Alpha-2-Macroglobulin in Inflammation, Immunity and Infections

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INTRODUCTION - THE MOLECULAR BASIS OF AN UNUSUAL PROTEASE INHIBITOR

Alpha-2-macroglobulin (A2M) is a member of MEROPS clan I39 which has seven members in humans and two in mice. All of these proteins can interact with a broad range of endopeptidases. A2M in particular is considered an inhibitor of active endopeptidases of all catalytic types (1). This broad inhibition range stems from its unique mechanism of action. Whereas most protease inhibitors directly interfere with the protease active site, the inhibitory mechanism of A2M works through the formation of a tetrameric cage around active proteases, thereby physically obstructing the interaction between proteases and substrates. This mechanism is sometimes referred to as the protease ‘snap-trap’ or ‘venus-flytrap’ mechanism (2). As a consequence, proteases ‘trapped’ by A2M are prevented from cleaving large substrate molecules (e.g. collagen), while the digestion of small peptides (sneaking into the A2M cage) remains intact (3). The ability of A2M to selectively capture only active proteases, relies on the presence of a ‘bait region’, which is a stretch of amino acids functioning as an exceptionally good substrate for endopeptidases of all catalytic types (4–6). Upon proteolytic cleavage of the bait region by a protease, A2M becomes ‘activated’ (A2M*) and undergoes a conformational change, thereby ‘trapping’ active proteases within its tetrameric cage.
cage (>720 kDa) (Figure 1). In addition to sterically capturing proteases, A2M* also exposes a reactive thioester which interacts with small primary amines in the protease to form covalent A2M/protease complexes (protease-A2M*) (7, 8). In vitro, small nucleophiles such as methylamine (MA) and other low-molecular-weight primary amines are used to cause a conformational rearrangement of the tetramer that is likely similar to the peptidase induced form (9). We will abbreviate this form as A2M**.

Another essential feature of A2M relies on the presence of the receptor binding domains (RBDs, Figure 1) that allow A2M to bind specific cell surface receptors. In native A2M, the RBDs are buried within the protein. However, during the conformational rearrangements associated with the transition into A2M*, the RBDs are exposed onto the surface of the protease-A2M* complex. Consequently, this ‘flags’ protease-A2M* complexes for uptake by cells through low density lipoprotein receptor–related protein-1 (LRP-1) (10). In human blood, binding of protease-A2M* complexes to LRP-1 is thought to result in rapid clearance by the liver and provides another level of protease regulation by A2M (11).

In addition, this domain is also thought to bind cell surface glucose-regulated protein (GRP)78, also called binding immunoglobulin protein (BiP) or heat shock 70 kDa protein 5 (HSPA5) (12, 13), thereby triggering several cell signaling pathways (see chapter on the endocytic and signaling receptors).

Since the first discoveries of A2M as a modulator of proteolysis, broader functions for A2M have been proposed, including the enhancement of immune and cancer cell migration and proliferation (13, 14), the promotion of antigen uptake, processing and presentation by antigen presenting cells (15, 16), the ability of A2M to function as a carrier molecule for cytokines and growth factors (17, 18) and the removal of damaged extracellular proteins (19). In this review manuscript, we place A2M in the context of the immune system and defense against invading microorganisms. We discuss the significance of A2M in neutrophil, monocyte/macrophage and lymphocyte biology, and its relevance in inflammatory diseases and infections.

### A2M AND THE COMPLEMENT SYSTEM

The complement system is a key part of the immune system that comprises an evolutionarily ancient component of the host defense. Central to this system is the cleavage of complement factors (C3 and C4), followed by conformational changes and the exposure of an internal thioester which binds hydroxyl or amino groups of neighboring glycoproteins proteins (Figure 2A). This process marks malignant cells for removal (opsonization) or generates new protein complexes dedicated to further activate the complement cascade (21). Several striking parallels between A2M and the complement system exist. Similar to C3 and C4, A2M undergoes proteolysis, a conformational change and exposes an internal thioester which binds and traps active protease (22). Hence, together with C3 and C4, A2M is classified as a member of the thioester-containing proteins (TEPs). Similarly, the A2M family of proteins (A2Ms, MEROPS clan I39) can be separated into two classes; the protease inhibitors and the complement factors (23). In addition, also a membrane associated member of the A2M family (CD109) exists (Figure 2B) (24). Interestingly, it is thought that C3 evolved from a gene duplication of A2M (20) and that all A2Ms evolved from a core structure of eight homologous domains (24). Indeed, comparison of the molecular models of A2M and C3 illustrates considerable similarities between both proteins (Figure 2C). For a comprehensive overview of the evolutionary origins of A2M and their structure and function the reader is referred to a manuscript by Garcia-Ferrer et al. (25).

In Limulus polyphemus (the Atlantic horseshoe crab), A2M acts as a protease inhibitor and, once activated, as an inhibitor of the haemolytic mechanism in Limulus haemolymph. A2M binds...
to erythrocyte surfaces and it was speculated whether it could act as an opsonin (22, 26–28). Therefore, it is tempting to wonder if A2M - being an ancestral thioester-containing protein - might have acted as the initial "opsonic" system, activated by proteases derived from invading organisms (e.g. virulence factors) and subsequently binding to the protease-producing organism (1, 29, 30). A main difference, however, between A2M and complement components is that the latter are secreted as monomeric proteins whereas human A2M forms a tetrameric structure, permitting physical entrapment of active proteases (29).

Several direct interactions between human A2M and proteins of the complement system have also been discovered. In human serum, a complex was found between mannose-binding protein (MBP), MBP-associated serine protease (MASP, a protease with complement activation activity in the lectin pathway) and A2M, suggesting a regulatory role for A2M in the lectin pathway of the complement cascade (31). Furthermore, direct inhibitory activity of A2M on MASPs was shown and others suggested that A2M is mainly an inhibitor of the ancient type protease MASP-1, whereas inhibition of MASP-2 is less efficient (32). An interaction between A2M and MASP-1 was also confirmed by Paréj et al., however, in this study A2M could not abolish lectin pathway activation (33). Also, a direct interaction between MBL and A2M was suggested (34, 35). When screening for human and mouse serum proteins with the capacity to bind to mannann-binding lectin (MBL), A2M was identified. This interaction occurred through the direct binding of MBL carbohydrate recognition domains to oligomannose glycans Man$_{5-7}$ present at Asn846 on A2M. This binding site remains accessible both on A2M and A2M*, and binding of MBL to A2M hardly interferes with the ability of MBL to bind mannann-coated surfaces. Interestingly, in the same study C3 and C4 were also identified as MBL-binders. Finally, it was suggested that an ancestral (glycosylated) A2M-like TEP might have generated "arrays" of oligomannose glycans on the surface of microorganisms through inhibition of cell surface proteases. Subsequently, MBL or other lectins could bind to the oligomannose layer, leading to opsonization and activation of the complement system (35). In a recent study, A2M was also identified as the possible antigen causing hexamerization/aggregation of IgG as seen in patients with chronic lymphocytic leukemia and chronic activation of the complement classical pathway. A2M was found to be part of the IgG hexamer complex and present at the cell surface of malignant B lymphocytes through binding with GRP78 (36). The mechanism through which such interaction would occur is not yet fully understood.

![FIGURE 2](image-url) The complement system and A2M. (A) Overview of the classical, lectin and alternative complement pathways. MAC, membrane attack complex; MASP, mannose-binding protein-associated serine protease; MBL, mannose-binding lectin. (B) Overview of the domain organization of a selection of human proteins belonging to the A2M family (MEROPS protease inhibitor family I39). A2ML1, A2M-like-1; ANA, anaphylatoxin domain; BRD, bait-region domain; C345C, C-terminal extension of C3-5; CUB, complement C1r/C1s, Uegf, Bmp1; GPI, glycosyl-phosphatidyl-inositol linker; MG, macroglobulin-like domains; TED, thioester domain. (C) comparison of the crystal structures of the A2M monomer (PDB structure: 4ACQ) (2) and C3 (PDB structure: 2A73) (23). Domain colors are based on the colors used in Panel (B) For A2M, part of the bait region and the MG8/RBD are not shown.
remains to be determined. In conclusion, several separate studies have provided clues for some involvement of A2M in activation of the complement system. However, the overall relevance of these findings in a biological context or the contribution to pathological processes largely remains to be explored.

