

A Physiological Function for Apolipoprotein(a): A Natural Regulator of the Inflammatory Response

JANE HOOVER-PLOW,¹ ERIKA HART, YANQING GONG, ALEKSEY SHCHURIN,²
AND TRACEY SCHNEEMAN³

*Joseph J. Jacobs Center for Thrombosis and Vascular Biology, Department of Cardiovascular Medicine
and Department of Molecular Cardiology, Cleveland Clinic Lerner Research Institute,
Cleveland, Ohio 44195*

Structural similarities between apolipoprotein(a) (apo(a)), the unique apoprotein of lipoprotein(a), and plasminogen, the zymogen of plasmin, can interfere with functions of plasmin (ogen) *in vitro*. The purpose of this study was to evaluate the role of apo(a) in inflammation *in vivo* using apo(a) transgenic mice and to determine if effects are plasminogen-dependent using backgrounds that are either plasminogen-replete or plasminogen-deficient. After administration of peritoneal inflammatory stimuli, thioglycollate, bioimplants or lipopolysaccharide, the number of responding peritoneal neutrophils and macrophages were quantified. Apo(a), in either wild-type or plasminogen deficient backgrounds, inhibited neutrophil recruitment but had no effect on plasminogen-dependent macrophage recruitment. Macrophage-inflammatory protein-2, a neutrophil chemokine, was reduced in apo(a) mice, and injection of this chemokine prior to thioglycollate restored neutrophil recruitment in apo(a) transgenic mice. In the lipopolysaccharide model, mice with apo(a), unlike mice without apo(a), did not increase neutrophil recruitment in response to the stimulus. In the bioimplant model, neutrophil recruitment and neutrophil cytokines were reduced in apo(a)tg mice but only in a plasminogen-deficient background. These results indicate for the first time that apo(a), independent of plasminogen interaction, inhibits neutrophil recruitment *in*

vivo in diverse peritoneal inflammatory models. Hence, apo(a) may function as a cell specific suppressor of the inflammatory response. *Exp Biol Med* 234:28–34, 2009

Key words: apo(a) transgenic mice; plasminogen deficient mice; thioglycollate and lipopolysaccharide induced peritoneal inflammation; neutrophil recruitment

Lipoprotein(a) [Lp(a)] was first described by Berg in 1963 (1) as a unique lipoprotein particle. Lp(a) is similar to low density lipoprotein (LDL), consisting of a lipid core and apolipoprotein B (2); but Lp(a) contains an additional apoprotein, apo(a), tethered to apoB by a disulfide linkage. Numerous clinical studies conducted over the past 40 years have identified Lp(a) as a risk factor independent from LDL for a variety of cardiovascular pathologies, including myocardial infarction (3), atherosclerosis (4), and peripheral vascular diseases (5). A meta-analysis (6) of 27 prospective studies demonstrated a clear association between elevated plasma Lp(a) and coronary heart disease (CHD). These disease associations suggest that apo(a) must impart distinct functions to the Lp(a) particle. Support for this contention is derived from studies (7, 8) of human and animal tissues showing distinct localizations of LDL and Lp(a) within the vessel wall under atherogenic conditions and the demonstration that mice expressing free apo(a) unlinked to apolipoprotein B display accelerated development of early atherosclerotic lesions (9, 10).

Much of the focus on the pathogenic activities of apo(a) has centered upon its strong resemblance to plasminogen (Plg) (11), the zymogen for plasmin, and the primary fibrinolytic enzyme for clot lysis. Apo(a) consists of a variable number of KIV kringle domains, and these domains are highly homologous to the kringle domains, KIV and KV of Plg. Many of the functions of Plg depend upon the lysine binding sites (LBS) associated with the kringles, and indeed, apo(a) also possesses LBS activity. As a result, Lp(a)

This study was supported by grants from NIH: T32 HL07914 (AS), HL 007914 (TS), HL17964 (EFP, JHP), HL65205 (JHP), HL 078701 (JHP) and the American Heart Association: 0625331B (YG). The authors have no conflicting financial interests.

¹ To whom correspondence should be addressed at Department of Molecular Cardiology, NB50, Cleveland Clinic Lerner Research Institute, 9500 Euclid Avenue, Cleveland, OH 44195. E-mail: hooverj@ccf.org

² Current address: Oakwood Healthcare System, Dearborn, MI.

³ Current address: Cleveland Public Schools, Cleveland, OH.

Received April 2, 2008.
Accepted August 29, 2008.

DOI: 10.3181/0804-RM-136
1535-3702/09/2341-0028\$15.00
Copyright © 2009 by the Society for Experimental Biology and Medicine

interferes with multiple functions of Plg, including its binding to fibrin, cells, and extracellular matrix proteins (12–14) *in vitro*. *In vivo* studies (9, 15) using apo(a)tg mice or rabbits have shown an increased formation of fatty streaks in the aorta. Transgenic mice with mutations (16, 17) in apo(a) inactivating its LBS have attenuated aortic fatty streak formation.

Apo(a)tg mice have been tested in various challenge models involving complex stimuli and multiple cell types, and a number of pathogenic activities have been ascribed to the apoprotein. For example, enhanced fatty streak formation (9, 10) reduced fibrinolysis (18), inhibition of TGF- β activation (19), and increased vascular smooth muscle cell activation (20). We have recently (21) used apo(a)tg mice in a Plg replete or deficient background in a carotid injury model. The apo(a)tg mice without Plg (apo(a):Plg $^{-/-}$) exhibited an increased incidence of thrombosis, compared to Plg $^{+/+}$, apo(a)tg:Plg $^{+/+}$, and Plg $^{-/-}$ mice. Also, collagen accumulation was increased in the presence or absence of Plg, raising the possibility that apo(a) may exert Plg-independent as well as Plg-dependent functions *in vivo*.

