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Title: Add necroptosis to your asthma action plan

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Running head: Add necroptosis to your asthma action plan

Necroptosis is one of several programmed lytic cell death processes for which key effector proteins have only been defined and experimentally dissected in the last decade. Unlike apoptotic cell death, where cellular contents undergo membrane-contained caspase-mediated disassembly,

necroptosis leads to the release of highly inflammatory intracellular components e.g. histones into the surrounding milieu[1].

Necroptotic cell death is thought to have originally evolved as a pathogen defence mechanism. As such, necroptosis can be induced by a series of cytokine- and pathogen detecting- receptors binding their appropriate ligands. Many of these signaling routes to necroptosis employ the apex kinase receptor-interacting serine/threonine protein kinase 1 (RIPK1). All upstream necroptotic signaling culminates in the activation of the effector protein Mixed Lineage Kinase domain-Like (MLKL) by its obligate activating kinase, receptor interacting protein kinase 3 (RIPK3). Following activation, MLKL associates with phospholipids to promote potassium efflux and eventual membrane bilayer destabilization, oncosis and cell death[1].

This lytic form of programmed cell death has been implicated in the etiology of a range of human pathologies. Specifically, its role in lung disease has recently come to the fore following prominent publications implicating necroptosis in lung epithelial cell damage and inflammatory neutrophil influx. Following a lethal dose of influenza A virus (IAV), the absence of necroptosis reduces mortality, protecting mice from lung epithelial cell damage and Neutrophil Extracellular TRAP (NET)-forming neutrophils influx[3]. Mice lacking necroptosis were also seen to be protected from airway remodelling and inflammation in a model of cigarette smoke-induced chronic obstructive pulmonary disease (COPD)[4]. These and other studies illustrate the contribution of necroptosis to the progression of lung pathologies and present an intriguing basis for recent work by Oikonomou *et al.*[2]

The relative contribution of necroptosis in specific tissues or cell types can be investigated using whole body or tissue-specific genetic modification of upstream and core necroptotic machinery. Genetic deletion of obligate necroptotic machinery (MLKL or RIPK3), or ablation of RIPK1 kinase activity, enables examination of necroptotic contribution through *elimination*. Conversely, genetic manipulation of certain upstream pathway components can be used to induce spontaneous *activation* of necroptosis. The most commonly used models of spontaneous necroptosis in mice are Fas-associated protein with death domain–(FADD) and Caspase-8 knockouts. In addition to their adapter and enzymatic roles in apoptosis pathways, FADD and

Caspase-8 also function as gate keepers to the necroptosis-inducing signalling platform comprising RIPK1 and RIPK3. The absence of FADD or Caspase-8 makes way for the unimpeded assembly of this necroptosis activation platform, the activation of MLKL and cell death. Whole body *FADD* or *Caspase-8* gene knockout mice die before or soon after birth. Limiting the ablation of FADD or Caspase-8 to specific tissues overcomes this impediment and this strategy has been used by the Pasparakis lab and others to demonstrate that dysregulated necroptosis in intestinal epithelial cells or keratinocytes alone are sufficient to induce the spontaneous development of inflammatory gut or skin damage[5-8].

Oikonomou *et al.* employ both airway epithelial cell (AEC)-specific ablation of FADD (necroptosis activation) and complementary whole body RIPK3 knockout, MLKL knockout or RIPK1 kinase activity knockin (necroptosis elimination) to dissect the role of this pathway in a model of asthma induced by house dust mite extract sensitization and challenge. Regulation of necroptosis in barrier tissues, such as skin and intestines, is known to be an important mechanism in maintaining immune homeostasis. Targeted gene deletion of FADD, Caspase-8, or RIPK1 sensitises epithelial cells to necroptosis. This strategy has been used by the Pasparakis lab and others to demonstrate that dysregulated necroptosis in intestinal epithelial cells or keratinocytes alone is sufficient to induce the spontaneous development of inflammatory gut or skin damage[5-8]. In barrier tissues like the gut or skin, where abundant microbially derived stimuli favour the expression and unmitigated assembly of the RIPK1-RIPK3, the specific genetic ablation of *FADD* alone is sufficient to trigger the cascade of necroptosis and inflammation. Oikonomou *et al.* report that mice with a FADD-deficient airway epithelium ($FADD^{AEC\text{-}KO}$) do not exhibit this same capacity for spontaneous necroptosis in the lung at steady state. They propose this may be due to a lower microbial load in the lung relative to the gut or skin. However, priming the airway epithelium with a physiologically-relevant stimulus in the form of house dust mite extract unmasks a capacity for an inflammatory response in the lungs of $FADD^{AEC\text{-}KO}$ mice that is over and above that of FADD-sufficient mice.

