

Mammalian N-Glycan Branching Protects against Innate Immune Self-Recognition and Inflammation in Autoimmune Disease Pathogenesis

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SUMMARY

Autoimmune diseases are prevalent and often life-threatening syndromes, yet the pathogenic triggers and mechanisms involved remain mostly unresolved. Protein asparagine linked-(N-) glycosylation produces glycan structures that substantially differ among the extracellular compartments of evolutionarily divergent organisms. Alpha-mannosidase-II (α M-II) deficiency diminishes complex-type N-glycan branching in vertebrates and induces an autoimmune disease in mice similar to human systemic lupus erythematosus. We found that disease pathogenesis provoking glomerulonephritis and kidney failure was nonhematopoietic in origin, independent of complement C3 and the adaptive immune system, mitigated by intravenous administration of immunoglobulin-G, and linked to chronic activation of the innate immune system. N-glycans produced in α M-II deficiency bear immune-stimulatory mannose-dependent ligands for innate immune lectin receptors, disrupting the phylogenetic basis of this glycomic recognition mechanism. Thus, mammalian N-glycan branching safeguards against the formation of an endogenous immunologic signal of nonself that can provoke a sterile inflammatory response in the pathogenesis of autoimmune disease.

INTRODUCTION

Autoimmune diseases arise when immune stimuli override mechanisms of self-tolerance and are often diagnosed by elevations in autoantibody titers. Pathogenesis is generally attributed to the effector functions of the adaptive immune system. Systemic lupus erythematosus (SLE) is an autoimmune syndrome in which autoantibodies to nuclear antigens and immune complex formation are induced with

a high prevalence of kidney disease (Jorgensen et al., 2004; Lauwerys and Wakeland, 2005). The etiology of the SLE-like syndrome that develops from the absence of alpha-mannosidase-II (α M-II) is puzzling (Chui et al., 2001). α M-II is conserved among mammals, and its deficiency in mice alters protein N-glycosylation in some cell types by blocking the formation of complex-type N-glycans that normally constitute the predominant N-glycan branching structure on the vertebrate cell surface (Chui et al., 1997).

Incomplete N-glycan branching in the Golgi apparatus results from α M-II deficiency, leading to the appearance of hybrid-type N-glycan structures at the cell surface. As mice age without α M-II function, signs of SLE invariably appear with an increase in anti-nuclear antibody (ANA) titers, a hematologic abnormality characterized as dyserythropoietic anemia, and glomerular deposition of immunoglobulins and complement component C3. Glomerulonephritis is the major pathologic feature in these animals leading to sclerosis, renal dysfunction, and kidney failure in a syndrome indicative of lupus nephritis (Chui et al., 2001). Remarkably, diminished N-glycan branching is observed among only a subset of cell lineages in α M-II deficiency resulting from the presence of another mannosidase, termed α M-IX, that can compensate to promote complex-type N-glycan branching (Chui et al., 1997; Akama et al., 2006). Although this compensation occurs among lymphoid and myeloid cells, it is absent from the erythroid lineage and variable in efficacy among multiple cell types (Chui et al., 1997, 2001; Akama et al., 2006). For example, T and B lymphocytes that lack α M-II retain high amounts of complex-type N-glycans. Not surprisingly, lymphoid cells develop normally in α M-II deficiency and respond to immunologic stimulation without alterations (Chui et al., 2001). These findings have suggested that α M-II-deficient autoimmune disease may reflect a distinct etiology encompassing a pathogenic mechanism that involves endogenous epitope modification (Wakeland et al., 2001).

We have used molecular and cellular approaches that discriminate among the roles of cell lineages to determine the origin and mechanism of the SLE-like autoimmune disease resulting from α M-II deficiency. Our findings show

a pathogenic trigger among nonhematopoietic cell types and glycoproteins that undergo altered protein N-glycan branching, indicating a sterile signal of infection and inflammation composed of extracellular mannose-dependent N-glycan ligands of innate immune lectin receptors. The adaptive immune system does not appear to play a pathogenic role and instead moderates the disease course, attenuating the inflammatory route that encompasses macrophage recruitment, glomerulonephritis, and kidney failure. Pathogenesis in α M-II deficiency arises by disrupting a mechanism of vertebrate nonself discrimination by which the innate immune system normally distinguishes the glycomes of lower eukaryotic and prokaryotic organisms.

RESULTS

Normal Kidney Morphogenesis in α M-II Deficiency

Tissue and organ damage in SLE is often focused and most severe in the kidney. To investigate the pathogenic basis of this autoimmune disease syndrome in α M-II deficiency, we first studied kidney development and early postnatal function. Among kidneys of animals analyzed at birth, we observed unaltered glomerular organization at the electron microscope level with normal glomerular basement membrane, epithelial foot processes, and filtration slits (Figure S1 in the Supplemental Data available online). In addition, kidney function in mice lacking α M-II was unimpaired during the first 6 months of life as determined by urinalysis measurements of hematuria and proteinuria. These findings indicate that the development of glomerulonephritis between 6 and 9 months of age is not associated with an ontogenic or functional disruption of the kidney *per se*.

Nonhematopoietic Origin of Autoimmune Disease in α M-II Deficiency

Autoimmune diseases may be of hematopoietic origin and resolved to abnormalities in lymphoid cell function, as evidenced in numerous bone-marrow transplantation studies (Ikehara, 1998). We subjected 8-week-old wild-type and α M-II null littermates to irradiation levels that were invariably lethal without hematopoietic reconstitution by bone-marrow grafts. At this age, disease pathology is not yet present and all mice are healthy. Syngeneic bone-marrow recipients that were fully reconstituted by donor-derived hematopoietic cells by 8–10 weeks and which attained normal peripheral leukocyte numbers in circulation were further studied up to 9 months after transplantation. Recipients analyzed remained donor derived throughout the course of the study as determined by hematopoietic cell-surface N-glycan markers and genomic DNA analyses (Figure 1A and data not shown). No complications reflecting graft-versus-host disease were noted in the experimental populations. Lymphoid lineage development, abundance, and activation responses were unaffected, as in systemic α M-II-deficient mice, because of the presence of the α M-Ilx isozyme (Chui

et al., 2001). Remarkably, elevated autoantibody titers to cellular and nuclear antigens, glomerular deposition of immunoglobulin-G and complement C3, as well as kidney inflammation and dysfunction were linked to α M-II-deficient recipients (Figures 1B–1F). Autoimmune disease pathogenesis was neither provoked by α M-II-deficient bone-marrow-derived cells nor attenuated by wild-type marrow grafts. Instead, the pathogenic trigger that leads to the induction of autoantibodies and kidney disease in α M-II deficiency resides among nonhematopoietic cell types.

Among disease markers, only the dyserythropoietic anemia was linked to α M-II-deficient bone-marrow-derived and hematopoietic lineages (Figure 1G). This is consistent with the erythroid dependence on α M-II, a cell lineage wherein no compensation by the α M-Ilx isozyme occurs, resulting in hybrid-type N-glycans at the cell surface and increased turnover of the anisotropic population of circulating erythrocytes (Chui *et al.*, 1997). It is evident from these studies that this erythroid defect does not contribute to the elevation of autoantibody titers or to kidney disease.

Attenuation of Disease by the Adaptive Immune System

The adaptive immune system fails to develop without recombination-activating gene-1 (RAG-1); mature lymphocytes, antibodies, and immune complexes do not exist in such animals (Mombaerts *et al.*, 1992). In the context of α M-II deficiency, it was possible to determine the contribution of the adaptive immune system to disease pathogenesis with a focus on the nephritic component. Unexpectedly, mice lacking both α M-II and RAG-1 were more severely affected with loss of weight, alopecia, and exacerbation of kidney disease, as compared with littermates lacking α M-II or RAG-1 alone (Figure S2). Glomerulonephritis was intensified and a high degree of nephron loss occurred concurrent with hematuria and proteinuria (Figures 2A and 2B).