Although the association studies in humans and the analyses of apo(a)tg mice point to many pathogenic functions of apo(a), physiological roles of Lp(a) have been more elusive, but must exist to account for its presence in human and non-human primates but not most other species (22). A deficiency of Plg (23, 24) markedly reduces leukocyte recruitment in a variety of inflammatory models. While Lp(a) and apo(a) interfere with Plg binding to cells and extracellular matrix substrates (12–14), the importance of this interference *in vivo* in leukocyte recruitment has not been investigated. In *in vitro* studies, Lp(a) and apo(a) stimulate the release of proinflammatory cytokines (25) from monocytes and endothelial cells (26) and can act as a chemoattractant for monocytes (27) suggesting a role of apo(a) in leukocyte regulation. In seeking to define a role of apo(a) in leukocyte recruitment, we have identified a novel activity of the apoprotein: apo(a) may function as a natural and cell specific suppressor of the inflammatory response. Furthermore, a mechanism for this novel function of apo(a) also has been identified: its selective regulation of cytokine production. We further show that these effects of apo(a) are independent of its molecular mimicry of Plg.

Materials and Methods

Mice. The Plg $^{-/-}$ mice were generated as previously described (28) and bred for eight generations into the C57BL/6J background. Apo(a)tg mice expressed an apo(a) 6 KIV construct (KIV 5–10, plus KV and a protease-like domain) with a liver specific promoter (29, 30) in a C57BL/6J background. The genotypes (Plg $^{+/+}$, Plg $^{+/-}$, Plg $^{-/-}$) of the offspring for litters without apo(a) and litters with apo(a) were determined by PCR analysis from a tail-clip or ear punch sample (21). A screening ELISA assay was also developed to facilitate apo(a) genotyping (21). Mice were

housed in sterilized isolator cages, maintained on a 12/12 hrs light/dark cycle, and provided sterilized food and water. All animal experiments were performed in accordance with a protocol approved by the Institutional Animal Care and Research Advisory Committee. Experiments were performed in 6–8 week-old mice that did not have any overt health problems of older Plg $^{-/-}$ mice (rectal prolapse or wasting).

Thioglycollate Induced Leukocyte Recruitment. The thioglycollate model has previously been described (23). Mice were injected intraperitoneally with 0.5 mL of 4% thioglycollate (Becton Dickinson, Cockeysville, MD). After 6 or 72 hrs, mice were injected with PBS into the peritoneal cavity and 2.5 mL of the peritoneal lavage removed. The number of neutrophils and macrophages accumulating in the lavage was determined from enzyme activity, myeloperoxidase for neutrophils (31), and non-specific esterase for macrophage/monocytes (32). The enzyme activity is a reliable indicator of the number of each cell type (33).

Bioimplant Induced Leukocyte Recruitment. As previously described (24), three polyethylene terephthalate (PET) 1.2 cm circular disks were inserted into the peritoneum. After 20 hrs, the disks were removed, and the peritoneal cavity washed with 4 mL of PBS and 2.5 mL of peritoneal lavage removed. The disks were rinsed in PBS and the adherent cells removed. The numbers of adherent leukocytes from the disks and in the lavage were determined by enzyme activities as described above. The total number of cell types responding was the sum of the cells in the lavage plus the cells on the disks.

LPS Induced Leukocyte Recruitment. The endotoxin, lipopolysaccharides (LPS) from *Escherichia coli* 011:B4 (Sigma-Aldrich), was used to induce inflammation. LPS was reconstituted in sterile PBS, and 25 or 200 μ g per mouse injected intraperitoneally. After 6 hrs the mice were injected intraperitoneally with PBS to collect cells, and serum was collected from the vena cava. Cells were counted in a hemocytometer, attached to slides by cytopspin, and identified with Wright's stain.

Cytokines. KC and MIP-2 were determined in the lavage 6 hrs after injection of thioglycollate with the Mouse KC Immunoassay Kit (#MKC00, R&D Systems, Minneapolis, MN) and Mouse MIP-2 Immunoassay Kit (#MM200, R&D Systems, Minneapolis, MN). Goat antibodies for KC (AF-453-NA, R&D Systems, Minneapolis, MN) and MIP-2 (AF-452-NA, R&D Systems, Minneapolis, MN) at 2 μ g or 20 μ g/mouse were injected IP in 0.5 mL of PBS 120 mins prior to the thioglycollate injection, and goat IgG was injected at 2 μ g or 40 μ g/mouse in 0.5 mL of PBS. Recombinant mouse KC (453-KC, R&D Systems, Minneapolis, MN) and recombinant MIP-2 (453-KC, R&D Systems) at 50 ng/mouse were injected IP in 0.5 mL of PBS 60 mins prior to the thioglycollate injection. Neutrophils were determined in the lavage after antibody or cytokine injections as described above.

Statistical Analysis. Statistical differences were determined by a Student's *t* test or a one-way ANOVA and a Newman-Kuels post-test. A *P* value < 0.05 was considered significant. Values are the mean $\pm$ SEM.

Results

Apo(a) Suppresses Neutrophil Recruitment in the Thioglycollate Peritoneal Inflammatory Model.

