Soluble α_2-macroglobulin receptor is increased in endotracheal aspirates from infants and children after cardiopulmonary bypass

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Objective: Cytokine dysregulation contributes to the systemic inflammatory response after cardiopulmonary bypass. Clearance of cytokine binding proteins may be important in the resolution of inflammation. Our aim was to determine whether the cytokine binding protein α_2-macroglobulin and its soluble receptor were upregulated in endotracheal aspirates from infants and children undergoing cardiopulmonary bypass.

Methods: Seventy tracheal aspirates were collected before and after cardiopulmonary bypass from 35 infants and children undergoing surgical correction of congenital heart defects. α_2-Macroglobulin and the soluble α_2-macroglobulin receptor were identified by Western blot. With the use of multi-analyte cytokine profiling, pro-inflammatory and anti-inflammatory cytokines were quantified, normalized to total protein, and expressed as ratios. Paired *t* tests and Wilcoxon signed-rank tests were performed between prebypass and postbypass samples. Correlations were examined among α_2-macroglobulin, soluble α_2-macroglobulin receptor, cytokine ratios, and the clinical variables of cardiopulmonary bypass, aortic crossclamp, and circulatory arrest times.

Results: α_2-Macroglobulin increased by 50% (mean densitometry increase 82,683 $\pm$ 184,594, $P = .012$), and soluble α_2-macroglobulin receptor increased by 17% (mean densitometry increase 506,148 $\pm$ 687,037, $P = .0001$) after cardiopulmonary bypass. The ratio of interleukin-8/interleukin-4 increased by 136% ($P = .0001$), and interleukin-8/interleukin-10 increased by 102% ($P = .001$). The increase in soluble α_2-macroglobulin receptor was positively correlated with the ratios of interleukin-8/interleukin-4 and interleukin-8/interleukin-10. There were no statistically significant positive correlations between the increase in α_2-macroglobulin or soluble α_2-macroglobulin receptor and measured clinical variables.

Conclusions: We report for the first time the upregulation of α_2-macroglobulin and soluble α_2-macroglobulin receptor in tracheal aspirates after cardiopulmonary bypass in infants and children. Soluble α_2-macroglobulin receptor correlates with increased α_2-macroglobulin and a disproportionate increase in pro-inflammatory to anti-inflammatory cytokine ratios.

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Eric A. Williams, MD, is a National Institute of Child Health and Human Development Fellow of the Pediatric Scientist Development Program (National Institute of Child Health and Human Development Grant Award K12-HD00850).

Salvatore V. Pizzo, MD, PhD, is funded by National Heart, Lung, and Blood Institute HL 24066.

Received for publication April 22, 2004; revisions received July 7, 2004; received for publication Aug 18, 2004.

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J Thorac Cardiovasc Surg 2005;129: 1098-103

0022-5223/$30.00

doi:10.1016/j.jtcvs.2004.08.020

CHD

Cardiopulmonary bypass (CPB) contributes to a systemic inflammatory response by activation of cellular and humoral cascades. Inflammation after CPB is similar to sepsis. It involves activation of immunocompetent cells, complement and coagulation, endothelial cell dysfunction, proteinase/antiproteinase imbalance, and increased cytokine production, and may lead to multiorgan dysfunction.[1-3] In particular, lung dysfunction after CPB can have detrimental effects on oxygen delivery and require ventilator management that may worsen cardiac output through unfavorable cardiorespiratory interactions. The progression to severe lung disease is believed to occur when regulatory mechanisms fail and inflammation becomes self-propagating.[4] This autoamplification may be the result of increased production or decreased clearance of

