<td>Osteoarthritis</td>
</tr>
<tr>
<td>OxLDL</td>
<td>Oxidized low-density lipoprotein</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PC</td>
<td>Phosphorylcholine</td>
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<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
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ABSTRACT OF THE THESIS

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The clearance of dying cells is critical for maintaining tissue homeostasis, the prevention of autoimmunity, and the control of inflammation. The body produces billions of apoptotic cells (ACs) everyday, which normally are cleared by cells of the innate immune system, such as dendritic cells. These phagocytic cells recognize markers on the AC surface. If there is
inefficient clearance, these cells progress to secondary necrosis and release pro-inflammatory factors, which can lead to inflammatory responses and autoimmune diseases.

Numerous studies have shown that natural antibodies (NAb) recognize ACs, but our understanding of their role in immune responses is limited. To evaluate whether antibodies are important for enhancing the phagocytosis of dying cells, mice were immunized with ACs to induce increased levels of anti-AC antibodies. An AC-binding assay showed that post-immunization sera had increased levels of both IgM and IgG antibodies binding to the surface of ACs, but not healthy cells, which was also seen with the NAb T15 IgM that recognizes phosphorylcholine determinants. To determine whether IgG antibodies are essential for apoptotic clearance, sera from AC immunized mice were depleted of IgG by passage on a protein-G column. In a phagocytic AC clearance assay, IgM antibodies were shown to be primarily responsible in aiding phagocytic cells to clear ACs, and there was no essential role for IgG in the process.

Therefore, the results showed that IgM-natural antibodies, the T15 NAb and IgM antibodies induced by AC immunization, recognize apoptotic cells and enhance the ability of immature dendritic cells to phagocytose these cells.
INTRODUCTION

The Innate and Adaptive Immune Systems

Regulation of autoimmune diseases and infectious pathogens requires a balance between the innate and adaptive immune systems. Over 100 billion apoptotic cells are generated and must be removed from the body every day (YuFeng, et al. 2007). Since these cells need to be cleared quickly, the apoptotic cells must be rapidly and efficiently recognized and engulfed by components of the innate immune system.

The innate immune system is the first line of defense against pathogens that invade the body. These cells express soluble and membrane associated molecules that are directly determined by the genes that are inherited from our parents. While these innate immune cells must be capable of fighting a broad range of infections, these cells cannot retain “memory” of past immune exposures. The innate immune system includes several types of myeloid cells, such as macrophages, dendritic cells, neutrophils, basophils, and eosinophils, which each have some level of phagocytic capacity (Beutler, 2003). When a pathogen invades the host’s tissues, soluble components of the innate immune system may recognize and mark these foreign cells for immune recognition. For example, the complement proteins C1q or Mannose Binding Lectin (MBL) are recognition molecules that can bind to the pathogens. The binding of C1q or MBL may be sufficient to facilitate their clearance by phagocytic cells such as macrophages and dendritic cells.
However, interactions with these same molecules can at times also result in the activation of the complement cascade, which can lead to an inflammatory response that mobilizes the immune system to destroy the pathogen. Phagocytic cells also express other innate immune receptors, such as Toll-like receptors (TLRs), can also recognize other components of the pathogen and elicit an inflammatory cascade (YuFeng, et al. 2007). During an infection, these pathways can result in antigen uptake, processing, and presentation in macrophages and dendritic cells that leads to the activation of the T cells in the adaptive immune system.

While the innate response helps to slow the ongoing infection, the B and T lymphocytes of the adaptive immune system are also recruited as part of the immune response. Unlike innate immune cells, which can access only a limited array with germline encoded receptors, lymphocytes can use immense sets of antigen receptors, which are created by combinatorial gene rearrangement and somatic hypermutation, to recognize and attack the invading pathogen (Janeway, et al. 2008). The adaptive immune system can also generate immunologic “memory”, which allows for a more rapid and stronger response to the next exposure of the same pathogen. While the initial (or primary) response of the adaptive system develops relatively slowly such responses may persist longer, while the innate response are generally more rapidly triggered but are often short lived. While the innate and adaptive
immune systems function quite differently, together they have inherent
synergies that provide more effective defensives against infectious pathogens.

Interestingly, there are significant overlaps in the processes by which
infectious agents are cleared from the body and those involved in the
recognition and clearance of our own cells when they undergo apoptotic
death. This suggests that the immune system may use overlapping pathways
to identify and clear self and non-self determinants within the body (YuFeng, et
al. 2007).

**Complement Activation**

The complement cascade can be activated through three different
pathways: the classical pathway, alternative pathway, and the lectin pathway.
However, interactions with the initiation molecules of the classical and lectin
pathway do not always lead to pro-inflammatory responses, and may in fact
under certain settings have an anti-inflammatory influence. An anti-
inflammatory pathway may be essential for the clearance of apoptotic cells by
professional phagocytic cells without causing tissue injury. The classical
pathway is initiated by C1q binding to the Fc domain of IgG or IgM antibodies
that are bound to a cell surface antigen (Arnold, et al. 2006). Although C1q
interactions were thought at one time to be restricted to antibody-antigen
immune complexes, recent studies have shown that C1q can also be directly
recruited to determinants that newly arise on apoptotic cells (Roos, et al.
Cellular interactions with C1q are reported to have different outcomes, as the uptake by dendritic cells of C1q-opsonized apoptotic cells which can lead to the production of IL-6, IL-10, and TNF-α (Nauta, et al. 2004), while in other settings C1q may suppress inflammatory responses (Bohlson, et al. 2007).

