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A Role of IgM Antibodies in Monosodium Urate Crystal Formation and Associated Adjuvanticity

Uliana Kanevets,* Karan Sharma,* Karen Dresser,† and Yan Shi2∗

Uric acid is released from injured cells and can act as an adjuvant signal to the immune system. Uric acid crystals invoke strong inflammatory responses in tissues. Although their biological effects are evident and the associated signaling mechanisms are becoming clear, it remains unexplained as to why uric acid precipitates rapidly in vivo, in sharp contrast to the minimal crystallization in vitro. We report in this study that a group of IgM Abs is able to bind to these crystals, which is interesting in light that B cell-deficient mice do not sense the proinflammatory adjuvant effect of uric acid. The titers of these Abs increase upon immunization with uric acid crystals. We have produced large quantities of such mAbs. The purified IgM Abs can significantly facilitate uric acid precipitation to form the inflammatory crystals in vitro. Infusion of these Abs into B cell-deficient mice significantly increases the basal level of inflammation in these recipients and restores the host’s ability to sense uric acid’s adjuvanticity. Therefore, we have identified a factor in determining uric acid precipitation and possibly its ability to function as an endogenous adjuvant. This finding suggests a new mechanism of the pathogenesis of gouty arthritis and uric acid-induced immune activation. The Journal of Immunology, 2009, 182: 1912–1918.

A s the causative agent of gout, uric acid crystal (monosodium urate (MSU))3-mediated inflammatory response has been a historic topic in medicine (1). It has gained recent interest in immunology because it was found to mediate danger signaling (2), and is reportedly involved in many immunological processes (3–5). The MSU crystal-mediated pathways are also being intensely investigated (6). However, one conceptual gap remains in contrast to the development concerning this research topic.

Uric acid only activates immune cells following its crystallization, a process similar to its pathogenesis in gout. Normally, uric acid is present in plasma at a concentration of 30–60 µg/ml. However, it has a very limited maximal solubility (70 µg/ml). Beyond 70 µg/ml (hyperuricemia), as seen during large-scale cell death in injury or chemotherapy, uric acid is at risk of crystalization. These crystals are the cause of gout, and to the best of our knowledge, form the structure recognized by the immune system as an endogenous danger (2). One of the most puzzling subjects in research on gout has been about the mechanisms that govern MSU precipitation. In neutral buffers, soluble uric acid takes 14 days or longer to reach its solubility equilibrium (70–100 µg/ml), even from highly supersaturated initial solutions (7–10) (our own observation). Therefore, there appears to be another important regulator in the blood/tissue that significantly accelerates the rate of MSU crystal formation. Furthermore, as a danger signal, MSU needs to be present at the microgram range to efficiently boost CTL responses (2). Whether there is a mechanism that retains this minuscule quantity of MSU on site and prevents instant diffusion is not known.

Intriguingly, there have been numerous reports that gout specimens isolated from acute phase of the attack were often covered with Ig-like structures (11–18). More specifically, it was reported that injection of MSU crystals into rabbits induced a serum factor that increased the rate of MSU crystal formation (19). Because nucleation is the rate-limiting step in crystal formation, it was proposed that this phenomenon was a result of Abs driving the equilibrium toward crystallization by stabilizing initial nucleating MSU monomers (20). In other words, the initial stacking of monomeric molecules is usually unstable under dispersion forces of the solvent. The binding of Ab is therefore expected to stabilize such a microscopic structure, tilting the balance toward precipitation. However, the concept of crystal-binding Abs and the associated crystallization in vivo have remained unproven, and research on the issue has stayed dormant since. This issue has become urgent in light of the rapidly growing interest in solid structure-mediated immune activation and inflammation (6, 21–24). Conceptually, gouty arthritis and MSU-mediated immune activation are similar events. We sought to study whether an underlying in vivo mechanism can explain the question of uric acid precipitation in the host.

