Supplementary Material

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Sustained NKG2D Ligand Expression in Pulmonary Epithelial Cells Promotes the Development of COPD Pathologies

SUPPLEMENTARY TABLE 1	DEMOGRAPHIC AND CLINICAL CHARACTERISTICS OF PATIENTS SUBMITTED TO BRONCHOSCOPIC SAMPLING				
		Total	**Non-COPD**	**COPD**	**p-value**
Patients	n (%)	93 (100)	26 (28)	67 (72)	
General characteristics					
Gender	M:F	73:9	12:14	63:4	0.000
Age (years)	x (SD)	66 (11)	65 (13)	67 (10)	0.389
Cigarette Smoking					
Never Smokers	n (%)	21 (23)	15(58)	6 (9)	0.000
Former Smokers	n (%)	30 (32)	4 (15)	26 (39)	
Current Smokers	n (%)	42 (45)	7 (27)	35 (52)	
Smoking Intensity, Pack-year	m (SD)	44 (35)	16 (21)	55 (33)	
Symptoms of chronic bronchitis					
No	n (%)	47 (51)	21 (81)	26 (34)	0.000
Yes	n (%)	46 (49)	5 (19)	41 (66)	
Pulmonary Function					
FEV_1, %pred	m (SD)	67 (23)	96 (12)	56 (16)	0.000
FVC, %pred	m (SD)	73 (20)	94 (11)	64 (16)	0.000
FEV_1/FVC, %	m (SD)	65 (12)	74 (7)	62 (12)	0.000
TLC, %pred	m (SD)	95 (19)	100 (16)	94 (21)	0.214
RV, %pred	m (SD)	130 (44)	111 (27)	137 (47)	0.024
TLco, %pred	m (SD)	78 (22)	94 (18)	71 (19)	0.000
Tlco/VA, %pred	m (SD)	88 (19)	96 (18)	84 (18)	0.016
Lung Cancer					
No Evidence of Lung Cancer	n (%)	31 (32)	25 (48)	6 (26)	0.004
Lung Cancer Confirmed	n (%)	62 (68)	31 (52)	31 (74)	
Bronchial MICA Expression					
No	n (%)	26 (28)	21 (38)	5 (14)	0.012
Yes	n (%)	67 (72)	35 (62)	32 (86)	

Abbreviations: (COPD): Chronic Obstructive Pulmonary Disease; (FEV_1): forced expiratory volume in the 1^{st} second; (FVC): forced vital capacity; (TLC): total lung capacity; (RV): residual volume; (Tlco): lung transfer capacity for CO; (MICA): Major histocompatibility complex-class-I-polypeptide–related sequence A protein.

SUPPLEMENTARY TABLE 2	MICA EXPRESSION IN BRONCHIAL BIOPSIES ACCORDING TO GENERAL CHARACTERISTICS, SMOKING STATUS AND PULMONARY FUNCTION IN COPD PATIENTS (ONLY)					
		MICA-		MICA+		p-value
N, (%)		35	(52)	32	(48)	
Age, Yrs.	m (SD)	67	(10)	66	(9)	0.802
BMI, kg/m^2	m (SD)	27	(4)	23	(3)	0.012
Smoking Status						
Never smoker	n (%)	6	(0)	0	(0)	0.034
Current smoker	n (%)	15	(46)	20	(54)	
Former smoker	n (%)	14	(54)	12	(46)	
Time from quitting (months)	m (SD)	64	(80)	81	(90)	0.337
Smoking Intensity, Pack-year	m (SD)	49	(35)	61	(29)	0.151
Symptoms of chronic bronchitis						
No	n (%)	18	(51)	8	(25)	0.027
Yes	n (%)	17	(48)	24	(75)	
Severity of COPD						
GOLD I	n (%)	2	(33)	1	(67)	0.930
GOLD II	n (%)	21	(50)	21	(50)	
GOLD III	n (%)	9	(56)	7	(44)	
GOLD IV	n (%)	3	(50)	3	(50)	
Pulmonary Function						
FEV_1, L	m (SD)	1.55	(0.58)	1.77	(0.57)	0.137
FEV_1, %pred	m (SD)	55	(16)	56	(15)	0.735
FVC, %pred	m (SD)	64	(15)	65	(16)	0.769
FEV_1/FVC, %	m (SD)	61	(13)	63	(10)	0.916
TLC, %pred	m (SD)	95	(21)	92	(19)	0.579
RV, %pred	m (SD)	140	(51)	134	(44)	0.651
Tlco, %pred	m (SD)	77	(22)	66	(16)	0.067
Tlco/VA, %pred	m (SD)	86	(18)	82	(19)	0.441

