430

The IL-33-ST2 pathway drives mast cell-dependent antiviral responses

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Recent studies have highlighted the new role of IL-33 as an "alarmin" in host defense. IL-33 has shown protective effects against infection with some pathogens in various organs such as the gut and peritoneal cavity, however, it has not been reported in the skin. We have previously reported that mast cells were critically involved in host defense at herpes simplex virus 2 (HSV-2)-infected sites through TNF-α and IL-6 production using a murine model of HSV encephalitis. We also found that IL-33 derived from HSV-infected keratinocytes induced TNF-α and IL-6 production by bone marrow-derived MCs (BMMCs) *in vitro* assay. Although the importance of mast cell-derived TNF-α and IL-6 was shown, it was unknown whether the IL-33-ST2 pathway contributes to antiviral host defense. Immunohistochemical staining for IL-33 in HSV-infected Hacat cells as well as Pam 212 cells showed that IL-33 was selectively expressed on damaged cells and the frequency of IL-33-expressing cells was increased in a MOI dependent manner. We also detected IL-33 positive cells in the epidermis of the labial herpes patient. Furthermore, supernatants of HSV-infected epidermis from wild type mice, but not IL-33$^{-/-}$ mice induced TNF-α and IL-6 production by BMMCs, demonstrating that IL-33 released from HSV-infected epidermis activates mast cells to secrete these proinflammatory cytokines. In addition, IL-33 receptor ST2$^{-/-}$ mice exhibited increased clinical severity and mortality following cutaneous HSV-2 infection, indicating that the IL-33 contributes to protective antiviral responses. Taken together, our findings suggest the involvement of the IL-33/ST2 pathway on mast cells in antiviral innate immunity.

431

Myeloid-lineage-restricted NLRP3 mutations cause variant Schnitzler's syndrome

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Schnitzler's syndrome is a late-onset autoinflammatory syndrome of unknown etiology, with clinical evidence of involvement of the interleukin-1 beta (IL-1β) pathway. Based on its late onset and absence of familial clustering, the syndrome was considered acquired rather than genetic. Here, we addressed the possibility of a genetic cause in classical and variant Schnitzler's syndrome by means of deep sequencing. Eleven patients with classical or variant Schnitzler's syndrome were included, and for the functional studies eight age- and sex-matched controls. Whole exome sequencing of whole blood was followed by targeted resequencing of NLRP3 in whole blood, leukocyte subsets, keratinocytes and fibroblasts. Spontaneous production of IL-1β and IL-6 by peripheral blood mononuclear cells (PBMCs) was measured during symptomatic and treatment episodes, and compared to controls. Whole exome sequencing, followed by targeted resequencing revealed NLRP3 mutations in whole blood DNA from two patients with IgG variant Schnitzler's syndrome. Remarkably, these mutations were exclusively present in neutrophils (13%/32%) and monocytes (6.5%/29%). Patient PBMCs showed excessive spontaneous in-vitro IL-1β production, consistent with the clinical response to IL-1β blockade. Identification of myeloid-lineage-restricted mosaicism of NLRP3 places Schnitzler's syndrome in the spectrum of cryopyrin-associated periodic syndromes, and clarifies the puzzling pathophysiology of this elusive disease. This is the first report on myeloid-lineage-restricted mosaicism in a non-malignant disorder. Importantly, these findings suggest that other late-onset disorders may have a genetic (mosaic) basis.

432

C-terminal filaggrin-2 targets the bacterial replication: a new antimicrobial mode of action?