We report in this study that mouse serum contains IgM Abs that bind to MSU crystals via their F(ab′)2 fragment. They also facilitate the precipitation of MSU crystals in uric acid solution. This precipitation effect, however, is dependent on an intact IgM structure. Of biological implication, B cell-deficient mice cannot sense uric acid as a danger or immune regulatory signal, which is readily restored with infusion of MSU-binding mAbs (uric acid-binding Abs (UBAs)). Our work explains the accelerated uric acid precipitation in vivo, aids in our understanding of the mechanisms of danger, and provides a testable hypothesis for the pathogenesis of gout.

*Immunology Research Group and Department of Microbiology and Infectious Diseases, University of Calgary, Calgary, Alberta, Canada; and †Department of Pathology, University of Massachusetts Medical School, Worcester, MA 01655

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Address correspondence and reprint requests to Dr. Yan Shi, 4A18 HRIC, 3330 Hospital Drive NW, University of Calgary, Calgary, Alberta, Canada T2N 4N1. E-mail address: yshi@ucalgary.ca

Abbreviations used in this paper: MSU, monosodium urate; DC, dendritic cell; MPO, myeloperoxidase; UBA, uric acid-binding Ab.

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Materials and Methods

Mice, cells, and reagents

All mouse strains were housed at the University of Calgary Animal Resource Centre under protocols approved by the University of Calgary Animal Research Review Committee. nu/nu mice were purchased from The Jackson Laboratory. Control IgM Abs were either purchased from Sigma-Aldrich or produced from similar MSU negative-staining mAb hybridomas (CD11b) (F11Tc). Abs were from eBioscience. Secondary goat anti-mouse IgG plus IgM (H + L, 115-095-068), IgM (μ-chain, 115-095-020), and F(ab′)1, (115-096-146) Abs were purchased from Jackson ImmunoResearch Laboratories. IgM fragmentation kit was from Pierce. SIINFEKL peptide was a gift from K. Rock (University of Massachusetts Medical School, Worcester, MA). CrCl was freshly ordered for each experiment from MF Biomedical. C5 complete ELISA kit was purchased from Sigma-Aldrich. Anti-CD86 antibody (5C11) was from Kamiya Biomedical. CD62L (PE) and CD11b (FITC) Abs were from eBioscience. Antibody/chemoab, mAb production, and HPLC Ig purification

C57BL/6 (B6) and BALB/c mice were immunized with 1 mg of suspended MSU crystal i.p. in PBS without any adjuvant, and were boosted with the same dose biweekly for 2 mo. Antiserum were collected at this time point for the FACS analysis. Tail blood (>100 μl) was mixed with 1 μl of heparin and spun in a microcentrifuge to remove blood cells. Splenocytes were then harvested and fused with A3A tumor cells using a standard hybridoma protocol (26), with 10^5 tumor partners fused with 4 × 10^5 splenocytes. Between weeks 2 and 4, 25 μl of cell supernatants from growing hybridomas were used to test for IgM secretion. All clones with moderate to high binding were kept, along with several negative-binding IgM controls.

Cytotoxic T cell lysis assay

C57BL/6 or nu/nu mice were s.c. immunized with 5 μg of OVA-coated 1-μm-diameter latex beads in 100 μl of PBS, as previously described (2). For those that received the Ab infusion, 500 μg of purified Abs was injected via tail vein on the same day before the immunization. The immunized mice were rested for 7 days and were then sacrificed, and 5 × 10^6 splenocytes were stimulated in 10 ml of culture medium in the presence of 10^−7 M SIINFEKL peptide. Five days later, 5000 35Cr-labeled EL4 cells pulsed with 10^−6 M SIINFEKL were used as target at the E:T ratios indicated. Unpulsed EL4 cells were used as a background control, which produced negligible reading.