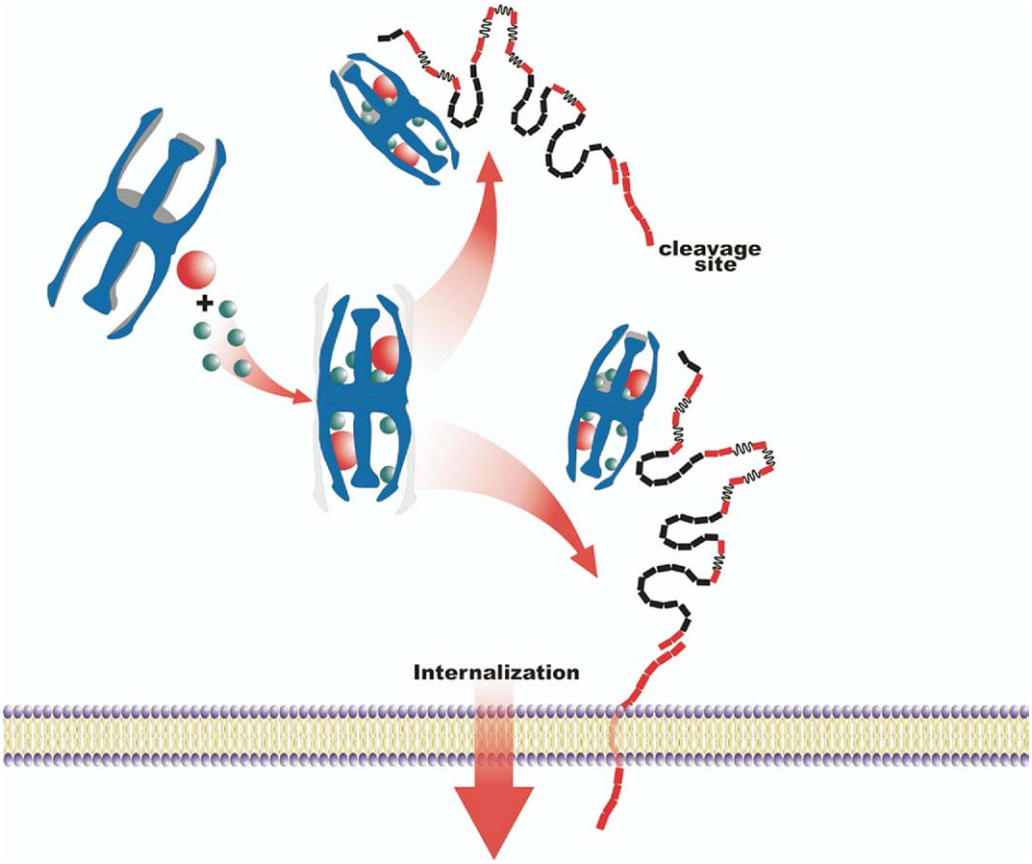

Figure 1. Transition of α_2-macroglobulin (α_2M) (blue molecule) to receptor-recognized form with exposure to proteinases *(red circles)* and binding of cytokines *(green circles)*. α_2M-cytokine complexes may bind to the α_2M receptor (α_2MR) at the cell surface with subsequent internalization and degradation or bind to the soluble form of the α_2MR. The soluble α_2MR (Sα_2MR) results from cleavage of the α_2MR near the cell surface and may competitively inhibit clearance of α_2M-cytokine complexes.

cytokines. Ultimately, the balance of pro-inflammatory and anti-inflammatory factors influences subsequent lung injury.

In addition to its role as a proteinase inhibitor, α_2-macroglobulin (α_2M) also functions as a cytokine binding protein.[5,6] At sites of inflammation, α_2M undergoes a conformational change to a receptor-recognized form, α_2M*. We have shown that α_2M* demonstrates increased binding to many cytokines and growth factors.[5,7] In vivo, α_2M* is increased in endotracheal aspirates (ETAs) from infants with lung disease,[8,9] and α_2M*-cytokine complexes have been identified in bronchoalveolar lavage (BAL) from adults with acute respiratory distress syndrome.[10,11] The increased α_2M may result from increased production/sequestration or decreased clearance. In general, cytokines and growth factors complexed with α_2M* retain their biologic activity. However, α_2M*-cytokine complexes are rapidly cleared when bound to the α_2M cell surface receptor (α_2MR).[7]

α_2MR is a classic scavenger receptor found on many cell types including hepatocytes, monocytes, and macrophages. Recent studies have identified a soluble form of α_2MR (Sα_2MR) with intact receptor binding that circulates in human plasma.[12,13] An Sα_2MR that maintains its ligand-binding properties should act as a competitive inhibitor for the cellular form of the receptor. Soluble α_2MR may inhibit the cellular internalization of α_2M*-cytokine complexes (Figure 1).

We evaluated the α_2M*-cytokine regulatory axis, and our study for the first time demonstrates the relative increase of Sα_2MR in ETAs from infants and children after the inflammatory insult of CPB. The increase in Sα_2MR correlated with increased levels of the ratio of pro-inflammatory to anti-inflammatory cytokines. Increased levels of Sα_2MR may prolong the course of inflammation within the lung after CPB by increasing the half-life of α_2M*-cytokine complexes.