**BINDING TO CYTOKINES AND GROWTH FACTORS**

As early as the 1970s, researchers discovered that macrophage activation factors and nerve growth factors were bound to A2M in human and mouse serum (37, 38). Next, many more cytokines and growth factors joined the list of A2M-binding proteins (see Table 1). Biochemical interaction studies revealed that most cytokines and growth factors preferentially bind protease-activated A2M* or chemically activated A2M**. For example, transforming growth factor (TGF)-β1 (K_D = 80 ± 11 nM), nerve growth factor (NGF)-β (K_D = 0.11 ± 0.01 μM), fibroblast growth factor (FGF)-2 (K_D = 0.59 ± 0.04 μM) and tumor necrosis factor (TNF)-α (K_D > 0.75 ± 0.10 μM) have increased binding affinity for A2M** and reach their binding equilibrium within 15 minutes. As an exception, TGF-β2 binds both forms equally well (K_D = 12 nM) (40, 41, 55, 64).

Based on competition experiments it was suggested that several binding sites for cytokines and growth factors exist and that these also depend on the A2M conformation. For example, FGF competes with TGF-β, but not platelet-derived growth factor (PDGF), for binding to A2M (41, 55). All PDGF-isoforms (AA, BB & AB) compete for binding to A2M and A2M**. However, they do not compete for native A2M with TGF-β1, TGF-β2, TNF-α, FGF2, interleukin (IL)-1β and IL-6, whereas for A2M** PDGF competes with TGF-β1 and FGF2 (50). Similarly, it was shown that vascular endothelial growth factor (VEGF) does not compete with TGF-β1 or PDGF (62). TGF-β1 competes for the binding of FGF-2 to A2M and A2M** (39). In addition, A2M only binds certain members of the FGF-family, including FGF-1, -2, -4 and -6, but not FGF-5, -7, -9 or -10 (39).

The nature of the interaction between A2M and cytokines/growth factors can be both non-covalent and covalent. Most often, initial complexes between A2M** and cytokines/growth factors are non-covalent and reversible, and are slowly converted into covalent interactions (40) (Table 1). Generally three mechanisms for A2M/cytokine/growth factor binding have been proposed: (i) non-covalent interaction through trapping in the A2M molecular cage, (ii) covalent interaction through thiol-disulphide exchange with a free thiol-group exposed upon A2M activation, (iii) covalent binding via the active thioester group, exposed upon protease activation of A2M (28). Interestingly, for some growth factors, an A2M-binding region was identified. A 20 kDa protein, comprising the A2M bait region and neighbouring sequences (amino acids 614-797), could interact with TGF-β1, TGF-β2, PDGF-BB and NFG-β (56). This sequence could also neutralize TGF-β1 and TGF-β2 activity in endothelial cell proliferation assays (fetal bovine heart cells) (56) and the binding of PDGF-BB to PDGF receptors on fibroblasts (NIH 3T3 cells) (49). Subsequently the growth factor binding site for TGF-β1 and PDGF-BB was narrowed down to a 16-amino acid peptide (WDLVVVNSAGVAEVGV), containing a high proportion of hydrophobic amino acids (51) and within this sequence one glutamic acid residue was shown to be crucial for binding to PDGF-BB (and not TGF-β1) (52).

For many cytokines/growth factors, the functional implications of binding to A2M remain unknown (18). One possibility is that A2M serves as a reservoir for cytokines and growth factors, and increases their half-life (18). For IL-8/A2M** complexes it was shown that IL-8 retained its potential to induce neutrophil chemotaxis and complexation rendered IL-8 less sensitive to proteolysis by neutrophil elastase (NE) (48). However, when analyzing the plasma clearance of TGF-β1, TGF-β1/A2M* and TGF-β1/A2M** complexes administered to mice, the complexes were efficiently cleared from the circulation through liver uptake (t1/2 of 4 min), whereas free TGF-β1 was also found in the lungs (58). This difference in clearance could be explained by receptor-mediated uptake of TGF-β1/A2M* complexes through interaction of the RBDs with LRP-1 (58). However, other researchers showed that blocking of the clearance receptor by excess administration of A2M** did not affect the half-life of TGF-β1 and that endogenous TGF-β1 is mainly bound to native A2M (65). Similarly, TNF-α/plasmin-A2M** complexes injected into mice were efficiently cleared and this clearance could be stopped by blocking the A2M-receptor (61). The same was true for PDGF-BB/A2M** complexes (54). These findings indeed suggest a role for A2M in cytokine regulation and the net effect might be context-dependent. For example, under physiological conditions, cytokines and growth factors might bind native A2M and remain in circulation. In contrast, under conditions with high protease activity, proteolysis of A2M and conversion into A2M*, results in rapid clearance of A2M*/cytokine/growth factor complexes. Unfortunately, in vivo evidence to support this hypothesis is limited.

Another possible impact of A2M complex formation is the modulation of growth factor/cytokine-receptor interactions and their associated functions. It was reported that TGF-β and A2M** work synergistically to promote proliferation of cultured smooth muscle cells (SMCs) (60). However, in another study, A2M reduced the ability of TGF-β2 to inhibit lung cell proliferation (CCL-63 mink lung cell line), whereas the activity of TGF-β1 remained unaltered (64). In case of TNF-α, the presence of A2M** or plasmin-A2M* did not affect its cytotoxic effects on cultured fibroblasts (mouse L929 cells) (61). FGF-2 incubation with A2M** reduced FGF-2–induced endothelial cell proliferation. In contrast, interaction with A2M** did not affect vascular tubule formation on Matrigel basement membrane matrix or collagen, likely due to exchange of FGF-2 with extracellular matrix components (39). Hence, the inhibitory effect of A2M** on FGF-2 function might be limited to the fluid phase and may serve to restrict FGF activity to sites of angiogenesis, inflammation, and tissue repair.
### TABLE 1 | A2M interaction with cytokines and growth factors.