Intraperitoneal injection of thioglycollate is a widely used agent to induce an inflammatory response. In this model, neutrophil recruitment occurs rapidly, leading to a maximal recruitment in 4–6 hrs, and macrophage recruitment follows a slower time course, peaking at 48–96 hrs. As previously reported (23) and verified in this study (Fig. 1A), macrophage recruitment 72 hrs after thioglycollate injection is suppressed in mice with a Plg deficiency (WT vs. Plg^{-/-} *P* < 0.0001 or apo(a)tg vs. apo(a):Plg^{-/-} *P* = 0.02). The presence of apo(a) in WT or Plg^{-/-} backgrounds had no effect (*P* > 0.05) on macrophage recruitment, indicating that apo(a) does not interfere with the functions of Plg in the recruitment of these cells. At 6 hrs (Fig. 1B), apo(a)tg mice exhibited a marked decrease (75% inhibition relative to WT) in neutrophil recruitment (WT vs. apo(a) *P* = 0.01). This difference was also observed in the Plg-deficient background (Plg^{-/-} vs. apo(a):Plg^{-/-} *P* = 0.001). The total number of leukocytes (cells $\times 10^6$ per mL blood, mean $\pm$ SEM) in the blood was not different in WT (14.0 ± 2.1 , *n* = 3) and apo(a)tg mice (9.8 ± 0.7 , *n* = 3) and the percent of leukocytes that were neutrophils was not different in WT ($55 \pm 4\%$ neutrophils, *n* = 3) and apo(a) mice ($63 \pm 8\%$ neutrophils, *n* = 3). The number of macrophages (cells $\times 10^6$) in the lavage in WT (2.3 ± 0.1 , *n* = 7) and apo(a)tg (2.5 ± 0.2 , *n* = 11) mice was similar at 6 hrs after thioglycollate injection, and no difference in leukocytes was found for WT and apo(a)tg mice after saline injection instead of thioglycollate (data not shown). Hence, apo(a) blunts neutrophil recruitment selectively in this inflammatory model, and this effect is unrelated to Plg.

Apo(a) Attenuates Neutrophil Recruitment in the Bioimplant and LPS Peritoneal Inflammatory Models. Two additional inflammatory models were implemented to determine if the effects of apo(a) on neutrophil migration applied broadly. In the first model, PET disks were implanted into the peritoneum. PET is a material used in Dacron vascular grafts, and the disk model was originally developed by Tang and Eaton to assess the inflammatory response to such biomaterials (33, 34). At 20 hrs after implantation, a robust recruitment of both macrophages and neutrophils occurs in the murine version of this model, and both cell types accumulate in the peritoneal cavity and on the implanted disks. There was no significant difference (*P* > 0.05) in total number of neutrophils or neutrophils attached to the disks between WT and apo(a)tg mice, and suggests that apo(a) does not have an independent role in leukocyte recruitment in this model. Busuttill *et al.* (24)

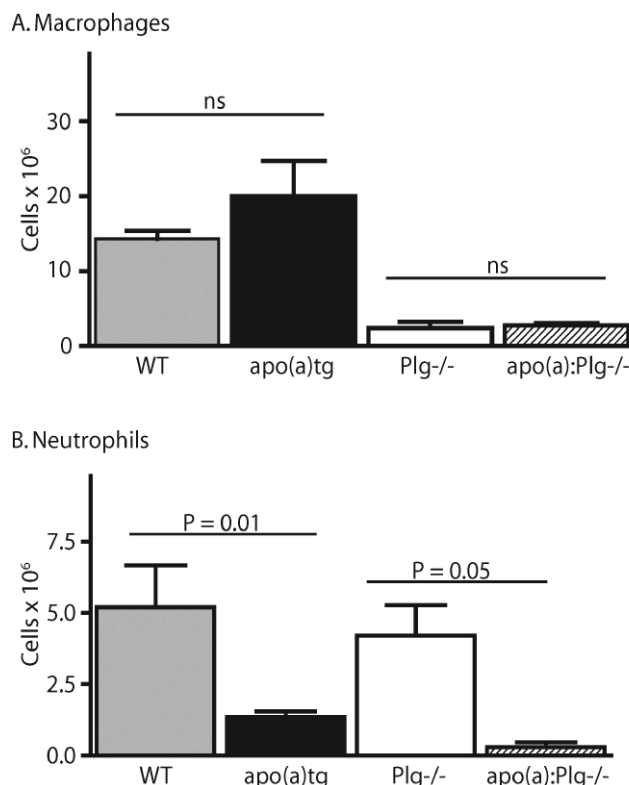


Figure 1. Leukocyte recruitment after thioglycollate injection in WT, Apo(a)tg, Plg^{-/-}, and Apo(a):Plg^{-/-} mice. **A.** Macrophage recruitment 72 hrs after injection of thioglycollate, *n* = 5–9 mice per genotype. **B.** Neutrophil recruitment to the peritoneal cavity 6 hrs after injection of thioglycollate, *n* = 4–11 mice per genotype. Values are the mean $\pm$ SEM. Statistical analysis was determined by a one-way ANOVA and a Newman-Kuels post-test. *P* values are indicated above the bars.

reported that neutrophil recruitment was Plg-dependent in the bioimplant model, and to test this in mice with apo(a), neutrophil recruitment was determined, and apo(a):Plg^{-/-} (2.4 ± 0.7 , cell number $\times 10^6$) compared to apo(a)tg (5.9 ± 1.6) mice had reduced (*P* < 0.05) neutrophil recruitment, but there was no difference in neutrophil recruitment between apo(a):Plg^{-/-} and Plg^{-/-} (3.3 ± 0.8) mice. Thus, neutrophil recruitment in this model apo(a) suppressed neutrophil recruitment only in the absence of Plg.