Molecules produced by lymphocytes that can modulate autoimmune disease include immunoglobulin-G (IgG), which is a ligand for Fc receptors expressed on various cell types (Nimmerjahn and Ravetch, 2006). Fc receptor function can decrease immune responses. For example, Fc γ RIIIb attenuates immune activation and mice lacking this Fc receptor spontaneously develop autoimmune disease (Takai *et al.*, 1996). We suspected that the absence of inhibitory signaling through Fc receptors among mice lacking RAG-1 might accelerate inflammation and nephritis in the α M-II null background. Cohorts of animals were given multiple intravenous injections of normal mouse IgG (IVIG) for a period of 4–6 months while disease signs were followed. In the absence of both α M-II and RAG-1, IVIG therapy diminished disease signs coincident with improved kidney function (Figure 2). The adaptive immune system therefore was not the source of the pathogenic trigger, nor was it required for disease progression, but instead appeared to moderate inflammation and glomerulonephritis.

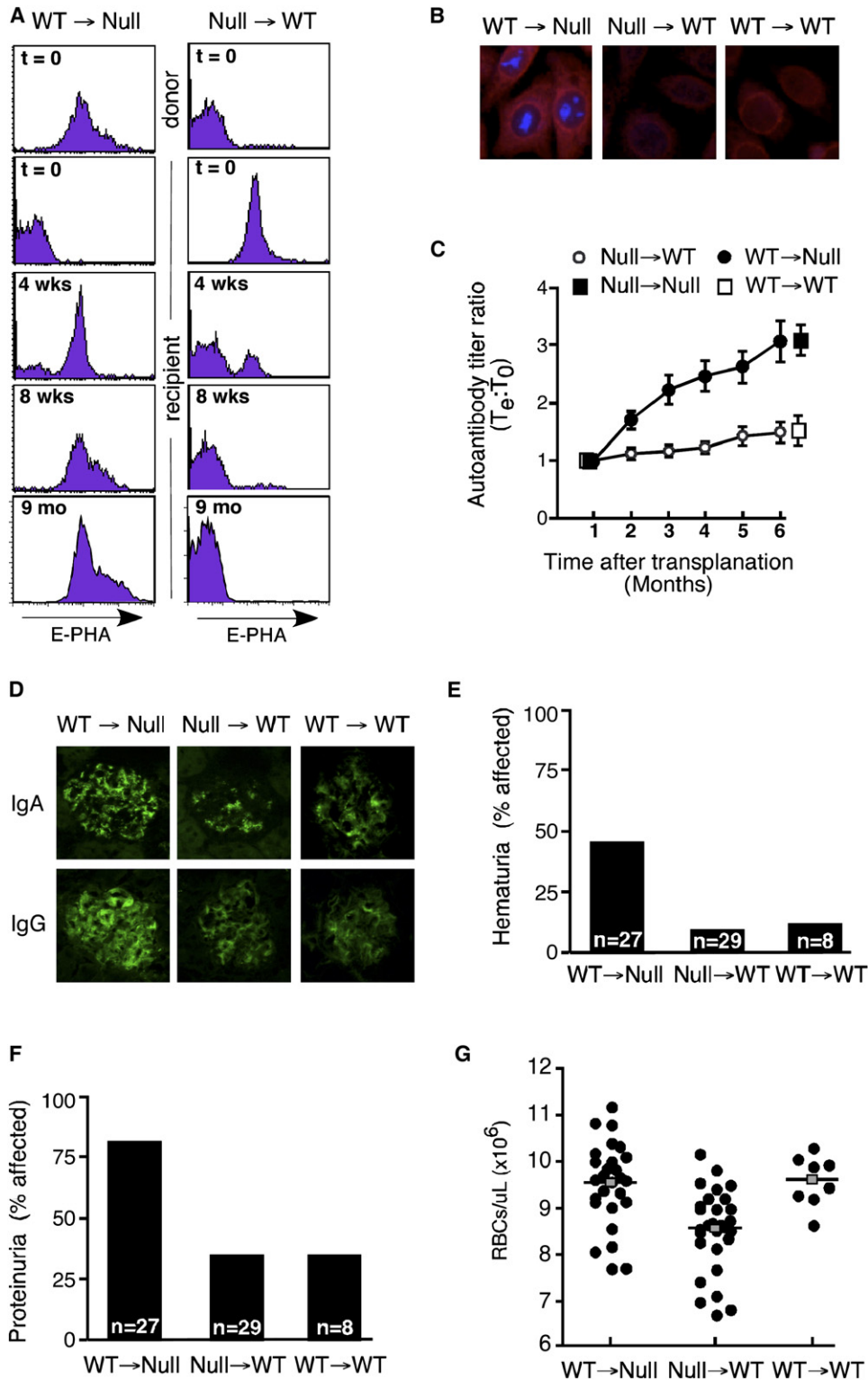


Figure 1. Nonhematopoietic Origin of Autoimmune Disease in α M-II Deficiency

(A) Bone-marrow transplantation to reconstitute the hematopoietic system was accomplished among 2-month-old recipients. At indicated times after transplantation, donor and recipient genotypes were detected by erythrocyte cell-surface binding of the erythroagglutinin (E-PHA) that binds complex N-glycan structures dependent upon α M-II function. Results were similar to hematopoietic cell DNA genotyping studies (not shown).

(B–G) Autoimmune markers were analyzed routinely for 6–9 months after transplantation, and disease occurred only in recipients that lacked α M-II function. These findings encompassed (B) anti-nuclear antibody (ANA) titers in sera diluted 1/200 with IgG binding (blue) to nuclear epitopes and IgM

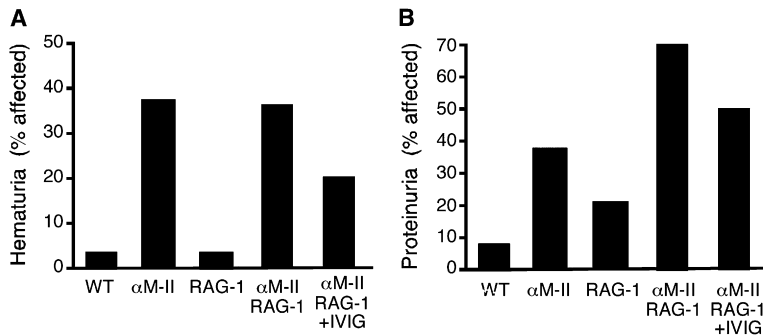


Figure 2. Disease in the Absence of the Adaptive Immune System and after Intravenous Administration of Immunoglobulin-G

Kidney function among mice of indicated genotypes, with or without intravenous IgG (IVIG) treatment, was measured by the frequency of animals exhibiting detectable levels of (A) blood (hematuria) and (B) protein (proteinuria) in the urine. Data are representative of 6 to 24 mice of each genotype and treatment regime.

Pathogenesis Is Independent of C3 Complement

Deposition of C3 complement in the kidney is elevated among glomeruli of α M-II-deficient mice concurrent with decreased amounts of C3 in circulation. Nevertheless, absence of complement C3 in α M-II deficiency failed to inhibit the development of SLE disease markers, including elevated autoantibody titers and nephritis, as well as anemia, indicating that deposition of C3 is not an important pathogenic factor (Figure 3). This finding unlinks C3 production and its glomerular deposition from disease pathogenesis in the absence of α M-II. Therefore, C3 deposition tracks with disease signs including glomerulonephritis but does not represent the pathogenic trigger. The persistence of nephritis in the absence of either C3 complement or the adaptive immune system focused attention on the possible role of the innate immune system in disease pathogenesis.