MBL, which is structurally and functionally similar to C1q, can recognize and bind to apoptotic cells through carbohydrate-specific lectin domains. Like C1q, the binding of MBL to the surface of an apoptotic cell can also enhance clearance by macrophages (Ogden, et al. 2001) and by dendritic cells (Nauta, et al. 2004). It is also known that there is a subpopulation of antibodies that express the appropriate carbohydrate ligands that bind MBL. This includes some IgA in its dimeric or polymeric form, IgM, and some isoforms of IgG (Arnold, et al. 2006).

Our lab has investigated the functional properties of a monoclonal antibody, T15 IgM, which can discriminate apoptotic cells based on their expression of accessible phosphorylcholine (PC) residues that are not exposed on healthy cells. To understand the potential functional properties of this anti-apoptotic cell IgM antibody, we postulated that the T15 IgM flags the phagocytic cells for clearance by increasing the local deposition of MBL and C1q. However, it was uncertain whether this might also result in downstream complement activation. We have wondered whether MBL binding to T15 IgM complexes might have also affect the immune system in other ways, as MBL
can also increase the production of IL-10, an anti-inflammatory cytokine, by some phagocytes (Sprong, et al. 2004).

Lastly, the alternative pathway of complement cascade results in spontaneous C3 activation without the necessity of prior upstream antibody binding. While only certain specific antigens are able to activate the pathway, this pathway generally initiates a faster response. Other studies in our lab have therefore focused on understanding the role of these initiation opsonins, MBL and C1q, in T15 IgM mediated clearance of apoptotic cells by innate immune cells (Chen, et al. in press).

Modification on the Apoptotic Cell Surface

To maintain homeostasis, cells in the body die in a natural, organized (i.e., programmed cell) death by apoptosis, but if these cells are not efficiently and rapidly cleared they may progress to secondary necrotic death that is associated with a loss of membrane integrity and the release of cellular contents. During apoptosis, soluble and membrane-associated chemical signals are expressed to help phagocytes find and engulf the dying cells, whereas during necrotic death pro-inflammatory molecules are released that can lead to tissue damage (Janeway, et al. 2008). Apoptosis is also associated with an early loss of cell membrane symmetry, during which phospholipids such as phosphatidylserine (PS) flip to the outer leaflet of the cell membrane, and become exposed to the extracellular environment.
Exposure of these membrane-associated apoptosis-induced ligands is critical for the binding of opsonins, such as Gas6, Protein S, and β-2 glycoprotein-I to the apoptotic cell (YuFeng, et al. 2007), which marks these cells as targets for clearance by professional phagocytic cells, such as macrophages and immature dendritic cells.

Phosphorylcholine (PC) is a breakdown product of phosphatidylcholine that is found in the outer plasma membrane. While phosphatidylcholine is inaccessible to antibody interaction, when the cell undergoes apoptosis, the PC residues become modified by processes that allow it to be exposed for antibody recognition. Other proteins, such as the soluble innate molecule, C-reactive protein (CRP), can also bind to the exposed PC residues (YuFeng, et al. 2007; Chen, et al. in press). Previous studies have shown that the T15 natural antibody displays reactivity with a range of modified PC determinants on the cell (Shaw, et al. 2003).

**Natural Antibodies**

Antibodies against PC have long been known to aid in the defense against systemic *Streptococcus pneumoniae* infections (Briles, et al. 1981). Many of these antibodies belong to a class of natural antibodies (i.e., those which naturally arise without immunization), and in mice the T15 idiotype dominates these responses (Sigal, et al. 1975). These antibodies, which arise from T-independent responses, are usually of the IgM or IgG3 subclass and
are secreted by the CD5+ B-1 cells, a self-replenishing type of B lymphocytes that constitutively produces these natural antibodies throughout life (Herzenberg, 1989; Masmoudi, et al. 1990). The B-1 cells arise spontaneously and become highly represented during the first week of life, even in mice raised under germ-free conditions, indicating that the antibody selection and expression is not solely mediated by microbial ligand stimulation (Sigal, et al. 1975). These antibodies also recognize the PC head group moiety of phosphatidylcholine that is exposed on oxidatively modified low density lipoprotein particles (oxLDL) (Shaw, et al. 2000). A previous study demonstrated that the natural levels of anti-PC IgM in LDL receptor-deficient hyperlipidemic mice increased when vaccinated with *S. pneumoniae* (Binder, et al. 2003). Furthermore, the increased antibody levels in these hyperlipidemic mice was associated with reduced progression of atherosclerosis, which suggests there may be a protective role of anti-PC IgM in this murine model (Binder, et al. 2003). However, these immunizations could also have induced active cellular responses that affected atherogenesis, so the mechanism for this protection was uncertain.

**Apoptotic Cell Clearance**

The removal of apoptotic cells by phagocytic cells prevents the release of toxic intracellular contents, such as HMGB-1, and there is evidence that this process is also important for the prevention of autoimmune diseases. The
“waste disposal hypothesis” states that if there is a failure in clearance of apoptotic cells by macrophages, these cells may progress to secondary necrosis, and this process may also lead to the recruitment of immature dendritic cells (iDCs) to the site. The phagocytosis of these secondary necrotic cells by activated iDCs could instead lead to the presentation of autoantigens to T cells, which could lead to autoimmune disease (Walport, 2001). Conversely, recent studies have shown that non-activated iDCs continuously sample self-antigens in apoptotic cells that may play an important role in maintaining immune tolerance (Steinman, et al. 2003). In health, when iDCs are exposed to apoptotic cells, cytokine production is suppressed, cell clearance is increased, and iDCs are prevented from maturing (Takahashi, et al. 2003). Therefore, these attributes may be pivotal in determining how iDC may suppress or facilitate autoimmunity.

