A specific DAMP profile identifies susceptibility to smoke induced airway inflammation

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Take home message:

A specific profile of DAMPs identifies susceptibility towards smoke induced neutrophilic airway inflammation in mice.

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To the editor:

Chronic obstructive pulmonary disease (COPD) is a major cause of morbidity and mortality, with a worldwide prevalence of 9-10%.[1] COPD is associated with chronic, neutrophilic inflammation in the lungs, causing destruction of lung parenchyma (emphysema) and/or remodeling of the airways with mucus hypersecretion (bronchitis).[2] Chronic exposure to noxious particles and gasses, e.g. cigarette smoke (CS) is the major risk factor for COPD, while susceptibility to the disease has a strong genetic component.[2] While the activity of the innate immune system increases with disease progression during early stages of COPD, the precise nature of the factors that trigger innate immune responses in COPD is currently unknown. Cell damage and death upon exposure to CS in COPD may induce the release of Damage Associated Molecular Patterns (DAMPs).[3] Elevated levels of several prototypic DAMPs, including High Mobility Group Box-1 (HMGB1), Heat Shock Proteins (HSPs) and S100A8 have been observed in bronchoalveolar fluid (BAL), serum and epithelial lining fluid (ELF) of COPD patients.[4 - 6] DAMPs activate cells of the innate immune system upon binding to Pattern Recognition Receptors (PRRs), such as Toll-Like Receptors (TLRs) and Receptor for Advanced Glycation End products (RAGE). Importantly, the *AGER* gene, which encodes the RAGE receptor, is a GWAS susceptibility gene for COPD.[7]

To date, studies investigating the role of DAMPs in COPD have focused on the analysis of a single DAMP. However, while each individual DAMP triggers specific receptors, the combination of DAMPs released upon CS-induced cell death likely determines the overall activation of the innate immune system.[8] Hence, we hypothesize that the signature of DAMPs released upon CS-exposure is critical in driving the early stages of lung inflammation in COPD, and as such constitutes a central component in the susceptibility for the disease. Therefore, we

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related the CS-induced DAMP profile to the susceptibility to develop neutrophilic airway inflammation *in vivo*. To this end, we used an experimental model where 30 inbred mouse strains were exposed to CS or air as a control (n=8 per group), for five consecutive days with two exposures per day and 1, 3 or 5 cigarettes per exposure *(Figure 1a)*. Two hours after the final CS or air exposure, BAL samples were collected to determine neutrophil counts. These 30 strains displayed the full range from susceptible to non-susceptible for CS-induced neutrophilic airway inflammation *(data not shown)*. For further analyses we selected four strains, with *BALB/cByJ* as the most susceptible strain, followed by *DBA/1J*, *C57BL/6J* and *C58/J*, the latter strain showing no induction of neutrophils after CS exposure at all *(Figure 1b)*. We determined the DAMP signature associated with susceptibility for CS-induced neutrophilic airway inflammation by measuring the BAL levels of a selected panel of six DAMPs consisting of Calreticulin (CRT), a Ca^{2+}-binding chaperone molecule that functions as a DAMP upon exposure on the cell membrane or release from secondary necrotic cells,[9] Galectin-3, a member of the β-galactoside-binding lectin protein family, which can activate leukocytes upon release from secondary necrotic cells, S100A8, a member of the Ca^{2+}-binding S100 protein family, which acts as a DAMP by binding RAGE and TLR-4,[3] dsDNA, which can bind to DNA sensors, including TLR-9,[10] HSP70, a prototypic heat-shock protein capable of binding TLR-2/4 and HMGB1, a nuclear non-histone chromatin-binding protein, that binds to TLR-2/4 and RAGE.[3] The magnitude of the CS-induced release varied widely between individual DAMPs, as well as between strains *(Figure 1c-h)*. BAL levels of CRT were increased upon CS-exposure in the highly susceptible *BALB/cByJ* strain, while no increase was observed in other strains *(Figure 1c)*. Galectin-3 release was increased in all mouse strains after CS exposure, with the strongest and significant effects in the two most susceptible strains *(Figure 1d)*. A strong and significant