To determine whether there was an independent effect of apo(a) on neutrophil migration in infection, a lipopolysaccharide (LPS) induced endotoxemia model was implemented. This model mimics sepsis caused by gram-negative bacteria. Neutrophils increase in the circulation from 1–24 hrs after an LPS challenge (35), and monocytes increase between 6–24 hrs (35). In this model, WT and apo(a)tg mice were administered either 25 μ g or 200 μ g LPS intraperitoneally, and, 6 hrs after injection, cells were recovered from the peritoneal lavage and quantified. Neutrophils in the lavage after PBS were significantly lower (*P* < 0.001) in WT than apo(a)tg mice (Table 1). Injection of 25 μ g LPS had no significant effect on neutrophil recruitment in either strain. However, in WT mice injected with 200 μ g LPS, neutrophil recruitment was

Table 1. Peritoneal Neutrophil Recruitment in LPS and Bioimplant Models^a

	WT	Apo(a)tg	P value
Bioimplant ^b			
Cell number $\times 10^6$			
Total neutrophils	4.4 $\pm$ 0.9	5.9 $\pm$ 1.6	ns
Disk neutrophils	0.34 $\pm$ 0.10	0.55 $\pm$ 0.11	ns
LPS ^c			
Cell number $\times 10^5$			
PBS	0.8 $\pm$ 0.2	2.1 $\pm$ 0.2	<0.001
25 μ g	1.2 $\pm$ 0.7	1.7 $\pm$ 1.2	ns
200 μ g	6.4 $\pm$ 0.8	2.6 $\pm$ 0.6	<0.02

^a Values are the mean $\pm$ SEM. Statistical difference between WT and apo(a) values determined by Student's *t* test. ns, not significant ($P > 0.05$).

^b Total neutrophils in peritoneum and disk-associated neutrophils 20 hrs after disk implantation, $n = 8-11$.

^c Neutrophils, 6 hrs after peritoneal injection of LPS or PBS, $n = 3-6$.

significantly ($P = 0.002$) higher than in PBS injected mice, and this increase was also significantly ($P < 0.02$) higher than for apo(a)tg mice. Specifically, recruited neutrophils were 2.5-fold higher in WT mice compared to apo(a)tg mice. Indeed, neutrophil number was not significantly increased in the apo(a)tg mice at either dose of LPS (Table 1) compared to the values in PBS injected mice, suggesting that apo(a) suppressed the response to the challenge. In addition, consistent with changes in the circulation after the endotoxin challenge (35), very few macrophages were present in the lavage fluid 6 hrs after the endotoxin challenge and there was no significant difference between WT and apo(a)tg mice. These results suggest that in this LPS model as in the thioglycollate model, apo(a), in the presence of Plg (apo(a)tg mice), has an independent role and suppresses neutrophil recruitment.

Neutrophil Chemoattractants Determine Role of Apo(a) in Neutrophil Recruitment. To determine whether apo(a) modulated cytokines, MIP-2 and KC (human IL-8 homolog), potent neutrophil chemoattractants, were measured in the lavage at 6 hrs after the thioglycollate injection in WT, apo(a)tg, Plg^{-/-}, and apo(a):Plg^{-/-} mice (Fig. 2). KC (Fig. 2A) was not significantly different in WT and apo(a) mice, but KC was significantly reduced in apo(a):Plg^{-/-} mice compared to Plg^{-/-} mice. Apo(a):Plg^{-/-} mice also had a reduced KC ($P < 0.01$) compared to WT mice, but Plg^{-/-} mice and WT had similar values, suggesting that apo(a) had a greater effect on KC than Plg deficiency. For MIP-2 (Fig. 2B), the difference between WT and apo(a) was significant ($P = 0.01$), and the difference between Plg^{-/-} and apo(a):Plg^{-/-} compared to WT was also significant ($P < 0.01$), suggesting that for MIP-2 both a Plg deficiency and apo(a) contributed to a reduction of MIP-2.

Since neutrophils were reduced in the apo(a)tg mice after an LPS challenge, KC and MIP-2 were also quantified in plasma and the peritoneal lavage before and 6 hrs after either treatment with 25 μ g or 200 μ g of LPS. The MIP-2 is undetectable in the plasma of either WT or apo(a) mice

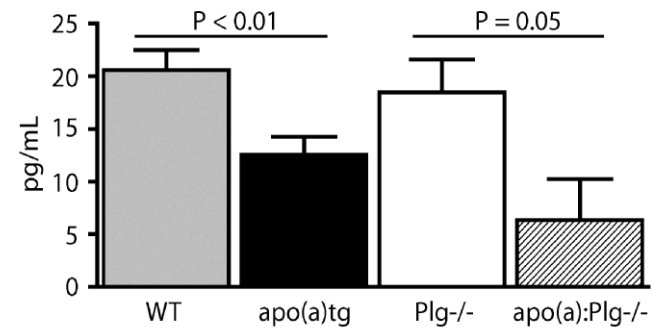
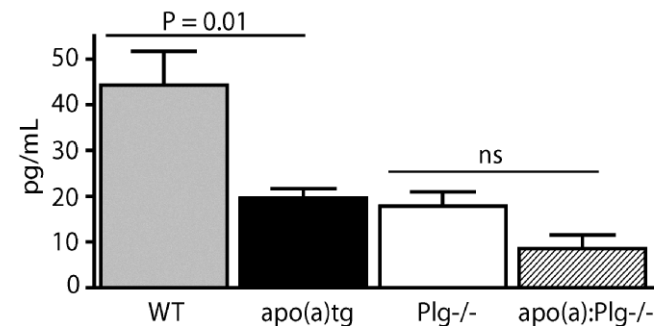
A. KC**B. MIP-2**

Figure 2. Lavage cytokines in WT, Apo(a)tg, Plg^{-/-}, and Apo(a):Plg^{-/-} mice. **A.** KC and **B.** MIP-2. Cytokines were determined in the lavage 6 hrs after the thioglycollate injection. Values are the mean $\pm$ SEM of 5–10 mice per genotype. Statistical analysis was determined by a one-way ANOVA and a Newman-Kuels post-test. *P* values are indicated above the bars.

before stimulation with LPS. KC values (pg/mL) are slightly higher ($P < 0.05$) in the apo(a) (171 ± 17 , $n = 7$) mice compared to WT mice (104 ± 16 , $n = 5$). In the WT mice, there was a significant ($P < 0.02$) increase in MIP-2 in plasma after the 200 μ g LPS challenge compared to the 25 μ g challenge (Table 2). The apo(a)tg mice were not able to respond to the higher dose of LPS and values at 25 μ g and 200 μ g were similar. There were no differences ($P > 0.05$) in MIP-2 in the peritoneal lavage or in KC in the plasma or peritoneal lavage (data not shown). Consistent with the lack of difference in neutrophil recruitment between WT and apo(a)tg mice in the bioimplant, there were no differences in the lavage cytokines in this model (Table 2).