Abbreviations: (COPD): Chronic Obstructive Pulmonary Disease; (MICA): Major histocompatibility complex-class-I-polypeptide–related sequence A protein. (FEV_1): forced expiratory volume in the 1st second; (FVC): forced vital capacity; (TLC): total lung capacity; (RV): residual volume; (Tlco): lung transfer capacity for CO.

SUPPLEMENTARY TABLE 3	DEMOGRAPHIC AND CLINICAL CHARACTERISTICS OF PATIENTS SUBMITTED TO THORACIC SURGERY	
Patients	n	18
Gender	M:F	8:10
Age, Yrs.	m (SD)	56 (9)
Presence of COPD		
Yes	n (%)	13 (72)
No	n (%)	5 (28)
Lung Cancer		
No	n (%)	6 (34)
Yes	n (%)	12 (66)
Lung Cancer		
No Evidence of Lung Cancer	Y:N	2:3
Lung Cancer Confirmed	Y:N	10:3
Smoking Intensity, Pack-year		
Non-COPD	x (SD)	0 (0)
COPD	x (SD)	56 (29)
Pulmonary Function		
FEV_1, % pred	x (SD)	76 (20)
FVC, % pred	x (SD)	80 (16)
FEV_1/FVC, %	x (SD)	74 (18.4)
TLC, %pred	x (SD)	99 (16.6)
Tlco, %pred	x (SD)	70 (15)

Abbreviations: (COPD): Chronic Obstructive Pulmonary Disease; (FEV_1): forced expiratory volume in the 1st second; (FVC): forced vital capacity; (TLC): total lung capacity; (Tlco): lung transfer capacity for CO.

Supplementary Figure Legends.

Supplementary Figure 1. Generation of inducible *Raet1a* transgenic mice. The Ccsp-rtta transgene consists of the 2.3-kb rat *ccsp* promoter, 1.0-kb *rtta* coding sequence, and a 2.0-kb fragment from the human growth hormone gene containing introns and a polyadenylation signal. The *(tetO)7-cmv-Raet1a* transgene consists of seven copies of the tet operator, a *CMV* minimal promoter, the mouse *Raet1a* coding sequence, and the bovine growth hormone polyadenylation signal (Supplementary Figure 1A). A single transgenic mice bearing the *Ccsp-rtta* transgene were bred to single transgenic mice bearing the *(Teto)7-cmv-RaeT1a* transgene to generate double transgenic progeny (Supplementary Figure 2B). We established three separate transgenic mouse lines (Lines 20, 22, and 32) bearing the target *(TetO)7-CMV-Raet1a* transgene. The copy numbers for the incorporation of the transgene were 11 (Line 32), 40 (Line 22), and 44 (Line 20) copies. All three founder lines were bred with the *Ccsp-rtta* activator transgenic mice to produce bi-transgenic progeny, and resultant bi-transgenic lines were screened for (*i*) *Raet1a* expression in response to DOX (1000 ppm in chow beginning at 6 weeks of age), and (*ii*) RAET1 immunohistochemistry (Supplementary Figure 2C). *Raet1a* transgene induction was measured by quantitative real-time RT-PCR in response to DOX for up to 60 days. We assessed transgene expression by calculating the transcript abundance of DOX-treated bi-transgenic mice compared to untreated bi-transgenic mice. None of the bi-transgenic mice expressed the *Raet1a* transgene in the absence of DOX. Additionally, none of the bi-transgenic mice exhibited any lung pathologies in the absence of DOX. Line 20 *(TetO)7-CMV-Raet1a* did not express significant levels of *Raet1a.* Line 32

(TetO)7-CMV-Raet1a expressed detectable levels of *Raet1a*. Line 22 *(TetO)7-CMV-Raet1a* transgenic mice exhibit robust *Raet1a* and is used in all the studies presented.