TABLE 1. Patient demographics

Diagnosis	Surgery	Age	Weight (kg)	CPB (min)	X-clamp (min)	CA (min)
HLHS	Norwood	2 d	2.7	185	76	15
HLHS	Norwood	4 d	2.9	166	60	10
HLHS	Norwood	6 d	2.8	165	59	12
HLHS	Norwood	7 d	2.7	177	68	4
		Mean		173	66	
AVSD	AV canal repair	1 mo	2.9	213	101	
VSD	VSD closure	2 mo	3.3	104	70	
TOF	1° repair	3 mo	5.3	52	28	
Mitral insufficiency	MVR	3 mo	4.6	89	54	
TOF	1° repair	3 mo	5.6	100	49	
HLHS s/p Norwood	Glenn	4 mo	5	181		
TGA	Glenn	4 mo	5.5	72		
Tricuspid atresia	Glenn	4 mo	5.7	80	5	
HLHS s/p Norwood	Glenn	4 mo	4.5	120	69	
DORV	Glenn	5 mo	6.1	60		
DORV	Glenn	6 mo	6.8	77		
TOF	1° repair	6 mo	7.5	98	58	
AVSD	AV canal repair	8 mo	4.9	108	67	
HLHS	Glenn	11 mo	7.3	82	10	
		Mean		103	51	
ASD	ASD closure	20 mo	10.4	51	40	
HLHS	Fontan	2 y	11	58		
ASD	ASD closure	2 y	14	51	37	
TOF s/p repair	Conduit change	2 y	13	96		
HLHS	Fontan	3 y	14.3	172	64	35
ASD, PAPVR	ASD closure	3 y	15.2	33	13	
PAPVR, ALCAPA, ASD	ASD closure, CAG	3 y	17.6	106	65	
ASD	ASD closure	3 y	16.7	39		
AV canal	Fontan	5 y	15.5	82		
TOF	PVR, conduit	5 y	18.7	103		
ASD	ASD closure	6 y	21.4	60	38	
Pulmonary atresia, PI	PVR	6 y	22.4	59		
Pulmonary stenosis	PVR	7 y	31	65		
ASD	ASD closure	7 y	26.5	72	42	
VSD	VSD closure	8 y	20	52	28	
RVOT obstruction	Conduit	8 y	28.3	57		
Ebstein	TVR	10 y	23	65	33	
		Mean		72	40	

CPB, Cardiopulmonary bypass; *X-clamp,* crossclamp; *CA,* circulatory arrest; *HLHS,* hypoplastic left heart syndrome; *AVSD,* atrioventricular septal defect; *VSD,* ventricular septal defect; *TOF,* tetralogy of Fallot; *MVR,* mitral valve replacement; *s/p,* status post; *TGA,* transposition of the great arteries, *DORV,* double-outlet right ventricle; *ASD,* atrial septal defect; *PAPVR,* partial anomalous pulmonary venous return; *ALCAPA,* anomalous left coronary artery from pulmonary artery; *CAG,* coronary artery graft; *PVR,* pulmonary valve replacement; *PI,* pulmonary insufficiency; *RVOT,* right ventricular outflow tract; *TVR,* tricuspid valve replacement.

Methods and Materials

Monoclonal antibodies directed against the β chain of human α_2MR were purchased from Pharmingen (San Diego, Calif). Polyclonal antibodies directed against α_2M were purchased from Sigma (St Louis, Mo). Reagents for sodium dodecyl sulfate-polyacrylamide gel electrophoresis, polyvinylidene difluoride membranes, and transfer reagents were purchased from Bio-Rad (Richmond, Calif). An enhanced chemifluorescent immunoblotting kit was purchased from Amersham (Buckinghamshire, United Kingdom). Complete protease inhibitors were purchased from Roche (Indianapolis, Ind).