In order to determine if reduction in these two cytokines was sufficient to reduce neutrophil migration in WT mice, antibodies to KC and MIP-2 were injected IP prior to the thioglycollate treatment (Fig. 3A). MIP-2 and KC antibodies (either separately or together at the higher dose of 20 μ g/mL) significantly decreased neutrophil recruitment compared to IgG injection in WT mice. Although there was a reduction of neutrophils with the lower dose of antibodies, the difference was not significant. Injection of MIP-2 antibody to apo(a)tg mice had no effect on neutrophil recruitment compared to the IgG injected apo(a)tg injected mice.

Table 2. Cytokine Levels After Bioimplant and LPS Challenge^a

	MIP-2 (pg/mL)		KC(pg/mL)	
	WT	Apo(a)tg	WT	Apo(a)tg
Bioimplant ^b	61 ± 9	62 ± 9	108 ± 39	97 ± 70
LPS 25 µg ^c	1221 ± 216*	1560 ± 182	3387 ± 157	3563 ± 75
LPS 200 µg ^c	1836 ± 23*	1602 ± 145	3628 ± 10	3597 ± 17

^a Values are the mean ± SEM, n = 3–6. Statistical difference determined by Student's *t* test (* *P* < 0.02) between WT MIP-2 values injected with 25 and 200 µg LPS.

^b Lavage cytokines 20 hrs after insertion, n = 3–5.

^c Plasma cytokines 6 hrs after peritoneal injection of 25 µg or 200 µg LPS, n = 6.

To determine if these cytokines could increase neutrophil migration in the apo(a)tg mice, KC and MIP-2 were injected separately or together and neutrophil recruitment determined at 6 hrs (Fig. 3B). MIP-2 had the most effect in restoring neutrophil recruitment (2.8-fold increase) in apo(a) mice compared to WT values, and injection of both cytokines together was only slightly higher than either cytokine alone and was not significantly different than the individual cytokines. Injection of KC or MIP-2 had no effect on neutrophil recruitment in WT mice compared to WT injected with saline.

Discussion

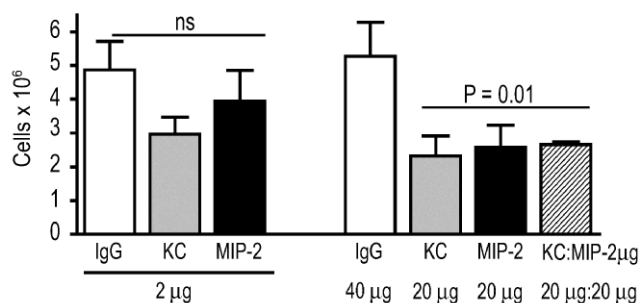
Leukocyte recruitment *in vivo* was assessed in an apo(a)tg mice model with a replete or deficient Plg background. This study demonstrates a role of apo(a) in the regulation of neutrophil recruitment in three different peritoneal inflammatory models. Suppression of leukocyte recruitment in chronic diseases, such as chronic obstructive pulmonary disease (36), inflammatory bowel disease (37), arthritis (38), and vascular disease (39), is one of the first therapeutic considerations. One of the confounding features of Lp(a) is evolution: expression being restricted primarily to higher primates where Lp(a)'s function appears to be the involvement in the pathogenesis of cardiovascular diseases. Our data support the possibility that Lp(a) and apo(a) have emerged as natural anti-inflammatory molecules to blunt the deleterious effects associated with excessive neutrophil accumulation at sites of inflammation.

Our model, apo(a)tg in a Plg replete and deficient background, has biological relevance and advantage in investigating the role of apo(a) in inflammation. The apo(a)tg mice express a small isoform in high concentrations. The small isoforms of apo(a) are reported (40, 41) to be more pathogenic than larger isoforms, and the concentrations of apo(a) found in our mice are at the pathogenic levels found in humans. Also, apo(a) fragments circulate in plasma (42), are found in the vessel wall with unstable atherosclerotic plaques (43), and exhibit functional differences from Lp(a) (44). Apo(a) fragments generated *in vitro* from neutrophils are more potent inhibitors of plasmin formation than intact apo(a) (45). In mice, apo(a) does not form a covalent bond with apoB (46) and precludes the

formation of Lp(a), minimizing the confounding influence of the LDL moiety in our model. In addition, the deletion of Plg in the apo(a)tg mice has provided a model to investigate the role of apo(a) without the interaction or interference of Plg.