**Extracorporeal Photopheresis**

Although the original intent may have been different, a related process may occur in patients undergoing a clinical treatment called extracorporeal photopheresis (ECP). In 1988, ECP became an FDA approved treatment for cutaneous T-Cell Lymphoma, which has also been used to treat Graft-versus-Host Disease and solid organ transplant rejections, amongst a long list of other pathologic conditions. In this procedure, a volume of blood is drawn from a patient, and the white blood cells are separated from the red blood
cells. The white blood cells are then mixed with 8-methoxypsoralen, a light-activating photosensitizing drug that binds to the nuclear DNA, allowing the lymphocytes to be highly sensitized to ultraviolet A (UVA) light. The cells are then irradiated with UVA light to promote the death of these host cells. These cells are washed to remove the psoralen before re-infusion of the irradiated cells into the body. When the white blood cells are returned to the body, recognition of these dying cells is believed to alter immune responsiveness (Marshall, 2006). It was hypothesized that such treatments with circulating T-cell Lymphoma (CTCL) cells might induce specific immune responses to these malignant cells. However, other studies have shown that ECP treatments can also down-regulate levels of pro-inflammatory cytokines, and the enhancement of anti-inflammatory responses has also been reported (Bladon and Taylor, 2006). Based on our findings in murine models, we have wondered whether this treatment may also affect the levels of natural antibodies to apoptotic cells, and such induced anti-AC antibodies may then be responsible for effects on innate immune responses that provide clinical benefits.

Based on previous studies showing the protective role of the anti-PC antibodies, in the current study we performed immunizations of healthy BALB/c mice with apoptotic cells and evaluated how this affected levels of anti-PC antibodies. We then tested the ability of these anti-AC antibodies to bind to apoptotic cells and aid in their clearance in an ex vivo model. To
explore the greater clinical relevance of our studies, we also assessed the baseline and post-treatment levels of IgM antibodies to PC and to the related malondialdehyde (MDA) antigen in a cohort of healthy adults and patients undergoing ECP treatments.
MATERIALS AND METHODS

**Mice.** Six-week old male BALB/c, or B-cell deficient muMT mice were purchased from The Jackson Laboratory (Bar Harbor, ME), and HuCD20 transgenic (kind gift of M. Shlomchik, Yale University), and congenic C4-/- C57BL/6 (kind gift of M. Carroll, Harvard University). All animal protocols were provided by the NIH Guidelines for the Care and Use of Laboratory Animals approved by the Institutional Animal Care and Use Committees of the University of California, San Diego.

**Apoptotic cell treatment.** Mice were injected with up to $2.3 \times 10^7$ apoptotic (etoposide-treated) thymocytes in PBS through a tail vein injection, although fewer cells were injected in some experiments. Thymocytes were cultured for 16 hours in RPMI-1640 media with 10% FBS, 1% penicillin/streptomycin/glutamine, 1% 1M HEPES, 0.1% 1000X 2-mercaptoethanol and 10 µM etoposide to induce apoptosis during incubation in 5% CO$_2$ at 37°C. These injections were performed weekly for a total of four injections. Baseline serum samples were obtained the day before immunizations. At the first immunization, 6.3 million cells were injected with apoptotic thymocytes intravenously through the tail vein. For the second immunization, 10.5 million cells were injected and the third injection 20.5 million cells were injected into each mouse. After a two week break, the mice
were immunized with a final 23 million cells each through the tail vein. Earlier studies in the lab have shown that optimal responses require at least $2.5 \times 10^7$ apoptotic cells. Due to the limited availability of mice for these studies, for this immunization three of the mice were injected with apoptotic thymocytes from C4 knockout B6 mice, while the other three had apoptotic thymocytes from HuCD20.B6 transgenic mice. A final bleed was taken a week later to check levels of antibody production. Two week following the final immunization, terminal bleeds were performed on the mice by cardiac puncture.

**ELISA.** Sandwich ELISAs were performed using PC-(16)-BSA, goat anti-ms IgM, or goat anti-ms IgG as precoats. Anti-ms Fc gamma or Fc mu chain specific-HRP conjugated antibodies were used for detection.

**Apoptotic cell binding assay.** Apoptotic cells were prepared as shown above. Cells were then re-suspended at $2 \times 10^7$ cells/ml containing 10 µl/ml Fc block in MBL binding Buffer (10 mM Tris-HCl, 5 mM CaCl$_2$, 1% BSA). Using $1 \times 10^6$ apoptotic cells, appropriate conditions were added and binding was allowed for 40-60 min at 37°C. Cells were then stained for 20 minutes at 4°C in the dark and data acquired on a FACSCaliber (Becton Dickinson) and analyzed with CellQuest software (Treestar).
**Dendritic cell cultures.** Bone marrow from mice femurs and tibias were collected by flushing bones with RPMI-1640 media. Single cell suspensions were created by homogenizing cells through a 70 µm nylon cell strainer. Cells resuspended in complete RPMI media and cultured at 1x10^6 cells/ml with 10 ng/ml GM-CSF for 3 days. Additional complete media and GM-CSF was added and further cultured for another 2 days. Non-adherent cells were collected on day 5. Dendritic cells were selected by use of a LS magnetic columns with anti-CD11c beads with Fc block. Cells were then washed and prepared in Serum Free media (Stem Cell Technologies) for the phagocytosis assay.

**Phagocytosis assay.** Dendritic cells were prepared as shown above. Thymocytes were labeled with CellTrace CFSE staining. Preparation of stock solution followed the manufacturers protocol. Cells were then quenched with cold media and incubated with 10 µM etoposide at 2x10^6 cells/ml in a 5% CO₂ 37°C incubator for 16 hours. Apoptotic cells were then washed with media and re-suspended in serum free media before use in assay. Equal amounts dendritic cells and apoptotic cells (1 x10^6 cells each) were incubated for 60 minutes at 37°C incubator with 5% CO₂. Cells were then stained with CD11c-APC or MHCII FITC for analysis on CellQuest flow cytometry for 20 minutes at 4°C in the dark. To quantitate phagocytosis of CFSE labeled apoptotic cells data analysis was done on FlowJo.
**ECP patient sera.** Patient sera samples were a kind gift of Dr. Alain Rook (University of Pennsylvania).