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The majority of cationic antimicrobial peptides is thought to kill bacteria by forming pores within their membranes. A C-terminal cationic fragment of filaggrin-2 (FLG24), a structural protein of human skin, has been found to be highly active against preferentially soil- and waterborne bacteria such as several *Pseudomonas* species and *Escherichia coli*. Transmission electron and confocal laser scanning microscopy analyses showed that FLG24 was not only able to induce bleb formation in *P. aeruginosa*, but also could be localized on or in those blebs. Both a lysozyme lysis assay as well as the SYTOX Green cell permeabilization assay did not reveal any pore formation in the bacterial membrane by FLG24. Strikingly, the latter indicated a competition in DNA-binding between the tested antimicrobial protein and the DNA-binding dye. showed a preferential binding of FLG2-4 to linear or open circular, relaxed bacterial DNA rather than supercoiled DNA. A relaxed DNA conformational state is essential for bacterial replication, suggesting that FLG24 can impede the bacterial replication process. In a simplified model for replication, FLG24 showed inhibiting effects on PCR reactions. The higher the FLG24 concentrations in the reactions, the weaker were the corresponding band intensities of PCR amplicons. Comparable amounts of hBD2 or BSA did not show any effects. Additionally, in an *in vivo* plasmid replication assay using chloramphenicol-treated *E. coli* harboring the plasmid pBR322, FLG24 was able to inhibit pBR322 replication in the translationally inactive *E. coli* compared to untreated bacteria. Taken together these results indicate that the antibacterial activity of FLG24 against *P. aeruginosa* and *E. coli* is based on the impairment of DNA replication, thereby inhibiting cell division and finally causing bacterial death.

433

A link between netting neutrophils and plasmacytoid dendritic cells in skin wound healing

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The mechanisms that regulate healing of the injured skin are not well understood. We have previously shown that plasmacytoid dendritic cells (pDCs) are normally absent from the healthy skin, but rapidly infiltrate both murine and human skin upon injury. Upon skin infiltration, pDCs sense nucleic acids via TLR7/TLR9 and are activated to produce type I IFN, a process that is crucial for reepithelization of skin wounds. However, the mechanisms that drive pDC recruitment and activation in injured skin remain unclear. We found that neutrophils, the first cells recruited to the injured skin, were directly responsible for pDC recruitement as neutrophil depletion abrogated pDC infiltration. In addition, we observed that infiltrating neutrophils release Neutrophil Extracellular Traps (NETs) composed of DNA filaments decorated with granule-derived proteins. Strikingly, blocking Netosis impaired pDC recruitment and subsequent activation, and injection of netting neutrophils in the dermis was sufficient for pDC infiltration and activation. Importantly, we found that neutrophil-driven pDC recruitment was dependent on CXCL10, constitutively expressed in neutrophils granules and released in the context of NETosis. Interestingly, CXCL10 was found to form complexes with DNA and to activate pDCs via TLR9, in addition to their chemotactic activity. Accordingly, when injected into skin, CXCL10 led to both pDCs infiltration and activation. Altogether, these data demonstrate that netting neutrophils control infiltration and activation of pDCs in the injured skin via CXCL10. Our findings provide new insights into the mechanisms of wound healing and open new avenues for potential therapeutic interventions to boost or inhibit wound repair in the skin.

434

Neutrophil extracellular trap-derived cathelicidin antimicrobial peptide: contribution to macrophage host responses

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Neutrophil extracellular traps (NETs), DNA-protein complexes that are released by dying neutrophils, trap pathogens. A characteristic feature of NETs is the expression of cathelicidin and other antimicrobial peptides. Accordingly, NETs are a source of self-DNA-antimicrobial peptide complexes. Phagocytes take up whole NETs, as well as DNA-antimicrobial peptide complexes. However, whether NETs and DNA-antimicrobial peptide complexes contribute to the cooperative antimicrobial responses by human neutrophils and macrophages against intracellular pathogens is not clear. Here, we investigate whether cathelicidin as part of NETs and/or as part of DNA-antimicrobial peptide complexes is taken up by human macrophages to provide direct antimicrobial activity against intracellular pathogens. To study cathelicidin as part of whole NETs we isolated NETs from activated primary human neutrophils. To study cathelicidin in the context of DNA-antimicrobial peptide complexes we generated DNA-cathelicidin complexes by incubating cathelicidin antimicrobial peptide with DNA. Subsequently, primary human M-CSF differentiated macrophages were incubated with whole NETs or DNA-cathelicidin complexes. Analyses by immunofluorescence showed that human macrophages internalized cathelicidin in whole NETs and cathelicidin complexed with DNA. Additionally, internalized cathelicidin partially colocalized with the lysosomal marker lysotracker, indicating that the internalized cathelicidin reaches lysosomal compartments. We are currently testing whether cathelicidin as part of whole NETs and DNA-cathelicidin complexes kills intracellular pathogens.