In our model, stimulation of inflammation with thioglycollate or LPS, apo(a)tg mice with Plg have suppressed neutrophil recruitment to the peritoneal lavage fluid. After thioglycollate stimulation, neutrophil recruitment was suppressed in apo(a)tg in either the Plg replete or deplete background, and neutrophil recruitment to the peritoneal lavage was also suppressed after LPS stimulation in apo(a)tg mice compared to mice without apo(a). Suppression of neutrophil recruitment in apo(a)tg mice could be attributed to differences in cytokine response. After thioglycollate

A. WT Mice



B. Apo(a)tg Mice

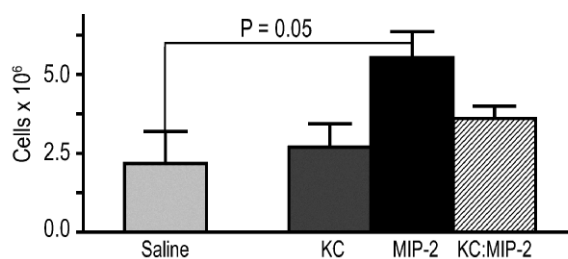


Figure 3. KC and MIP-2 neutralization and cytokine administration 6 hrs after thioglycollate injection. **A.** Inhibition of neutrophil recruitment with KC and MIP-2 antibodies in WT mice, n = 5–7 mice per genotype. **B.** Cytokine injection of KC and MIP-2 to apo(a)tg mice, n = 5–7 mice per genotype. Values are the mean ± SEM. Statistical analysis was determined by a one-way ANOVA and a Newman-Kuels post-test. *P* values are indicated above the bars.

stimulation, the neutrophil chemoattractants, KC and MIP-2, were reduced in the apo(a)tg mice, and injection of the MIP-2 to apo(a)tg mice restored neutrophil recruitment. To explain this, in the apo(a)tg mice, cellular sources of cytokines may be reduced or apo(a) may inhibit the release of the cytokines or their activation. Monocytes and macrophages are a major source (47) of the neutrophil cytokines, KC and MIP-2, but the number of macrophages in the lavage at the six hour time point was similar in mice with or without apo(a), suggesting that the major source of cytokines was not limiting in the apo(a)tg mice. Release of cytokines from macrophages may be impaired. In addition, the number of neutrophils in the blood was similar in the apo(a)tg and WT mice, but other sources of the neutrophil cytokines have been suggested (48), such as resident cells in the tissue, but apo(a) mice injected with PBS alone have increased leukocytes rather than a decrease compared to WT mice. These results suggest that if the source of cytokines is not limiting, then apo(a) may alter the release or activation of the cytokines.

In a third inflammatory model, the bioimplant model, apo(a) suppressed neutrophil recruitment or cytokine release only in the Plg deficient background. In the bioimplant model, neutrophil recruitment was plasminogen-dependent and when Plg was absent, apo(a) also suppressed neutrophil recruitment. In pathological situations PAI-1, the major inhibitor of Plg activation, is often elevated and has been shown to reduce plasmin activity influencing function. When apo(a) and PAI-1 are elevated, apo(a) may also suppress neutrophil recruitment. While there are several common features of the implant and thioglycollate inflammatory models, including the leukocyte recruitment to the peritoneal cavity, adhesion molecules (49, 50) complement activation (51, 52), and Plg activation (23, 24), there are notable differences in the models, such as the time course of the neutrophil accumulation, a role for fibrinogen (33) in the bioimplant model, and a role of various cytokines, MCP-1 (53), KC, and MIP-2 (54) in the thioglycollate model. Thus, an apo(a)-independent role in neutrophil recruitment may depend on the mechanism of the inflammatory model.

In conclusion, this study demonstrates for the first time a role in apo(a) *in vivo* in neutrophil recruitment. Using apo(a)tg with a replete or deficient background, our results demonstrate that apo(a) can inhibit leukocyte recruitment by a mechanism independent of Plg in the thioglycollate and LPS models. In the bioimplant model neutrophil recruitment is also suppressed by apo(a) but in a Plg dependent fashion. Thus, in patients with elevated Lp(a), apo(a) could play a beneficial role by suppressing inflammation and can do so independent of Plg.

The authors thank N. Klimczak and R. Lewis for assistance with the manuscript preparation and Dr. Edward Plow for helpful discussions.