This augmented inflammatory response in $FADD^{AEC\text{-}KO}$ mice manifests in both enhanced inflammatory cytokines and immune cell infiltrates. Oikonomou *et al.* used genetic deletion of *Ripk3* to assess the contribution of FADD-deficient AEC necroptosis in house dust mite-induced

airway inflammation. FADD^{AEC-KO},*Ripk3*$^{-/-}$ mice exhibited limited immune cell infiltration and similar inflammatory cytokines levels when compared to control mice. Consistent with this finding, FADD^{AEC-KO},*Ripk1*^{D138N} kinase dead, and FADD^{AEC-KO}, *Mlkl*$^{-/-}$ mice also showed an attenuation of this house dust mite-induced pathology.

FADD^{AEC-KO} mice were protected from induced airway hyperresponsiveness, resembling mice that had not been treated with house dust-mite extract. Despite exaggerated inflammation induced by house dust mite extract, FADD^{AEC-KO} mice exhibited reduced mucus production stemming from reduced numbers of mucus-producing goblet cells in the lung relative to *fadd*$^{fl/fl}$ controls. This loss of goblet cells was not attributed to enhanced necroptosis in these mice, but to the expression of Cre-recombinase itself. While this confounding experimental artifact precluded examination of the full physiological impact of enhanced airway epithelial cell necroptosis in this model, it still stands that inflammation, mucus production and airway hyperresponsiveness certainly go hand-in-hand in real life. It is thus reasonable to predict that necroptosis-enhanced airway inflammation would also enhance mucus production and airway hyperresponsiveness. These carefully controlled experimental data reveal an important caveat to the future use of the Scgb1a1 (AEC-specific promoter)-Cre transgene for the study of airway epithelial cell death itself, with airway epithelial cell death unrelated to FADD activity being observed on the microscopic level. This study by Oikonomou *et al.* also lends further credence to the effectiveness of targeting downstream mucus hypersecretion for the amelioration of airway hyperresponsiveness[9], but certainly does not detract from the potential benefits of stopping the train further up the etiological line at necroptosis.

An enhanced propensity for airway epithelial cell necroptosis (through conditional FADD ablation) quantitatively intensifies inflammation in the lungs. While unable to directly demonstrate or quantify necroptotic epithelial cell death *in situ* in FADD^{AEC-KO} mice, the protein level of the MLKL-activating kinase, RIPK3, is clearly upregulated in the lungs. Detection of dead or near-dead necroptotic cells remains highly challenging in mouse tissues however, improvements in necroptosis-detecting tools and techniques will soon permit a more reliable detection in such scenarios[10].

The work of Oikonomou and colleagues prompts further questions regarding the potential role of necroptotic cell death in human asthma: Is the propensity for necroptosis what distinguishes patients with severe life-long chronic asthma from those with milder forms? Is it the activation and perpetuation of necroptosis that underpins the role of common respiratory viruses in inducing asthma? Is the enhanced propensity for airway epithelial cell necroptosis mediated purely by infection history and the adaptive immune response, and/or is there a direct genetic influence on propensity for necroptosis that contributes to asthma risk in humans? Etiology aside, this work provides an important precedent for further preclinical exploration of whether asthmatics could benefit from the emerging class of necroptosis-inhibitor drugs currently in development.

Conflict of interest

JMM and JMH contribute to a protect developing necroptosis inhibitors in collaboration with Anaxis Pty Ltd.

