Review

Defensins: Potential Effectors in Autoimmune Rheumatic Disorders

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Abstract: Defensins are small cationic peptides with antimicrobial properties. They constitute a highly conserved innate immune defense mechanism across species. Based on the arrangement of disulfide-bonds, α- and β-defensins are distinguished in humans. Both types of defensin comprise several distinct molecules that are preferentially expressed at epithelial surfaces and in blood cells. In the last decade, multiple immunomodulatory functions of defensins have been recognized, including chemotactic activity, the promotion of antigen presentation, and modulations of proinflammatory cytokine secretion. These findings suggested a role for defensins not only as a first line of defense, but also as connectors of innate and adaptive immune responses. Recently, increasingly accumulating evidence has indicated that defensins may also be involved in the pathogenesis of autoimmune rheumatic disorders such as systemic lupus erythematosus and rheumatoid arthritis. The current review summarizes the data connecting defensins to autoimmunity.

Keywords: defensin; antimicrobial peptide; rheumatology; autoimmune disease

1. Review

1.1. Introduction to Defensins

The generation of peptides with antimicrobial properties is a highly conserved mechanism of innate immune defense [1,2]. Across species, over 800 distinct molecules have been included into this group [3,4]. Amongst them, the family of defensins have been identified in nearly all species from plants over insects to higher mammals [2]. Defensins are cationic peptides which contain 12–50 amino acids in length and primarily form β-sheets [1]. In humans, α- and β-defensins are distinguished based on the arrangement of disulfid bonds between their 6 cysteine-residues. While α-defensins display a 1–6, 2–4, 3–5 pattern, β-defensins show a 1–5, 2–4, 3–6 arrangement [5]. So far, 6 α-defensins and 4 β-defensins have been differentially characterized, but computational genomic analyses have revealed that 28 potential β-defensin genes are present in the human genome [6]. The expression of defensins varies in different tissues, which is thought to reflect microbial challenges at the expression sites [1,2,7]. Human α-defensin 1–4 (commonly referred to as human neutrophil peptides (HNP)) are the major protein species within azurophilic granules of neutrophils, comprising up to 70% of their content [8], while human defensins (hD) 5 and 6 are almost exclusively found in paneth cells of the small intestine [7,9]. Human β-defensins (hBD) are predominantly expressed at epithelial surfaces, but hBD1-4 were also detected in various blood-cells such as monocytes, macrophages and dendritic cells [2,3]. While hBD1 seems to be a constitutively expressed defensin, most other defensins are consistently upregulated by proinflammatory stimuli such as lipopolysaccharides, tumor necrosis factor α (TNFα), and Interleukin-1b (IL1β) [2]. Signal transduction mechanisms were reported to predominantly depend upon nuclear factor κB [3]. All defensins exert antimicrobial effects against a wide range of pathogens including bacteria, fungi, viruses and parasites [3,4]. Their positive charge facilitates association to membranes followed by the generation of pores that disrupt electrolyte homeostasis, thereby leading to cell death [4]. The antimicrobial activity to a certain pathogen varies with each individual defensin [3,4]. Their potent antimicrobial functions and their highly conserved structure across species suggest that the role for defensins initially consisted in direct pathogenic activity. In the evolutionary process, however, this group of peptides has developed additional, *i.e.* immunomodulatory, functions, a process also known as protein promiscuity [10]. While this review focuses on the potential role of defensins in autoimmune rheumatic disorders, excellent previous reviews cover defensin biology, antimicrobial properties and highlight their various impacts on the immune system [1,2,5,8,11].

1.2. Immunomodulatory Effect of Defensins

Defensins modulate both adaptive and innate immune mechanisms by targeting a wide array of cells (Table 1). For instance, monocytes (Mo), macrophages (MΦ) and immature dendritic cells (iDC) are attracted to sites of inflammation by defensins [12-16], where the secretion of proinflammatory cytokines by these cells is modulated, although there is still dispute to whether HNP1-3 are predominantly pro- [17,18] or anti-inflammatory [19-21]. Moreover, defensins have an impact on antigen presentation by influencing processes of differentiation: HNP1-3 was shown to inhibit M-CSF induced macrophage differentiation of monocytes [22], while maturation of iDC with upregulation of the co-stimulatory molecules CD80 and CD86 as well as MHCII is promoted by hBD3 [5,12,23].

