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The role of complement in the modulation by fluid-phase IgG of the production of reactive oxygen species by polymorphonuclear leukocytes stimulated with IgG immune complexes

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Abstract

The production of reactive oxygen species (ROS) by polymorphonuclear leukocytes (PMN) can be induced by immune complexes and is an important component of phagocytosis in the killing of microorganisms, but can also be involved in inflammatory reactions when immune complexes are deposited in tissues. We have observed that fluid-phase IgG can inhibit the generation of ROS by rabbit PMN stimulated with precipitated immune complexes of IgG (ICIgG) in a dose-dependent manner, acting as a modulatory factor in the range of physiological IgG concentrations. This inhibitory effect is compatible with the known affinity (Kd) of monomeric IgG for the receptors involved (FcRII and FcRIII). The presence of complement components in the immune complexes results in a higher stimulation of ROS production. In this case, however, there is no inhibition by fluid-phase IgG. The effect of complement is strongly dependent on the presence of divalent cations (Ca^{2+} or Mg^{2+}) in the medium, whereas the stimulation of ICIgG (without complement) does not depend on these cations. We have obtained some evidence indicating that iC3b should be the component involved in the effect of complement through interaction with the CR3 receptor. The absence of the inhibitory effect of fluid-phase IgG in ROS production when complement is present in the immune complex shows that complement may be important *in vivo* not only in the production of chemotactic factors for PMN, but also in the next phase of the process, i.e., the generation of ROS.

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- Immune complex
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Introduction

It is well known that polymorphonuclear leukocytes (PMN) can undergo a rapid metabolic change, known as the respiratory burst, when exposed to a variety of stimuli (1); this includes an increased rate of oxygen consumption, a marked activation of the pentose-phosphate pathway and the generation of reactive oxygen species (ROS; O_2^-, H_2O_2, $OH^\bullet$, singlet oxygen, etc.). The production of these highly reactive compounds which have microbicidal properties is a component of the process of phagocytosis, being thus im-

plicated in the mechanisms of defense. However, in some instances it may be involved in inflammatory reactions, since these compounds may be released from the cells and contribute to tissue damage (2).

It is well established that the interaction of particles or surfaces with leukocytes mediated by Fcγ receptors (immune complexes of IgG - ICIgG) can trigger the respiratory burst. However, divergent findings have been reported as to the ability of complement (C3b and iC3b) to induce the generation of ROS upon interaction with the cells. Some experiments have shown that C3b or iC3b is not able to induce the generation of ROS by human monocytes and PMN (3); other observations, however, have indicated an active role of these complement components in triggering the respiratory burst (4,5). Also, some evidence has been presented indicating that the production of ROS by PMN is dependent on a synergic mechanism involving Fcγ and complement receptors (6).

Regardless of the importance of determining which kind of receptors are able to trigger this mechanism by themselves, another relevant issue is to know the behavior of these cells in the production of these compounds under physiological conditions, when the phagocytes interact with immune complexes which contain IgG or IgG and complement components, since *in vivo* there is a high concentration of fluid-phase IgG which possibly could compete for the Fcγ receptors. We analyze this question by *in vitro* experiments with rabbit PMN using as a stimulus two types of immune complexes in the form of precipitates: ICIgG and ovalbumin and the same immune complexes with complement components incorporated by previous incubation with whole serum (ICIgG-C).

Material and Methods

The preparation of the antigen (chicken ovalbumin), the purification of rabbit IgG anti-ovalbumin antibodies, the preparation of ICIgG with ovalbumin in the equivalence zone, as well as the isolation of rabbit blood PMN were performed as described (7).

Media and solutions

Phosphate-buffered saline (PBS) containing 0.9% NaCl and 8 mM sodium phosphate buffer, pH 7.2, was used. Hanks' medium was prepared as described (8). Luminol (Sigma, St. Louis, MO, USA) was dissolved in dimethylsulfoxide (DMSO) at a concentration of 2 mM; for the experiments this stock solution was diluted with Hanks' medium (the final concentration of DMSO was 0.45%, v/v).

Animals

New Zealand rabbits were used as blood donors for the isolation of PMN as well as of normal or immune serum.