**Statistical analysis.** Values are reported as mean ± SD unless otherwise stated. Significance was assigned for P<0.05 by two-tailed t test, with Welsh correction, or ANOVA, as appropriate (Instat, San Diego, CA).

These studies were performed in the Laboratory of Dr. Gregg J. Silverman and were supported by funding from NIH-NIAID, Alliance for Lupus Research, and the Research Education Foundation of the American College of Rheumatology Within Our Reach program.
RESULTS

Immunization of BALB/c mice

Immunization of mice with healthy immune systems with apoptotic cells has been shown to induce increased levels of antibodies to certain autoantigens, which include cardiolipin and DNA (Mevorach, et al. 1998). Apoptotic cell immunizations have been reported to induce the production of antibodies to phosphorylcholine (PC), which gets exposed following apoptotic death, and also other oxidation-associated neo-determinants, such as malondialdehyde (MDA) (Chen, et al. in press). To study the properties of AC-induced antibodies, a series of four immunizations were administered through weekly tail vein injections. Weekly bleeds were taken from the submandibular site using an animal bleeding lancet 24 hours prior to the next immunization. As a source of apoptotic cells, thymocytes from mice aged 6-11 weeks (see Methods). The levels of anti-PC IgM were then assayed by sandwich ELISA. The results showed that the mice averaged an increase in the levels of antibodies from 14.46 µg/ml to 38.08 µg/ml (P=0.001, paired \( t \) test with Welsh correction), having at least a two-fold increase post-immunization for most mice (Table 1). While we found that the induced antibody titers were lower than seen in previous studies in our lab that used injections of \( 2.5 \times 10^7 \) cells, this likely was due to our immunization with only \( 6.3 \times 10^6 \) to \( 2.3 \times 10^7 \) apoptotic cells. In addition, the low induced antibody levels could also be due to
incomplete infusions into the tail vein. In any case, in all treated mice consistent increases from baseline levels were documented. Sera from the mouse that showed the greatest AC-induced increases of anti-AC antibody levels were subsequently used in the antibody-dependent functional assays (Figure 2B).

**IgM binding to apoptotic cell surfaces**

To understand the binding properties of antibodies to apoptotic cells, I first tested the reactivity of the purified monoclonal T15 IgM, which is a natural antibody product of a B-1 cell clone. My studies confirmed that the T15 antibody had strong reactivity to a PC-BSA conjugate (data not shown), which is consistent with previous reports that this antibody is PC-specific, and reacts with a range of PC-containing antigens, such as purified pneumococcal cell wall polysaccharide (C-PS), copper oxidized low density lipoprotein (Ox-LDL), and the pneumococcal polysaccharide vaccine, Pneumovax. (Shaw et al. 2003, Chen, et al. *in press*). As a negative control, a B-1 cell derived isotype control IgM, NC17-D8, was used that showed little or no reactivity with any of the PC-determinants (Chen, et al. *in press*).

To test the binding capacity of the immunized sera with apoptotic cells, the different sera samples were incubated with apoptotic thymocytes for 40 minutes at 4°C and binding was assessed by flow cytometry. Binding of IgM or IgG and MBL to the apoptotic cells was also assayed in studies that compared
the levels of apoptotic cell binding by the T15 IgM antibody with the isotype control NC-17D8 IgM, or with muMT sera alone, no sera (buffer alone), post-immunized sera, and naïve sera. The sera of muMT mice were used because these mice are B-cell deficient and thus incapable of making immunoglobulins (Igs) (Kitamura, et al. 1991). Therefore, this Ig-deficient serum was used to supplement opsonins that may be required for phagocytosis in the absence of a possible influence of antibodies.

Compared to the level of binding associate with incubation with sera from naïve mice (mean 14.75 ± 4.89%), the post-immunized mice conveyed significantly higher levels of IgM binding to apoptotic cell surfaces (mean 38.1 ± 3.36%, p<0.001). The level of binding of the post-immunized sera was similar to that of the purified T15 IgM, when used at 20 ug/ml (mean 46.40 ± 3.68%, p>0.05) (Figure 3). Notably, T15 IgM and IgM in post-immune sera recognized a distinct subset of apoptotic cells. These findings showed that the T15 IgM and the IgM antibodies induced by AC immunization recognize similar subsets of these apoptotic cells. However, these studies did not specifically assess whether the antibody binding interactions with the apoptotic surface by post immune sera is PC-specific, or whether it may instead involve the recognition of different epitopes. These data do confirm that apoptotic cell immunization induces high levels of IgM antibodies that bind apoptotic cells.

The levels of AC binding were evaluated after sera were diluted at a 1:20 dilution and at a 1:100 dilution. While comparisons of naïve and post-
immunization sera at a 1:20 dilution showed little difference in the levels of antibody binding to the AC surface; at the 1:100 dilutions there were more significant and consistent differences in levels of the binding of IgM in naïve and post-immune sera. It is also possible that incubation with the more concentrated sample resulted in the complete saturation of potential apoptotic cell binding sites, while with the more dilute samples the levels of binding were not saturating, which enabled better demonstration of the differences in the levels of anti-AC antibodies in naïve and post-immunized sera.

**Anti-AC Abs enhance phagocytosis of apoptotic cells by immature dendritic cells**

Next, I wanted to see how the anti-AC antibodies may affect the clearance of apoptotic cells by professional phagocytic cells. To evaluate phagocytosis, I used bone-marrow derived purified CD11c+ conventional dendritic cells from C57BL/6 mice. We compared the abilities of naïve sera, post-immune sera and the T15 natural antibody to promote phagocytic clearance by immature dendritic cells. I used flow cytometry to assess the interactions of purified immature dendritic cells after incubation with CFSE-labeled apoptotic thymocytes. The immature dendritic cells were identified using gating based on their larger size (FSC) (Figure 4A), and was also confirmed by CD11c staining (Figure 4B). This gating strategy also allowed
the identification of the dendritic cell population that shifted in FL1 because they had ingested CFSE-labeled apoptotic cells.