Hybridoma culture, typing, fragmentation, and purification

B cell hybridomas were cultured in RPMI 1640 with 10% FBS plus 1 mM HEPES, 25 μM 2-ME, penicillin/streptomycin antibiotics, and hypoxanthine/aminopterin/thymidine medium. As a standard operation, the mAb from the supernatant was isolated from 800 ml of the supernatants collected. An equal volume of the supersaturated solution of ammonium sulfate was slowly added to the supernatant to precipitate the Ab. The mixture was centrifuged, and only the precipitate was collected and dissolved using 30–50 ml of PBS. This solution was then dialyzed three times against PBS overnight. The solution was then analyzed using HPLC. The HPLC analysis was performed with a Shimadzu Prominance system with a CBM-20A controller, a LC-20AB fluid pump, and a SPD-M20A diode array. A total of 500 μl of the Ab solution was injected and analyzed on an Alltech Macrophere GPC 300A 7u column (Alltech 88181) with an isotropic buffer containing 10 mM phosphate and 120 mM NaCl (pH 7.2). The samples were run at a rate of 1 ml/min over 40 min. The fractions collected were tested for the ability to bind MSU crystals, and then the active fractions were collected. This mixture was then further subjected to concentration via centrifugation with a Macrosep 300 K filter (Pall Filtron). The leftover fraction was tested for protein concentration using the bicinchoninic acid protein assay kit (Pierce). The Abs were further purified with a UNO 12 column (Bio-Rad) with a Bio-Rad FPLC, with 10 mM ethanamine (pH 9.6) as buffer A, and buffer A plus 1.5 M NaCl as buffer B, resolved at a rate of 1 ml/min in 25 min from 0% buffer B to 80% buffer B. IgM fragmentation and the associated analysis were performed with a kit and instructions from Pierce (ImmunoPure IgM Fragmentation Kit).

Flow cytometric analysis of MSU crystals

The flow analysis for MSU crystals was developed in our laboratory by modifying standard cell-based FACS analysis. A total of 100 μl of 1 mg/ml MSU crystal suspension was mixed with 100 μl of mAb supernatant or 1 μg of purified mAb (or as indicated otherwise) and incubated at room temperature for 20 min. The crystals were then washed twice with PBS and incubated with 0.5 μl of 1 mg/ml second Ab (FITC) in 100 μl for 10 min. The crystals were then washed again before FACS analysis. On a BD FACScan (BD Biosciences), MSU crystals showed a smaller size (~5–10% on a linear scale) on the forward scatter, and higher granularity (1 to several folds higher on a linear scale) on the side scatter. Typically, 40,000–50,000 total events were collected with CellQuest, and data were analyzed with Flowjo (Tree Star). Xanthine crystals were produced by first incrementally adding 1 N NaOH into 5 ml of xanthine suspension to dissolve the powder at high pH. Once completely dissolved, 1 N HCL was added to bring pH back to 7.0. The crystals thus precipitated were washed in 95% ethanol twice, and once in acetone. The crystals were dried before use.

Uric acid precipitation assay

A supersaturated uric acid solution (1 mg/ml) was incubated with various concentrations of a control protein (OVA) and quantified (both by sizing and analyzing exchange columns, 99% pure by HPLC analysis) Ab, either UBA 11, UBA E6, UBA fragment, or a control in 1 ml at the indicated concentrations for 6 h. The supernatants were removed and wells were flushed. A total of 1 ml of 0.01 N NaOH solution was added to the wells, and 20 μl of the solution was added to 1 ml of water and read at 292 nm with a spectrophotometer. The conversion to the quantity of uric acid was achieved using a standard curve for UV absorbance from a set of uric acid solutions with known concentrations, as follows: Y (uric acid in μg) = 0.0766 × (UV 292 absorption) + 0.0298; R^2 for the curve is 0.9937.

Serum uric acid measurement

Mice were injected i.v. with 500 μg of indicated Abs or PBS for 3 days, with or without the coinjection of 1 mg of soluble uric acid each day. Twenty-four hours after the last injection, blood from mice was collected into vials with 1 μl of heparin, and serum was isolated via centrifugation. A total of 2 μl of serum was mixed with 198 μl of buffer A, which contained 10 mM ethanolamine and 120 mM NaCl adjusted to pH 9.6. This mixture was passed through a nanosep 30 K filter (Pall Filtron), which would allow the uric acid in the sample to pass through, but would prevent possible protein contaminants from the sample. A total of 100 μl of the sample was then injected into the HPLC. Uric acid was resolved on a PolyWAX LP column (The Nest Group) by a gradient of 0–60% B (10 mM ethanolamine and 1.12 M NaCl) adjusted to pH 9.6) at 0.5 ml/min over 15 min. The HPLC analyses with EZ-start software were done at UV 292 nm. Peak area at 292 nm as a percentage of total area of the sample was used for the calculation of uric acid levels in the serum to minimize run-to-run or injection volume variability, as reported previously (25).