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induction of S100A8 release was observed upon CS exposure in *BALB/cByJ* and *DBA/1J*, while no significant induction was observed in the less susceptible strains *(Figure 1e)*. For dsDNA, a strong induction was observed in *BALB/cByJ* mice, while a smaller increase was observed in *DBA/1J* and *C57BL/6J* mice and no increase was observed in the non-susceptible *C58/J* mice *(Figure 1f)*. HSP70 levels were increased in *BALB/cByJ*, although the levels in the air-exposed group were relatively low when compared to the intermediate susceptible strains *DBA/1J* and *C57BL/6J (Figure 1g)*. Finally, a relatively small but significant increase in HMGB1 levels was observed upon CS exposure compared to air exposure in *BALB/cByJ* and *C58/J*, but not in *DBA/1J* and *C57BL/6J* mice *(Figure 1h)*. Using the Spearman's ρ test, we observed a significant correlation (p≤0.01) between the increase in DAMP levels and neutrophil counts upon CS exposure for CRT (ρ=0.4518; p=0.0034), Galectin-3 (ρ=0.4415; p=0.0049), S100A8 (ρ=0.4390; p=0.0052), dsDNA (ρ=0.4813; p=0.0001) and HSP70 (ρ=0.4276; p=0.0059), but not for HMGB1 (ρ=0.3153; p=0.0475). To evaluate the difference in DAMP pattern released upon CS exposure in susceptible versus non-susceptible strains, we plotted the relative increase of all six DAMPs for each mouse strain *(Figure 1i)*. Here, a specific combination of DAMPs, i.e. CRT, Galectin-3, S100A8 and dsDNA showed an association of CS-induced release with increasing susceptibility for neutrophilic airway inflammation, while the CS-induced increase in HMGB1 and HSP70 did not associate with susceptibility.

Together, this study shows for the first time that genetic susceptibility for CS-induced neutrophilia is significantly associated with a specific profile of DAMPs released into the BAL. From the panel of selected DAMPs it appears that the combination of CRT, Galectin-3, S100A8 and dsDNA is a reliable marker for susceptibility towards CS-induced innate immune activation as measured by neutrophilic airway inflammation. Our data indicate that the increase of a

specific profile of DAMPs rather than that of HMGB1 alone, which has been implicated in COPD development,[6] may be an important determinant of the susceptibility towards neutrophilic airway inflammation upon cigarette smoking.

In future studies it will be of interest to confirm whether a similar DAMP release signature is present in COPD patients, whether this is related to the susceptibility of smoking individuals to develop COPD and whether this signature can be used for the early detection of susceptibility to or presence of COPD.