α M-II Deficiency Stimulates the Innate Immune System

We examined the innate immune system in mice lacking α M-II and found that macrophage infiltration in the kidneys is an early and consistent disease marker that is further elevated by the absence of RAG-1 (Figure 4A). IVIG treatment reduced macrophage recruitment, coincident with improved kidney mass, appearance, and function. Macrophages lacking α M-II retained normal functions and activation responses when analyzed ex vivo, as might be expected because they can continue to express complex-branched N-glycans ascribed to the α M-IIx isozyme (Figure S3; Chui et al., 2001; Akama et al., 2006). Macrophages infiltrating the kidneys of α M-II-deficient mice expressed activation markers including elevated MHC class-II expression and were often found proximal to the glomerular capsule and adjacent to mesangial cells (Figure 4B). The abundance of activated macrophages in the kidneys of α M-II-deficient mice was correlated with the degree of inflammation and tissue damage. This relationship extended to the amount of inducible nitric oxide

synthase (iNOS) that generates nitric oxide, which is a reactive oxygen species associated with inflammation after an innate immune response and increased glomerular injury in SLE (Tachtman, 2004; Oates and Gilkeson, 2006). iNOS expression in the kidneys was evident in α M-II-deficient mice but not among wild-type littermates. Amounts of iNOS were substantially elevated by RAG-1 codeficiency and moderated by IVIG treatment (Figure 4C). The cell types that expressed iNOS were predominantly activated macrophages and epithelial cells of the proximal tubules (Figure 4D). These findings revealed that α M-II deficiency induced macrophage infiltration and activation among kidney tissue in a pathogenic process associated with iNOS induction and that was moderated by IVIG treatment.

Mesangial Cells of the Innate Immune System Are Activated in α M-II Deficiency

Mesangial cells in the glomeruli possess innate immune modulatory activity and when activated, they produce cytokines and chemotactic factors such as monocyte chemoattractant protein-1 (MCP-1) that recruits and activates macrophages (Matsushima et al., 1989; Largen et al., 1995; Gomez-Guerrero et al., 2005). MCP-1 expression has been linked with inflammation and glomerulonephritis in SLE and lupus nephritis (Rovin et al., 1994; Largen et al., 1995; Zoja et al., 1997; Tesch et al., 1999; Kim et al., 2002; Hasegawa et al., 2003; Shimizu et al., 2004; Gomez-Guerrero et al., 2005). The activation of mesangial cells appeared early in α M-II-deficient mice with the onset of hyperplasia and glomerular inflammation. Markers of mesangial cell proliferation (Ki-67) and inflammation (IL-1 β) were prominent by 3–4 months of age. By 6–9 months of age, mesangial cell apoptosis measured by active caspase-3 was consistently observed coincident with increased tissue sclerosis, nephron loss, and kidney dysfunction (Figure 5A). MCP-1 was absent from wild-type adult kidney tissue at all ages but was induced among mesangial cells of α M-II-deficient littermates (Figure 5B).

binding (red) to membrane and cytosolic proteins of HEP-2 cells (1000 \times), (C) autoantibody titers at 1/400 dilution to wild-type cellular proteins (including Sm and dsDNA, not shown), (D) glomerular immunoglobulin deposition (400 \times), as well as kidney dysfunction detected by the frequency of mice exhibiting (E) hematuria and (F) proteinuria after transplantation. In contrast, anemia (G) as detected by low red blood cell (RBC) numbers in circulation occurred in mice transplanted with α M-II-deficient marrow. Autoantibody titers in (C) were calculated as a ratio at the experimental time point (T_a) divided by the initial titer (T_0). Results are representative and encompass 8 to 29 mice of indicated ages and genotypes. Standard errors of the means are indicated in (C) and (G) as vertical black lines and gray bars, respectively.

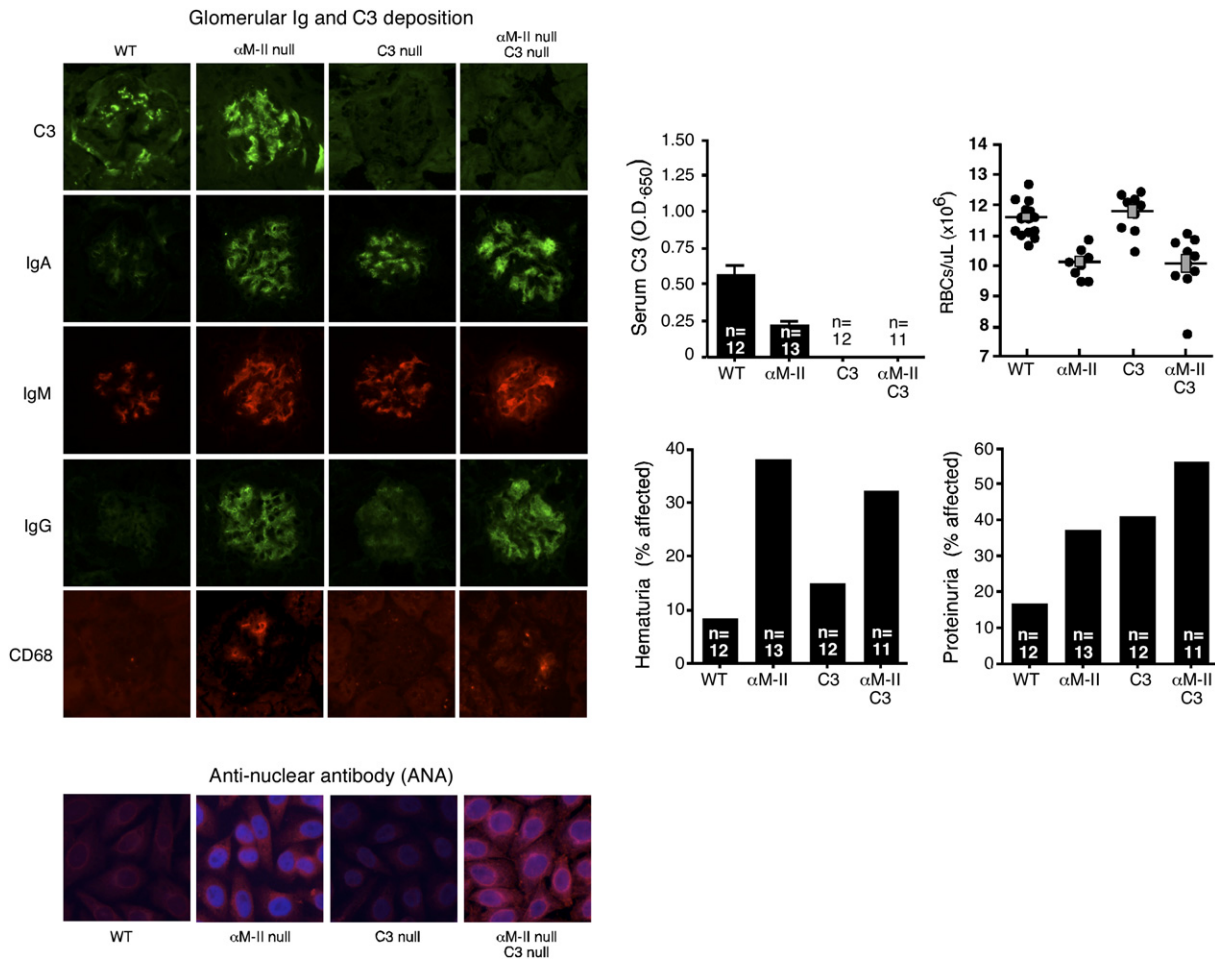


Figure 3. Pathogenesis in the Absence of Complement C3

Among mice lacking α M-II, complement C3, both α M-II and C3, as well as wild-type littermates, disease signs were measured at 6–9 months of age including glomerular immunoglobulin deposition (400 \times), the presence of anti-nuclear antibodies (ANA) (400 \times), reduced RBC numbers (anemia), and kidney dysfunction assessed by frequencies of hematuria and proteinuria. Analyses depicted are representative of results with six or more littermate pairs of indicated genotypes. Standard errors of the means are indicated as gray bars in the RBC analysis.

Glomerular expression of MCP-1 was moderated by IVIG treatment, as was the abundance of activated macrophages found adjacent to mesangial cells and the glomerular capsule (Figure 5C). These studies revealed that the activation of mesangial cells in the absence of α M-II led to the production of proinflammatory proteins that are known pathogenic factors in the etiology of SLE and kidney disease.