MPO measurement

MPO measurements were performed by a method adapted from Bradley et al. (27) on lung tissue isolated on day 4 from mice injected with Ab and uric acid solution. The tissue was homogenized with a 50 mM potassium phosphate buffer that contained 0.5% hexadecytrimethyl ammonium bromide (pH 6.0). The samples were then vortexed, and the supernatant was centrifuged for 4 min at 5000 rpm. The 7-μl supernatant samples were placed in a 96-well plate, and 200 μl of 0.167 mg/ml o-dianisidine hydrochloride solution containing 0.0005% (w/v) of hydrogen peroxide was added and the changes in absorbance at 450 nm were measured using a microplate reader. The absorbance measurements were then converted to MPO units. The MPO values were generated by multiplying the UV reading by a factor of 0.2528 per a standard protocol. One MPO unit of activity is defined as amount of MPO required to degrade 1 μmol of peroxide per minute at 25°C.

Complement C3 reading

The blood from mice i.v. injected with Ab and uric acid was isolated using the cardiac puncture technique, heparinized, and spun down for 10 min at 7500 rpm. The supernatant was collected. This mixture was then further subjected to concentration via centrifugation with a Macrosep 300 K filter (Pall Filtron). The leftover fraction was tested for protein concentration using the bicinchoninic acid protein assay kit (Pierce). The Abs were further purified with a UNO 12 column (Bio-Rad) with a Bio-Rad FPLC, with 10 mM ethanamine (pH 9.6) as buffer A, and buffer A plus 1.5 M NaCl as buffer
microplate reader. The absorbance was then converted to C3 concentration in mg/ml using a standard curve fitting.

Neutrophil activation

Blood samples were collected from mice injected with UBA E6 or control Ab as in the serum uric acid reading, and were subjected to hemolysis treatment and then stained with CD11b (FITC) and CD62L (PE) Abs. The samples were then analyzed with flow cytometry.

Statistical analysis

All results were reproduced in at least three independent assays. All error bars are 1 SD of the test sample groups. Student’s t test (2 tail) was used to produce the p values shown in the graphs.

Results

B cells are required for the uric acid adjuvant effect

We hypothesized that some serum factor, most likely Abs, facilitated and enhanced the immune activation associated with MSU crystals. As such, the proposal predicts that the MSU-mediated immune activation should be substantially decreased in the absence of Igs in vivo due to the reduced MSU crystal precipitation. We tested the validity of this prediction using muMT (IgH) mice, which lack an important domain of the Ig μ-chain, and therefore lack mature B cells (28). We used a well-established protocol of cytolytic T cell (CTL) induction by s.c. immunization of OVA-coated latex beads (2, 29). Splenocytes from the immunized mice 7 days after the immunization were stimulated with an OVA CD8 epitope peptide (SIINFEKL). Five days later, the resulting T cells were counted, and the target EL4 (H-2B) cells were labeled with 51Cr and pulsed with the same peptide. A 5-h CTL assay was performed at the E:T ratio indicated, and as described in Materials and Methods. The two lines in each panel represent two independent mice. The data are representative of three independent repeats. Most of the error bars (1 SD) were covered by the symbols.

that other factors associated with B cells, most likely Abs, played a role in the sensing of uric acid by the host.