Previous studies in our lab have shown that when no sera or antibodies were added to the cultured DCs with serum-free media for the 1 hour incubation at 37°C, only a low level of phagocytosis of the apoptotic cells was observed. Yet, compared to serum-free media alone the addition of Ig-deficient sera alone increased the level of phagocytosis, indicating the importance of serum opsonins in the clearance of apoptotic cells. These studies also showed that the addition of T15 IgM along with the Ig-deficient sera resulted in a significant increase in the levels of phagocytosis (Chen, et al. *in press*). In my studies, the addition of T15 IgM at 20 μg/ml also increased the level of phagocytosis (mean 7.41 ± 0.1%, p>0.05) (Figure 4B), however due to difference in technique and cell ratios the relative increase in the levels of clearance in my studies were not as great as those shown in other studies in our lab (not shown).

To assess the potential roles in enhancing iDC phagocytic function of the antibodies induced from the apoptotic cell immunizations, I compared the influence of sera from naïve mice and post-immunized mice. There was a significantly lower level of phagocytosis associated with the naïve sera (mean 4.94 ± 0.45%) than with the post-immunized sera (mean 7.58 ± 0.79%, N=6, p<0.001) (Figure 4B). I also found that the level of apoptotic cell clearance was comparable after incubation with the post-immunized sera and the T15
IgM, and both were greater than with naïve sera. This further suggested that the properties of the anti-AC antibodies that were induced by apoptotic cell immunizations were similar to those of the T15 IgM monoclonal antibody.

**Depletion of IgG from post-immunized sera**

To assess for a role of IgG antibodies in these responses, an aliquot of post-immunization sera with the highest increase in anti-PC IgM levels was passed through a Protein G column in order to remove all IgG antibodies due to specific binding via the Fc gamma region of IgG, but not the IgM antibodies, resulting in the selective removal of IgG antibodies from the sera. To isolate these IgG antibodies from the column, after washing with a neutral pH buffer, a low pH elution buffer was then applied, which resulted in the disruption of the interaction between the antibodies and the protein G beads. The eluted IgG antibodies were then collected in fractions containing neutralization buffer.

With ELISA assays, I confirmed that the eluate from this column had increased levels of IgG antibodies. Indeed, the passage of the sera over the column resulted in over 95% depletion of total IgG content (Table 2). Passage over the protein G column also completely removed all the anti-PC IgG antibodies (Table 3). The specificity of these interactions was also confirmed by comparisons with an aliquot of the same sera that was instead passed through a control sham sepharose column. Here, I found that there was no significant change in the levels of IgG after passage over the control
sepharose column (data not shown). Taken together, this study showed that passage over the protein G column selectively removed IgG from post-immunized sera.

The role of IgG in apoptotic cell binding and phagocytic clearance

To assess for a role of IgG in post-immune responses, the AC binding assay was used to compare the influence of the IgG depleted sera with the IgG enriched eluted sample of post-immunized sera. While earlier studies have shown that IgM antibodies that bind apoptotic cells can result in increased levels of opsonin binding and enhanced apoptotic clearance, the potential contribution of IgG antibodies to these processes has not been well characterized. The IgG depleted serum sample was therefore incubated with apoptotic cells for 1 hour at 4°C to allow for binding. I found that incubation with the IgG-depleted sera resulted in similar levels of IgM binding to the apoptotic cell surface, which indicated that the presence of IgG was not required for IgM binding to apoptotic cells. However, I also confirmed that the IgG depleted sera had little or no binding of IgG antibodies to the apoptotic cells, as I had expected (Figure 5A). When looking at anti-IgG stained apoptotic cells, there was almost no IgG binding to the cells in the IgG depleted samples while the IgG eluted sample had levels of IgG binding to the apoptotic cells, with levels similar to that associated with the unfractionated post-immunization sera (Figure 5B). In contrast, the control sepharose column
did not alter the binding to the apoptotic cells of IgG in these same post-immunization sera sample (Figure 5B). Nevertheless, I found that AC immunization of mice increased the levels of IgG content in the sera, akin to that also seen with the IgM antibodies.

To find out if the IgG antibodies were essential for the increased apoptotic cell clearance occurring after incubation with post-immune sera, I compared the aliquot of sera that was passed though the protein G-sepharose column and the unfractionated post-immunized sera. To compensate for the dilution of the sample that occurred during passage through the column, sera were added at 20% instead of the 5% sera for the other conditions. It was shown that there was no statistically significant change in apoptotic cell clearance by the dendritic cells with (7.58 ± 0.79%) or without IgG (7.22± 0.89%) or in sera passed through the control column (7.89 ± 0.55%, N=3, p>0.36) after incubation for 1 hour at 37°C (Figure 6). These results indicated that the depletion of IgG did not affect the efficiency of phagocytosis of apoptotic cells, and that IgM antibodies, which are the dominant isotype of anti-AC antibodies induced by immunization, appear to be the major contributor to the enhanced clearance of apoptotic cells.

**Apoptotic clearance in human samples treated with ECP**

To determine whether humans also have similar antibody responses, I evaluated the levels of anti-PC IgM and anti-MDA IgM antibodies in the sera of
forty-two patients with a range of disease states, including graft-versus-host disease (GVHD), cutaneous T-cell Lymphoma (CTCL), cardiac allograft rejection, and chronic arthritis, osteoarthritis (OA) and ankylosing spondylitis (AS). In some cases, longitudinal sera samples were available. Most of these patients received extracorporeal photopheresis (ECP) treatments that involve infusions of ACs. As a control, samples were also obtained from healthy volunteers from the lab and from the San Diego Blood Bank.