Preparation of antigen-antibody-complement complexes

Immune complexes of ovalbumin and IgG antibodies were prepared at equivalence (7). Complement was added to ICIgG for the preparation of the ICIgG-C complexes as follows: after centrifugation and washing three times with PBS, the antigen-antibody precipitates were incubated with fresh rabbit serum at the proportion of 182 μg of immune complex per ml of serum for 30 min at 37ºC (7,9). The immune precipitates were then washed three times with PBS by centrifugation at 4ºC and resuspended in Hanks' medium containing 15 mM HEPES, pH 7.2, and 0.1% gelatin. The quantity of immune complexes is always reported as the mass of protein before the addition of complement. Protein concentrations in the immune precipitates were determined by the micro-biuret method. We found that after formation of ICIgG-C the mass of the immune complex

increased by 44%. To test for the complement-fixing capacity of the ICIgG, the residual CH_{50} (50% hemolytic unit of complement) was determined (10) in the supernatant after incubation of the immune complexes with serum. It was found that 182 µg of ICIgG consumed 29.8 CH_{50} units.

Treatment of rabbit serum to inactivate some complement components

Rabbit serum used as a source of complement was subjected to three types of treatment in order to selectively inactivate complement components: a) heat inactivation at 56ºC for 30 min to block the activity of C1, C2 and factor B; b) depletion of C3 by treatment of serum with zymosan using 15 mg of zymosan per ml of serum at 37ºC for 1 h (10); c) inactivation of C3 and C4 by treatment of serum with 50 mM hydrazine, incubated with serum at 37ºC for 2 h, followed by dialysis with PBS containing Ca^{2+} and Mg^{2+} at the concentrations of Hanks' medium (11).

Measurement of the production of ROS by chemiluminescence

The production of ROS by PMN was assayed by the generation of chemiluminescence in the presence of luminol (12). This is an indirect measurement of the amount of superoxide produced: the stimulation of the cells leads to the activation of NADPH oxidase, resulting in the formation of O_2^- which, upon the action of superoxide dismutase (SOD), undergoes a dismutation to H_2O_2 which, in the presence of Cl^- and myeloperoxidase, forms OCl^-. Some of these compounds react with luminol bringing the molecule to an excited state whose decay results in light emission (13). The kinetics of chemiluminescence was recorded with a BioOrbit luminometer, model 1251 (Bio-Orbit Oy, Turku, Finland). PMN suspensions (10^6 cells) in Hanks' medium containing 15 mM HEPES, pH 7.2, 0.1% gelatin and 10 µM luminol were added to the cuvettes and preincubated for 15 min at 37ºC. The cuvettes were then inserted into the luminometer, and the stimulus (immune complex) was added to start the experiment; the final volume was 1.1 ml. The cuvettes were maintained at 37ºC and the rate of photon emission (reported in mV) was recorded every 51 s. The first measurement was made after 25 s.

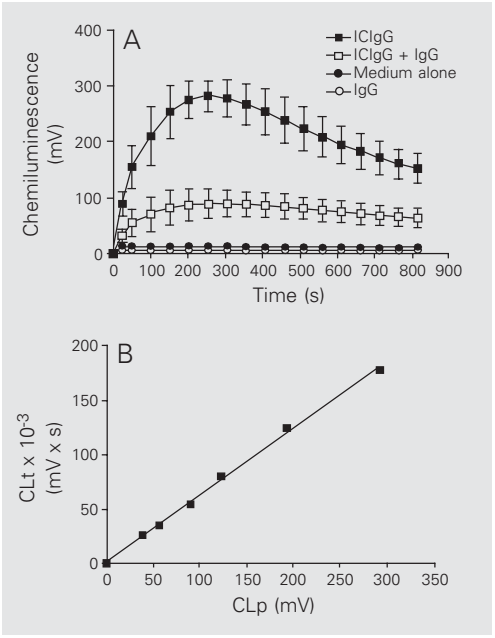

Figure 1. Kinetics of chemiluminescence, a measure of the rate of reactive oxygen species production, by rabbit polymorphonuclear leukocytes stimulated with IgG immune complexes (ICIgG) in the absence and in the presence of fluid-phase IgG. *A*, Cells (10^6) were preincubated in Hanks' medium alone or containing 10 mg/ml IgG for 15 min at 37ºC and then stimulated with 300 µg ICIgG. Chemiluminescence was recorded in a final volume of 1.1 ml. Two controls are shown: only cells (medium alone) and cells plus 10 mg/ml IgG (IgG). Data are reported as means ± SD for N = 4 (cells from different animals). *B*, Correlation between chemiluminescence in the peak (CLp) and total chemiluminescence (CLt) for 13.6 min (calculated by integration of the peak). The six points correspond to six different concentrations (9.4 to 300 µg/ml) of ICIgG. Each point corresponds to the mean value of 4 independent experiments (different animals).