Innate Immune Lectin Receptor Modulation and Proinflammatory Ligand Formation

Cells of the innate immune system, including mesangial cells and macrophages, express pattern-recognition receptors including lectins that bind to mannose-enriched glycan structures typical of microbial and pathogen cell surfaces (Barton and Medzhitov, 2003; McGreal et al., 2004). For example, the macrophage mannose receptor (MMR) and the mannose-binding lectins (MBL-A and MBL-C in the mouse) bind to glycan structures bearing

mannose linkages typical of lower eukaryotic and microbial glycans (Sharon, 1987; Ezekowitz et al., 1990; Sastry et al., 1995; Hansen et al., 2000; Gordon, 2002; McGreal et al., 2004). Mammalian lectins of the innate immune system therefore appear to distinguish evolutionarily disparate organisms based in part upon unique differences in mannose linkages expressed among cellular glycomes.

Mesangial cells are exposed to a variety of stimuli that exist in vascular circulation and which enter the kidney glomerulus during normal filtration processes. Although macrophages are recruited and activated by MCP-1, macrophages were not directly stimulated by hybrid N-glycans produced in the absence of α M-II (Figure S3). The source of the mesangial cell activation stimulus was investigated among N-glycans in circulation. Serum from wild-type mice induced some MCP-1 production in isolated cultures of glomeruli, whereas the addition of the yeast cell wall constituent mannan induced a significantly greater response. MCP-1 production was also markedly

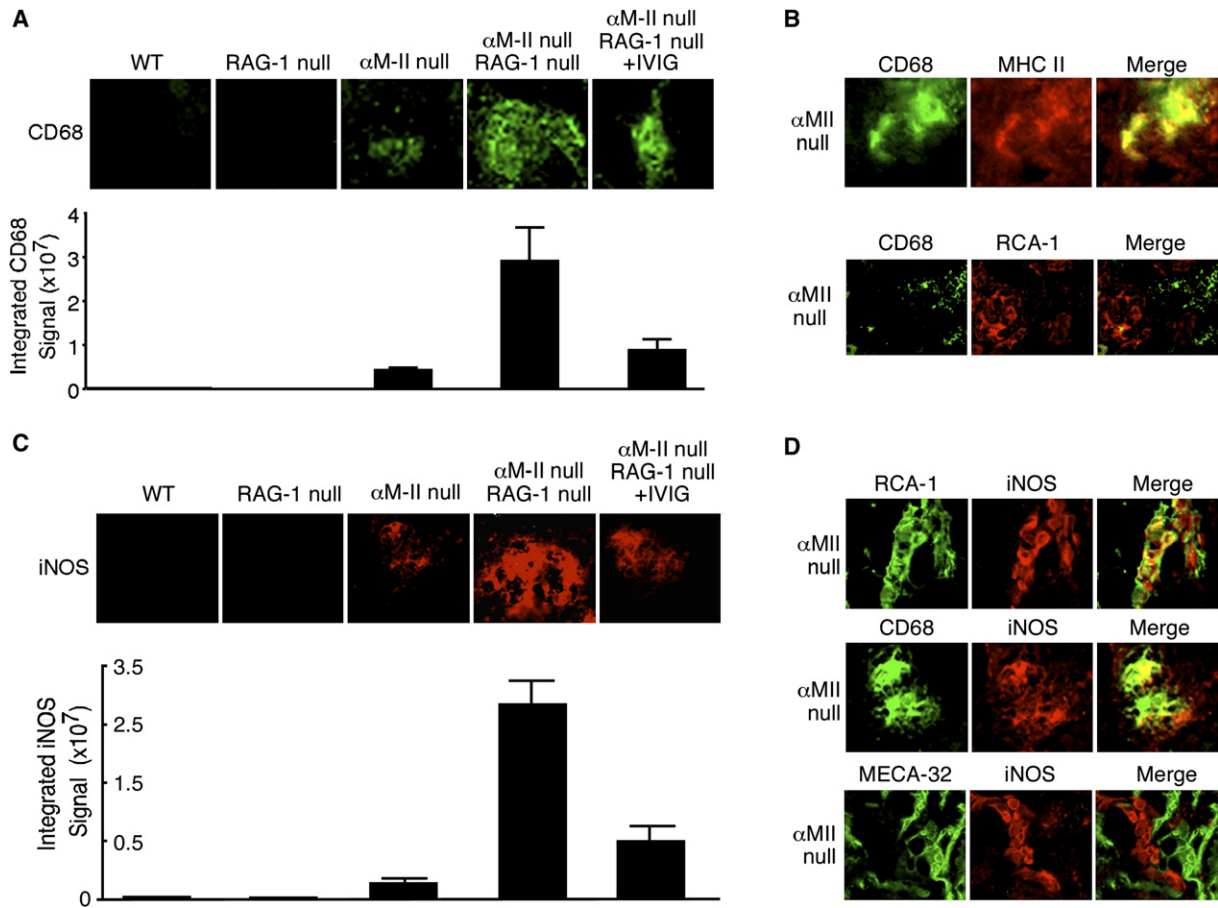


Figure 4. Innate Immune Activation and Kidney Inflammation

(A) Macrophage recruitment was detected by means of anti-CD68 antibody, and fluorescent signals were quantified among multiple kidney sections. Results were plotted from five 8-month-old mice of indicated genotypes.

(B) Expression of MHC class II among CD68⁺ macrophages in the kidneys of αM-II null mice (top, fluorescent colocalization in yellow, effective magnification 2000×). RCA-1 (ricinus communis agglutinin-1) lectin binding in the glomerulus marks mesangial cells adjacent to CD68⁺ macrophages (bottom, magnification 200×).

(C) iNOS protein expression was measured by fluorescence in kidney sections from mice of the indicated genotypes.

(D) iNOS expression was analyzed among various cell types including kidney proximal tubular epithelial cells (nonglomerular RCA-1⁺ binding, top), activated macrophages associated with the glomerular capsule (middle), and endothelial cells via the MECA-32 marker (bottom). Data are representative of three results with more littermate pairs; means and standard deviations are indicated in (A) and (C).

increased in cultures of isolated glomeruli treated with serum from αM-II-deficient mice. Moreover, this response, like that of mannan, was inhibited in the presence of the mannose analog and mannose lectin binding inhibitor alpha-methylmannoside (αMM) (Figure 6A). These findings indicated that hybrid N-glycans present in the sera of αM-II-deficient mice induced mesangial cell activation and proinflammatory cytokine production by a mannose-dependent binding mechanism.

Among innate immune lectins that exhibit mannose-binding activity, the expression of the MMR, MBL-A, and MBL-C was analyzed. In αM-II-deficient mice, we observed that MMR protein expression was induced on mesangial cells in the kidney and was present among a proportion of intercalating macrophages (Figure 6B and data not shown). In studies of MBL protein abundance, we noted reduced serum concentrations of both MBL-A and

MBL-C that correlated with their increased deposition in the kidney of αM-II-deficient mice (Figures 6C and 6D). The modulation of mannose-binding lectin expression observed in αM-II deficiency implicated the presence of endogenous glycan ligands.