Supplementary Figure 2. Binomial quantification of MICA immunoreactivity. (A) Indirect immunperoxidase staining was utilized to quantify the expression of MICA in bronchial biopsies. Supplementary Figure 1A shows representative MICA staining in a non-smoker, current smoker without COPD, former smoker with COPD, and a current smoker with COPD. Images are shown for (a) isotype control-stained sections, (b) MICA-stained sections and (c) computer-modified MICA-stained sections as described below. Bronchial samples were immediately fixed in 10% neutral buffered formalin at 4°C for 24 hr, and then processed for paraffin sectioning. Six-µm-thick tissue sections were stained by IHC using an indirect immunoperoxidase method (The Binding Site, UK). All biopsies were processed using and automatic tissue processor. Sections were rehydrated and blocked for endogenous peroxidase using 0.3% hydrogen peroxide. Specificity of immunoreactivity was assessed using an appropriate isotype-matched, non-relevant control antibody (IgG_1, BD Pharmingen), which was included as a negative control. Control monoclonal antibody estimated non-specific binding (*i.e.*, background) of target primary antibodies to cell surface antigens because they showed negligible cross-reactivity with cell surface antigens on tissue sections. Isotype controls were used at identical concentrations and staining conditions as the target primary antibody. In addition, HeLa cell smears (HeLa cells are positive for MICA) were included as positive staining controls. Microscopic images were evaluated using a microscope and an image-

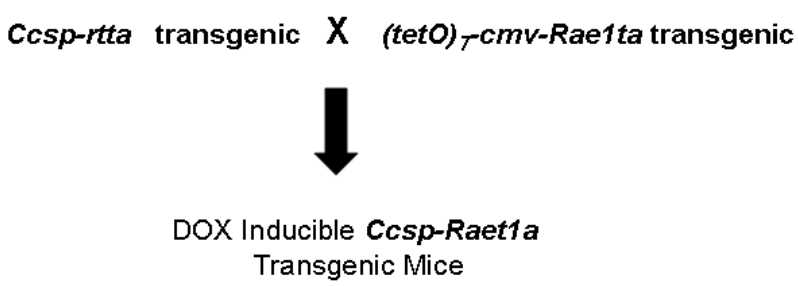
digitizing camera. Micrographs of bronchial biopsies were obtained at a final magnification of 40x. Profile measurements were performed using computer-assisted image analysis (CAIA) software *(ImageJ 1.37, Wayne Rasband, National Institutes of Health, USA. http://rsb.info.nih.gov./ij).* Supplementary Figure 1B depicts the various steps involved in the quantification of MICA in bronchial biopsies by CAIA. CAIA is considered an effective method of quantification when comparing staining characteristics among experimental groups. CAIA allows extraction of 2D feature data such as area fraction (total stained area) and distribution. CAIA relies on the ability to cleanly separate or segment a structure of interest from its background using a physical difference, such as color, to facilitate segmentation of red/brown stained epithelial cells in the blue nuclear counterstained tissue sections. The method converts the RGB (red, green, and blue) composites into HSI (hue, saturation, and intensity). Immunoreactivity was quantified using the hue (color wavelength) and saturation (color amount) of the reaction on the epithelial cells. Thresholding was used to segment images into stained epithelial area and background on the basis of gray levels. Threshold was automatically based on the histogram of the current selection. When thresholding was enabled, positive epithelial areas were displayed in black and background in white. With this method, positive descriptions of color were broad enough to include all the features of interest and strict enough to exclude background. Due to varying color hues among different biopsies, MICA immunoreactivity analysis was restricted only to a binomial scale according to stained epithelial area normalized to the overall length of epithelium in each biopsy. The rationale is that MICA immunoreactivity is positive when 40% and more of the epithelial area showed a red/brown reaction pattern greater than background

threshold. The rationale for negative immunoreactivity is that immunoreactivity was equivalent to, or below of background (*i.e.*, structures such as interstitium labeled with a similar intensity), and/or less than 40% of epithelial area.