1. Berg K. A new serum type system in man—the Lp system. *Acta Pathol Microbiol Scand* 59:369–382, 1963.

2. Fless GM, Rolih CA, Scanu AM. Heterogeneity of human plasma lipoprotein(a): isolation and characterization of the lipoprotein subspecies and their apoproteins. *J Biol Chem* 259:11470–11478, 1984.
3. Rhodes GG, Dahlén GH, Berg K, Morton NE, Dannenberg AL. Lp(a) lipoprotein as a risk factor for myocardial infarction. *J Am Med Assoc* 256:2540–2544, 1986.
4. Terres W, Tatsis E, Pfalzer B, Beil FU, Beisiegel U, Hamm CW. Rapid angiographic progression of coronary artery disease in patients with elevated lipoprotein(a). *Circulation* 91:948–950, 1995.
5. Groves P, Rees A, Bishop A, Morgan R, Ruttle M, Lewis N, Lane I, Hall R. Apolipoprotein(a) concentrations and susceptibility to coronary artery disease in patients with peripheral vascular disease. *Br Heart J* 69:26–30, 1993.
6. Danesh J, Collins R, Peto R. Lipoprotein(a) and coronary heart disease—meta-analysis of prospective studies. *Circulation* 102:1082–1085, 2000.
7. Nachman RL, Gavish D, Azrolan N, Clarkson TB. Lipoprotein(a) in diet-induced atherosclerosis in nonhuman primates. *Arteriosclerosis* 11:32–38, 1991.
8. Cushing GL, Gaubatz JW, Nava ML, Burdick BJ, Bocan TM, Guyton JR, Weilbaecher D, DeBakey ME, Lawrie GM, Morrisett JD. Quantitation and localization of apolipoproteins(a) and B in coronary artery bypass vein grafts resected at reoperation. *Arteriosclerosis* 9:593–603, 1989.
9. Lawn RM, Wade DP, Hammer RE, Chiesa G, Verstuyft JG, Rubin EM. Atherogenesis in transgenic mice expressing human apolipoprotein(a). *Nature* 360:670–672, 1992.
10. Svinland A, Berg K, Eliassen K, Lawn RM, Djurovic S, Aleström P, Noren T, Smith A. Histopathology of arterial lesions in LPA transgenic mice on cholesterol-enriched chow. *Atherosclerosis* 153:349–354, 2000.
11. McLean JW, Tomlinson JE, Kuang WJ, Eaton DL, Chen EY, Fless GM, Scanu AM, Lawn RM. cDNA sequence of human apolipoprotein(a) is homologous to plasminogen. *Nature* 330:132–137, 1987.
12. Hoover-Plow JL, Miles LA, Fless GM, Scanu AM, Plow EF. Comparison of the lysine binding functions of lipoprotein(a) and plasminogen. *Biochemistry* 32:13681–13687, 1993.
13. Miles LA, Sebald MT, Fless GM, Scanu AM, Curtiss LK, Plow EF, Hoover-Plow JL. Interaction of lipoprotein(a) with the extracellular matrix. *Fibrinolysis Proteolysis* 12:79–87, 1998.
14. Miles LA, Fless GM, Scanu AM, Baynham P, Sebald MT, Skocir P, Curtiss LK, Levin EG, Hoover-Plow JL, Plow EF. Interaction of Lp(a) with plasminogen binding sites on cells. *Thromb Haemost* 73:458–465, 1995.
15. Fan J, Shimoyamada H, Sun H, Marcovina S, Honda K, Watanabe T. Transgenic rabbits expressing human apolipoprotein(a) develop more extensive atherosclerotic lesions in response to a cholesterol-rich diet. *Arterioscler Thromb Vasc Biol* 21:88–94, 2001.
16. Boonmark NW, Lou XJ, Yang ZJ, Schwartz K, Zhang JL, Rubin EM, Lawn RM. Modification of apolipoprotein(a) lysine binding site reduces atherosclerosis in transgenic mice. *J Clin Invest* 100:558–564, 1997.
17. Hughes SD, Lou XJ, Ighani S, Verstuyft J, Grainger DJ, Lawn RM, Rubin EM. Lipoprotein(a) vascular accumulation in mice—in vivo analysis of the role of lysine binding sites using recombinant adenovirus. *J Clin Invest* 100:1493–1500, 1997.
18. Palabrica TM, Liu AC, Aronovitz MJ, Furie B, Lawn RM, Furie BC. Antifibrinolytic activity of apolipoprotein(a) in vivo: human apolipoprotein(a) transgenic mice are resistant to tissue plasminogen activator-mediated thrombolysis. *Nature Med* 1:256–259, 1995.
19. Grainger DJ, Kemp PR, Liu AC, Lawn RM, Metcalfe JC. Activation of transforming growth factor- β is inhibited in transgenic apolipoprotein(a) mice. *Nature* 370:460–462, 1994.
20. Lawn RM, Pearle AD, Kunz LL, Rubin EM, Reckless J, Metcalfe JC,