| Binding partner | Proposed interaction | Biological significance and impact on cytokine/growth factor activity | ref. |
|-----------------|----------------------|---------------------------------------------------------------------|------|
| **FGF1**        | binds to A2M and increasingly to A2M** | – | (39) |
| **FGF2**        | non-covalent contact with A2M**, slowly converts into a covalent interaction | • complex forms in human plasma  
• reduced binding to FGF-receptors (BHK-21 cells)  
• reduced ability to stimulate plasminogen activator production (bovine epithelial cells)  
• A2M** inhibits FGF-2-dependent fetal bovine heart endothelial cell proliferation  
• does not affect FGF-2-induced vascular tubule formation on Matrigel or collagen matrix | (39–41) |
| **FGF-4**       | binds A2M, increased binding to A2M** | | (39) |
| **FGF-6**       | binds A2M, increased binding to A2M** | | (39) |
| IFN-γ           | covalent binding to A2M** and protease-A2M* | • no impact on antiproliferative activity (bladder tumor cell line)  
• no influence on induction of MHC class II | (42) |
| **IL-1β**       | • covalent binding to A2M** and trypsin-A2M*  
• increased binding to MAC | • complex found in human plasma  
• IL-1β/A2M retains IL-1-like activity (mouse thymocytes) | (43–45) |
| **IL-2**        | binds oxidized A2M | | (48) |
| **IL-4**        | binds A2M and preferentially A2M** | | (44) |
| **IL-6**        | • binds A2M** and MAC  
• better binding to oxidized A2M | • complex found in human plasma  
• IL-6 receptor binding intact  
• Stimulation of IL-6-dependent hybridoma cells remains intact  
• protects IL-6 from proteolysis | (44, 46, 47) |
| **IL-8**        | non-covalent binding to A2M** | • IL-8/A2M isolated from lungs of ARDS patients  
• no effect on neutrophil chemotaxis  
• protects IL-8 from proteolysis | (48) |
| **IL-18**       | binds mostly to MAC | | (44) |
| **NGF-β**       | • non-covalent with A2M**, slowly converts into a covalent interaction  
• binds less to oxidized A2M  
• binds A2M between AA 614-797 | | (40, 46, 49) |
| **PDGF**        | • non-covalent binding to A2M**, slowly converts into a covalent interaction  
• binds less to oxidized A2M  
• 2 x PDGF per A2M  
• all isoforms bind native A2M and A2M**  
• binds to the growth factor binding site | • complex found in human plasma  
• retains mitogenic activity  
• not detected by anti-PDGF antisera  
• blocks receptor binding  
• clearance of PDGF-BB/A2M** from mouse plasma through uptake via LRP-1  
• fusion protein containing the A2M binding site blocks binding to PDGF-β receptor (NIH 3T3 cells) | (17, 40, 49-54) |
| **TGF-β1**      | • non-covalent contact with A2M**, slowly converts into a covalent interaction  
• binds less to oxidized A2M  
• binds to the growth factor binding site | • complex found in human plasma  
• TGF-β1/A2M is the 'latent' TGF-β form in plasma  
• A2M impedes binding to cell surface receptors  
• Fast clearance of TGF-β1/A2M* and TGF-β1/A2M** complexes from mouse plasma through liver uptake  
• TGF-β and A2M** synergistically promote SMC proliferation (cultured rat aorta cells)  
• protein with A2M binding site neutralizes TGF-β1 activity (endothelial cell proliferation assays) | (38, 51, 55-60) |
| **TGF-β2**      | • non-covalent contact with A2M**, slowly converts into a covalent interaction  
• binds equally well A2M and A2M**  
• binds less to oxidized A2M  
• binds A2M between AA 614-797 | • A2M inhibits binding to cell surface receptors  
• protein containing the A2M binding site neutralizes TGF-β2 activity in endothelial cell proliferation assays (fetal bovine heart cells) | (40, 56) |
| **TNF-α**       | • non-covalent contact with A2M**, slowly converts into a covalent interaction  
• binds to plasmin-A2M* and less to native A2M, trypsin-A2M* or thrombin-A2M*  
• increased binding to MAC and oxidized A2M | • TNF-α binding to MAC suppresses inflammation by inhibition of MAPK p38 phosphorylation  
• TNF-α retains cytotoxic effects on fibroblasts (L929 murine fibroblasts)  
• no effect on antiproliferative activity (bladder tumor cell line) | (40, 42, 44, 46, 61) |
| **VEGF**        | • covalent binding  
• does not bind at growth factor binding site  
• binds the interior of A2M** and the exterior of native and protease-activated A2M* | • reduced binding to VEGF receptor  
• VEGF/A2M** complexes are internalized and degraded by macrophages (LRP-1-mediated)  
• A2M does not impact VEGF-induced cell proliferation or Ca²⁺ increases (HUVECs) | (62, 63) |

A2M*, protease-activated A2M; A2M**, A2M activated through reaction with low molecular-weight primary amines; AA, amino acid; ARDS, adult respiratory distress syndrome; FGF, fibroblast growth factor; HUVECs, Human umbilical vein endothelial cells; IFN, interferon; IL, interleukin; MAC, A2M activated for cytokine binding; NGF, nerve growth factor; PDGF, platelet-derived growth factor; SMC, smooth muscle cell; TGF, transforming growth factor; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.
The preference of some cytokines/growth factors to bind A2M*/A2M** has been exploited by efforts to generate stable conformational intermediates of A2M*/A2M** with optimized cytokine and growth factor binding capacities (66). Human A2M, treated with a cross-linking reagent to lock the A2M conformation and subsequently with methylene blue, bound TGF-β1 and TGF-β2 with higher affinity than other A2M forms. This form successfully inhibited TGF-β1-mediated inhibition of endothelial cell proliferation (66). Similarly, Webb et al. generated a modified form of A2M named ‘macroglobulin activated for cytokine binding’ or MAC. MAC had increased binding affinity with pro-inflammatory cytokines TNF-α and IL-1β (67). Administration of MAC (intraperitoneal) prior to lipopolysaccharide (LPS)-challenge in mice (intravenous injection), increased the survival rate (67). In a model for peripheral nerve injury, MAC could also suppress inflammation. Hence, it has been suggested that MAC has an anti-inflammatory function through binding inflammatory cytokines (44). Interestingly, in a different study it was shown that oxidation of A2M or A2M** by hypochlorite (an oxidizing agent secreted by neutrophils), increases the affinity of A2M and A2M** for TNF-α, IL-2, and IL-6, but decreases the affinity for β-NGF, PDGF-BB, TGF-β1, and TGF-β2. This implicates that oxidative modification of A2M during inflammation might alter the repertoire of A2M-binding cytokines and growth factors (46). Interestingly, mice deficient in A2M have significantly lower levels of plasma TNF-α and develop a short-term attenuated fever in response to LPS administration. Therefore, it has been suggested that the A2M chemokine-binding capacities might be important for the development of fever (68).

THE ENDOCYTIC AND SIGNALING RECEPTORS

LRP-1/the A2M Receptor/CD91

LRP-1 (also called the A2M receptor or CD91) is a large, multifunctional receptor, composed out of a non-covalently bound 515-kDa extracellular domain (also called the ‘heavy domain’ or α-chain) and an 85 kDa transmembrane domain (also called the ‘light chain’ or β-chain) (Figure 3). These two domains are formed from a 600 kDa precursor protein after cleavage by furin (69). After its biosynthesis, LRP-1 interacts with a 39 kDa receptor-associated protein (RAP) which can also be found associated with LRP-1 at the cell surface (70). RAP efficiently blocks LRP-1 function and is therefore an endogenous regulator of LRP-1 activity (71). The extracellular LRP-1 α-chain holds four regions with cysteine-rich complement-type repeats (CR) (72), also referred to as clusters I-IV or ligand binding domains, and which are the main interaction interface for extracellular ligands. Clusters II and IV were identified as the most widely used interaction sites for LRP-1 ligands (73).

The best known function of LRP-1 is its role as a scavenging receptor for many proteins including protease, protease inhibitors, complement proteins and even toxins and viruses (74). After ligand binding, LRP-1 undergoes efficient endocytosis via clathrin-coated pits and subsequent recycling. This process is mainly mediated through the YxxL motif and dileucine repeats, present in the intracellular part of the LRP-1 β-chain (75) (Figure 3). Activated A2M* undergoes efficient cellular uptake through interaction with LRP-1. For example, in mouse fibroblasts (Balb 3T3 cells), cellular uptake and presence of A2M in cytoplasmic vesicles occurs within 5 minutes, and after 15-30 minutes the content of these vesicles is found in the lysosomes (76). Upon proteolysis of the A2M bait region by active proteases or chemical activation, the A2M receptor binding domains (RBD, Figure 1) are exposed, which form the interface between LRP-1 and A2M*/A2M** (77).
evaluating variants of the receptor binding domain, two lysine residues (Lys1393 and Lys1397) in the A2M receptor binding domain were found to be crucial for receptor binding (78–80). Moestrup (81) et al. found that trypsin-A2M* can bind with low (kd = 2 nM) and with high (kd = 40 pM) affinity to LRP-1 and proposed that binding efficiency depends on the availability of the receptor (receptor density). For example, at low receptor density only one subunit of the A2M tetramer will interact with one LRP-1, whereas at high receptor density two or more LRP-1 receptors will bind per A2M tetramer (81). Electron microscopy visualization showed that up to three LRP-1 receptors could bind to chymotrypsin-A2M* (82). In addition, cooperative binding to several LRP-1 clusters has been reported, for example, A2M*- trypsin complexes cooperatively bind to cluster I and cluster II (83).