T-cells that interact with antigen-presenting cells to promote adaptive immune responses [24] are attracted by defensins [13,15,16,25]. Costimulatory molecules on T-cells (*i.e.*, CD28) are increased in response to HNP1-3 [26,27] and the secretion of various proinflammatory peptides is promoted [26,28]. In accordance with the notion that defensins are capable of inducing the maturation/activation of antigen-presenting cells, enhanced antigen-specific humoral and cellular immune responses were found when antigen was administered in combination with HNP or mouse-homologues of beta-defensins [12,26]. Furthermore, defensins were demonstrated to inhibit the classic and lectin pathway of complement activation by binding C1q and mannose-binding lectin [29,30], they increase proliferation, migration, and proinflammatory cytokine secretion in keratinocytes [31-33], and enhance antigen-presentation and proinflammatory cytokine secretion in lung epithelial cells [27,34-36].

Table 1. Immunomodulatory functions of human defensins.

Target	Immunomodulatory Action	Defensins	Ref.
Mo	increase of TNFα and IL1β release from stimulated cells	HNP1-3	[17]
	inhibition of IL1β release from stimulated cells	hD5, HNP1	[21]
	inhibition of M-CSF induced macrophage differentiation	HNP1-3	[22]
	chemotaxis	hBD1-4, HNP1-3	[37-40]
MΦ	inhibition of TNFα, IL8, IL6, IL1β and NO release	HNP1-3, hBD3	[19,20]
	increase of TNFα and IFNγ release	HNP1-3	[18]
	enhancement of phagocytosis	HNP1-3	[18]
	chemotaxis	hBD1-4, HNP1, -3, hD5	[13,14,39]
iDC	maturation (upregulation of CD40, CD80, CD86, MHCII)	hBD3	[5,12,23]
	chemotaxis	hBD2-3, HNP	[12,15,16,39]
granulocytes	Anti-chemotactic	HNP1	[41]
	Chemotactic for TNFα activated cells	hBD2	[31]
	inhibition of apoptosis	hBD3	[42]
mast cells	increase of mast cell degranulation	hBD2	[43,44]
	chemotaxis	HNP1-3, hD5, hBD1-4	[14,44]
T-cells	Increase of IFNγ, TNFα, IL10, IL1β, IL6, and IL22, inhibition of IL17 secretion	hBD2	[26,28]
	increase of CD28, CD11a, decrease/increase of CTLA4	HNP	[26,27]
lung epithelial cells	increase apoptosis dose dependently	HNP1, hBD2	[35,36,45]
	increase IL8, IL1β, MCP1	HNP1	[34-36]
	enhancing antigen-presentation (upregulation of CD54, CD80, CD86)	HNP1-3	[27]
endothelial cells	stimulation of angioneogenesis	hBD2	[46]
	COX2 and endothelin-1 production increased	HNP	[47]
	inhibition of TNFα-mediated upregulation of VCAM1 > ICAM1	HNP	[17]
keratinocytes	increase of IL1β, IL6, IL10, IP10, MCP1, MIP3α, RANTES, IL18	hBD2-4	[31-33]
	increase of migration and proliferation	hBD2-4	[31]
fibroblasts/epithelia	increase in proliferation, dose-dependent cytotoxicity	HNP1	[48]
complement	inhibition of classical and lectin pathway by binding to C1q and MBL	HNP1	[29,30]
miscellaneous	inhibition of T-cell response by competetive binding to MHC-molecules	HNP1-2	[49]
	enhancing antigen-specific humoral and cellular immune response	HNP	[12,26]

Legend: Monocyte (Mo), Macrophage (MΦ), Tumor necrosis factor α (TNFα), immature dendritic cells (iDC), Interleukin (IL), macrophage-colony-stimulating-factor (M-CSF), Interferon (IFN), Cluster of differentiation (CD), Major Histocompatibility Complex (MHC), Cytotoxic T-Lymphocyte Antigen (CTLA), Monocyte Chemotactic Protein (MCP), Cyclooxygenase (COX), Vascular Cell Adhesion Molecule (VCAM), Inter-Cellular Adhesion Molecule (ICAM), Macrophage inflammatory protein (MIP), Regulated upon Activation, Normal T-cell Expressed, and presumably Secreted (RANTES), mannose binding lectin (MBL).