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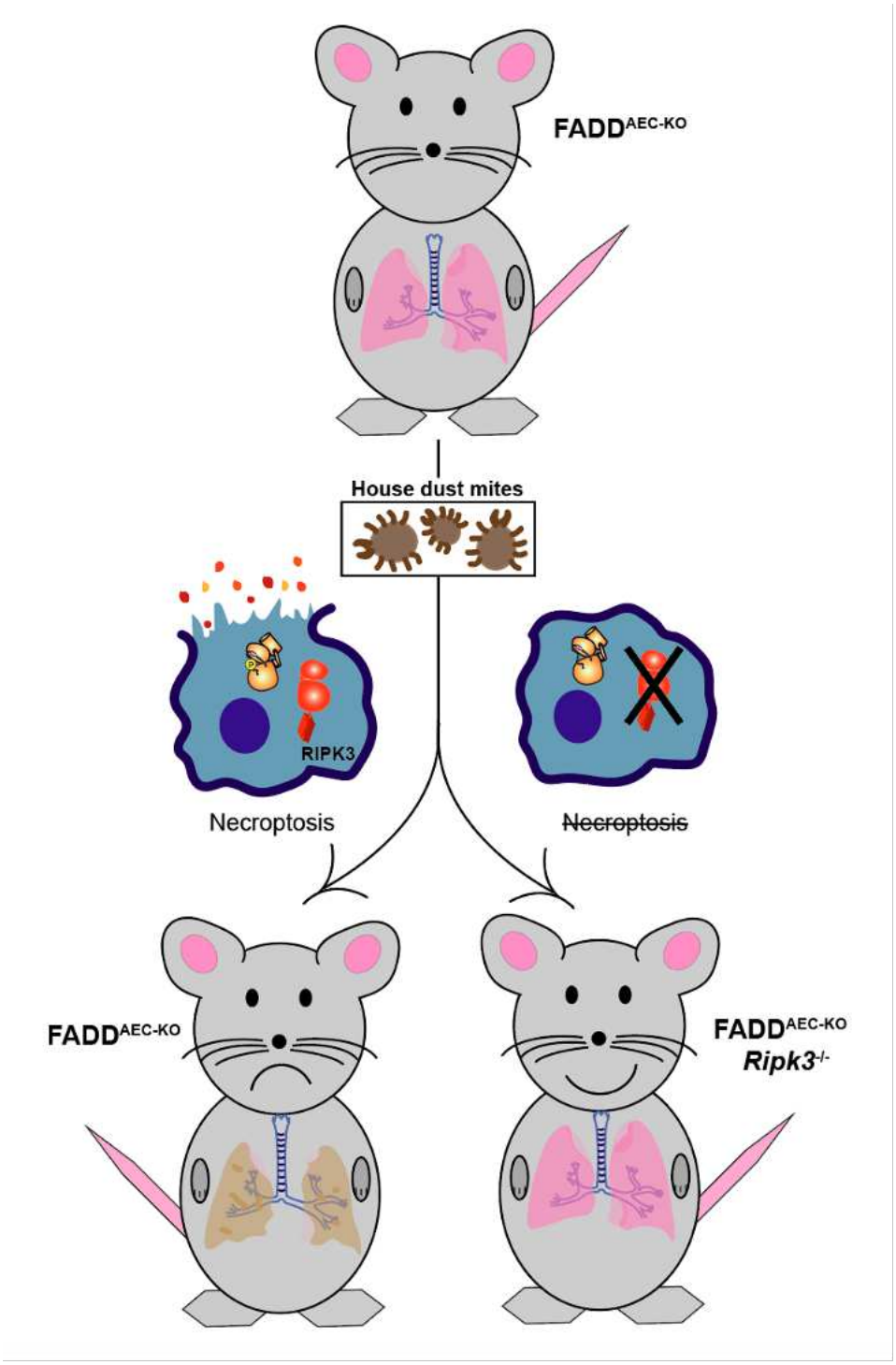
Figure 1. Relative to *fadd*$^{fl/fl}$ controls, FADD$^{AEC\text{-}KO}$ mice demonstrate exacerbated lung inflammation when exposed to house dust-mite extract. This exacerbation is dependent on an intact necroptosis pathway.



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