435

Post-septic immune-suppression following Gram positive sepsis is mediated by TLR dependent induction of myeloid derived suppressor cells

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Systemic Gram$^+$ bacterial infection (sepsis) possibly starting as skin infection is a leading cause of death among critically ill patients. Treatment of acute sepsis has been improved, but secondary infections due to post-septic immune suppression still have high mortality rates and underlying mechanisms are poorly understood. Therefore, we established a mouse model of Gram$^+$ sepsis. Mice were *iv.* infected with *Staphylococcus aureus* SA113. Weight and bacterial CFU (kidneys) indicating sepsis severity, immune cell populations and cytokines were determined at different time points. Post-septic immune status was assessed measuring a cutaneous T-cell mediated recall response to FITC (FITC-contact hypersensitivity; CHS). Indeed, immune suppression in these mice was evident as FITC-CHS was significantly reduced. Strikingly, we found a massive expansion of Gr1$^+$CD11b$^+$ so called myeloid derived suppressor cells (MDSC). We found induction of Ly6G$^-$CD11b$^+$ granulocytic MDSC (grMDSC) but stronger and more sustained upregulation of Ly6C$^+$Ly6G$^-$CD11b$^+$ monocytic MDSC (mMDSC) paralleled by reduced numbers of plasmacytoid, CD8$^-$ and activated dendritic cells (DC), suggesting inhibition of DC differentiation to be associated with MDSC accumulation. Importantly, mMDSC and grMDSC differed in their functional properties. Compared to grMDSC, mMDSC showed a more immature phenotype during early infection with increased differentiation capacity later (expression of CD115, MHCII and F4/80). Only mMDSC, but not grMDSC completely blocked T-cell activation *ex vivo,* depending on NO and oxygen radicals. Elimination of MDSC by anti-Gr1 antibody or blocking their differentiation by vitamin A abrogated post-septic immune suppression. MDSC induction after sepsis *in vivo* was clearly reduced in IL-6-ko mice and when mice deficient in TLR-adaptor protein MyD88 or in TLR2/3/4/7/9 were infected. In summary, we showed for the first time post-septic immune suppression after Gram$^+$ sepsis to be mediated by mMDSC induced via MyD88 and TLR signaling and dependent on IL-6.

436

MiR-146a is a potent regulator of TLR2- and IL-1β-induced inflammatory responses in keratinocytes

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Keratinocytes represent the first line of defense against pathogens in skin; they recognize pathogens by pattern-recognition receptors such as Toll-like receptors (TLRs) and initiate an inflammatory response. Although much is known about the innate immune functions of keratinocytes, the mechanisms that prevent excessive inflammation are not well understood. MicroRNAs are short, endogenous RNAs that regulate gene expression. Here, we investigated the role of miR-146a during the innate immune response of keratinocytes. Stimulation of primary human keratinocytes with Toll-like receptor 2 (TLR2) ligands or IL-1β resulted in an NF-κB and MAPK-dependent induction of miR-146a expression. Surprisingly, a single stimulation with TLR2 ligands or IL-1β resulted in long-lasting up-regulation of miR-146a, contrasting the rapid and transient expression of inflammatory mediators (e.g. IL-8, CCL20, TNF-α). Overexpression of miR-146a suppressed the production of IL-8, CCL20 and TNF-α, which functionally suppressed the chemotactic attraction of neutrophils by keratinocytes. Inhibition of endogenous miR-146a induced the production of inflammatory mediators even in resting, non-stimulated keratinocytes, and potentiated the effect of TLR2-stimulation. Transcriptomic profiling revealed that miR-146a suppresses the expression of a large number of immune-related genes in keratinocytes, including cytokines, chemokines, and components of immune-related signal transduction pathways. Mechanistically, miR-146a down-regulated IRAK1 and TRAF6, two key adapter molecules downstream of TLR and IL-1 signaling, and suppressed NF-κB-promoter binding activity, as shown by promoter-luciferase experiments. Together, these data identify miR-146a as a novel regulatory element in keratinocyte innate immunity, which prevents the production of inflammatory mediators under homeostatic conditions and serves as a potent negative feedback regulator after TLR and IL-1 stimulation.