Competition experiments with fluid-phase IgG

PMN suspensions were preincubated with fluid-phase IgG at 37ºC for 15 min in Hanks' medium with the desired concentrations of IgG before stimulation with the immune complexes, also in the presence of IgG. The IgG solution used (prepared by DEAE cellulose chromatography) was tested for the presence of aggregated molecules by polyacrylamide gel electrophoresis using 3.5% acrylamide gels with 0.5% agarose as described previously (14), and no aggregation was found. In addition we demonstrated that centrifugation of IgG solution at 100,000 *g* for 30 min just before these experiments had no appre-

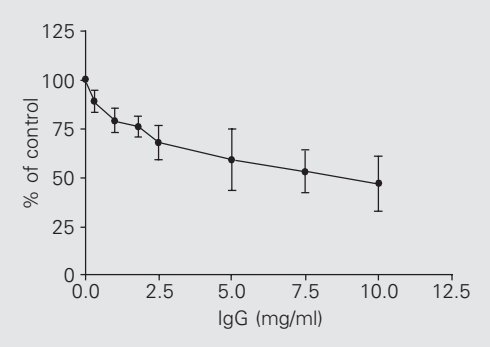

Figure 2. Inhibition of reactive oxygen species production by increasing concentrations of fluid-phase IgG. Cells (10^6) were preincubated with fluid-phase IgG for 15 min at 37ºC and then stimulated with 300 µg ICIgG. The control cells were preincubated in the medium without IgG. Results are reported as percent chemiluminescence in the peak in relation to control values (mean ± SD, N = 4 with cells from different animals).

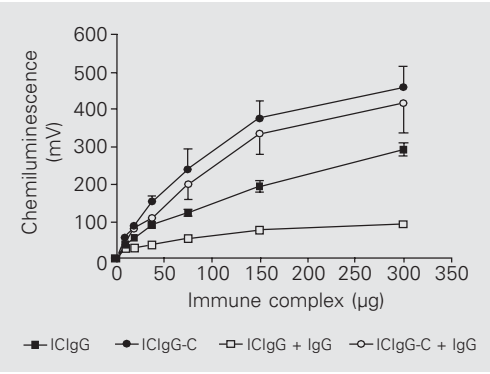

Figure 3. Effect of complement components incorporated into immune complexes of IgG (ICIgG-C) on the stimulation of reactive oxygen species production by polymorphonuclear leukocytes and the influence of the presence of fluid-phase IgG. Cells (10^6) were preincubated in Hanks' medium with or without 10 mg/ml fluid-phase IgG for 15 min at 37ºC before stimulation with different amounts of immune complexes. For ICIgG-C the mass of immune complexes indicated on the abscissa corresponds to its mass before the addition of complement. ICIgG-C was prepared using 182 µg ICIgG per ml of serum. Data are reported as means ± SEM for N = 4 (different animals).

ciable effect on the inhibitory action of fluid-phase IgG on ROS production by PMN stimulated with ICIgG. In view of this result, this previous centrifugation was performed in some, but not all, experiments.

Results

The kinetics of chemiluminescence production by PMN induced by ICIgG is presented in Figure 1A. The presence of fluid-phase IgG at a concentration representative of that existing in plasma (10 mg/ml) greatly reduced the stimulatory effect of the immune complex. The chemiluminescence with PMN alone as well as with PMN plus 10 mg/ml fluid-phase IgG was very low and negligible compared with that induced by the immune complex. Figure 1B shows the correlation between the values of chemiluminescence in the peak and the total chemiluminescence after 13.6 min (calculated by integration over this period of time) using six different concentrations of ICIgG (from 9.4 to 300 µg/ml). Since there was a close correlation between the two measurements, we used only the chemiluminescence in the peak, that corresponds to the maximum rate of ROS production, for quantification in the subsequent experiments.