Study Subjects

After parental informed consent and Duke University Institutional Review Board approval were obtained, ETAs were collected from children (aged 2 days to 10 years) undergoing corrective congenital heart surgery requiring CPB. In each patient, ETAs were collected after intubation but before CPB. During CPB the lungs were not ventilated, but the patient's endotracheal tube remained attached to the ventilator with humidified fresh gas flow. After inflation, a second sample was collected immediately before the separation from CPB, before the institution of modified ultrafiltration. Samples were obtained with a sterile endotracheal suction

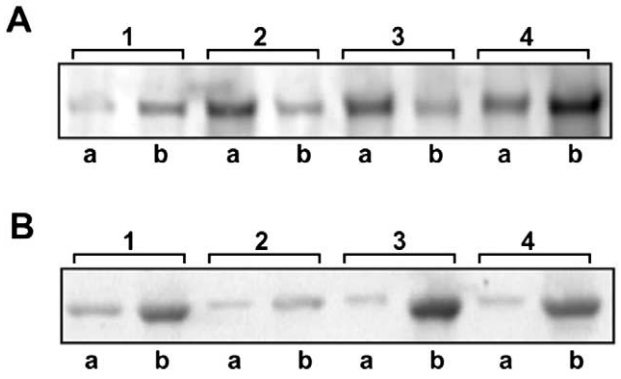

Figure 2. A, Western blot analysis probing α_2M within 4 patients' endotracheal aspirate (ETA) samples. The ~180 kDa band is the α_2M subunit, 4 of which make up the native molecule. Pre-CPB (a) and post-CPB (b) time points. B, Western blot analysis probing Sα_2MR within 4 patients' ETA samples. The ~55 kDa band represents the surface-cleaved extracellular portion of the ~85 kDa β chain. Pre-CPB (a) and post-CPB (b) time points.

catheter. If necessary, patients received 0.5 to 1 mL of normal saline to assist aspiration of secretions.

Anesthesia and CPB were per institutional protocol. All patients aged less than 1 month received intravenous methylprednisolone 10 mg/kg (q6 × 2) preoperatively in addition to 30 mg/kg on pump prime. Children aged more than 1 month received methylprednisolone 10 mg/kg on pump prime.

Sample Processing

After sample collection, 2.5 mL of Complete protease inhibitor (dilution 1:1) was added to prevent protein degradation, and the sample was placed on ice. Within 4 hours, samples were centrifuged at 1000 rpm for 8 minutes to remove cellular debris. Subsequently, samples were centrifuged at 100,000*g* for 30 minutes to isolate soluble components and stored at −80°C for further analysis.

Immunoblotting

Total protein analysis was performed with the BCA Protein Assay Kit (Pierce, Rockford, Ill). Polyacrylamide gel electrophoresis was performed at 150V for 3.5 hours per the manufacturer's instructions. All gels were loaded with identical total protein for each pre-CPB and post-CPB pair. Western blotting was performed for α_2M and Sα_2MR. After application of fluorescent dye, blots were dried and digitized on the Storm 860 gel and blot imaging system (Molecular Dynamics, Sunnyvale, Calif). Image densitometry was performed with ImageQuant v5.2 (Molecular Dynamics, Sunnyvale, Calif). Quantitative multi-analyte cytokine profiling was performed on all samples per standard protocol with 50-μL samples on the Luminex 100 (LuminexCorp, Austin, Tex).

Statistical analysis was performed by Microsoft Excel 2002 (Redmond, Wash) and SAS (Cary, NC) and consisted of paired hypothesis tests between pre-CPB and post-CPB samples within each patient for image densitometry and cytokine ratios. Paired *t* tests were used for normally distributed data, and Wilcoxon signed-rank tests were used for non-normally distributed data. Spearman correlations were examined between Sα_2MR, α_2M, and cytokine ratios as well as the clinical variables of CPB, aortic crossclamp, and circulatory arrest times.

TABLE 2. Cytokine analysis

Cytokine	Pre (pg/mL) ± SD	Post (pg/mL) ± SD	*P*
INF-γ	1362 ± 3242	1917 ± 3401	.052
IL-1β	21.6 ± 30.9	15.1 ± 10.5	.435
IL-8	97.3 ± 182	205 ± 316	.0045
IL-10	5.6 ± 3.2	6.3 ± 3.2	.14
IL-6	29.9 ± 62.7	6.01 ± 9.03	.0002
IL-12	14.2 ± 52	41.8 ± 134	.384
IL-4	112 ± 100	86 ± 34	.449
IL-8/IL-4	0.83 ± 1.39	1.96 ± 2.7	.0001
IL-8/IL-10	13.1 ± 21.1	26.5 ± 36.7	.001

IL, Interleukin; *INF,* interferon.