The LRP-1 β-chain also holds several signature sequences that connect LRP-1 with adaptor proteins involved in cell signaling and protein trafficking (84). Two NpxY domains are located in the C-terminal part of LRP-1 (Figure 3) and these provide binding sites for several adaptor and scaffolding proteins containing phosphotyrosine-binding (PTB) domains [e.g. disabled homolog 1 (DAB1), C-jun-amino-terminal kinase-interacting protein 1 & 2 (JIP1-2)] (84) or proteins containing Src homology 2 (Sh2) domains [e.g. Sh2-containing protein tyrosine phosphatase 2 (SHP2)] (85). In addition, several adaptor proteins containing PDZ (post synaptic density protein containing phosphotyrosine-binding (PTB) domains [e.g. PSD95), drosophila disc large tumor suppressor and zonula occludens-1 protein) domains were found to bind the LRP-1 intracellular tail [e.g. PSD-95, protein carboxy-terminal PDZ ligand of nNOS (CAPON)] (84). Hence, LRP-1 has the potential to activate different signaling pathways, driving processes such as cytoskeletal reorganization, cell proliferation, apoptosis and cell adhesion (84). Interestingly, it appears that phosphorylation of the distal NpxY site is mostly involved in the interaction with signaling proteins, whereas the proximal phosphorylation site is more involved in the process of receptor recycling, suggesting non-redundant properties (73, 84, 85).

Whereas the multifunctional potential of LRP-1 is clear, knowledge on how each of these pathways in triggered by different LRP-1 ligands and how LRP-1 selects among these pathways remains incomplete. One possible way is through the interaction with a co-receptor (86). For example, LRPI functions as a single system with the N-methyl-D-aspartate (NMDA) receptor and tyrosine receptor kinase (trk) receptor, to activate cell signaling [extracellular signal–regulated protein kinase (ERK)-1/2] in response to tissue plasminogen activator (tPA) and A2M**. In contrast, myelin-associated glycoprotein affects LRP-1 differently and results in recruitment of p75 neurotrophin receptor (p75NTR) into a complex with LRP-1 and activated Ras homolog family member A (RhoA) (86).

LRP-1 has generally low tissue specificity and is expressed ubiquitously, including in hepatocytes, neurons, astrocytes, epithelial cells of the gastrointestinal tract, SMCs, fibroblasts, Leydig cells in testis, granulosa cells in ovary, and dendritic interstitial cells of the kidneys (87). In addition, monocytes and macrophages express more LRP-1 (88). One method of LRP-1 regulation is proteolytic shedding of its ectodomain from the cell surface. Membrane-associated proteases including membrane-type 1 matrix metalloproteinase (MT1-MMP) and a disintegrin and metalloprotease 17 (ADAM17) are capable of shedding LRP-1 in chondrocytes (89) and the prevalence of soluble LRP-1 (sLRP-1) was shown to correlate with pro-inflammatory conditions (90). Following LPS injection, increases in sLRP can be found in mouse plasma. In conditions of chronic inflammation, increased levels of sLRP-1 have also been found (90). Interestingly, stimulation of macrophages with purified sLRP results in a pro-inflammatory effect and activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), c-Jun N-terminal kinases (JNK) and p38 mitogen-activated protein kinases (p38 MAPK) (90). In contrast, other studies found anti-inflammatory functions for sLRP-1. For example, sLRP-1 could reduce the pro-inflammatory effects of TNF-α (91).

**GRP78/Bip/HSP70**

LRP-1 was identified as the first receptor for activated A2M, but the evidence for a second receptor was published in 1994 by Misra (12) et al. In this study, the LRP-1 inhibitor RAP could not block A2M**-mediated increases in intracellular calcium andinositol 1,4,6-triphosphate in macrophages, which led the authors to suggest the existence of a second receptor (12). The newly identified receptor functioned through a pertussis toxin-sensitive G-protein and contrasts with LRP-1 mediated signaling, which occurs through a pertussis toxin-sensitive G protein (12, 92). From the membrane fraction of mouse macrophages and 1-LN prostate cancer cells, GRP78 (78 kDa) was identified as the second A2M-receptor (13). This interaction was confirmed when purified GRP78 was found to bind A2M** with high affinity (Kd ~ 150 pM) (13). In addition, LRP-1 was co-purified during this study, suggesting a co-receptor relationship between GRP78 and LRP-1 (13) (Figure 3).

Surprisingly, GRP78 is mainly known as an intracellular protein and member of the heat shock protein 70 (HSP70) family involved in correct translocation and folding of newly synthesized polypeptides across the endoplasmic reticulum membrane (93). Glucose-regulated proteins are induced under conditions of cellular stress, such as glucose starvation or agents affecting calcium stores or glycosylation patterns (94). Consequently, GRPs are typically increased in conditions involving tissue starvation and stress such as ischemia, vascular dysfunction, inflammation, apoptosis and necrosis. Under healthy conditions they are thought to protect against cell death, whereas the anti-apoptotic functions might also support the survival of neoplastic cells and their resistance to treatments (94–96). Based on co-immunoprecipitation in macrophage membrane fractions, it was shown that DnaJ homolog subfamily C member 1 (DnaJc1, also called MTJ-1) interacts with GRP78 at the cell membrane and enables cell-surface localization of GRP78 (97). DnaJc1 belongs to the family of J domain proteins (JDPs) which bind and activate Hsp70 proteins through their J domain (98). The interaction of GRP78 with DnaJc1 might thus explain the association of GRP78 with cell membranes.
Functionally, it was shown that A2M** signaling through GRP78 triggers pro-proliferative and anti-apoptotic behavior in macrophages and cancer cells (99). In prostate cancer cells, binding of A2M** to GRP78 causes an increase in prostate-specific antigen (PSA). PSA is secreted as an active serine protease which again binds and activates more A2M, resulting in a positive feedback loop where PSA-A2M* complexes bind GRP78, activate mitogen-activated protein kinase (MEK)-1/2, ERK1/2, S6 kinases S6, and Akt resulting in the promotion of DNA and protein synthesis and increased cell proliferation (100). A detailed overview of A2M*/A2M** signaling through GRP78 on macrophages is provided in the next chapter.

### EFFECTS OF A2M ON LEUKOCYTES

In this section we will discuss the effects of A2M on leukocytes, specifically neutrophils, monocytes, macrophages, and lymphocytes. Generally, the roles of A2M in leukocyte biology relate to all previously mentioned aspects of A2M function including inhibition of protease activity, binding of immunologically important molecules (e.g. cytokines/growth factors) and binding of cell-surface receptors for clearance or to trigger diverse pathways relevant for cell function.

#### Neutrophils

A2M inhibits the activity of the main proteases released from stimulated human neutrophils (101), specifically NE (101–104), proteinase 3 (P3) (105), cathepsin G (catG) (101) and matrix metalloproteinase-9 (MMP-9) (106, 107). However, inhibition of total neutrophil proteolysis - in *in vitro* assays with isolated neutrophils or their full degranulates - is often incomplete (102, 103). For example, only 73.5% of fibronectin proteolysis by neutrophils can be inhibited by A2M (102). Several explanations for this phenomenon have been proposed. First, proteases trapped by A2M remain functionally active against substrates that can access the interior of the A2M molecular cage (104). For example, in bronchoalveolar lavage fluid from patients with adult respiratory distress syndrome, NE activity against a low molecular weight substrate could be measured despite a 30-fold excess of the anti-proteases alpha-1-antitrypsin and A2M. Whereas most NE was complexed to these inhibitors, NE activity against small substrates could be associated with NE-A2M complexes (104). Second, as part of the antimicrobial host defense, stimulated neutrophils produce and release a range of reactive species (ROS) modifying biological components. Therefore, it was suggested that neutrophils inactivate A2M by the release of reactive species which again binds and activates more A2M, resulting in a positive feedback loop where PSA-A2M* complexes bind GRP78, activate mitogen-activated protein kinase (MEK)-1/2, ERK1/2, S6 kinases S6, and Akt resulting in the promotion of DNA and protein synthesis and increased cell proliferation (100). For example, in bronchoalveolar lavage fluid from patients with adult respiratory distress syndrome, NE activity against a low molecular weight substrate could be measured despite a 30-fold excess of the anti-proteases alpha-1-antitrypsin and A2M.