Figure 2 shows the inhibitory effect of increasing concentrations of fluid-phase IgG on the stimulation of ROS production by PMN induced by ICIgG (300 µg/ml). The inhibition was progressive with increasing IgG concentration and started to be demonstrable at around 0.9 mg/ml IgG; with 10 mg/ml IgG the effect reported as percent of control (without fluid-phase IgG) was in the range of 34 to 64% in four independent experiments (47 ± 14%, mean ± SD).

In another set of experiments we analyzed the possible effect of complement incorporated into the immune complex of IgG on its stimulatory effect on ROS production, as well as the susceptibility to inhibition by the soluble immunoglobulin, using several immune complex concentrations (Figure 3). In the range of immune complex concentrations from 9.4 to 300 µg/ml, the presence of IgG greatly reduced ROS production by ICIgG; however, when complement was present in the immune complex, no inhibition was observed. The stimulatory effect of ICIgG-C was higher than the effect of ICIgG, albeit it was not affected by the presence of soluble IgG (the two curves were practically coincident in the entire range of immune complex concentrations).

The experiments in Figure 4 show the influence of extracellular divalent cations (Ca^{2+} and Mg^{2+}) on ROS production induced by ICIgG and ICIgG-C. For each type of immune complex the control was the value of chemiluminescence in the peak obtained in complete Hanks' medium (1.26 mM Ca^{2+} and 0.9 mM Mg^{2+}).

Stimulation with ICIgG was practically independent of the presence of these cations in the medium. The effect of ICIgG-C, how-

ever, was strongly dependent on them, with Ca^{2+} alone being sufficient for the full effect, whereas Mg^{2+} produced a lower stimulation (around 60% of control). Moreover, when fluid-phase IgG was present (10 mg/ml) the same dependence on Ca^{2+} and Mg^{2+} was obtained for the effect of ICIgG-C.

In order to have some indication of which components of complement incorporated into the immune complex were responsible for the effect of ICIgG-C, we prepared this immune complex using as a source of complement rabbit serum previously treated by three different procedures: heat inactivation at 56ºC for 30 min blocking the activity of C1, C2 and factor B, depletion of C3 by treatment with zymosan, and inactivation of C3 and C4 by treatment with hydrazine. The experiments in Figure 5 were done in the presence of 10 mg/ml fluid-phase IgG.

All three treatments abolished the effect of complement (in these experiments the control was the effect of ICIgG without competition with soluble IgG). These results indicate that iC3b must be involved in the effect of complement incorporated into immune complexes.

Discussion

Fcγ receptors, which recognize the Fc domain of IgG, are involved in a number of cellular functions such as phagocytosis, superoxide production and cytokine release. Three types of Fcγ receptors have been described in human and mouse PMN: FcRI, FcRII and FcRIII (15). FcRI is a high affinity receptor for monomeric IgG with a dissociation constant ranging from 10 to 0.1 nM, and is expressed in INF-γ-treated neutrophils. FcRII and FcRIII are normally present in non-activated PMN and have a lower affinity for IgG, with a dissociation constant ranging from 10 to 0.1 μM (16-18). The same types of receptors may be present in rabbit PMN. Thus, in our experiments the low affinity receptors FcRII and FcRIII might