Results

All measured variables except α_2M were not normally distributed and required the Wilcoxon signed-rank test to determine statistical significance and are expressed as mean ± SD. Patient demographics are tabulated in Table 1. In our patients, 17 of 35 (49%) were aged more than 1 year and 18 of 35 (51%) were aged less than 1 year. Of the patients aged less than 1 year, 4 of 18 (22%) were aged less than 1 month. The longest CPB and aortic crossclamp times were required in this same subpopulation. In addition, they received hypothermic circulatory arrest. Aprotinin was administered to only 2 of 35 patients (6%).

Representative Western blots for α_2M and Sα_2MR are shown in Figure 2, *A* and *B,* respectively. The relative increases after CPB of the α_2MR β-chain image density and α_2M image density for all patients was 17% (mean densitometry increase 506,148 ± 687,037, $P = .0001$) and 50% (mean densitometry increase 82,683 ± 184,594, $P = .012$), respectively.

Total protein increased before CPB to after CPB from 346 ± 420 to 507 ± 563 μg/mL ($P = .009$). The results of the multi-analyte cytokine profiling are shown in Table 2. Interleukin (IL)-2, tumor necrosis factor-α, and granulocyte-macrophage colony-stimulating factor were below the limits of detection of the multi-analyte cytokine analysis. Cytokines normalized to total protein are shown in Table 3. The pro-inflammatory to anti-inflammatory ratios of IL-8/IL-10 and IL-8/IL-4 were increased after bypass. Specifically, mean IL-8/IL-10 increased from 13.1 to 26.5 (102%) ($P = .001$), and IL-8/IL-4 increased from 0.83 to 1.96 (136%) ($P = .0001$).

The increase in Sα_2MR was positively correlated with the increase in α_2M (Spearman coefficient $R = 0.52$, $P < .01$). The increase in Sα_2MR was also positively correlated with the ratios of IL-8/IL-4 (Spearman coefficient $R = 0.50$,

TABLE 3. Normalized cytokine analysis

Cytokine/TP	Pre (pg/μg) ± SD	Post (pg/μg) ± SD	P
INF-γ/TP	2.96 ± 6.2	3.84 ± 5.6	.218
IL-1β/TP	0.46 ± 1.3	0.07 ± 0.17	.0001
IL-8/TP	0.88 ± 2.9	0.42 ± 0.67	.43
IL-10/TP	0.10 ± 0.21	0.027 ± 0.03	.028
IL-6/TP	1.48 ± 5.9	0.06 ± 0.21	.0001
IL-12/TP	0.19 ± 0.78	0.04 ± 0.11	.70
IL-4/TP	4.35 ± 17.2	0.43 ± 0.64	.002

INF, Interferon; *IL,* interleukin; *TP,* total protein.

$P < .01$) and IL-8/IL-10 (Spearman coefficient $R = 0.39$, $P < .05$). There were no statistically significant positive correlations between the increase in Sα_2MR or α_2M and the clinical variables of CPB, aortic crossclamp, and circulatory arrest times.

Discussion

During CPB, serum α_2M has been found to decrease, most likely because of dilutional effects.[14,15] However, a critical component of the α_2M-cytokine regulatory axis is the α_2M receptor, a scavenger receptor located on many cell types.[16] This study for the first time describes the increase of Sα_2MR in ETA from infants and children after the inflammatory insult of CPB. The increase in Sα_2MR had a positive correlation with α_2M and the proinflammatory to anti-inflammatory ratios of IL-8/IL-4 and IL-8/IL-10. Previous in vivo research identified the presence of a soluble form of α_2MR circulating in human plasma that maintained its ligand-binding properties and was increased in inflammatory states.[12,13] Further in vitro modeling suggested that the release of Sα_2MR was through cleavage of α_2MR at a membrane proximal region by a membrane-tethered metalloproteinase (Figure 1).[13,17] In vitro treatment with a metalloproteinase inhibitor decreased the cleavage of α_2MR, thereby decreasing the Sα_2MR available to compete with the cellular α_2MR. Thus, mechanisms that affect the clearance of α_2M*-cytokine complexes, such as receptor competition, could play a role in the modulation of inflammation after CPB.