- Grainger DJ. Feedback mechanism of focal vascular lesion formation in transgenic apolipoprotein(a) mice. *J Biol Chem* 217:31367–31371, 1996.
21. Sha J, McCullough B, Hart E, Nassir F, Davidson NO, Hoover-Plow J. Apo(a) promotes thrombosis in a vascular injury model by a mechanism independent of plasminogen. *J Thromb Haemost* 3:2281–2289, 2005.
22. Lawn RM, Boonmark NW, Schwartz K, Lindahl GE, Wade DP, Byrne CD, Fong KJ, Meer K, Patthy L. The recurring evolution of lipoprotein(a)—insights from cloning of hedgehog apolipoprotein(a). *J Biol Chem* 270:24004–24009, 1995.
23. Ploplis VA, French EL, Carmeliet P, Collen D, Plow EF. Plasminogen deficiency differentially affects recruitment of inflammatory cell populations in mice. *Blood* 91:2005–2009, 1998.
24. Busuttill SJ, Ploplis VA, Castellino FJ, Tang L, Eaton JW, Plow EF. A central role for plasminogen in the inflammatory response to biomaterials. *J Thromb Haemost* 2:1798–1805, 2004.
25. Klezovitch O, Edelstein C, Scanu AM. Stimulation of interleukin-8 production in human THP-1 macrophages by apolipoprotein(a). Evidence for a critical involvement of elements in its C-terminal domain. *J Biol Chem* 276:46864–46869, 2001.
26. Haque NS, Fallon JT, Taubman MB, Harpel PC. The chemokine receptor CCR8 mediates human endothelial cell chemotaxis induced by I-309 and Kaposi sarcoma herpesvirus-encoded vMIP-I and by lipoprotein(a)-stimulated endothelial cell conditioned medium. *Blood* 97:39–45, 2001.
27. Syrovets T, Thillet J, Chapman MJ, Simmet T. Lipoprotein(a) is a potent chemoattractant for human peripheral monocytes. *Blood* 90:2027–2036, 1997.
28. Ploplis VA, Carmeliet P, Vazirzadeh S, Van Vlaenderen I, Moons L, Plow EF, Collen D. Effects of disruption of the plasminogen gene in mice on thrombosis, growth and health. *Circulation* 92:2585–2593, 1995.
29. Bonen DK, Hausman AML, Hadjiagapiou C, Skarosi SF, Davidson NO. Expression of a recombinant apolipoprotein(a) in HepG2 cells: evidence for intracellular assembly of lipoprotein(a). *J Biol Chem* 272:5659–5667, 1997.
30. Fan J, Ji ZS, Huang Y, de Silva H, Sanan D, Mahley RW, Innerarity TL, Taylor JM. Increased expression of apolipoprotein E in transgenic rabbits results in reduced levels of very low density lipoproteins and an accumulation of low density lipoproteins in plasma. *J Clin Invest* 101:2151–2164, 1998.
31. Himmelhoch SR, Evans WH, Mage MG, Peterson EA. Purification of myeloperoxidases from the bone marrow of the guinea pig. *Biochemistry* 8:914–921, 1969.
32. Torres JL, Rush RS, Main AR. Physical and chemical characterization of a horse serum carboxylesterase. *Arch Biochem Biophys* 267:271–279, 1988.
33. Tang L, Ugarova TP, Plow EF, Eaton JW. Molecular determinants of acute inflammatory responses to biomaterials. *J Clin Invest* 97:1329–1334, 1996.
34. Tang L, Eaton JW. Fibrin(ogen) mediates acute inflammatory responses to biomaterials. *J Exp Med* 178:2147–2156, 1993.
35. Sharma L, Melis E, Hickey MJ, Clyne CD, Erlich J, Khachigian LM, Davenport P, Morand E, Carmeliet P, Tipping PG. The cytoplasmic domain of tissue factor contributes to leukocyte recruitment and death in endotoxemia. *Am J Pathol* 165:331–340, 2004.
36. Cazzola M, Ciapri C, Page CP, Matera MG. Targeting systemic inflammation: novel therapies for the treatment of chronic obstructive pulmonary disease. *Expert Opin Ther Targets* 11:1273–1286, 2007.
37. Ardizzone S, Bianchi PG. Biologic therapy for inflammatory bowel disease. *Drugs* 65:2253–2286, 2005.
38. Adams A, Lehman TJ. Update on the pathogenesis and treatment of systemic onset juvenile rheumatoid arthritis. *Curr Opin Rheumatol* 17:612–616, 2005.
39. Morgan BJ. Vascular consequences of intermittent hypoxia. *Adv Exp Med Biol* 618:69–84, 2007.
40. Simo JM, Joven J, Vilella E, Ribas M, Pujana MA, Sundaram IM, Hammel JP, Hoover-Plow JL. Impact of apolipoprotein(a) isoform size heterogeneity on the lysine binding function of lipoprotein(a) in early onset coronary artery disease. *Thromb Haemost* 85:412–417, 2001.
41. Linden T, Taddei-Peters W, Wilhelmssen L, Herlitz J, Karlsson T, Ullstrom C, Wiklund O. Serum lipids, lipoprotein(a) and apo(a) isoforms in patients with established coronary artery disease and their relation to disease and prognosis after coronary by-pass surgery. *Atherosclerosis* 137:175–186, 1998.
42. Mooser V, Marcovina SM, White AL, Hobbs HH. Kringle-containing fragments of apolipoprotein(a) circulate in human plasma and are excreted into the urine. *J Clin Invest* 98:2414–2424, 1996.
43. Fortunato JE, Bassiouny HS, Song RH, Kocharian H, Glagov S, Edelstein C, Scanu AM. Apolipoprotein (a) fragments in relation to human carotid plaque instability. *J Vasc Surg* 32:555–563, 2000.
44. Edelstein C, Italia JA, Klezovitch O, Scanu AM. Functional and metabolic differences between elastase-generated fragments of human lipoprotein[a] and apolipoprotein[a]. *J Lipid Res* 37:1786–1801, 1996.
45. Lamanuzzi LB, Mtairag EM, Pepe G, Angles-Cano E. Neutrophils stimulated by apolipoprotein(a) generate fragments that are stronger inhibitors of plasmin formation than apo(a). *Thromb Haemost* 92:1066–1075, 2004.
46. McCormick SPA, No JK, Taylor S, Flynn LM, Hammer RE, Young SG. Mutagenesis of the human apolipoprotein B gene in a yeast artificial chromosome reveals the site of attachment for apolipoprotein(a). *Proc Natl Acad Sci U S A* 92:10147–10151, 1995.
47. Flesch IE, Barsig J, Kaufmann SH. Differential chemokine response of murine macrophages stimulated with cytokines and infected with *Listeria monocytogenes*. *Int Immunol* 10:757–765, 1998.
48. Garcia-Ramallo E, Marques T, Prats N, Beleta J, Kunkel SL, Godessart N. Resident cell chemokine expression serves as the major mechanism for leukocyte recruitment during local inflammation. *J Immunol* 169:6467–6473, 2002.
49. Bogen S, Pak J, Garifallou M, Deng X, Muller WA. Monoclonal antibody to murine PECAM-1 (CD31) blocks acute inflammation in vivo. *J Exp Med* 179:1059–1064, 1994.
50. Tang L, Jiang W, Welty SE. The participation of P- and E-selectins on biomaterial-mediated tissue responses. *J Biomed Mater Res* 62:471–477, 2002.
51. Segal BH, Kuhns DB, Ding L, Gallin JJ, Holland SM. Thioglycollate peritonitis in mice lacking C5, 5-lipoxygenase, or p47(phox): complement, leukotrienes, and reactive oxidants in acute inflammation. *J Leukoc Biol* 71:410–416, 2002.
52. Tang L. Mechanisms of fibrinogen domains: biomaterial interactions. *J Biomater Sci Polym Ed* 9:1257–1266, 1998.
53. Brodsky JA, Brody FJ, Endlich B, Armstrong DA, Ponsky JL, Hamilton IA. MCP-1 is highly expressed in peritoneum following midline laparotomy with peritoneal abrasion in a murine model. *Surg Endosc* 16:1079–1082, 2002.
54. Remick DG, Green LB, Newcombe DE, Garg SJ, Bolgos GL, Call DR. CXC chemokine redundancy ensures local neutrophil recruitment during acute inflammation. *Am J Pathol* 159:1149–1157, 2001.