By using recombinant mammalian MMR and MBL chimeras, mannose-dependent ligands of these lectins were detected in the sera of αM-II-deficient mice (Figure 6E). Multiple glycoproteins exhibited ligands that were recognized by both lectins, and this binding was competed by αMM. The presence of endogenous ligands among various cell and tissue types was further investigated and revealed accumulation of mannose-dependent MMR ligands primarily among kidney tissue and mesangial cells within the glomerular capsule, but not among various other organs and cell types (Figure 6F). Ligands of MBL-A and MBL-C lectins were also present among glycoproteins

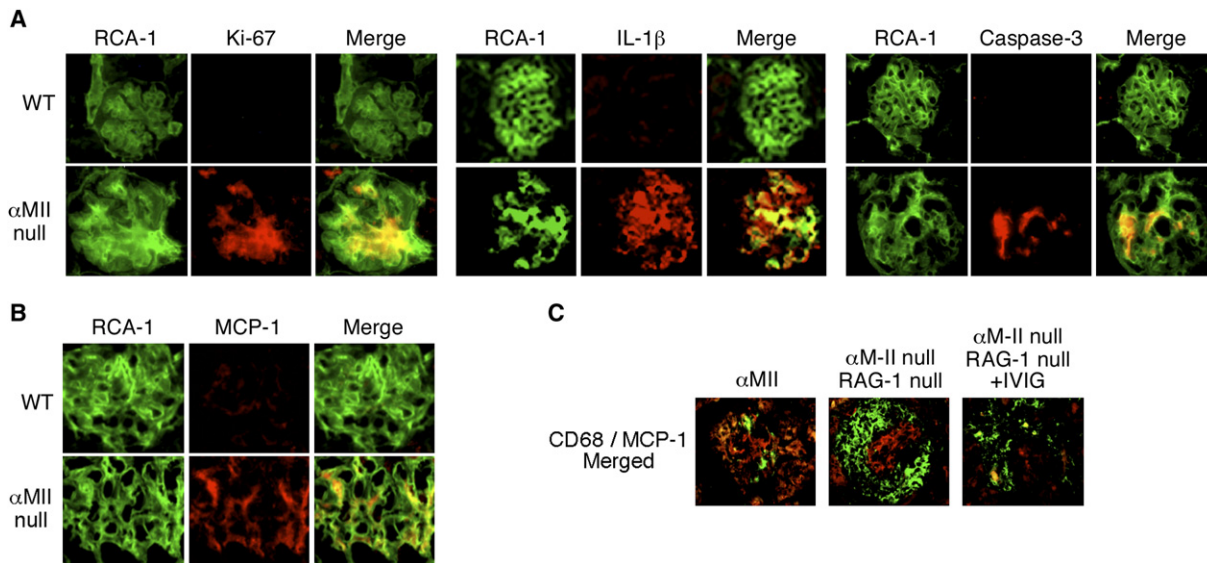


Figure 5. Mesangial Cell Activation and Apoptosis

(A) Detection of Ki-67, IL-1 β , and activated caspase-3 proteins among mesangial cells in the glomeruli of kidney sections from 3- to 6-month-old mice of indicated genotypes.

(B) Expression of monocyte chemoattractant protein-1 (MCP-1) in mesangial cells at 3–6 months of age.

(C) MCP-1 expression (rhodamine, red) and macrophage recruitment (FITC, green) in the absence or presence of previous IVIG treatment. Magnifications are 400 \times unless indicated. Data are representative of results obtained with three or more littermates of the indicated ages and genotypes.

in the kidneys of α M-II-deficient mice as well as among glycoproteins expressed on the erythrocyte cell surface (not shown). In further studies, inherited deficiency of the MBL-A lectin with the coabsence of α M-II continued to induce autoimmune disease (Figure S4; and data not shown). The absence of disease modulation by a single lectin deficiency may represent cell-type-specific functions and compensation involving a growing list of mannose-binding lectins expressed among cells of the innate immune system including Dec-205, Endo180, DC-SIGN, Langerin, Dectin-2, and BDCA-2. Our findings have shown that altered N-glycosylation because of loss of α M-II activity led to the production of proinflammatory mannose-dependent glycan ligands for multiple innate immune lectin receptors. These endogenous ligands have structural similarity with glycan linkages on the cell surface of lower eukaryotes and microbes (Figure S5).

DISCUSSION

Protein N-glycosylation is altered in α M-II deficiency resulting in the formation of hybrid-type N-glycans bearing mannose-dependent ligands of innate immune lectin receptors that induce inflammation. Endogenous ligands of innate immune lectins do not normally accumulate among extracellular compartments of vertebrates, and those produced in the absence of α M-II appear to act as a pathogenic trigger of nonhematopoietic origin consistent with the etiologic features of the resulting SLE-like autoimmune disease syndrome. We found that disease progression tracked with chronic stimulation of the innate

immune system involving mesangial cell activation and macrophage recruitment in a sterile milieu lacking an infectious and causative pathogen. Inflammation and cell death in the kidney was accompanied by markers of nitric oxide production and increased antigen presentation, yet neither elevated autoantibody titers nor complement C3 were factors in disease progression, and neither appeared to play a substantial pathogenic role.

The increased severity of kidney disease among animals lacking both α M-II and RAG-1 indicated an etiology independent of peptide antigen-presentation mechanisms and the adaptive immune system. On balance, the adaptive immune system provides a moderating effect in disease progression. This moderation does not appear to reflect alterations in lymphoid cell subpopulations including regulatory T cells, which were similar between mutant and wild-type genotypes (data not shown). Among genetic lesions that alter the immune system and induce kidney disease, a lymphocyte-independent mechanism of pathogenesis has been similarly detected by loss of RAG-1 function among mice lacking the Fyn tyrosine kinase (Yu et al., 2001). Fyn deficiency accelerated autoimmune disease symptoms induced by the absence of the Lyn tyrosine kinase, appearing to reflect a kidney-intrinsic role of Fyn. SLE-like disease signs induced by α M-II deficiency followed normal kidney development and early postnatal function and may be augmented by immunologic disease processes similarly engendered by other genetic lesions.

Administration of IVIG in α M-II deficiency reduced macrophage recruitment and disease severity evoked by RAG-1 codeficiency, providing the moderating effect

ascribed to the adaptive immune system. Autoantibodies detected in α M-II deficiency do not exhibit N-glycan binding (Chui et al., 2001), implying that conserved regions within the IgG molecule participate in disease attenuation. IVIG is a therapeutic treatment in human diseases of inflammation and autoimmunity including myasthenia gravis, Guillain-Barre syndrome, graft-versus-host disease, Kawasaki's syndrome, as well as SLE, in which it may operate by promoting inhibitory signaling of Fc receptors (Ott et al., 2001; Kazatchkine and Kaveri, 2001; Sherer and Shoenfeld, 2006; Park-Min et al., 2007). It is possible that loss of Fc γ R11b in the α M-II-deficient background may also elevate innate immune inflammatory responses and increase disease severity. Our findings support the view that the therapeutic efficacy of IVIG treatment reflects at least in part a reduction in innate immune activation, and we suggest that genetic or spontaneous variation in human protein N-glycan branching frequency may also alter IVIG efficacy in disease intervention.

The nonhematopoietic origin of innate immune activation and autoimmune disease in α M-II deficiency can be further resolved into a molecular and pathogenic trigger. Alterations in the functions of N-glycoproteins that normally regulate the innate immune system remain possible but unlikely, because inflammation did not appear systemically and immune cell response parameters were unaffected by the absence of α M-II. Moreover, a pathogenic trigger was detected among the hybrid N-glycans bearing exposed mannose linkages resulting from the loss of α M-II function. These proinflammatory N-glycans reside on circulating glycoproteins and perhaps immune complexes that accumulate among cells of the kidney filtration apparatus where they can induce MCP-1 production and an organ-selective disease process. The nonhematopoietic and cellular origin of these ligands may include various cell types that secrete N-glycoproteins into circulation and may also involve the kidney should some cells within this organ lack sufficient compensation afforded by expression of the α M-IIx isozyme.

Innate immune mannose-binding lectins, of the C-type family, include endocytotic and phagocytic receptors that promote foreign antigen uptake, intracellular processing, and antigen presentation (McGreal et al., 2004; Robinson et al., 2006). At least some stimulate intracellular signaling pathways involving tyrosine kinases and phosphatases (Lopez-Herrera et al., 2005; Robinson et al., 2006; Sheng et al., 2006). The stimulation of MCP-1 production among isolated glomeruli by α M-II-deficient sera was blocked by α MM, implying that a mannose-dependent binding event induces inflammatory signaling by mesangial cells. Elevated expression of MCP-1 is common in glomerulonephritis among humans and mice wherein this cytokine recruits and activates macrophages (Matsushima et al., 1989; Largen et al., 1995; Gomez-Guerrero et al., 2005). Moreover, MCP-1 is linked to pathogenesis and nephritis in some autoimmune syndromes (Rovin et al., 1994; Largen et al., 1995; Zoja et al., 1997; Tesch et al., 1999; Kim et al., 2002; Hasegawa et al., 2003; Shimizu et al., 2004; Gomez-Guerrero et al., 2005).