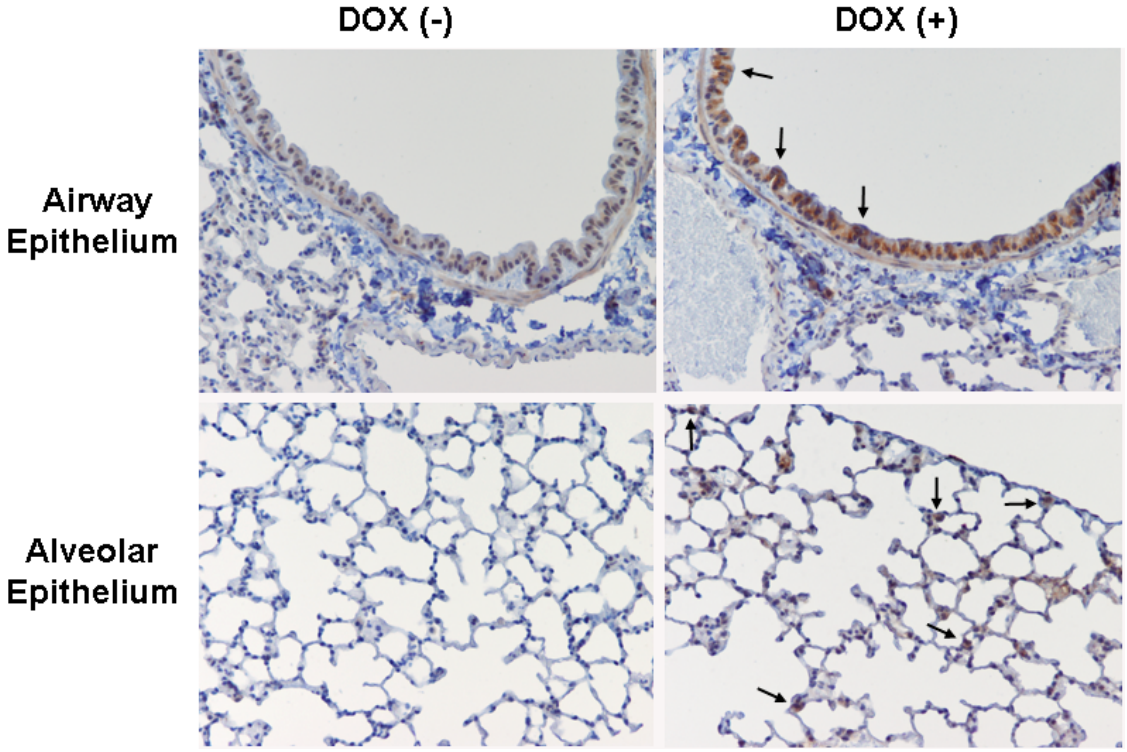
Supplementary Figure 1.