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Figure 1
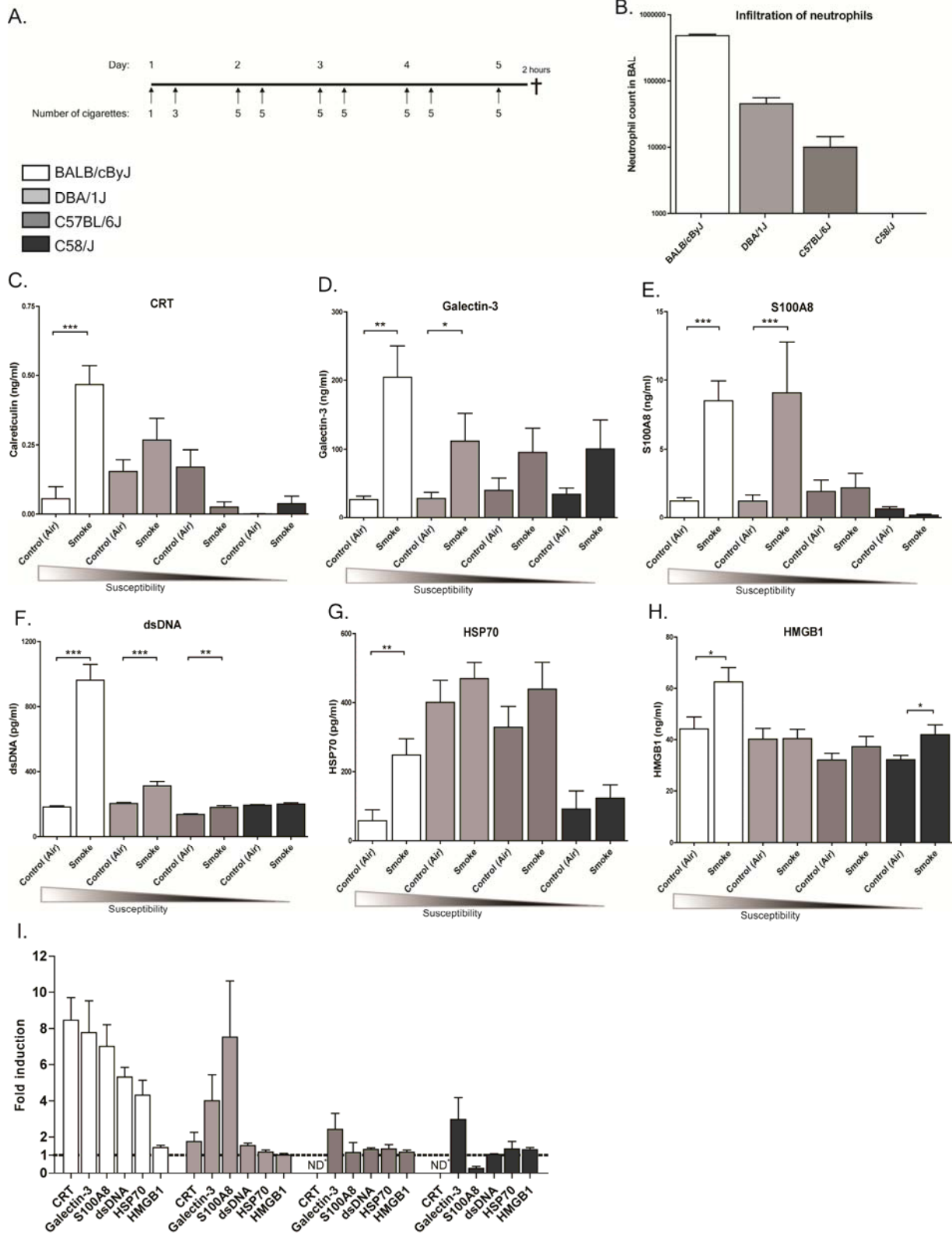
CS-induced increase in neutrophil counts and CS-induced DAMP release signature in BAL fluid of susceptible and non-susceptible mouse strains. **(A)** Schematic representation of the CS-exposure experiments in mice. **(B)** The numbers of neutrophils measured in BAL fluid two hours after the final CS- or control air-exposure. Values depict the average number of neutrophils of CS-exposed mice (n=8) minus the average number of neutrophils of control air-exposed mice (n=8). **(C/H)** CRT, Galectin-3, S100A8, dsDNA, HSP70 and HMGB1 levels (mean ± SEM) measured by commercial ELISA kits in BAL fluid of susceptible and non-susceptible mice two hours after the final CS exposure (n=8) or control air-exposure (n=8). Significance tested by Mann-Whitney-U test, * = $p<0.05$, ** = $p<0.01$, ***=$p<0.001$. **(I)** The DAMP signature visualized by the fold induction of CS-exposed mice compared to the average levels of the air-exposed control mice. ND* indicates that more than half of the values were below the detection limit of the ELISA.