The presence of MSU-binding Abs

We immunized B6 and BALB/c mice with preformed crystals to generate MSU crystal-binding Abs. The mice were immunized with 1 mg of MSU crystals biweekly for 2 mo in the absence of any additional adjuvant. The sera collected from the tail blood of the immunized mice strongly stained MSU crystals as determined by FACS analysis (Fig. 2A). Unimmunized mice also had MSU-binding Abs, albeit at titers somewhere between half to 1 log lower than the immunized mice (Fig. 2A). As a control, the MSU immune sera failed to stain xanthine crystals (Fig. 2B). We produced over 300 mAbs from the immunized mice by fusing the splenocytes with a tumor partner A3A. Approximately 30% of total clones showed various degrees of staining of the MSU crystals, with 9% showing substantial binding (over a log shift compared with the second Ab alone). Of the mAbs that showed binding, we tested their subtypes by analyzing the supernatants with an Ab subtyping kit, and determined that 85% of them are IgM κ (Fig. 2D), whereas IgGs and one IgM A made up the rest. This indicates that MSU-binding B cells do not frequently undergo Ab class switching. This is a common feature of T-independent B cell responses, as expected for a nonprotein Ag (MSU crystals) that would not have a cognate T cell response. The lack of adjuvant in our immunization protocol could also explain the absence of class switch.

Additional controls showed that serum from muMT mice had very low staining of MSU even after a similar immunization schedule, suggesting that the substances bound to MSU are Abs, in line with Fig. 2 that they were detectable by anti-Ig Abs (Fig. 3A). The low staining of muMT serum of MSU seems to suggest that other serum factors can also interact with the crystal surface, albeit

FIGURE 1. B cell-deficient mice do not sense uric acid as an adjuvant. Wild-type or muMT mice were immunized s.c. in the hinder flanks with 5 μg of OVA/latex beads mixed with PBS, 100 μg of MSU crystals, or uric acid solution (100 μl total volume). Seven days later, the mice were sacrificed, and 50 million splenocytes were prepared and stimulated with the 10^{-10} SIINFEKL peptide. Five days later, the resulting T cells were counted, and the target EL4 (H-2B) cells were labeled with 51Cr and pulsed with the same peptide. A 5-h CTL assay was performed at the E:T ratio indicated, and as described in Materials and Methods. The two lines in each panel represent two independent mice. The data are representative of three independent repeats. Most of the error bars (1 SD) were covered by the symbols.

FIGURE 2. The cloning and analysis of MSU crystal-binding Abs. A. Unimmunized B6 or immunized BALB/c or B6 serum (25 μl) was used to stain 500 μg of MSU crystals, followed by a second FITC-conjugated Ab recognizing mouse IgG/IgM H + L chains. The FACS analysis is similar to a typical cell-based assay, except the crystals are smaller on the forward scatter. The lightly shaded area in this and following assays is second Ab only control. B. Identical with A, except xanthine crystals were used in place of MSU. C. Supernatant from a representative high binding UBA (UBA 11, 100 μl) was used to stain MSU crystals as in A. IMM, MSU immune serum. D, Schematic representation of the frequencies of various Ig subtypes within MSU-binding mAbs.
to a much lesser extent than the Abs. Whether such a low binding had any functional consequence was not further studied in this report. Likewise, the binding of two control Abs (against mouse and human MHC class I molecules) showed minimal staining as well (Fig. 3B). These results and the finding that some similarly produced IgM did not stain the MSU crystals suggest that the binding is likely to be specific. The strong affinity and binding of these Abs suggest a possible involvement of Ig-mediated cell activation in the biological functions of MSU crystals, especially in light of the possibility of IgM immune complex formation.

**UBAs facilitate uric acid crystallization**

In the process of generating UBAs, we fortuitously discovered that the presence of these Abs altered MSU precipitation (data not shown). To investigate the issue, we incubated supersaturated uric acid (1 mg/ml, pH 8.0) solution with a control protein and purified UBAs (UBA 11 or UBA E6) at different concentrations (Fig. 4 and data not shown). After 6 h (Fig. 4) or overnight (data not shown), the MSU crystals settled at the bottom of the tissue culture plate. After carefully washing the plate to remove residual soluble uric acid, the crystals were resolubilized with 0.01 N NaOH, and the amount of precipitation was quantified with UV 292 absorbance. Fig. 4 shows that the rate of crystal precipitation was in fact a function of the concentration of UBAs, and was less affected by a control protein (OVA). This result indicates that uric acid precipitation is significantly facilitated by UBAs. This observation seems to suggest that nonspecific interaction with other proteins might alter uric acid precipitation to a certain extent, as implicated in an earlier report (9).