S1A. Constructs

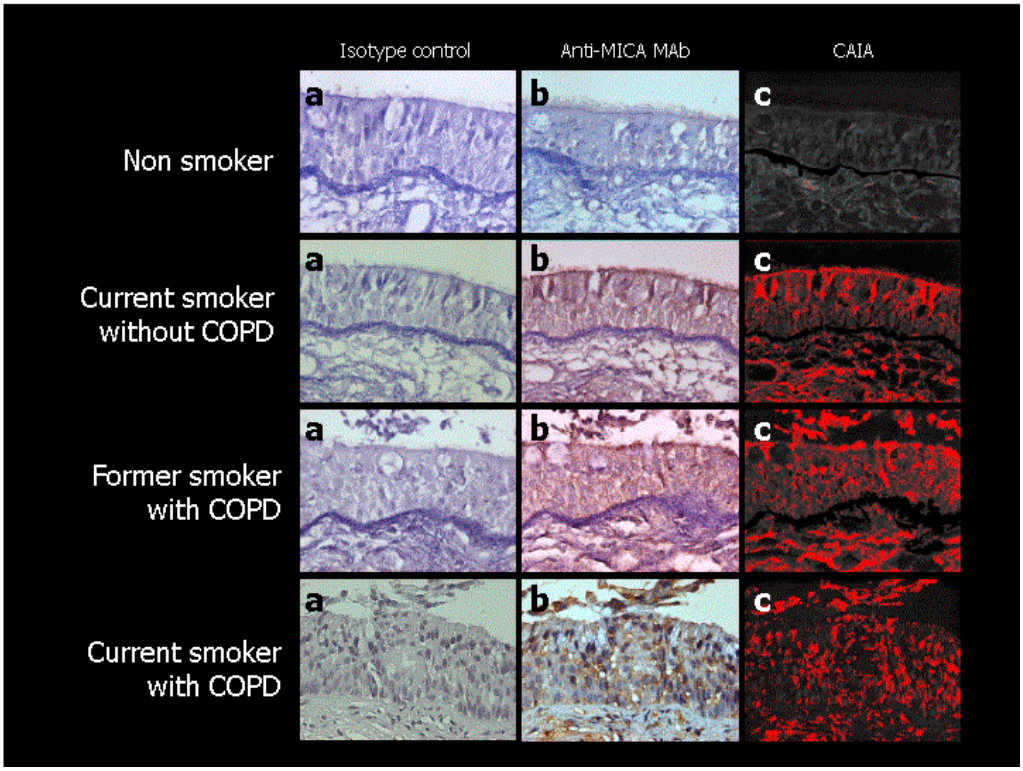
S1B. Transgenic Model



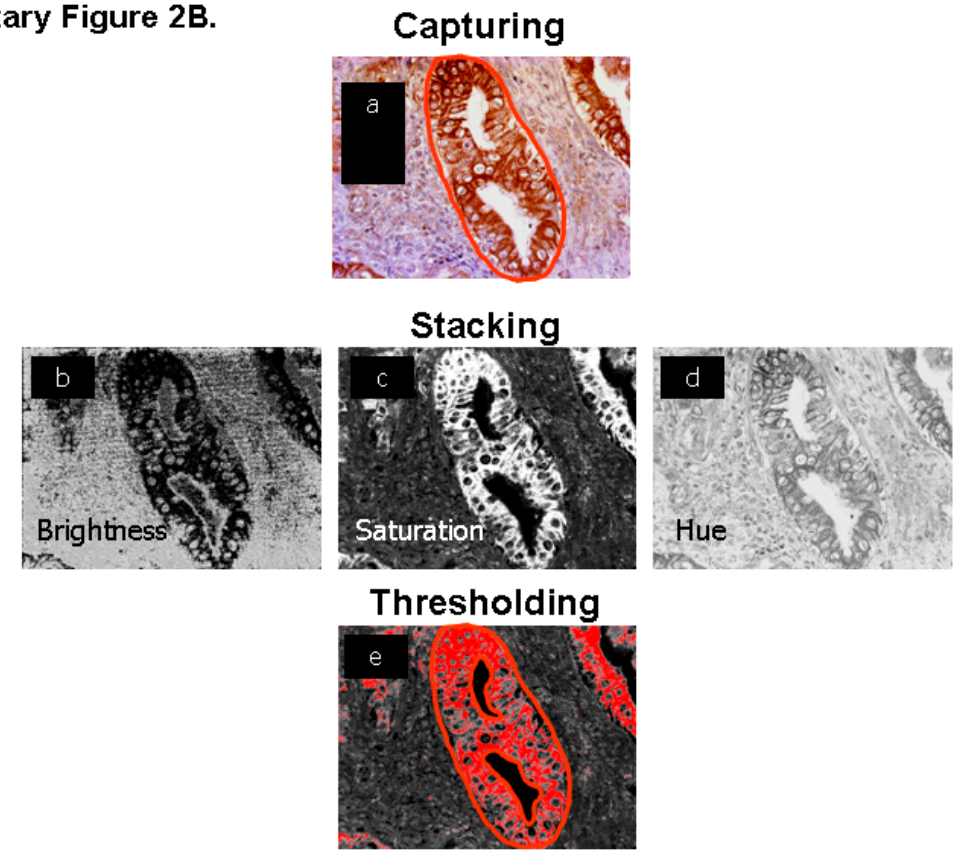
S1C. RAET1 Immunohistochemistry



Supplementary Figure 2A.



Supplementary Figure 2B.



Computer assisted calculations: stained epithelial area (according to selected threshold) and expressed as % of total epithelial area.