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### Figure 4

A2M and neutrophil function. A2M* aids neutrophils through stimulation of their capacity to bind to endothelial cells (1), to migrate (2) and to phagocytose and kill pathogens (3). For example, the chemotactic potential of neutrophils is increased in the presence of A2M* by reducing LPS-induced G-protein-coupled receptor kinase 2 (GPRK2) expression, which subsequently results in increased C-X-C motif chemokine receptor 2 (CXCR2) expression and increased neutrophil migration towards IL-8 (2). A2M* also induces CD11b in neutrophils challenged with LPS, thereby increasing the interaction with ICAM-1 and aiding neutrophil adhesion to the vascular endothelium (1). A2M* augments phagocytosis and bacterial killing by neutrophils, possibly through interaction with LRP-1 (4). Finally, A2M can inhibit all major proteases secreted by neutrophils and reduce their proteolytic activity against large substrates (5). However, in the presence of reactive oxygen species (ROS), such as hypochlorite, A2M dissociates thereby switching its activity from protease inhibition to an extracellular chaperone and cytokine carrier (6).

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these complexes undergo efficient receptor-mediated uptake (e.g. by monocyte/macrophage RAW 264.7 cells), thereby avoiding the build-up of toxic protein aggregates (112, 114).

In human neutrophils, LRP-1 is found at the cell surface as well as intracellularly. Upon stimulation with LPS, LRP-1 is mobilized from intracellular stores to the cell surface, which suggests a role for LRP-1 in anti-microbial defense (115). Interestingly, A2M is also a major component of neutrophil-derived microparticles. In a proteomics study of neutrophil-derived microparticles, microparticles of human umbilical vein endothelial cell (HUVEC)-adhering neutrophils were found to be enriched in A2M (116). In the presence of active A2M, LPS-challenged neutrophils increasingly expose the adhesion and migration marker CD11b. This results in increased interaction with ICAM-1, likely increasing neutrophil adhesion to vascular endothelium (Figure 4). Likewise, A2M is thought to improve the capacity of endothelial cells to interact with neutrophils. Pretreatment of TNF-α-stimulated HUVECs with A2M-enriched microparticles or microcapsules also results in an increase in neutrophil adhesion onto the endothelial cell monolayer (115, 117). No difference in the expression of endothelial adhesion molecules was found, but it was suggested that A2M is delivered onto the endothelial cells plasma membranes where it engages LRP-1 on the neutrophils promoting firm leukocyte adhesion (115).

In vitro, A2M enhances the migration of polymorphonuclear cells towards neutrophil-derived eosinophil chemotactic factor (118). Activated A2M quickly (1h stimulation) reduces LPS-induced G-protein-coupled receptor kinase 2 (GRK2) and increases CXCR2 leading to increased migration of LPS-induced HUVECs with A2M-enriched microparticles or microcapsules also in an increase in neutrophil adhesion onto the endothelial cell monolayer (115, 117). No difference in the expression of endothelial adhesion molecules was found, but it was suggested that A2M is delivered onto the endothelial cells plasma membranes where it engages LRP-1 on the neutrophils promoting firm leukocyte adhesion (115).

Finally, A2M has also been implicated in neutrophil phagocytosis and bacterial killing. A2M binds to the surface of Streptococcus pyogenes (groups A, C and G) (120, 121). So far, the identified protein interaction partners are either the N-terminal region protein G (groups C and G) (122) or the protein G-related A2M-binding protein (GRAB) (group A) (123). Whereas earlier studies report that binding of A2M to S. pyogenes enhances its phagocytosis by human neutrophils (124), more recent studies propose that S. pyogenes-bound A2M protects this bacteria against host proteases and is thus a virulence factor in S. pyogenes infections. In a separate study, neutrophils pre-treated with activated A2M or A2M-enriched microparticles, had an increased capacity to phagocytose E. coli and produced increased amounts of ROS and bactericidal cathelicidins, a process which was LRP-1-dependent (115).

**Monocytes and Macrophages**

Macrophages are specialized in sensing and attacking invading pathogens, in modulating the immune response by secretion of inflammatory mediators and finally in contributing to healing of damaged tissues (125). Given that macrophages secrete A2M (126, 127) and also express both A2M receptors (128–130) it is not surprising that protease-A2M complexes are efficiently taken up by macrophages (131) and that several roles for A2M* in macrophage function have been proposed (Figure 5).

Comparable to neutrophils, A2M increases the phagocytic and anti-microbial capacity of macrophages. For example, Trypanosoma cruzi, the parasite causing Chagas’ disease, undergoes more efficient uptake by mouse macrophages when pre-treated with A2M (132). Human monocyte-derived macrophages pre-treated with activated A2M or A2M-enriched microparticles or microcapsules also increase bacterial phagocytosis (E. coli), phagocytosis of zymosan and ROS production (115, 117). This effect can be blocked by anti-LRP-1 antibodies and is thus thought to be mediated through LRP-1 (115). Interestingly, several lines of research support the idea that the activation of anti-bacterial or antiviral pathways in macrophages leads to the suppression of A2M function and its associated pathways. For example, endotoxin stimulation suppresses A2M secretion, and stimulation with LPS or interferon (IFN)-γ results in a reduced mRNA expression of LRP-1 (128, 133). In the macrophage-derived cell line J774, effects triggered by A2M* and occurring though LRP-1, could also be abrogated when cells were first challenged with LPS (134). Even microglia, the resident macrophages of the central nervous system, express LRP-1 and internalize A2M**, a process which is decreased upon stimulation with LPS or IFN-γ (135).

In a series of studies on mouse macrophages, Misra *et al.* performed a detailed analysis of the cell signaling cascades activated by A2M*/A2M** and mediated through GRP78. When stimulated with A2M**, mouse macrophages undergo a rapid increase in intracellular Ca**+ and in signaling molecules such as inositol trisphosphate (IP3), diacyl glycerol (DAG), arachidonic acid (AA), lysophosphatidylcholine (lysoPC) and cyclic adenosine monophosphate (cAMP) (14, 136). In addition, a gradual increase in cytosolic pH occurs (130). Several pathways were identified including phosphorylation of phospholipase Cγ1 (PLCγ1) and an increase in phosphorylation, activity and membrane/nuclear translocation of cytosolic phospholipase A2 (cPLA2) (130, 137, 138). Activation of several mitogen-activated protein kinase (MAPK) cascades was also suggested based on the finding of increased phosphorylation of MEK 1/2, ERK 1/2, p38 MAPK, and JNK (137). Phosphorylation of the transcription factor cAMP response element-binding (CREB) protein was also reported (139). Activation of these pathways may have several important consequences for macrophage biology. For example, increased protein and DNA synthesis was reported, which improved cell division (139, 140). Synthesis and metabolism of platelet activating factor (PAF) was altered, resulting in increased release of this potent inducer of acute inflammation (141). Trypsin-A2M* and A2M** could also induce the secretion of eicosanoids [*e.g.* prostaglandin E2 (PGE2)] from cultured mouse macrophages (142, 143). This occurs through upregulation of total cellular and nuclear cyclooxygenase-2 [COX2, also known as prostaglandin-endoperoxide synthase-2 (PTGS2)], and requires participation of both the p21-ras dependent MAPK pathway.
and PI 3-kinase signaling pathways (144). Finally, given the fact that these pathways were resistant to inhibition by the LRP-1 antagonist RAP, it was suggested that these are triggered mainly through the GRP78 receptor (12, 92, 130). In addition, silencing of LRP-1 did not have an effect on A2M**-induced IP3 synthesis (13). Nevertheless, based on the co-immunoprecipitation of GRP78 with LRP-1 a co-receptor relationship was also suggested (13). In a different study increased cell proliferation by A2M** (in the J774 macrophage-derived cell line) also relied on MAP kinase phosphorylation (Mek1-ERK1/2 pathway), but was dependent on LRP-1 (134). Furthermore, in a follow-up study it was shown that A2M** also induces the expression and secretion of MMP-9 and involved PKC and extracellular calcium influxes (145).