Ligands of C-type lectins with mannose-binding activity have been proposed to consist of repetitive arrays of terminal mannose residues that normally reside among pathogen-derived oligomannose structures. A significant but limited degree of molecular spacing among mannose linkages is believed to be important in pathogen recognition by facilitating lectin domain interactions (McGreal et al., 2004). Nevertheless, low levels of endogenous ligands exist among few mammalian glycoproteins including the lysosomal hydrolases that are secreted in inflammation (Stahl et al., 1978; Sheperd et al., 1985). Mammalian N-glycoproteins that are trafficked to the lysosome by the mannose-6-phosphate signal are also unprocessed by α M-II (Varki and Kornfeld, 1980), and therefore such N-glycoproteins display mannose linkages also present in the absence of α M-II. Nevertheless, substantial binding specificity is evident among mannose-binding lectins of the mammalian innate immune system that normally precludes extensive self-recognition.

Although erythrocytes bear hybrid N-glycans at their cell surface in α M-II deficiency that are also endogenous MBL and MMR ligands, they do not contribute to kidney disease or to the elevation of autoantibody titers. This may reflect a lesser degree of coclustering involving exposed mannose linkages among N-glycoproteins when tethered to the cell surface or perhaps the sequestering of erythrocyte accumulation and turnover in the spleen. Interestingly, α M-II-deficient mice bear an increased number of splenic macrophages concurrent with an elevation in circulating monocyte numbers in the blood. Although humans deficient in α M-II activity may exist among some cases of congenital dyserythropoietic anemia type II (also known as HEMPAS), genetic linkage studies of this syndrome have excluded the α M-II gene (Iolascon et al., 1997; Lanzara et al., 2003). Clinical diagnosis is inexact at present, and whether N-glycosylation defects associated with human HEMPAS are identical to those induced in the absence of α M-II, extend to all cell types, and provoke autoimmune disease as these patients age remains to be established.

The phylogenetic and structural contexts of mannose linkages are important. Although a portion of total cellular N-glycans in the Golgi of wild-type mammalian cells are hybrid N-glycans, these are predominantly synthetic intermediates and do not normally reach the cell surface in substantial amounts. In phylogeny, vertebrates are unique as compared with invertebrates and lower eukaryotes in the mannose linkages exposed among extracellular glycans. Those high-mannose N-glycans found on mature glycoproteins in vertebrates, as well as those on budding viruses, typically bear terminal α 2 mannose linkages emanating from and masking the underlying α 3 and α 6 mannose linkages of the core N-glycan structure. The fraction of N-glycans with α 2-linked terminal mannose is unaltered by α M-II deficiency (Chui et al., 2001). Instead, extracellular hybrid-type N-glycans bearing terminal α 3 and α 6 mannose linkages are produced at the expense of complex-type N-glycan branching, mimicking structural features among the exposed terminal mannose linkages typical

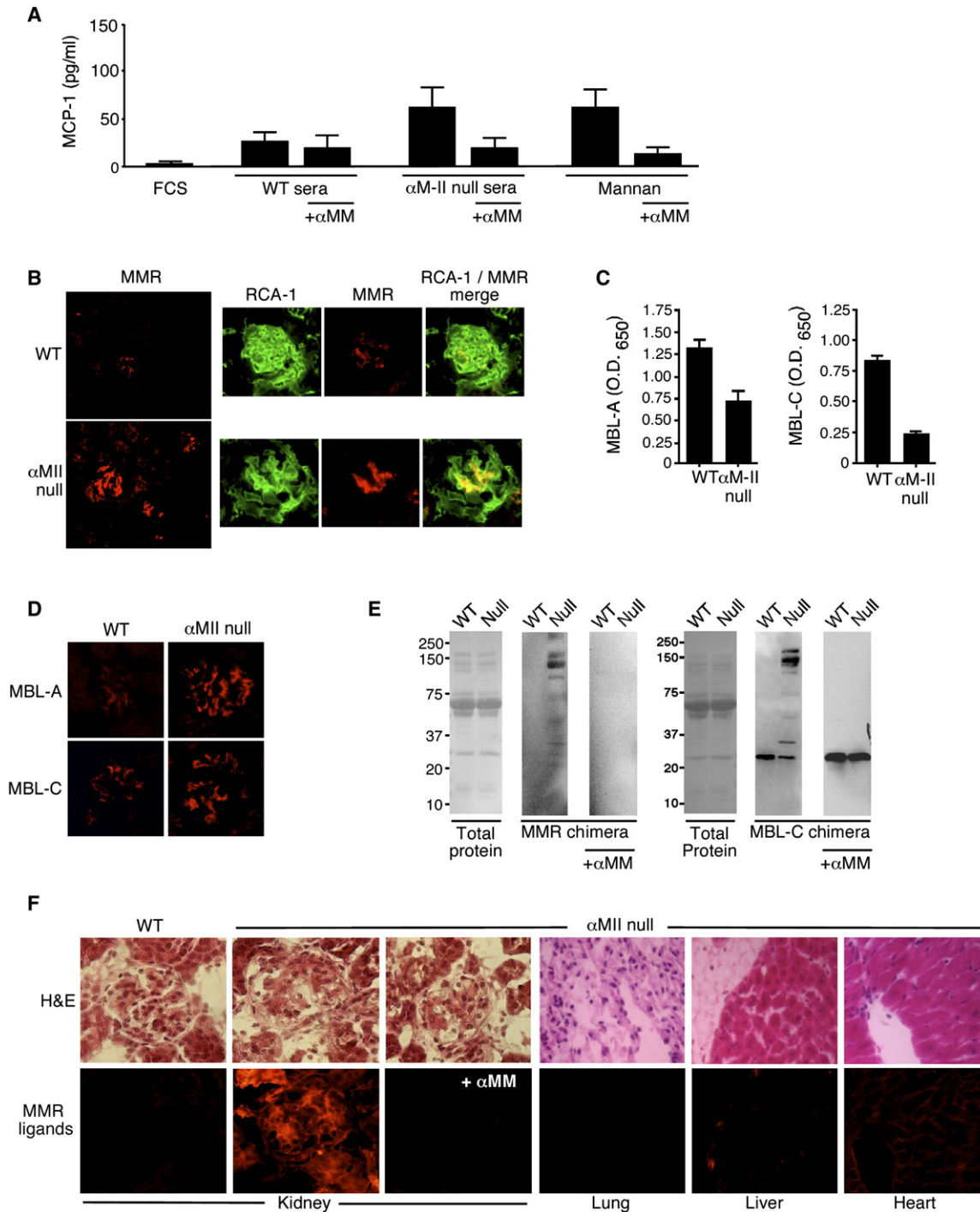


Figure 6. Mannose-Binding Lectin Expression and Endogenous Ligand Formation

(A) MCP-1 production among isolated glomeruli after incubation with sera from 6- to 9-month-old mice of indicated genotypes. Differences observed between α M-II null and wild-type sera as well as between α M-II null sera with or without pretreatment with 1 mg/ml of alpha-methylmannoside (α MM) were significant ($p = 0.038$ and $p = 0.016$; respectively). MCP-1 levels in culture upon addition of mouse sera and FCS at $t = 0$ were 2 pg/ml or less. Data shown are the mean and standard error from six or more independent experiments with each condition.

(B) Macrophage mannose receptor (MMR) expression among mesangial cells in kidney sections (RCA-1⁺) (colocalization in yellow, 400 \times). Large panels at left are magnified 200 \times .