437

Effects of *S.aureus* and *S.epidermidis* from atopic children microbiota on TCD4+ cell-subsets

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Atopic dermatitis (AD) has been recently revisited with respect to composition of skin microbiota. Interactions between the immune system and pathogen or commensal skin bacteria is of major interest to the pathophysiology of AD. To assess the role of skin microbiota in TCD4+ polarization, we designed a cohort of children with AD sensitized to HDM allergens (anti-Derp1 sIgE) (N=22, 22.9 mo, mean SCORAD 26) and its non-AD counterpart (N=17, 24.9 mo). We analyzed the microbiota in inflamed areas by culture followed by MALDI-TOF MS identification and the phenotype of peripheral TCD4+ to Derp1 by ELISPOT (IL-4, IFN-γ). Shannon diversity was similar between AD (2.75) and non-AD (2.44) but *S.aureus* was identified only in AD (32% *vs* 0%). ELISPOT showed a prevalence of IL-4 *vs* IFN-γ-producing T cells in AD, a Th2 feature confirmed by qRT-PCR on inflamed skin samples by upregulation of GATA3, IL-13, CCL22 and CCL17. To understand how *S.aureus* (*SA*) and *S.epidermidis* (*SE*) could influence TCD4 polarization, monocyte-derived dendritic cells (MoDC) from blood donors were stimulated with bacteria secretion products prepared from AD and non-AD. *SA*-conditioned MoDC secreted only IFN-γ while *SE*-MoDC produced only IL-10. HLA-DR expression increased on *SA*-MoDC but decreased on *SE*-MoDC. Treatment with a mixture of *SE* and *SA*, blunted the effect of *SA*-induced IFN-γ. Overall, our data suggest that *SA*-secreted products, including superantigen (SAg), induced IFN-γ secretion by MoDC, increasing HLA-DR expression thus providing more binding sites for SAg to exacerbate proliferation of resident Th2. On the opposite, besides its tolerogenic properties, induction of IL-10 secretion by *SE* could block SAg-mediated effects of *SA*.

438

Anti-TNF promotes type I interferon-driven psoriasis-like skin inflammation

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Acute flares of psoriasis are a well-known side-effect of anti-TNF therapy, although the pathogenic mechanism has remained elusive. We analyzed a series of 33 cases of paradoxical psoriasis induced by all anti-TNF agents available. Underlying diseases, clinical presentation and histological patterns varied considerably among patients. However, we found a striking, uniform, and selective upregulation of type I interferons (IFN) in all skin samples of paradoxical psoriasis as compared to classic chronic psoriasis. This overexpression was paralleled by the accumulation of significantly higher numbers of plasmacytoid dendritic cells (pDCs) within the dermal infiltrate of paradoxical psoriasis. Accumulation of dermal pDCs correlated significantly with expression of type I interferons. Furthermore, anti-TNF increased IFN-alpha production by activated pDCs in vitro, and in a skin injury mouse model, TNF inhibition led to an increased and prolonged accumulation of pDCs and IFN-alpha expression within the skin. Intriguingly, in the mouse model, anti-TNF treatment induced a psoriasis-like skin inflammation with acanthosis, epidermal hyperproliferation, and aberrant keratinocyte differentiation one week after skin injury. The development of a psoriatic phenotype was critically dependent on type I IFN as blockage of IFN signaling completely abrogated the effect of TNF inhibition. These data suggest the yin-yang model, in which TNF controls IFN-alpha expression by pDCs and, that acute psoriasis-like skin inflammation results from persistent unabated IFN-alpha production by pDCs, completely independent of underlying disease.