A2M can also influence macrophage morphology and their capacity to migrate. For example, A2M*/A2M** are able to counter IFN-γ-induced morphological changes in mouse peritoneal macrophages (146). Furthermore, in a macrophage-derived cell line (Raw264.7 cells), A2M** increased cell migration in an in vitro wound-migration assays, and induced the formation of enlarged cellular protrusions containing MT1-MMP. This process was dependent on LRP-1 and was mediated through activation of PKC (147). In contrast, in a study by Misra et al., mouse macrophages stimulated with A2M** had increased tyrosine phosphorylation of GRP78, autophosphorylation of p21-activated protein kinase-2 (PAK-2), phosphorylation of LIM kinase and coflin, causing cytoskeletal rearrangements (148). Finally, a role for A2M in promoting antigen presentation by macrophages was suggested. Antigens conjugated to A2M are efficiently taken up by mouse macrophages which results in efficient activation of murine T-cells, which suggests that A2M could aid in antigen presentation (149). For example, hen egg lysozyme (HEL), complexed to elastase-A2M* (ELA-A2M*-HEL), is taken up by mouse macrophages more efficiently than free HEL, a process which relies on receptor-mediated endocytosis. Subsequently, the threshold for antigen presentation is lowered, allowing for more efficient antigen processing and presentation.

FIGURE 5 | A2M and macrophage function. Macrophages produce A2M and express both A2M receptors. Hence, several functions for A2M have been proposed in macrophage biology. A2M promotes bacterial phagocytosis, killing and the production of ROS (1) and this effect might be mediated through interaction with LRP-1. Triggering of LRP-1 and/or GRP78-associated signaling pathways leads to several cellular effects. A first effect is increased cell growth, which is mediated through activation of MAPK pathways, phosphorylation of cAMP response element-binding (CREB) protein and activation of protein kinase C (PKC) (2). A second effect is increased cell migration, which might occur through increased phosphorylation of coflin, activation of actin polymerization and the formation of enlarged cellular protrusions containing MT1-MMP (3). Binding to LRP-1 and/or GRP78 also results in a rapid increase in intracellular calcium which is thought to contribute to the release of mediators of inflammation including platelet activating factor (PAF), prostaglandin E2 (PGE2) and matrix metalloproteinase-9 (MMP-9) (4). Finally, A2M also promotes antigen presentation by macrophages where both MHC-I and MHC-II presentation have been reported (5).
presentation to (and activation of) HEL-specific T-hybridoma cells was 2.2 to 2.7 log units lower than free HEL (15). In rabbits, subcutaneous injection of ELA-A2M*-HEL complexes also resulted in 10 to 500-fold higher IgG titers compared to unconjugated controls (150). Furthermore, this adjuvant-like effect could also be established with microbial antigens. Proteins isolated from Kirsten murine sarcoma virus and subsequently conjugated to A2M (by activation with trypsin), were more efficiently taken up by mouse thioglycolate-induced peritoneal exudate cells compared to unconjugated viral proteins. Subsequent co-culturing with spleen cells resulted in higher amounts of antibodies against viral proteins (151).

Similarly, complexes between cruzipain, a cysteine protease from *Trypanosoma cruzi*, and A2M* were more efficiently taken up by human monocytes and resulted in enhanced (MHC-II-dependent) presentation of cruzipain peptides to CD4+ T cells from patients with Chagas’ disease (152).

A new insight in A2M-mediated antigen presentation was achieved with the identification of LRP-1 as the receptor for the heat shock protein gp96/CD91 (153). Gp96 is known for its immunogenicity through antigen binding, followed by uptake and MHC-I dependent presentation by antigen presenting cells. Given the striking similarities with A2M, the same research group subsequently confirmed that both gp96 and A2M act as T-cell adjuvants that can facilitate the transition of exogenous antigens into the endogenous pathway for antigen presentation (154). This effect was corroborated in a study where an A2M*-delivered antigen enhanced the expansion of a CD8+ T cell population, resulting in a 25-fold greater secretion of IFN-γ and IL-2, and induced cell-mediated cytotoxicity (16). Hence, both MHC-I and MHC-II-dependent processes can be promoted by antigen conjugation to A2M.

**Lymphocytes**

As discussed previously, binding to activated A2M increases the ability of antigens to be presented to T-cells by macrophages and this can happen both in MHC-I and MHC-II context (16, 149–152, 154). Hence, A2M can have an indirect effect on lymphocyte proliferation. In addition, A2M can bind several cytokines, including IL-2 which is a produced by activated T-cells. Due to its small size, the cytokine IL-2 can still be degraded by A2M*-captured trypsin, resulting in the loss of its capacity to stimulate the proliferation of mouse cytotoxic T lymphocytes (CTLL-20 cells) and primary human lymphocytes (155, 156). A2M can capture proteases ranging from approximately 20 to 100 kDa and allows substrates of up to approximately 17 kDa to enter its molecular cage (depending on their overall structure) (4). Hence, the proteolytic activity of A2M-bound proteases remains highly relevant in the context of immune-modulation by small soluble cytokines. Given the important role for cytokines such IL-2 in lymphocyte function, one might wonder what the net contribution of A2M is taking the potential proteolysis and degradation of cytokines into account. Furthermore, A2M might switch proteolysis to smaller substrates while inhibition of large or cell surface-bound substrates remains intact. To that regard, Petersen *et al.*, reported that native A2M blocks T-cell-mediated cytotoxicity and that this effect relies on the anti-proteolytic activities of A2M (157). Direct effects of A2M on lymphocyte biology have, however, not yet been described. Perhaps this is due to the fact that the expression of the LRP-1 receptor in T-cells and B-cells is limited, albeit, recent RNA-Seq studies profiling human PMBC immune subsets demonstrated an expression level of LRP-1 similar (or even higher than) neutrophils in certain T-cell and B-cell subsets (87, 158).

**A2M IN INFLAMMATION AND INFECTION**

In this part we discuss the established relevance of A2M in a selection of immune-mediated pathologies and its contribution to infections. Most disease-related studies focus on the ability of A2M to inactivate active proteases and its ability to bind cytokines, whereas other contributions of A2M remain unexplored.

**A2M in Sepsis Syndromes**

In patients with sepsis or animal models for sepsis, plasma native A2M is decreased whereas activated A2M is present at higher levels. This change is likely due to the formation of complexes with proteases released by activated neutrophils or proteases that are part of the fibrinolytic or coagulation cascade. Hence, complexes such as plasmin-A2M*, thrombin-A2M*, cathepsin-G-A2M*, and elastase-A2M* can be found (159, 160). In guinea-pigs, depletion of A2M results in high lethality upon administration of pseudomonal elastase or *Pseudomonas aeruginosa* culture supernatants, an effect that appears to rely on activation of coagulation factor XII (also known as Hageman factor), which is a serine endopeptidase part of the coagulation cascade. Furthermore, restoration of A2M levels could abrogate this effect (161). In a different study, A2M-deficient mice were also more sensitive to endotoxin, but induction of a lethal Gram-negative infection with *Klebsiella pneumoniae* rendered A2M-deficient mice more resistant. A2M-deficient mice more efficiently cleared *K. pneumoniae* from major organs in comparison with control mice (162). This finding is highly surprising given that fact that A2M promotes phagocytosis and killing of bacteria by neutrophils and macrophages.