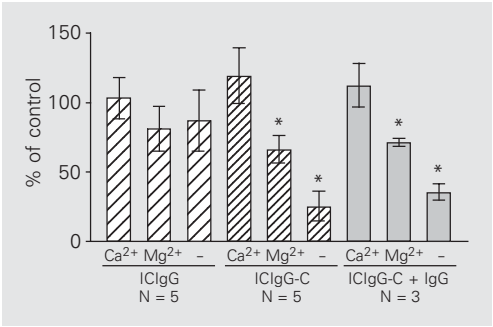

Figure 4. Effect of extracellular Ca^{2+} and Mg^{2+} on reactive oxygen species production induced by ICIgG and ICIgG-C. Polymorphonuclear leukocytes (10^6 cells) were suspended in Hanks' medium without Ca^{2+} and Mg^{2+} (–), or in medium containing either one of these cations at the concentrations used in Hanks' medium (1.26 mM Ca^{2+} and 0.9 mM Mg^{2+}). The chemiluminescence in the peak with 300 μg ICIgG was measured and the results are indicated as % of control; for each of the three groups the control value was that obtained with complete Hanks' medium. ICIgG-C + IgG indicates the experiments in the presence of 10 mg/ml fluid-phase IgG. The values obtained with only Ca^{2+} in the medium were not statistically different from the corresponding controls (with Ca^{2+} and Mg^{2+}). Data are reported as means ± SEM. *$P < 0.02$ for comparison of the conditions with Mg^{2+} or (–) with the corresponding values with Ca^{2+} in each of the three groups (*t*-test).

mediate the interaction between ICIgG and the cells. In competition experiments using 10 mg/ml fluid-phase IgG we had an IgG concentration of 67 μM, a value in the range of the dissociation constant for FcRII and FcRIII (10 to 0.1 μM). It is thus reasonable to assume that fluid-phase IgG could compete with the immune complexes for the binding to the receptors, resulting in partial inhibition of the stimulation of ROS production, as observed here.

We have shown that the presence of complement components in the immune complexes of IgG appreciably increases the capacity of ROS production by PMN. Also, when complement is present in the immune complexes, fluid-phase IgG has no effect on the induction of ROS production. Complement receptors would not be affected by the occupation of Fcγ receptors by fluid-phase IgG, so that the ICIgG-C can by-pass the competitive inhibition that is observed with ICIgG. In this case, the interaction mediated by complement receptors may increase the binding of immune complexes to the cells or may by itself also stimulate ROS generation.

One possibility we should consider is that the immune complex could be partially solubilized upon the addition of serum for the preparation of ICIgG-C, as previously reported (19,20). In our experiments this was improbable since the relation between serum volume and the ICIgG mass used by us was much smaller than that needed to solubilize the immune complexes. Compared to the volume used by the cited investigators with IgG antibody and BSA, we used a se-

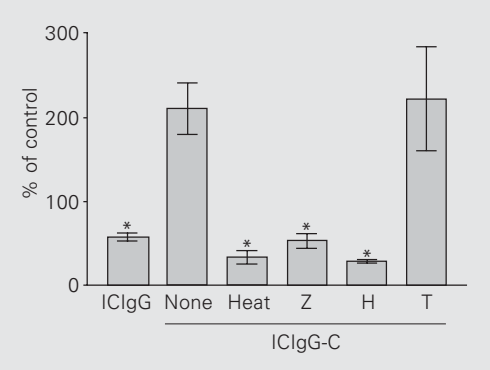

Figure 5. Effect of three kinds of treatment of rabbit serum used as a source of complement in the preparation of ICIgG-C on the experiments of competition with fluid-phase IgG. Polymorphonuclear leukocytes (10^6 cells) were stimulated with the immune complexes (300 µg) and the chemiluminescence in the peak was recorded (general conditions were the same as described in previous experiments). The values presented were obtained in the presence of 10 mg/ml fluid-phase IgG, and are reported as percent of control (the chemiluminescence in the peak obtained with ICIgG without fluid-phase IgG). Serum treatments: H = treatment with hydrazine, Heat = incubation at 56ºC for 30 min, T = the same conditions for treatment with hydrazine but without this reagent, Z = absorption with zymosan. Data are reported as means ± SEM for N = 4-6 with different cell preparations (with the exception of H and T in which 2 cell preparations were used (2 determinations for each one). *P < 0.004 compared to ICIgG-C (*t*-test).

rum volume about 18 to 20 times smaller than that required for solubilization. But, even in the case of a small amount of immune precipitate being solubilized (despite the increase in mass of the immune complex by incorporation of complement components), this does not interfere with the interpretation of the results since ICIgG-C induces a higher ROS production than ICIgG (obviously it would interfere if the reverse were obtained).