(C) Abundance of mannose-binding lectins MBL-A and MBL-C in the sera of mice of indicated genotypes. Six or more mice of indicated genotypes were evaluated.

(D) Glomerular MBL deposition in kidney glomeruli of 6-month-old mice of the indicated genotypes (400 \times).

of invertebrates and many lower organisms. Among yeast mannan mutants comprised of differing proportions of mannose linkages, the $\alpha 3$ and especially the $\alpha 6$ linkages are the most potent in assays of mannose receptor ligand binding and glycoprotein clearance (Stahl et al., 1978).

The SLE-like syndrome that develops in αM -II deficiency originates from an endogenous nonhematopoietic and organ-selective pathogenic stimulus composed of hybrid-type N-glycans that promote chronic inflammation, leading to the elevation of systemic autoantibody titers, cell activation and apoptotic death, and kidney failure. Autoinflammatory diseases such as Muckle-Wells and familial cold urticaria bear some etiologic similarities, because they also arise from the dysfunction of endogenous cellular components (Sutterwala et al., 2006). Although the disease trigger in αM -II deficiency appears to mimic a foreign entity, our findings are consistent with a pathogenic mechanism of autoimmune disease in which endogenous cellular signals of danger and infection may contribute in the absence of an exogenous pathogenic stimulus (Matzinger, 2002). Other glycans from various sources that include chitin, N-acetylglucosamine residues, and fragments of hyaluronan also activate the innate immune system and may in some cases contribute to disease pathogenesis (Malhotra et al., 1995; Jiang et al., 2006; Reese et al., 2007). We infer that the development of complex N-glycan branching during vertebrate evolution in part enabled the immune system to acquire and retain lectin receptors to detect and signal the presence of nonself upon encountering the extracellular glycomes of potential pathogens. This interaction may be exploited in immunization protocols should terminal $\alpha 3$ and $\alpha 6$ mannose linkages provide an adjuvant function that explains the heightened immune responses to some N-glycoprotein antigens synthesized in yeast and insect cells. The transfer of mammalian N-glycosylation pathways into microbial organisms may usurp this mechanism of nonself discrimination and perhaps alter the outcome of infection. Transcriptional networks and mutagenic events that depress the formation of complex N-glycan branching among mammals can induce chronic inflammation and may serve as the pathogenic bases of some autoimmune and autoinflammatory diseases.

EXPERIMENTAL PROCEDURES

Mice

Strains included those deficient in αM -II (Chui et al., 1997), RAG-1 (C57BL/6J-Rag-1^{tm1Mom}; Mombaerts et al., 1992), MBL-A (B.6129Mb19; Hansen et al., 2000; Liu et al., 2001; kindly provided by T. Kawasaki, Kyoto University, Japan), and C3 (B6.129S4-C^{3tm1Crr}; Wessels et al., 1995). Mutations were analyzed in the C57BL/6J background after 10 or more generations of breeding prior to experimental

tion. All animals were housed and maintained in compliance with ethical and institutional guidelines.

Flow Cytometry

Flow cytometric experiments were carried out as previously described (Chui et al., 2001) with the following modifications: whole blood was collected and 5×10^5 erythrocytes were incubated with fluorescein (FITC)-conjugated E-PHA (1 μ g/ml) (Vector Laboratories, Burlingame, CA) in a final volume of 100 μ l. All incubations and washes were performed on ice in FACS buffer (2% fetal calf serum in phosphate-buffered saline). Cells were analyzed with a FACSCalibur flow cytometer and CellQuest software (Becton Dickinson, Mountain View, CA).

Sera Collection and Hematology

Mice were anesthetized with methoxyfluorane (Medical Developments, Smithvale, Australia) and blood was collected from the tail vein into EDTA- or heparin-coated polypropylene tubes (Becton Dickinson). A Hemavet 850FS (Drew Scientific, Oxford, CT) programmed for normal mouse parameters was used to acquire hematology data. For sera preparation, blood was collected from the tail vein into BD Microtainer tubes with serum separator (Becton Dickinson) and incubated at 22°C for 45 min. Samples were then centrifuged on a bench-top centrifuge at 14,000 rpm. Serum was transferred to eppendorf tubes prior to analysis and storage at -80°C .

Bone-Marrow Transplantation

Recipient mice of each relevant genotype were administered 10–11 Grays of gamma-radiation from a Cs137 source at 6–8 weeks of age for optimal transplant engraftment by donor cells (Cui et al., 2002). Donor cells were isolated from femurs of 8- to 10-week-old sex-matched nonirradiated donors. 24 hr after irradiation, recipient mice were injected via the tail vein with 10^7 bone-marrow cells resuspended in 200 μ l phosphate-buffered saline (PBS). In addition to RBC analyses of donor engraftment (Roy et al., 1990; Down et al., 1991), upon termination of the study, genomic DNA from bone-marrow cells of recipients (Purgene, Genra Systems) were analyzed by polymerase chain reaction.

Autoantibody Analyses

For membrane protein isolation, tissues were homogenized in ice-cold buffer (10 mM HEPES [pH 7.4], 1 mM EDTA, protease inhibitor cocktail). Homogenates were centrifuged at $600 \times g$ for 10 min at 4°C. Pellets were discarded, and supernatant was centrifuged at 32,000 rpm for 1 hr at 4°C. Supernatants were discarded and pellets were resuspended in lysis buffer on ice for 30 min. After a final centrifugation at 14,000 rpm for 10 min at 4°C, supernatant was transferred to new tubes and stored at -80°C until further analysis.

For detection of auto-reactivity toward cellular proteins, 96-well microplates (Nunc, Rochester, NY) were incubated with isolated membrane protein fractions at 10 μ g/ml in PBS for 2 hr at 37°C. Plates were washed three times with 200 μ l PBS + 0.05% NP-40, and wells were then blocked with PBS + 2% BSA for 2 hr at 22°C. Plates were washed and serial dilutions (1:400–1:1600) of wild-type or mutant sera (in PBS with 1% BSA) were added at 100 μ l/well for 2 hr at 22°C. Plates were washed and wells were incubated 45 min at 22°C with 100 μ l of an alkaline phosphatase-conjugated or HRP-conjugated anti-mouse Ig kappa light-chain monoclonal antibody (PharMingen, San Diego, CA) diluted 1:5000 in PBS with 1% BSA. Plates were washed again and then developed with 100 μ l of p-nitrophenyl

(E) Detection of endogenous ligands of innate immune mannose-binding lectins in the sera of mice via MMR and MBL-C chimeras. MBL-C lectin in the sera is also detected by this approach (~ 30 kDa). Total sera protein is shown stained by Ponceau S. Binding of MBL-C and MMR chimeras to glycoprotein ligands was inhibited by 1 mg/ml of alpha-methyl-mannoside (α MM).

(F) Endogenous MMR ligands, detected by binding of the MMR-his chimera, among various tissues of mice of indicated genotypes (800 \times). MMR-his binding is also prominent among proximal tubule cells and their brush borders in αM -II-deficient mice (not shown). Binding was competed with 150 mM α MM, as well as either 1 mg/ml of mannan or 10 mM EDTA (not shown). Results are representative of findings with three or more littermate pairs of indicated genotypes unless otherwise stated.

phosphate (Sigma) for 15 min at 22°C, and reactions were stopped with 50 μ l of 0.1 M EDTA. Signals were measured with a VersaMax microplate reader at 405 nm (Molecular Devices Corporation, Sunnyvale, CA). For HRP-conjugated antibody, plates were developed by adding 100 μ l Tetramethylbenzidine (TMB) liquid substrate solution (Sigma) and signals measured at 650 nm.