One outstanding issue in the UBA-mediated MSU precipitation is the mode of contact between the crystal surface and the Ab. To study the binding mechanism between UBAs and the crystal surface, we digested UBAs with an IgM fragmentation kit and produced various Ig domains. Using the same amount of UBA (E6) as the starting point, the resulting F(ab\textsubscript{2}) completely retained the binding, nearly indistinguishable from the original IgM (Fig. 5A). Fc\textsubscript{5} (the Fab-deleted pentameric H chain) demonstrated no staining at all (Fig. 5A). Therefore, MSU binding is F(ab\textsubscript{2}) dependent.

However, the binding itself is not equivalent to the actual crystal precipitation. We mixed a supersaturated uric acid solution (1 mg/ml) with 50 \(\mu\)g/ml E6 or its fragments, and incubated the solution for 6 h. Surprisingly, despite the unabated binding to MSU crystals, the F(ab')\textsubscript{2} fragment completely failed to precipitate the soluble uric acid (Fig. 5B). Other control structures, including Fc5 and a control IgM, MOPC that does not bind MSU, had no effect at all. It is clear from this assay that Ab affinity is necessary, but not sufficient for the uric acid precipitation. The pentameric IgM configuration appears to be a crucial structure for the nucleation and growth of the MSU crystals.

As with other Abs, one concern was the ability of these Abs to block MSU crystal interaction with immune cells. We reported early on that MSU crystals are potent stimulators of DC activation (2). However, it was possible that MSU-mediated activation of DCs might be blocked in the presence of these Abs. We tested the effect of UBAs on DC activation by coincubating DCs with MSU and either UBAs or control Abs, as indicated. In the presence of 200 \(\mu\)g/ml MSU crystals, 7-day B6 bone marrow DCs induced by GM-CSF and IL-4 were activated to express surface CD86, a characteristic DC activation marker. None of the individual UBA or their combination altered MSU-mediated DC activation, similar to that of irrelevant control Abs (Fig. 5C). These data argue that the presence of these Abs does not interfere with the strong adjuvant activities of MSU crystals, at least with regard to DCs.
Serum uric acid levels were analyzed by HPLC, as described in Materials and Methods.

UBA restores the sensing of uric acid as an immune adjuvant

The notion that UBA mediates uric acid precipitation predicts that blood uric acid levels should drop following the injection of the Ab. We therefore injected soluble uric acid i.v., followed by an infusion of UBA (see Materials and Methods) (25). Three days later, we collected the sera from tail blood. The sera were passed through a 30 K cutoff filter to reduce the UV absorption interference. The pass-through was then injected into a weak anion exchange HPLC column, and the uric acid peak absorption at 292 nm was recorded as a percentage of total UV absorption of each run (a method to minimize run-to-run human injection error). Fig. 6A shows that in the presence of UBA, the recipient mice demonstrated reduced serum uric acid levels, whereas a control IgM failed to do so. This result was consistent with our hypothesis that UBAs may indeed cause a phase change of uric acid in vivo. As an outcome, the soluble uric acid available in the serum was reduced.