In these studies, the anti-PC response from healthy sera (587.6±343.84 relative units RU, N=13) were significantly higher than the levels seen in the GVHD patients (188.4±66.65 RU, N=4, p=0.0392) and in the cardiac allograft rejection patients (187.1±126.37 RU, N=4, p=0.0387) (Figure 7). The levels of anti-PC IgM also appeared to change over time, depending on the disease. In CTCL and the cardiac allograft rejection patients, there was little or no change over time in the levels of anti-PC IgM. However, the GVHD patients, who had low baseline levels, had significant increases in antibody levels over the course of the treatment (Table 4). Therefore, these data may indicate that AC infusions in humans can induce higher levels of IgM anti-PC antibodies.

When the anti-MDA IgM levels were examined, I found no significant differences in patients with any of diseases from the healthy controls (Figure 8). However, although not statistically significant, the levels of antibodies in the CTCL and GVHD patients (2872.5 ± 2444.5 RU N=16 and 2876.3 ±
1459.04 RU N=4, p>0.05) were almost double those found in the healthy subjects (1435.7 ± 1790.6 RU N=14), while levels in the cardiac allograft rejection patients (444.5 ± 94.7 RU N=4) were below half the mean values found in healthy adults. Taken together, these studies showed that there can be significant differences in the relative levels of anti-PC IgM antibodies in CTCL, GVHD, and cardiac allograft rejection patients treated with ECP compared to healthy controls. While I found that levels for CTCL and cardiac allograft rejection had limited change over time, with the same treatment when used for GVHD there seemed to be increases in the levels of anti-PC antibodies. For the anti-MDA antibodies, there seemed to be almost no difference between patients with different diseases. Although these studies are limited and only show the levels of antibodies present, and cannot assess rates of production or consumption, these findings may suggest there is clinical relevance of anti-PC IgM antibody levels that is linked to the progression of disease or response to ECP therapy.
DISCUSSION

Immune responses have evolved to allow the host to fight off infections and tissue injuries, but we have found evidence that some IgM antibodies may play a role in regulating a key functional activity of the innate immune system, which is involved in immune tolerance and the control of inflammation. The phagocytic clearance of dying cells is one of the most fundamental functions of the innate immune system, and defects in the clearance of dying cells can cause an increase in the level of autoantibodies and of pro-inflammatory signaling that can lead to the development of autoimmune disease. Therefore, this study shows that although naïve mice have modest baseline levels of IgM anti-AC antibodies that aid phagocytic cells in the clearance of apoptotic cells, the level of clearance can be enhanced by immunizing mice with apoptotic thymocytes, which increased the levels of circulating anti-AC antibodies. Furthermore, I now show that, akin to the murine monoclonal T15 IgM, these IgM anti-AC antibodies from post-immunized sera bind to the AC surface and help to increase the levels of phagocytic clearance in the presence of serum opsonins. My results also suggested that the antibodies that signal the phagocytic cells for clearance are mostly of the IgM isotype and there appears to be no essential contribution from anti-AC IgG antibodies to this process. These findings therefore provide evidence for the important roles of natural antibodies in apoptotic cell clearance.
Along with these studies, other experiments done in the lab have shown that naïve mice already have low levels of circulating natural antibodies to apoptotic cells that allow for the clearance of dying cells by professional phagocytic cells (Figure 4B, Chen, et al. in press). We therefore hypothesized that increasing the levels of these antibodies to apoptotic cells could help immature dendritic cells and macrophages clear the dying cells more efficiently and also prevent their contribution to an inflammatory response.

After a series of four weekly immunizations of BALB/c mice with apoptotic cells, there was a significant increase in the levels of anti-PC IgM antibodies (Table 1). C57BL/6 mice were previously used for immunization in other studies in the lab; however, in my hands, the anti-PC IgM induction of these mice was not as great as seen in other studies from our lab. A previous study by Cancro, et al. (1978) showed that immunization with S. pneumoniae induced significantly greater increases in the concentrations of serum T15-idiotype bearing antibody in BALB/c mice than the C57BL/6 mice, which is why I shifted to use BALB/c mice. My studies confirmed that the anti-PC responses induced by apoptotic cells were greater with the use of BALB/c mice than when I used C57BL/6 mice, but it was still lower than in previous studies in our lab. The heterogeneity in these induced responses could also be attributed to the lower levels of apoptotic cells that I used for the first two intravenous challenges. My inexperience and technical variability in the
number of apoptotic cells actually injected into the tail veins of these mice were also likely contributors to the low induced antibody levels.

My studies were designed to test the hypothesis that AC immunization, which results in increases in anti-AC antibodies, also results in higher binding to the apoptotic cell surface and concordant increases in the level of immature dendritic cell phagocytosis. A binding assay with labeled apoptotic cells was therefore developed, which showed that following immunization the binding characteristics of the induced IgM response appeared very similar to that of the T15 IgM NAb, and the level of binding of IgM in post-immune sera was greatly increased compared with naïve serum (Figure 3). To test the ability to aid in cellular clearance, a phagocytosis assay was also performed. The post-immunized sera along with the T15 IgM had the highest levels of CFSE-labeled apoptotic cell clearance, suggesting that in the presence of serum opsonins these antibodies facilitated iDC phagocytosis (Figure 4B). However, the cell ratios used in these studies were lower than those used in previous studies as I used fewer apoptotic cells. Nonetheless, these studies did show that with in vivo AC challenge, there was induction of IgM anti-AC antibodies that also increased rates of phagocytosis by immature dendritic cells. However, although these antibodies did increase apoptotic clearance, these studies did not directly assess whether the antibodies induced by AC immunization are PC-specific. It remains likely that the binding to the apoptotic cells involves a range of other epitopes.
An important component of the study was the finding that antibody isotype is a determinant of the efficiency of phagocytosis. Passage over a protein G column was shown to deplete over 95% of the IgG from the post-immunized sera. Without IgG in the sera, the levels of IgM binding to the apoptotic cell surface were not altered (Figure 5A), and the IgG depleted sample also had no effect on the amount of phagocytosis by dendritic cells although no IgG is bound to the cell surface (Figure 6). This provided evidence that IgG is not essential for binding to apoptotic cells or for their clearance. Together, these studies show the possible roles of the natural antibodies in aiding clearance of apoptotic cells by immature dendritic cells. However, it remains possible that some anti-AC IgG antibodies, induced by AC immunization, also have the capacity to enhance phagocytosis under certain settings.