Our current understanding of inciting events in autoimmune disorders such as systemic lupus erythematosus or rheumatoid arthritis crucially involves presentation of autoantigens by antigen-presenting cells to T-cells which subsequently promote adaptive immune responses including the generation of autoantibodies. Furthermore, an important role is attributed to a proinflammatory cytokine milieu [50,51]. The above delineated actions of defensins on these processes render the hypothesis of their involvement in autoimmune processes plausible and were a rationale to explore their role in autoimmune rheumatic disorders. Furthermore, defensins may provide a link between adaptive immune responses to autoantigens and innate immune mechanisms in autoimmune disorders.

1.3. Rheumatoid Arthritis (RA)

RA is the most common inflammatory rheumatic disorder, affecting approximately 1% of the population and ultimately leading to destruction of joints by a chronic inflammation which affects cartilage and bone [51]. In patients with RA and other inflammatory arthritic disorders, HNP1-3 was identified in synovial fluid by spectrometry. HNP1-3 discriminated synovial fluid from patients with RA and osteoarthritis (OA) with a sensitivity of around 70% and a specificity of 56%. According to receiver operating characteristics analysis, the discriminative value was moderate (area under the curve of 66–70%) [52]. Importantly, HNP-levels in the synovial fluid were clearly higher in RA patients with erosive disease as opposed to non erosive disease in one study (4,374 ± 805 *vs.* 2,391 ± 946 ng/mL) [53]. Besides further suggesting a potential involvement in the pathogenesis, these findings indicate that HNP1-3 in the synovial fluid may also function as a biomarker for RA [52,53]. Interestingly, there was no correlation between synovial neutrophil counts and HNP1-3-levels in RA patients [52]. This was speculated to be attributable to an increased load of HNP1-3 in azurophilic granules, increased degranulation and apoptosis of neutrophils in synovial fluid, or to increased production of HNP1-3 by sources other than neutrophils within the RA joint [53]. Indeed, HNP1-3 is present in the synovial membrane [54,55], and was immunhistochemically located to neutrophils in, or close to the synovial layer [55]. While no significant differences in immunohistochemical stains for HNP1-3 were detected between RA and OA-synovial membranes, explanted synovial tissue of RA-patients secreted more HNP1-3 [55]. Thus, besides neutrophils in the synovial fluid, neutrophils within the synovial membrane contribute to HNP1-3 production in RA joints. Data concerning the regulation of HNP1-3 in synovial tissue is scarce. In RA synovial membranes, in contrast to tissues from OA patients, the secretion of HNP1-3 was not lowered by incubation with the proinflammatory TNFα. This was speculated to be attributable to TNFα-receptor desensitization by already high levels of TNFα present in RA. So far, there is no proof of this concept, however [55]. Although HNP1-3 levels in the synovial fluid of RA patients are clearly superior to their concentration in blood [53], HNP1-3 were identified to be upregulated in peripheral blood mononuclear cells of RA patients by mRNA micro-array analysis when compared to healthy controls [56]. This increases the attractiveness of HNP1-3 as a biomarker for RA, because peripheral blood is more easily accessible than synovial fluid. However, more studies are needed in order to establish the usefulness of HNP1-3 in distinguishing RA from relevant differential diagnoses, or to demonstrate their usefulness as a follow-up parameter to assess disease activity.

Besides defensins from neutrophils, β-defensins in joints were also described. HBD1 and hBD2 are expressed in articular cartilage and synovial membranes of joints [54,57,58]. The expression of hBD2

in chondrocytes can be induced by the proinflammatory cytokines TNFα, IL6, IL1β [58,59], which have all been strongly implied in the pathogenesis of RA [60]. In a study employing RT-PCR and immunohistochemistry of synovial membranes from 10 patients each with RA, OA, pyogenic arthritis or healthy controls, there was a tendency towards a preferential detection of hBD2 in RA [54]. Similar data exist for hBD3: although largely absent in healthy articular cartilage, hBD3 can be found in OA or RA [54,61]. Proinflammatory cytokines such as TNFα and IL1β are potential stimuli for hBD3 induction [61]. Interestingly, besides its immunomodulatory properties, hBD3 may also have a role in cartilage destruction present in RA, because it activates matrix metalloproteinases which degrade the extracellular matrix of cartilage [61,62]. Thus, hBD3 is a potential candidate for therapeutical interventions to inhibit cartilage destruction and further studies are clearly warranted.