Microparticles enriched with A2M (as produced by adherent neutrophils) are found in plasma samples from patients suffering from severe sepsis and vary according to the source of infection (116, 163). A2M-containing microvesicles are associated with survival in community acquired pneumonia-associated sepsis, but not with sepsis caused by fecal peritonitis (163). Furthermore, the amount of A2M-containing microparticles in plasma from sepsis survivors is higher than in plasma from non-survivors and healthy volunteers (116). In the CLP mouse model for bacterial sepsis, administration of soluble A2M or A2M-enriched human microvesicles (A2M-E-MV) improves survival rates. A2M-E-MV also protects against hypothermia and reduces bacterial loads by improving bacterial phagocytosis. Surprisingly, both A2M and A2M-E-MV reduce total proinflammatory lipid mediator levels (e.g. PGE2), increase IFN-γ levels, and A2M-E-MV alone increase pro-resolving lipid mediator levels.
Furthermore, mice receiving A2M-E-MV had lower levels of peritoneal leukocytes and lung myeloperoxidase levels (115). Finally, a conjugate between the LPS-binding antibiotic Polymyxin B and A2M was also able to decrease the lethality of both LPS-induced acute inflammation and polymicrobial sepsis induced by cecal ligation and puncture (CLP) in mice, when administered before or shortly after start of the model (164). Overall, it appears that supplementation with A2M or A2M-microparticles, has beneficial outcomes in sepsis and might have therapeutic potential in these syndromes.

**Lung Inflammation**

Lung inflammation is characterized by abundant secretion of extracellular proteases which contribute to lung damage (165). A logical role for A2M is therefore the inhibition of excessive proteolysis. Indeed, during acute respiratory distress syndrome (ARDS), A2M binds active NE. However, whereas A2M-captured NE is no longer able to cleave insoluble elastin, it remains active against small substrates and this activity can no longer be inhibited by alpha-1-antitrypsin (104). NE captured by A2M might therefore no longer contribute to connective tissue injury, but is still able to modify smaller substrates such as cytokines. This again supports the hypothesis that A2M switches proteolysis towards the catalysis of small substrates. Another particularly interesting discovery is the finding of complexes between IL-8 and activated A2M in lung fluids from patients with ARDS. Whereas these complexes might not be detectible by standard IL-8 immunoassays, IL-8 complexed to A2M retains its ability to attract and activate neutrophils and even protects IL-8 against proteolysis by NE (48, 166). As a result, the complexes might account for an underestimation of biologically active IL-8.

**Rheumatic Diseases**

In the inflamed joint, A2M is associated with synovial fluid monocytes, cells of the synovial lining and perivascular cells. Furthermore, the abundance of A2M correlates with the degree of inflammation (167-169). Levels of inactive A2M correlate with neutrophil numbers and are thought to be generated by intraarticular reaction with proteases or by oxidating agents. Whereas A2M is found complexed to elastase-like and chymotrypsin-like proteases (presumably NE and cathepsin G), the majority of inactive A2M is thought to be generated through reaction with ROS (170). A first consequence of A2M oxidation is the decreased capacity of oxidized A2M to inhibit proteases. Synovial fluids from patients with rheumatoid arthritis (RA) contain a 2-fold higher amount of A2M compared to that from osteoarthritis (OA) patients, but the amount of oxidized A2M in RA is significantly higher and the ability of A2M to inhibit proteolysis is lower than that with OA (111). A2M oxidation also influences the capacity of A2M to bind cytokines and growth factors. It has also been proposed that A2M oxidation down-regulates inflammation by shifting the A2M cytokine/growth factor binding profile towards TNF-α, IL-2, and IL-6, while up-regulating the development of tissue repair by reduced binding to bFGF, β-NGF, PDGF, and TGF-β. To that regards, it was shown that A2M from synovial fluid of RA patients, had a decreased capacity to bind TGF-β compared to A2M from synovial fluid of OA patients (46). Finally, hypochlorite-induced oxidation of A2M also induces the chaperone function of A2M. It remains to be determined what the contribution of this effect would be in arthritis.

Another finding is that NE-A2M* complexes can still degrade proteoglycan in sections of human articular cartilage (171). Furthermore, injection of collagenase-A2M* or trypsin-A2M* complexes into the joint cavity of healthy rabbits, also causes experimental synovitis which is more severe than trypsin or collagenase alone (167). Nevertheless, administration of A2M appears to have merit. In rats undergoing anterior cruciate ligament transection, intra-articular injection of A2M or A2M variants (with altered bait regions) attenuates OA damage, presumably by inhibiting cartilage degrading enzymes (172). In a collagen II-induced arthritis model in mice, injection of A2M into the ankles, significantly reduced ankle thickness and improved disease scores (173). Finally, miR-146b, a microRNA associated with OA, was shown to negatively regulate A2M again supporting an overall beneficial role for A2M in arthritis (174).

**Infections**

Given the unique mechanism and the broad substrate/inhibition repertoire of A2M, it is no surprise that it also binds proteases from microbial origin such as *Vibrio vulnificus* derived metalloprotease (175) and *Trypanosoma cruzi* cruzipain (176, 177). Cruzipain is the main cysteine protease present in all life-cycle stages of *T. cruzi* and in the presence of A2M, *T. cruzi* is more efficiently taken up by mouse macrophages (132). Furthermore, A2M also reduces *T. cruzi*-induced apoptosis of host cells and A2M** reduces DNA fragmentation of infected mouse macrophages (178). Finally, mice surviving *T. cruzi*-infection have higher levels of A2M compared to non-surviving mice (179). Whereas mice deficient in A2Ms have a lower blood parasitemia, analysis of mouse hearts revealed more amastigote nests and inflammatory infiltrates (180). Altogether, this suggests a protective role for A2M in *T. cruzi* infection.

A2M can also directly bind to pathogens and thereby influence the course of infections. A2M binds to the cell wall of *Streptococcus* through binding of protein GRAB, a cell surface protein discovered on *Streptococcus pyogenes* and several clinical isolates. Surprisingly, A2M bound to *S. pyogenes* GRAB remains capable of inhibiting microbial and host proteases. Given that *S. pyogenes* mutants lacking GRAB have reduced virulence, it is hypothesized that *S. pyogenes* uses A2M as a protecting factor against host proteases (123, 181). This hypothesis was confirmed by the discovery that SpeB, a cysteine protease secreted by *S. pyogenes*, binds GRAB-bound A2M and protects these bacteria against the host antibacterial peptide LL-37 (182).

A role for A2M in the innate immune defense against viruses has also been proposed. In a proteomics study to identify the components of saliva responsible for inhibition of the H1N1 swine origin influenza A virus (virus-induced erythrocyte hemagglutination assay), A2M was identified as an essential inhibitor. The proposed mechanism involves the inhibition of host proteases responsible for influenza virus hemagglutinin activation and competition with the virus for binding 2,6-sialylated glycoprotein receptors on the host (183). Recently, it
was also hypothesized that A2M might confer some protection from COVID-19 through its ability to protect the vascular endothelium and potential antithrombin activity. In addition, children have considerably higher plasma levels of A2M, which might correspond to the fact that children remain relatively resistant to severe COVID-19 (184, 185). However, the analysis of A2M protein levels in plasma samples from patients with COVID-19 revealed no significant differences or correlations with other disease parameters (186).

DISCUSSION

A2M is a unique macromolecule that interacts with a broad range of endopeptidases. Based on this feature and its ability to inhibit these proteases, its potential biological relevance is enormous. During inflammation, A2M protects against structural damage by inhibition of proteases released by activated leukocytes (e.g. neutrophils) (172). At the same time, A2M also inhibits proteases secreted by invading microorganisms (161). While A2M is mainly known as a general protease inhibitor, the discovery of many exceptions to this principle has indicated that A2M function is far more sophisticated than just protease inhibition. The mechanism through which A2M imposes protease inhibition provides the first clue for other important functions of A2M. A2M inhibits active proteases by forming a molecular cage around the protease and shielding the protease from its substrates (Figure 1) (2).