Our work presented above led to the proposal that the presence of UBA determines the precipitation of uric acid and the associated inflammation and adjuvanticity. We first studied whether the presence of UBAs alters the overall inflammatory state in vivo, because it is expected that the precipitation of MSU crystals will cause certain inflammatory reactions. We measured two common indicators, MPO in the lung and total serum complement C3. MPO is a product of neutrophils, and its level indicates the accumulation of these cells and tissue inflammation (27). The serum C3 is produced in response to inflammation as well and can be regarded as a general indicator of inflammatory state (31). Fig. 6, B (MPO measurement) and C (complement C3 concentration), shows that, following the injection of uric acid and UBA, both of the factors were significantly elevated after 3 days. The control IgM Abs did not have such an effect. One interesting observation was that in both cases, injection of UBA (E6C7) alone led to higher readings, although the C3 elevation with UBA alone was not statistically significant in comparison with the control (Fig. 6C). It is possible that UBAs can cause endogenous uric acid precipitation without the additional infusion. It is also possible that injection of UBA causes stress in the recipient, and thus increases the uric acid levels in the animal in response to this sudden infusion of great amounts of Abs. Because the increases in MPO measurements suggested the activation/migration of neutrophils into the tissues, we analyzed their CD62L (L-selectin) level by FACS. Neutrophil activation in inflammation is usually accompanied by the loss of this surface adhesion molecule as the neutrophils migrate into the tissue. UBA injection substantially reduced the expression of CD62L on blood neutrophils, whereas the control IgM appeared to have a smaller, but noticeable effect (Fig. 6D).

The most critical prediction for our study was that the availability of UBA restores the sensing of uric acid as an adjuvant in B cell-deficient mice. We therefore injected UBA and a control IgM i.v. into muMT mice and repeated the assay of Fig. 1. If the presence of UBAs enables uric acid precipitation, then the defect in sensing uric acid shown in Fig. 1 should be restored. Fig. 7 shows that the addition of UBA significantly elevated the CTL activity to the levels similar to that of the wild-type mice (Fig. 1). Interestingly, the control Ab also occasionally provided an enhancing effect, although to a lesser extent (data not shown). This may suggest that the presence of IgM itself is an important means of local immune response, which may come in addition to uric acid precipitation. Overall, the results were in line with our hypothesis that MSU-binding Abs are a critical factor in determining the uric acid-mediated adjuvant effect and inflammation. These UBAs most likely achieve such an effect via their ability to cause MSU crystallization.

Discussion

We report in this study that IgM Abs enhance the precipitation of uric acid from solution at relatively low concentrations. Gout has been a recognized disease for centuries (32), and the underlying cause was discovered many years ago (33). To date, many mathematical models have been proposed to delineate the rate of crystallization in relationship with environmental factors, such as the temperature, pH, salt, vibration, and even the material of the container in which the experiments were conducted (7–10). Yet a formula for predicting the rate of crystal formation remains elusive. The robust onset of gouty arthritis therefore also remains a peculiar event.

Our discovery that uric acid is a danger signal released by dying cells is another example of uric acid’s biological effect (2). We reported earlier that a crystallization event is required for this adjuvant effect. We have made substantial progress in understanding
how MSU crystals, once formed, interact with DCs by a nonreceptor-dependent, chemical response with the cellular membrane (23). But no underlying mechanisms with regard to the actual phase transition have been revealed, particularly in vivo. It has long been known that uric acid is one of the principal antioxidative systems in mammals, and higher species progressively lose the ability to process this substance, in a likely attempt to gain benefits from its protective functions (34, 35). Primates have completely lost any enzymatic activities to process uric acid to allantoin due to a mutational silencing (36, 37). This progression permits the increasing likelihood of uric acid accumulation and possible crystallization. Our work suggests that UBAs are an important facilitating factor that results in the actual crystal precipitation.

In gout, it is likely that sustained high uric acid levels lead to macroscopic MSU precipitation. There is some clinical evidence to support our hypothesis. In humans, Ig-coated MSU crystals are found in gouty specimens (16–18, 38). Electron microscopy revealed that the Fab portion of the Ab is attached to the crystal surface with Fc pointing away from the clusters (14). This is the correct configuration of immune complexes, which frequently triggers activation and inflammatory phagocytosis of accessory cells and APCs. A clear exception is that in humans, IgGs are found to bind MSU (16–18, 38), and the role of MSU-binding IgM has not been studied. A potential explanation for this observation in humans is that gout is a recurring condition with robust cytokine production. Certain IgM responses may be driven to switch as a result of repeated stimulation and using cytokines as a class switch trigger (39). Another important distinction is that to date we have not been able to directly visualize gout-like MSU crystals in mouse tissues, because the intrinsic residual uricase activities prevent the formation of macroscopic crystals. In studying the underlying mechanism of uric acid-mediated immune activation, a uricase knockout mouse model would work best; however, these mice are not viable due to acute kidney failure (40). To us, it has also been proven futile to visualize the microscopic MSU crystal formation in situ. Before our production of UBAs, there had been no immunological reagents to directly detect MSU crystals in the tissue. The preparation steps for histological or other microscopic analysis, such as Gomori’s methenamine-silver method, are incompatible with the microcrystals because they dissolve or dismount quickly in washing steps (our own observation). This report further extends the call for a suitable animal model for this important immunological topic.