Furthermore, these studies also showed that not all IgM antibodies from B-1 cells can bind and assist in the clearance of apoptotic cells. The isotype control IgM, NC-17D8, used in these studies, is a B-1 cell derived antibody shown in other studies to recognize bromelain-treated red cells, and similar antibodies have been implicated in the removal of senescent red cells (Mercolino, et al. 1989). However, my studies showed that this IgM neither bound efficiently to the cell surface of apoptotic thymocytes (Figure 3) nor did it facilitate apoptotic thymocyte clearance (Figure 4B). Therefore, while this
IgM may have other roles in immune defenses, it does not appear to have significant activity for apoptotic thymocyte clearance.

Previous studies within our lab have also looked at the role of serum opsonins, particularly MBL and C1q, in assisting apoptotic cell clearance. Interestingly, some settings interactions with C1q can result in DC maturation and the release of inflammatory cytokines (Csomor, et al. 2007). Studies have also shown that without C1q, the defect in recognition of dying cells results in impaired removal of apoptotic cells, and this can lead to inflammation. These studies have shown that both C1q and MBL, independent of their downstream role in activating the classical complement pathway, play important roles in the regulation of professional phagocytic cells and the clearance of apoptotic cells, therefore preventing the release of inflammatory factors (Chen, et al. in press). Others in the lab have also shown that that the addition of purified C1q or MBL to cultures of apoptotic cells with dendritic cells and T15 IgM further enhances the levels of apoptotic cell clearance (Chen, et al. in press). Although I performed similar studies with the post-immunization sera, I did not evaluate the amount of MBL or C1q recruited T15 IgM binding to the apoptotic cell surface. In the future, additional studies should be performed to further assess the roles of these opsonins. Purified C1q and MBL could be added to determine if their presence together and separately induces higher levels of eating by phagocytic cells.
My studies on natural antibodies and apoptotic cell clearance have led to many new questions regarding the pathogenesis of autoimmune and inflammatory diseases. While our studies suggest that these antibodies may be protective against disease activity in certain conditions, it has also been reported that the level of the anti-PC IgM antibodies can be decreased in patients with certain pathogenic conditions (Padilla, et al. 2004; Su, et al. 2008). In specific, patients with severe tissue damage from limb ischemic injury have been reported to have very low levels of IgM anti-PC antibodies presumably due to local IgM deposition in the injured tissues (Padilla, et al. 2004). Our studies also showed that patients with GVHD and cardiac allograft rejection also had significantly lower levels of anti-PC IgM when compared to healthy individuals or other diseased patients. It could be possible that the chronic inflammation in these patients may contribute to the consumption of the anti-PC antibodies in vivo. We wonder whether this consumption is part of a physiologic process that acts to enhance local clearance of apoptotic cells and reduce the local inflammatory process. In support, our murine studies have shown that increases in anti-PC IgM antibodies results in more efficient clearance of apoptotic cells and blebs by phagocytic cells. Such a process may therefore explain reports from our clinical collaborators that patients with GVHD and cardiac allograft rejection commonly benefit from ECP treatments, while such benefits are less predictable with other conditions. While our studies provided evidence of modest ECP induced increase in IgM anti-PC
responses in several cases, our studies could not determine the relative rates of production or consumption of these antibodies. In addition antibody levels in these patients could have been altered by the disease itself, by other co-morbid conditions, or by other therapies they may be getting. Our studies are therefore not conclusive. It is also difficult to determine the underlying mechanisms of the immune system because insufficient clinical data is known about the patients before and after treatment. Therefore additional studies should be performed to determine what may be causing the decrease in levels of in anti-PC IgM in these patients and the potential roles in chronic inflammation.

Another documented role of anti-PC IgM antibodies involves binding to oxLDL and atherosclerotic plaques (Shaw, et al. 2003). Further studies should determine what function these antibodies have in the disease progression of atherosclerosis and how they may be developed into therapeutic agents. In conclusion, additional research should be done to uncover more about the potential roles of natural antibodies to apoptotic cells in other aspects of the immune system, and how such mechanisms may be involved in the control of inflammation and the progression of autoimmune diseases.
Figure 1. Role of cell surface modification in recognizing and clearance of apoptotic cells. Phosphatidylserine (PS) flipping from the inner to outer leaflet of the apoptotic cell allows for the binding of certain opsonins, which can then mediate binding the receptors on the phagocytic cell. The modification of membrane phospholipids during apoptotic death also exposes phosphorylcholine (PC) which allows certain IgM antibodies to bind. The binding of specific IgM can also enhance the level of C1q and/or MBL binding, which provides “eat me” signals for the phagocytic cell.
Figure 2. Immunization of BALB/c mice protocol. Mice immunizations on days 0, 7, 14, and 28 were done by tail vein injection of up to $2.5 \times 10^7$ apoptotic cells in 30 µl PBS. Mice sera were then studied on day 35 with comparisons to prebleeds.