However, this also implies that small proteins with the ability to access the A2M cage can still be subject to proteolysis (3). At the same time, the A2M cage also protects proteases from further inhibition by other protease inhibitors. For example, NE captured by A2M is no longer able to cleave insoluble elastin but remains active against small substrates. In addition, this activity can no longer be inhibited by alpha-1-antitrypsin (104). Whereas this finding is particularly important for conditions involving lung inflammation, similar effects can be anticipated in other conditions where major effects relate to the activity of small proteins. As an example, inflammatory chemokines are very susceptible to proteolysis and this modification can inactivate or even increase chemokine activity (187). Hence, it remains to be determined what the contribution of A2M could be in processes relying on chemokine activity. Along the same line, A2M (in particular A2M*) can bind to several cytokines and growth factors. Whereas this interaction seems to have no influence on IFN-γ, IL-1β, IL-8 and IL-6 activities, binding to FGF2, PDGF, TGF-β1 and TGF-β2 results in functional inhibition (see Table 1). Furthermore, for IL-6 and IL-8, A2M was shown to protect against proteolytic inactivation (47, 48). For many other cytokines and growth factors the functional relevance of this interaction remains to be determined. In addition, oxidative modification of A2M also alters the cytokine/growth factor binding capacity of A2M, presumably lowering pro-inflammatory mediators and aiding tissue healing (46).

In in vitro assays, A2M contributes to phagocytosis and killing of bacteria by neutrophils and macrophages. In both cell types, A2M increases the production of ROS or facilitates phagocytosis either indirectly through binding to the bacterium or directly through interaction with LRP-1 (115). Surprisingly, in A2M-deficient mice, K. pneumoniae was cleared more efficiently from major organs in comparison with control mice (162). Hence, the ‘net’ contribution of A2M in active bacterial infections in vivo remains to be determined. Furthermore, the exact contribution of A2M to pathogen clearance might also be pathogen-dependent. For example, in case of S. pyogenes, A2M functions as a virulence factor by binding to the bacterial surface and by capturing the bacterial protease SpeB and using it as protection against antibacterial peptides produced by the host (182).

Another shared contribution of A2M to neutrophil and macrophage function relates to increasing their motility (Figure 4). In macrophages, A2M* mainly appears to induce chemokinesis by activation of signaling pathways associated with cytoskeletal rearrangements (147, 148). In neutrophils, this effect mainly relates to chemotaxis and endothelial cell adhesion by inducing or stabilizing the presence of CXCR2 and CD11b (Figure 3) (115). These functions appear to be mediated through LRP-1, GRP78 or a combination of both receptors acting in a co-receptor relationship.

One discrepancy between in vitro and in vivo functions of A2M relates to the secretion of mediators of inflammation. In vitro stimulation of macrophages with A2M*/A2M** resulted in increased production of predominantly pro-inflammatory molecules such as PAF, PGE2 and MMP-9 (141–143, 145). However, in a mouse model for sepsis, administration of A2M reduced total proinflammatory lipid mediator levels (e.g. PGE2) (115). Hence, further investigation seems necessary. Administration of A2M, in particular A2M-enriched microparticles, appears to be beneficial in models for sepsis and increased levels of A2M are associate with better outcomes for sepsis patients. Hence a therapeutic use for A2M in sepsis syndromes or other types of systemic inflammations was suggested (188). Another potential application for A2M relates to its ability to enhance antigen presentation by macrophages. Antigens bound to A2M* are more efficiently presented to T-cells by macrophages. Meanwhile, a technique for the rapid and efficient incorporation of non-proteolytic antigens into A2M was developed (tradename, SynerVaxTM) and this technique was proposed as a novel adjuvant technology for vaccine development or antibody production (189).

The recent discovery of hypochlorite-treated A2M as an extracellular chaperone is particularly interesting in the context of neutrophilic inflammations (113). Hypochlorite secreted by activated neutrophils results in oxidation of A2M and its dissociation into dimers. Whereas these dimers no longer have the capacity to capture active proteases, they are able to form stable complexes with other ‘stressed’ proteins generated by oxidative modification. Subsequently, A2M aids their removal through receptor-mediated endocytosis (112). These mechanism might contribute to our understanding of how disordered proteins or cell debris generated by aggressive inflammatory environments are cleared and how build-up of pathological
protein aggregates are avoided (19). Finally, being a highly conserved protein sharing structural similarities with complement factors C3 and C4 (23), it is interesting to see how some of the functions of A2M might relate to functions of the complement system such as opsonization and complement-mediated phagocytosis (190).

In conclusion, the potential contribution of A2M to inflammation, immunity and infection is clear. However, more studies are needed to understand which are the major mechanisms through which A2M contributes to pathology. To that regard, mechanistic studies using up-to-date technologies or applying in vivo models with cell specific deletion of A2M might provide crucial new insights and lead to new applications for A2M or new A2M-derivatives.

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AUTHOR CONTRIBUTIONS

JV wrote the manuscript and YI provided crucial feedback and modifications. All authors contributed to the manuscript and approved the submitted version.

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## GLOSSARY

| Abbreviation | Definition |
|--------------|------------|
| A2M          | alpha-2-macroglobulin |
| A2ML1        | A2M-like-1 |
| AA           | arachidonic acid |
| ADAM17       | a disintegrin and metalloprotease 17 |
| ANA          | anaphylatoxin domain |
| ApoE         | apolipoprotein E |
| BiP          | binding immunoglobulin protein |
| BRD          | bait-region domain |
| C345C        | C-terminal extension of C3-5 |
| Uegf         | Bmp1 |
| cAMP         | cyclic adenosine monophosphate |
| CatG         | cathepsin G |
| CLP          | cecal ligation puncture |
| COX2         | cyclooxygenase-2 |
| cPLA2        | cytosolic phospholipase A2 |
| CR           | complement-type repeats |
| CUB          | complement C1r/C1s |
| CXC2         | C-X-C motif chemokine receptor 2 |
| FGF          | fibroblast growth factor |
| DAB1         | Disabled homolog 1 |
| DAG          | diacyl glycerol |
| GPI          | glycosyl-phosphatidylinositol linker |
| GRAB         | G-related A2M-binding protein |
| GRK2         | G-protein-coupled receptor kinase 2 |
| GPR          | cell surface glucose-regulated protein |
| HEL          | hen egg lysozyme |
| HSP70        | heat shock protein 70 |
| IL           | interleukin |
| IP3          | inositol trisphosphate |
| JDP          | J domain protein |
| JIP          | G-Jun-amino-terminal kinase-interacting protein |
| JNK          | c-Jun N-terminal kinases |
| LysoPC       | lysophosphatidylcholine |
| LRP-1        | low density lipoprotein receptor-related protein-1 |
| MA           | methylamine |
| MAC          | macroglobulin activated for cytokine binding |

| Continued |
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| MAPK       | mitogen-activated protein kinases |
| MASP       | mannose-binding protein-associated serine protease |
| MBP        | mannose-binding protein |
| MBL        | mannose-binding lectin |
| MG         | macroglobulin-like domain |
| MMP        | matrix metalloproteinase |
| MT1-MMP    | membrane-type 1 matrix metalloproteinase |
| NE         | neutrophil elastase |
| NF-κB      | nuclear factor kappa-light-chain-enhancer of activated B cells |
| NGF        | nerve growth factor |
| NMDA       | N-methyl-D-aspartate |
| OA         | osteoarthritis |
| P3         | proteinase 3 |
| p75NTR     | p75 neurotrophin receptor |
| PAK2       | p21-activated protein kinase-2 |
| PDGF       | platelet-derived growth factor |
| PGE2       | prostaglandin E2 |
| PLCγ1       | phospholipase Cγ1 |
| PSA        | prostate-specific antigen |
| PSD        | post synaptic density protein |
| PTB        | phosphotyrosine-binding |
| PTGS2      | prostaglandin-endoperoxide synthase-2 |
| RA         | rheumatoid arthritis |
| RAP        | receptor-associated protein |
| RBD        | receptor binding domain |
| RhoA       | Ras homolog family member A |
| Sh2        | Src homology 2 |
| Shp-2      | Sh2-containing protein tyrosine phosphatase 2 |
| ROS        | reactive oxygen species |
| SMCs       | smooth muscle cells |
| TED        | thioester domain |
| TEP        | thioester-containing protein |
| TGF        | transforming growth factor |
| TNF        | tumor necrosis factor |
| trk         | tyrosine receptor kinase |
| tPA        | tissue plasminogen activator |
| VEGF       | vascular endothelial growth factor |

(Continued)