Our goal has been to evaluate the inflammatory response within the lung in hopes of understanding the more clinically severe forms of acute lung injury and acute respiratory distress syndrome.[18] We chose CPB as a model of inflammation because of its predictable onset. This is in contrast with the poor predictability of lung disease secondary to sepsis, in which clinical samples before the onset of inflammation may be impossible to obtain. We confirmed inflammatory upregulation after CPB by measuring the ratios of inflammatory mediators IL-8/IL-4 and IL-8/IL-10 from the lung and found them increased.

Systemic inflammation is upregulated by CPB with activation of plasma protein systems: contact, intrinsic and extrinsic coagulation, fibrinolysis, and complement, as well as cellular systems: platelets, endothelial cells, neutrophils, monocytes, and lymphocytes.[3] Despite a complex inflammatory response, the majority of patients are extubated early after CPB with minimal ventilatory requirements. However, in patients requiring prolonged mechanical ventilation, positive pressure can exacerbate the inflammatory response because of overdistension of lung tissue.[19] Thus, both the inflammatory insult and supportive therapy may potentiate lung injury. To date, studies have implemented novel therapies directed at reducing inflammatory cytokines and the associated potential for prolonged inflammation (eg, steroids, modified ultrafiltration, complement inhibitors, and proteinase inhibitors).[20-23] Despite these efforts, the physiologic response to CPB in infants and children can be exaggerated and lead to organ dysfunction in the postoperative period, clinically evidenced in the lung.[24]

Clinically, lung disease may become exaggerated with dysfunction of cytokine-binding proteins.[25] Found in abundance at sites of inflammation, α_2M* binds a host of proinflammatory cytokines and growth factors, and we previously showed that in vitro binding to α_2M* is specific.[5] α_2M* is increased in ETA from intubated infants with lung disease.[8,9] Studies have measured α_2M* levels from BAL fluid in infants and adults and found increases in those patients with inflammatory lung diseases.[26,27] The increase in α_2M* in BAL fluid has been presumed secondary to leakage from plasma after lung injury and localized production by alveolar macrophages and fibroblasts.[25] We found α_2M increased in the inflammatory state associated with CPB. Decreased clearance of α_2M*-cytokine complexes may contribute to the increase in α_2M*.

ETA samples were obtained as representative of the milieu of the alveolar compartment. To date, ETA samples have been used to measure components within the alveolar compartment of intubated neonates and infants.[8,9,28] Dilutional effects on Sα_2MR measurements have been taken into account by loading identical protein concentrations on the gels for pre-CPB and post-CPB samples. By examination of the ratio of pro-inflammatory to anti-inflammatory cytokines at any time point, the dilutional variability can also be removed. Here, the data indicate a disproportionate increase in pro-inflammatory to anti-inflammatory mediators before to after CPB. Despite these attempts, it is unclear how well tracheal aspirates can represent the acellular components of the lower respiratory tract and how well we can use these measurements to distinguish temporal changes resulting from pathology or therapy.

Interpretation of our results requires careful consideration. Although we understand that steroids may alter the inflammatory response to CPB, our study was not designed

to determine this effect. Because of the inherent variability of patient demographics and the influence of varying amounts of immunomodulators, our current sample size does not provide the statistical power required to delineate the contributions of these effectors. For example, in addition to having the lowest temperatures, all of the neonates (n = 4) received 2 additional doses of methylprednisolone before their surgery on CPB. The lack of a statistically significant increase in $S\alpha_2MR$ in the neonatal subgroup may not be solely attributable to the small sample size of 4. Recent clinical data indicate that combined preoperative and intraoperative steroids can reduce inflammatory mediator expression greater than intraoperative steroids alone.[29] Thus, we cannot rule out the potential contribution from the extra doses of steroids in this subgroup.

We report for the first time the presence of $S\alpha_2MR$ in ETAs from infants and children and the increase of $S\alpha_2MR$ after the inflammatory insult of CPB. On the basis of our findings, we hypothesize that therapy directed at decreasing cleavage of α_2MR, by inhibiting metalloproteinases, will lead to increased α_2MR-dependent cellular internalization and degradation of α_2M^*-cytokine complexes. This hypothesis supports therapy directed at reducing or inhibiting metalloproteinases within the lung to reduce inflammation.[30] Future studies will be directed toward understanding the mechanisms of α_2MR release and the effect of $S\alpha_2MR$ on the clearance of α_2M^*-cytokine complexes in vitro.

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