It is known that, upon activation of the complement system by immune complexes, several activated proteins (derived from C1, C4, C2, and C3) become tightly bound to the complexes (9,21,22). PMN have been shown to have receptors for some of these components which may be involved in various biological functions (23). Thus, interaction through C3b/iC3b can promote the binding of immune complexes; it was shown, however, that the interaction through C3b and iC3b cannot induce phagocytosis (24). C1q bound to latex beads stimulates the respiratory burst and the hexose phosphate pathway in these cells (25). As to the capacity of C3b/iC3b to trigger the generation of ROS, some studies have led to conflicting results (3-6). Possibly the differences in physical form of the stimulus could explain these differences. None of these studies on the function of complement receptors used precipitated immune complexes as done in our experiments, a fact that might be important in inflammatory reactions involving PMN. Our finding that complement can reverse the inhibition by fluid-phase IgG suggests that its components incorporated into the immune complexes could have a stimulatory effect by themselves. However, one cannot exclude the possibility that Fc receptors could be involved because, if complement promotes the binding of the immune complex to the cell membrane, the IgG molecules present in the immune precipitate would be forced to interact with its receptors and the Fc-mediated triggering of stimulation would be feasible.

We have observed that the effects of the presence of complement in the immune complexes on ROS generation are strongly dependent on divalent cations (Ca^{2+} or Mg^{2+}), whereas the stimulation of ROS production by ICIgG practically does not require these cations in the medium. This is evidence for the involvement of other kinds of receptors (CR3 and CR4 which bind to iC3b or CR1 which binds to C3b). This is consistent with the observation that neutrophil activation by IgG immune complexes was less dependent on extracellular Ca^{2+} than that induced by C5a (26).

It is interesting to note that in the experiments with Ca^{2+} and Mg^{2+} (Figure 4) the effect of ICIgG-C in the absence of these cations was very low even without IgG in the fluid phase. At first this would not have been expected since IgG is present in the immune complex and could interact with Fcγ receptors, and this interaction, as shown in the same experiment, does not depend on these ions. One possible explanation would be that the incorporation of complement into ICIgG blocks to some extent the binding site for the receptor in the Fc domains. This is plausible since it was found that FcRII and FcRIII bind to IgG in the lower hinge region (27), which is also close to the site of fixation of C1q to the immunoglobulin (28).

The experiments illustrated in Figure 5, in which the serum used as a source of complement was subjected to some treat-

ments in order to selectively inhibit complement components, indicate that the components involved in the interaction of ICIgG-C with PMN should be C3b (involving CR1) or iC3b (involving CR3 and CR4). The direct participation of C1q, for which there is a specific receptor in PMN (25), may be excluded since treatment with hydrazine or absorption of serum with zymosan does not interfere with this component. It is most likely that iC3b may be responsible for the binding to the cells, since C3b (which binds to CR1) has a half-life of about 90 s (29) and, in the preparation of ICIgG-C, ICIgG was incubated with serum for 30 min. So, the receptor involved should be CR3. Further evidence for excluding CR1 in the interaction is that, although this receptor can also recognize iC3b, it does not require divalent cations for binding (15). It is also interesting to note that CR3 has a long extracellular domain containing three calmodulin-like consensus Ca^{2+}-binding sequences (30), in agreement with the effect of this cation in our experiments.

It is known that PMN can migrate to diverse sites of the organism where variable concentrations of IgG might exist. Thus we may say that the fluid-phase IgG present at these sites can exert an important modulation of the process of ROS production by PMN induced by IgG immune precipitates.

PMN have been implicated in many diseases as possible mediators of tissue damage. The production of ROS by these cells upon stimulation may be one of the mechanisms involved in this effect (2). Since ICIgG is a powerful stimulant of ROS generation, it is possible that the formation of these compounds by PMN could be involved in several pathological states dependent on deposition of ICIgG on tissues. Complement has been implicated in several conditions of tissue injury induced by immune complexes (31-33), although it may not be necessary, depending on the animal species and tissue site (34,35). It has been well established that activation of complement results initially in the production of the potent chemotactic molecule (C5a) which promotes PMN recruitment to sites of inflammation (36). Our results indicate another important role for complement mediated by the incorporation of its components into ICIgG in the next phase of the process, i.e., the stimulation of ROS production. Although the presence of complement in the immune complex may not be an absolute requirement for this function (because the competition by physiological concentrations of IgG in tissue fluids cannot be complete), it is quantitatively most relevant. This result is also consistent with previous observations (37) showing the same important role of complement in the induction of lysosomal enzyme release by PMN, which might be another factor involved in inflammatory reactions.

Acknowledgments

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