**Study Mice**
1. Terminal Bleed
2. Binding to Apoptotic cells
3. Phagocytosis by iDCs
Figure 3. Serum reactivity of BALB/c mice with PC-antigens. ELISA plate wells were coated with PC-BSA and developed with enzyme-conjugated anti-IgM antibody. Sera were obtained after bleeds each week following treatment with apoptotic cells on day 0, day 7, day 14, day 28, and final bleeds were taken on day 35.
Figure 4. IgM binding to the surface of apoptotic cells is enhanced in post-immunized sera. The results compare the binding of T15 IgM, isotype control, Ig-depleted sera, naïve or post-immunized sera when incubated at 4°C for 40 minutes with apoptotic thymocytes.
Figure 5. Post-immunized sera and T15 IgM binds apoptotic cells and enhances the level of phagocytosis by immature dendritic cells. (A) Flow cytometric gate on purified bone marrow-derived immature dendritic cells after incubating with CFSE-labeled apoptotic thymocytes. Larger cells are healthy iDCs, and the smaller are apoptotic cells. Apoptotic thymocytes have been CFSE labeled to allow determination of iDCs that have ingested apoptotic cells. (B) Using this same gating, results are compared after 1 hour incubation at 37°C of DC and AC, with T15 IgM or isotype control at 20 µg/ml, 10% Ig-deficient sera, naïve sera and post-immunized sera at 1:10 dilution. Phagocytosis of apoptotic thymocytes is increased by T15 and post-immunized sera addition, compared to the absence of Ig or with naïve sera.
**Figure 6.** IgM antibody binding to apoptotic cells is not affected by IgG depletion of post-immunization sera. Gating on all significant events, the results compare IgG depleted sera binding to apoptotic cell surfaces when incubated at 4°C for 40 minutes with apoptotic thymocytes. The cells were stained with anti-IgM APC to see relative IgM binding to the cell surface.
Figure 7. IgG antibody binding to apoptotic cells is not affected by IgG depletion of post-immunization sera. Same gating methods and experimental methods were used to compare post-immunized sera to IgG depleted sera using an anti-IgG FITC stain to visualize IgG binding to the cell surface. However, gating could have been shifted to the right no clearly mark the negative IgG population when no sera or antibodies were added.
Figure 8. The depletion of IgG from post-immunization sera does not significantly diminish the enhancement of phagocytosis by immature dendritic cells. Same flow cytometric gating of purified bone marrow-derived immature dendritic cells with CFSE-labeled apoptotic thymocytes as in figure 4. Phagocytosis was unaffected by the removal of IgG, in sera normalized based on levels of IgM anti-AC antibodies.
Figure 9. Anti-PC IgM levels in healthy controls and diseased patients. Data complied from a series of ELISAs. Plates were coated with PC-BSA (5 µg/ml). Samples from various patients were added and incubated for 4 hours at RT. Detection by HRP-conjugated anti-hu IgM to obtain relative values of anti-PC IgM levels in healthy and diseased patients.
Figure 10. Changes in anti-PC IgM levels over time in diseased patients. Data compiled from a series of ELISAs. Only patients that were measured at multiple time points were included.
Figure 11. Anti-MDA IgM levels in healthy controls and diseased patients. Data compiled from a series of ELISAs. Plates were coated with MDA-BSA (5 µg/ml). Samples from various patients were added and incubated for 4 hours at RT. Detection by HRP-conjugated anti-hu IgM to obtain relative values of anti-MDA IgM levels in healthy and diseased patients.
Table 1. Levels of anti-PC IgM induced from immunization with apoptotic cells. Pre-immunization and post-immunization levels of each mouse after four i.v. injections.

<table>
<thead>
<tr>
<th></th>
<th>pre-immunization</th>
<th>post-immunization</th>
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<tbody>
<tr>
<td>Mouse 2</td>
<td>19.71 µg/ml</td>
<td>37.40 µg/ml</td>
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<td>Mouse 3</td>
<td>17.18</td>
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<td>Mouse 20</td>
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<td>Mouse 30</td>
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<td>27.80</td>
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<td>Mouse 50</td>
<td>22.01</td>
<td>84.74</td>
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<tr>
<td>Average</td>
<td>14.46 µg/ml</td>
<td>38.09 µg/ml</td>
</tr>
<tr>
<td>Std. Dev.</td>
<td>6.47</td>
<td>23.50</td>
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Table 2. Levels of IgG antibodies. ELISA plate wells were coated with goat anti-mouse IgG, γ chain specific, molecules and developed with HRP–conjugated anti-ms IgG antibody for detection of total IgG concentrations (µg/ml) of fractions after Protein G column passage. Values were determined by comparison to a monoclonal IgG anti-PC standard curve.

<table>
<thead>
<tr>
<th>Total IgG</th>
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<tbody>
<tr>
<td>Original Sample</td>
<td>8649.18</td>
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<tr>
<td>Flow Through 1</td>
<td>154.23</td>
</tr>
<tr>
<td>Flow Through 2</td>
<td>73.07</td>
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<tr>
<td>Eluant 1</td>
<td>409.45</td>
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<td>Eluant 2</td>
<td>246.33</td>
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Table 3. Levels of IgG anti-PC antibodies. ELISA plate wells were coated with PC-BSA and developed with HRP–conjugated anti-ms IgG antibody to detect the total levels of anti-PC IgG in fractions. Values were determined by comparison to a monoclonal IgG anti-PC standard curve.

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Masmoudi, H., T. Mota-Santos, F. Huetz, A. Coutinho, and P.A. Cazenave. 1990. All T15 Id-positive antibodies (but not the majority of VHT15+ antibodies) are produced by the peritoneal CD5+ B lymphocytes. Int. Immunol. 2(6): 515-520.