1.4. Systemic Lupus Erythematosus (SLE)

SLE is a multisystem inflammatory autoimmune disorder characterized by autoantibodies to nuclear antigens and causing a wide variety of organ damage including glomerulonephritis and arthritis [50]. Based on the current understanding of SLE, there is reasonable ground to suspect a role for defensins in SLE pathogenesis: presentation of autoantigens by antigen-presenting cells and epithelial cells to autoreactive T-cells, apoptosis, and complement deficiency are major mechanisms driving the disease [50,63,64].

Micro-array experiments suggested an increased HNP3-gene expression in peripheral blood of SLE patients as compared to healthy controls [65,66]. This finding was later confirmed by demonstrating elevated peptide levels in two SLE patient cohorts [67,68]. Furthermore, although the subgroup analyses contained only few patients, some correlation to clinical data was suggested, including higher levels in patients with lupus nephritis, skin rash, or arthritis [67]. Moreover, disease activity measured by the SLEDAI (Systemic Lupus Erythematosus Disease Activity Index) correlated to HNP-levels on an individual patient basis [67]. Correlations to disease activity parameters, *i.e.*, SLEDAI and antibody-concentration to double-stranded DNA, were also observed for hBD2 [68]. Besides elevated HNP-protein levels, antibodies against HNP have been found in SLE patient sera and were elevated when compared to healthy controls. Patients with active disease (SLEDAI ≥ 6) had higher values than those in remission. In the same cohort, serial HNP-antibodies were available from two patients and were demonstrated to decline after initiation of effective therapy with glucocorticosteroids [69]. The role of these antibodies in SLE is not clear and warrants further attention. Interestingly, no β-defensin antibodies were detected in a search of over 300 patients with connective tissue disorders including SLE [70].

SLE commonly also involves the skin and leads to a variety of rashes. These manifestations may also occur separately from systemic signs of the disease [71]. Skin infections are rarely observed in patients with cutaneous lupus, even though inflammatory skin lesions may be extensive [72]. This prompted Kreuter *et al.* to analyze if an increase in defensin-expression may be found [72]. RT-PCR revealed that hBD1 and hBD3 were found to be significantly higher in subacute cutaneous lupus erythematodes (SCLE) than in clinically distinct subsets of the disease termed Lupus erythematosus tumidus (LET), discoid Lupus erythematosus (DLE), and in healthy controls. HBD2 was increased in SCLE and DLE when compared to healthy controls. Immunohistochemical analysis confirmed that

hBD1-3 were predominantly expressed in SCLE skin. The upregulation of defensins may reflect the cytokine milieu of cutaneuous lupus with the presence of TNFα and IFNγ which upregulate hBD2 and 3-expression [72,73]. Alternatively, the distinct expression patterns in SCLE and DLE found in this study were interpreted as hints for a pathogenetical involvement of defensins in cutaneous lupus [72].

1.5. Sjögren's Syndrome (SS)

SS is an autoimmune disorder which features lymphocytic infiltration of various organs, especially exocrine glands [74]. In minor salivary glands, the expression of hBD1 and especially hBD2 was reduced when compared to control tissues as assessed by immunohistochemistry. This was surprising, because the authors initially hypothesized that increased expression of defensins would be found due to the inflammatory processes at salivary glands. It was speculated that a reduction in defensin expression might be found even before infiltration of salivary glands with lymphocytes [75], which in turn might suggest that defensin-deficiency might contribute to SS pathogenesis.

1.6. Wegener's Granulomatosis (WG)

WG (Granulomatosis with Polyangiitis [76]) is a vasculitis of small to medium sized vessels characterized by the formation of destructive granulomas in the respiratory tract and vasculitis affecting various organs such as the kidneys leading to rapid progressive glomerulonephritis [77]. Antineutrophil cytoplasmatic antibodies (ANCA) are implied in the pathogenesis of WG and can be found in 90% of patients with generalized disease [77]. Cross-linking of Fcγ-receptors by these ANCA leads to activation of neutrophils, degranulation, and HNP-release [78]. The chemotactic properties of HNPs on various leukocytes (Table 1) may then contribute to the formation of typical granulomas [78]. This model is further supported by the finding that increased serum levels of HNPs can indeed be found in WG [79]. Moreover, four patients with serious organ involvement requiring treatment with cyclophosphamid had higher HNP concentrations than WG patients without cyclophosphamid treatment. Although hampered by small patient number, this finding hints at increased values in more severe disease states. Besides HNP, hBD2, but not hBD3 was found to be increased in WG and was especially high in four patients with meningeal granulomas [79].