HEp-2 anti-nuclear antibody (ANA) assays were performed as described (Chui et al., 2001) with minor modification. For anti-nuclear antibody detection, sera from mice were diluted (1:40–1:320) in PBS and incubated with HEp-2 cell substrate slides (Immuno Concepts, Sacramento, CA) in a covered, humidified chamber for 30 min at 22°C. Slides were then rinsed in PBS for 20 min, and antibody binding was detected with the use of AMCA-conjugated or FITC-conjugated anti-mouse IgG and a Rhodamine-conjugated anti-mouse IgM (Jackson ImmunoResearch, West Grove, PA) at 1:400 for 30 min at 22°C. Slides were next washed in PBS and mounted with coverslips for fluorescent microscopy with a Zeiss Axioplan fluorescent microscope (Zeiss, Gottingen, Germany).

Kidney Histology and Function

Tissues were frozen in O.C.T. medium (Sakura Finetech USA Inc., Torrance, CA) sectioned to 5 μ m (Leica Microsystems CM3050 S, Wetzlar, Germany) and dried 2 hr at 22°C. Sections were fixed in -20°C acetone for 2 min, allowed to dry, hydrated in PBS, and rinsed 3 \times with PBS, prior to incubation in PBS with 2% BSA with or without Fc receptor-blocking antibody for 1 hr before further incubation with primary antibodies or lectins used to detect tissue proteins. Slides were washed 4 \times between each step (PBS with 1% BSA, with or without 0.05% Tween-20). In studies of C-type lectin binding, 2 mM CaCl₂ was included in all buffers. Incubation with primary antibodies was carried out overnight in a dark, humidified chamber at 4°C. Secondary antibody incubation proceeded for 1 hr in a dark humidified chamber at 22°C. After a final wash, slides were mounted in Prolong Gold (Invitrogen, Carlsbad, CA) and observed with a Zeiss Axioplan fluorescent microscope (Zeiss). Images were obtained with a Photometrics CoolSNAP ES CCD camera (Roper Scientific/Photometrics, Tucson, AZ) and analyzed by AxioVision (Carl Zeiss) or MetaMorph (Molecular Devices Corporation, Downingtown, PA) software. All images were acquired and processed identically in comparative studies among tissues of different genotypes.

Antibodies and lectins (with dilutions or concentrations) included: CD68 (1:200), CD68-FITC (1:200), CD206 (1:100), MHC Class II I-Ab (1:25) (Serotec, Raleigh, NC), iNOS (1:100) (Upstate, Lake Placid, NY), IL-1 β (1:200), Ki-67 (1/100) (Abcam), Caspase 3 active form (1/100) (PharMingen), MCP-1 (1/100) (Serotec), anti-IgA-FITC (1:200), CD16/32 (0.5 μ g/ml) (PharMingen, San Diego, CA), MBL-A (2 μ g/ml), MBL-C (2 μ g/ml) (gift from S. Thiel, University of Aarhus, Denmark), anti-mouse C3 (1:200), goat anti-hamster-FITC (1:200) (ICN/Cappel, Aurora, OH), anti-IgG-FITC (1:400), anti-IgM-Rhodamine (1:400), mouse F(ab')₂ anti-Rat IgG-FITC (1:400), mouse F(ab')₂ anti-rat IgG-Rhodamine (1:400), mouse anti-Rat IgG-CY5 (1:200), goat anti-rabbit IgG-rhodamine (1:200), mouse anti-goat IgG-FITC (1:400) (Jackson). Ricinus communis agglutinin-1 (RCA)-1 lectin FITC conjugate (1/2000) (Vector Laboratories) was used to visualize mesangial cells and proximal tubules (Laitinen et al., 1987).

Kidney function assessed by hematuria and proteinuria was accomplished after isolation of urine with Multistix 9SG reagent strips (Bayer, Elkhart, IN). Hematuria (trace-large) and proteinuria (trace-2000 mg/dl) were quantified by color. For proteinuria, a positive result was determined as a minimum value of 100 mg/dl.

IVIG Treatment

Total murine IgG (Equitec, Kerrville TX) was resuspended in sterile PBS and administered to 4- to 8-week-old mice via tail-vein injection. IgG concentrations in circulation were maintained at approximately 200 μ g/ml for the duration of treatment. Sham treatments were carried out in parallel with PBS or PBS + BSA.

Lectin and Ligand Analyses

For analysis of MBL protein levels in serum, sera were diluted 1:200 in Tris-buffered saline (TBS [pH 7.5], 1% BSA, 5 mM Ca²⁺) and incubated in wells of microplates (Nunc). Biotinylated anti-MBL antibody (gift from S. Thiel) was detected with a Streptavidin-HRP secondary (PharMingen) and TMB liquid substrate solution (Sigma). Plates were read at 650 nm with a VersaMax microplate reader (Molecular Devices).

For analysis of circulating lectin ligands, serum protein (20 μ g) was separated by SDS-PAGE via a 4%–15% Tris-HCl gradient gel (Bio-Rad) and transferred to a PVDF membrane (Bio-Rad). Blots were incubated overnight at 4°C with 2% IgG-free BSA (Jackson ImmunoResearch) in TBST with 2 mM CaCl₂. Blots were then incubated in either 50 pg/ml of the MBL-C chimera or the MMR-his chimera overnight at 4°C. For binding competition analyses, 150 mM α MM was used. Recombinant MMR and MBL-C were detected with either biotinylated anti-MMR or biotinylated anti-MBL-C (R&D Systems) (250 ng/ml), respectively, followed by incubation with Avidin-HRP (BD PharMingen) (1:2000). All washes and reactions were done with 2% BSA in TBS plus 2 mM CaCl₂. Peroxidase was detected with ECL plus (GE Healthcare) and visualized with EpiChem3 Darkroom (UVP Bioluminescence Systems).

To detect MMR ligands in the kidney, the polyhistidine-tagged recombinant mouse MMR chimera (R&D Systems, Minneapolis, MN) was incubated (2.5 μ g/ml) on 5 μ m tissue sections and detected with rabbit polyclonal antibody to 6-Histidine (2.5 μ g/ml) (Novus Biologicals, Littleton, CO) followed by goat anti-rabbit IgG-rhodamine (1:100) (Jackson). Mannan or α MM (Sigma) was used at indicated concentrations to confirm mannose-dependent binding.

Macrophage Isolation and Activation Assays

Isolation and activation of peritoneal macrophages was performed as described (Fortier, 1994; Fortier and Falk, 1994).

Isolation of Glomeruli and MCP Production

Isolation of glomeruli was performed as previously described (Takemoto et al., 2002). Glomeruli from wild-type mice were cultured in 200 μ l of DMEM plus L-glutamine, penicillin, and streptomycin. Either 5% heat-inactivated fetal calf serum, 20% wild-type mouse sera, 20% α M-II null mouse sera, or 10 mg/ml of mannan was added. α MM (1 mg/ml) was added 2 hr prior to the above stimuli where indicated. MCP-1 concentration in cell-culture media was measured by ELISA (eBiosciences).

C3 Measurements

Microplates (Nunc Maxisorp) were coated with 100 μ l/well of rat anti-mouse C3 mAb (at 2 μ g/ml) (HyCult Biotechnology, Uden, Netherlands) overnight at 4°C. Plates were washed 4 \times with PBS + .05% Tween-20 (PBST). Plate wells were then incubated with 200 μ l PBST with 2% BSA (0.45 μ m filtered) for 2 hr at 22°C, and then washed 4 \times with 250 μ l PBST. Sera was diluted (1:40,000) in PBS, with 1% BSA then added to plates at 100 μ l/well and incubated 2 hr at 22°C. Plates were washed 4 \times and goat anti-mouse C3 (ICN/Cappel) was added at 1:2000 in PBS with 1% BSA (100 μ l/well) for 1 hr at 22°C. Plates were washed and HRP-conjugated anti-goat was diluted (1:5000) in PBS with 1% BSA, prior to incubation at 100 μ l/plate well for 1 hr at 22°C. Binding was detected with TMB substrate solution (Sigma) and measured at 650 nm with a VersaMax microplate reader (Molecular Devices).

Statistical Methods

Unless otherwise indicated, the Student's t test was performed to determine statistical significance.

Supplemental Data

Five figures and Experimental Procedures are available at <http://www.immunity.com/cgi/content/full/27/2/308/DC1>.

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