439

Role of neutrophils in the pathogenesis of imiquimod-induced psoriasis-like skin lesions

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Psoriasis is a common cutaneous disorder characterized by chronic inflammation and erythematous plaques. The thickening of the skin has been denoted by enhanced epidermal proliferation, which is critically dependent on IL-23-IL-17/22 axis. However, we also noted significant thickening of the dermis both in the lesional skin of psoriasis patients and in the psoriasis-like skin lesions induced by topical application of imiquimod (IMQ), a TLR7/8 agonist, on murine skin, indicating the possible role of dermal fibroblasts in the development of psoriatic skin lesions. However, it remains unclear how fibroblasts are functionally regulated in the psoriatic skin lesions. Here, we show that skin-infiltrating neutrophils regulate fibroblast functions including collagen production in IMQ-induced psoriasis-like skin inflammation. Depletion of neutrophils by injecting anti-Gr-1 antibody significantly attenuated the IMQ-induced thickening of the dermis but not of the epidermis of lesional skin. Attenuation of dermal thickening was associated with reduced skin collagen content. In addition, dermal fibroblasts isolated from IMQ-induced skin lesions in anti-Gr-1-treated mice showed altered expression of genes possibly associated with psoriatic skin inflammation, compared with control-treated mice. However, IMQ-induced expressions of *Il23*, *Il17 and Il22* mRNA in lesional skin were not affected by neutrophil depletion. Collectively, these results suggest that neutrophils regulate dermal fibroblast functions, including collagen production, independently of IL-23-IL-17/22 axis. We further investigated the direct effects of neutrophils on fibroblast function by assessing gene expression profiles of mouse embryonic fibroblasts cultured with or without neutrophils. Our findings provide insight into the interaction between immune system and dermal fibroblasts in inflammatory skin diseases.

440

Activation of Toll-like receptors alters microRNA expression in keratinocytes

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Keratinocytes in the skin represent the first line of defense against pathogens. They recognize microorganisms by pattern recognition receptors, among others Toll-like receptors (TLRs), and initiate a cascade of signaling events that lead to an inflammatory response. The role of miRNAs in this process is as yet unexplored. Here we aimed to identify miRNAs that are regulated in keratinocytes upon treatment with different TLR ligands. MicroRNA expression profiling of keratinocytes treated with zymosan (a ligand of TLR2), flagellin (a ligand of TLR5) and the viral RNA-analogue poly(I:C) (a ligand of TLR3), identified specific sets of miRNAs significantly regulated by these TLR ligands. MiRNAs were regulated in a concentration-dependent and TLR-ligand-specific manner. One miRNA was outstanding regarding its expression as it was strongly induced by all treatments in most time points: miR-146a. Our results provide a basis for further studies to unveil the role of miRNAs in keratinocytes and may lead to a better understanding of skin innate immune responses, which are often altered in inflammatory skin diseases.

441

Adipose tissue response to IL-17 combined with TNF-α exposure

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Psoriasis is a chronic inflammatory skin disease characterized by the activation of antigen presenting cells and T cells, inducing a tissue response. As occurs with other immune-mediated inflammatory diseases, psoriasis is associated with a higher risk of developing "systemic" comorbidities including cardiovascular diseases, dismetabolic disorders, hypertension, and depression. Pathogenically, psoriasis is characterized by complex pathogenic mechanisms, involving multiple cytokines (i.e., IL-23, IL-17, TNF-α, IFN-γ), chemokines (CCL20), mitogens, and other pro-inflammatory mediators, which may participate to psoriasis skin inflammation and to the pathogenesis of its comorbidities. Among psoriasis-signature cytokines, IL-17 and TNF-α are recognized as central to the key pathogenic inflammatory circuits. Their in vitro effects have been tested in normal human keratinocytes, but no data are available regarding their combined effect on normal adipose tissue. Thus, we treated whole human adipose tissue with IL-17 or TNF-α alone, and with the combination of both cytokines, investigating their effects on gene expression by RT-PCR and microarray analysis. We seek to determine: *(i)* the potential contribution of adipocytes as source of inflammatory products that are implicated in psoriasis inflammation, and *(ii)* the pathogenic role of psoriasis-signature cytokines in the development and progression of adipocyte-related disorders. We firstly tested few key inflammatory genes, namely CCL20, IL-23, IL-8, IL-6, which are known to be involved in the psoriasis pathogenic circuits. We observed that IL-17 and TNF-α induced an additive or synergistic effect on adipose tissue gene expression. These preliminary results suggest a relevant role of the adipose tissue in the pathogenesis of psoriasis as source of pro-inflammatory mediators. We will perform a comprehensive gene expression analysis investigating the overall alterations in human adipose tissue induced by cytokine stimulation.