The situation may be different in the uric acid immune adjuvant effect. The influx of IgM at the site of tissue stress (41, 42) may precipitate as well as anchor the microcrystals in situ of uric acid release. Because the formation of the crystals is at the source of tissue destruction by injury or infection, this would establish a situation in which DCs and other APCs may come into the close proximity of Ag (self or nonself) and adjuvant (MSU crystals), efficiently enabling immune induction. On another note, it has long been reported that IgM is attracted to the site of injury and is a potent mediator of subsequent tissue damage (42). Whether MSU is one source of the attraction remains to be determined.

It is important to note that the source of the MSU-binding Abs is unknown. We can probably exclude the involvement of B2 B cells due to the lack of cognate protein Ag presentation. B1 and marginal zone B cells differ in the target locations that they protect (43). B1 B cells are mostly located in body cavities, whereas marginal zone B cells tend to engage pathogens that penetrate the bloodstream. In humans, MSU crystals appear most often in joints. Furthermore, from the substantial amounts of UBAs present in the uninimmunized mice, it seems to suggest that B1 cells are the source of UBAs, because B1 Abs are produced without antigenic stimulation. Signaling-wise, we have recently gained a comprehensive understanding of how MSU crystals can trigger immune cell activation by directly engaging cell surface lipids, particularly cholesterol. This leads to plasma membrane lipid sorting and nonspecific aggregation of ITAM-containing receptors and downstream activation essentially similar to a phagocytic event (23). However, we have no evidence to rule out immune complex-mediated activation. It is known that IgM complexes trigger complement fixation, which is ~1000-fold more efficient than IgG complexes (44).

Small peptides released during complement fixation, such as C3a and C5a, are anaphylatoxins (45). Additionally, it has been suggested that there might exist a mouse μ-chain FcR (46, 47), which would presumably trigger immune activation akin to the better defined FcR-mediated inflammatory responses (48, 49).

It should be prudentely noted that without direct visualization of crystals in vivo, this report lacks an important piece of evidence to support our central hypothesis. The interpretation of our data, particularly the possibility of Ab-induced MSU crystal formation in vivo, remains speculative. With advent of new imaging technologies, we remain hopeful that MSU crystal formation in vivo will be directly monitored in due time. Such a methodological development will either substantiate or nullify the main hypothesis of this report, although our proposal that Abs are a substantial component in uric acid-mediated inflammation would still stand.

Our work has added a new angle in the study of urate precipitation and the subsequent inflammatory responses. In recent years, the interest in solid structure-mediated immune activation has grown significantly. However, one question has remained unresolved: where do the endogenous crystals come from? Our work is therefore a timely finding. One interesting fact to consider is that in the original report by Addadi and colleagues (19, 20), the authors tested several immunization crystals and found that each immune serum produced in rabbit led only to the precipitation of the original immunizing crystal. This type of specificity and the common presence of the crystal-binding tissue factors among tested mammals may indicate that MSU-specific Abs are not an oddity. Other crystal depositions in humans could involve Abs as well, for precipitation and subsequent inflammatory responses.

In summary, our work implicates a role of IgM Abs in MSU crystal formation and potentially subsequent adjuvanticity/inflammation, and provides evidence that it is a biologically relevant event in vivo. It may add a line of investigation on other crystal-related arthropathies and pathogeneses, in addition to addressing a lingering question in MSU crystal-related immunological research in general.

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Disclosures
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