1.7. Behçet Disease (BD)

BD is a vasculitis which involves arteries and veins of all sizes, typically leads to mucocutaneous ulcerations and is geographically distributed along the silk route [80]. HNP1-mRNA was identified to be overexpressed in peripheral blood mononuclear cells of BD patients and the HNP1-level in blood of patients was increased when compared to healthy controls. Especially high values were found in patients with arthritis or active disease [81]. Expressional changes, but also copy number variations of individual genes (CNV) may contribute to varying defensin levels [82]. In one study, CNV of the hBD2-gene in 197 patients with BD were compared to matched controls. Although there was a tendency towards lower CN in patients, the difference was not statistically significant and there was no correlation to clinical data. The authors thus concluded that CNV of the hBD2-gene do not contribute

to the pathogenesis of BD [83]. This does not exclude the contribution of hBD2 in BD altogether, however, because CNV are not the only determinate for levels of biologically active peptides [82].

1.8. Microscopic Polyangiitis (MPA)

MPA is a necrotizing vasculitis of small vessels with predominantly pulmonary and renal involvement. Antineutrophil cytoplasmatic antibodies with perinuclear staining in indirect immunofluorenscence assays is noted in the majority of patients [84]. The target antigen of these antibodies most commonly is myeloperoxidase. Another potential target for ANCAs are HNPs because they are predominantly present in neutrophil granulocytes. Indeed, elevated HNP-ANCA were described in MPA [69]. The function of these antibodies is unknown, however. Conversely, no ANCA to hBD1 or hBD2 were detected in sera of patients with ANCA-associated vasculitis [70], reflecting that alpha but not beta defensins are preferentially expressed by neutropils.

1.9. Sarcoidosis

Sarcoidosis is a systemic disorder characterized by a non-caseating granulomatous inflammation which can affect virtually any organ, including lung, joints and skin [85]. In broncheoalveolar lavage fluid of sarcoidosis patients, HNP-levels were increased approximately 10-fold. Moreover, patients with stage II or III disease had higher values than patients suffering from stage I [86]. Interestingly, alveolar macrophages of sarcoidosis patients released more TNFα than alveolar macrophages of control patients when stimulated with HNP. The authors thus concluded that HNP may have a role in sustaining pulmonary inflammation in sarcoidosis [86].

1.10. Cardiovascular Morbidity in Rheumatic Diseases and Defensins

Patients with autoimmune disorders are known to suffer from an increased cardiovascular morbidity which is not completely attributable to a higher burden of cardiovascular risk factors. Disease inherent mechanisms and a chronic systemic inflammation are thought to contribute to the observed effects [87]. In this context, it is interesting to note that defensins have recently been linked to atherosclerosis. HBD2 was shown to possess angiogenetic properties similar to vascular endothelial growth factor, as demonstrated by the ability to induce tube formation of HUVEC cells in a matrigel-assay. Furthermore, endothelial cells proliferate in the presence of hBD2, albeit to a lesser extent than in response to VEGF. Of note, the mechanisms of action are distinct from VEGF and at least in part mediated via the vitronectin receptor avβ3 [46]. Beyond *in vitro* experiments, there is clinical proof of concept: In a cohort of SLE patients, baseline hBD2 values were higher in patients experiencing cardiovascular events during follow-up and hBD2 was of additional benefit to traditional cardiovascular risk factors in predicting cardiovascular events in the cohort (unpublished data). HNPs also confer endothelial cell dysfunction and are increased in atherosclerotic plaques [88]. In a clinical study, the risk of cardiovascular mortality in patients with intermittent claudication who had HNP-levels in the upper tertile was shown to be elevated threefold. When highly sensitive CRP-values were combined into a model also incorporating HNP-values, predictions for cardiovascular mortality

could be achieved with a moderate discrimination (area under the curve in receiver operating characteristics analysis of 0.71) [89].

2. Conclusions

Over recent years, it has become increasingly clear that defensins take part in innate and adaptive immune responses. Their immunomodulatory actions provide the rationale for the assumption that they play a role in autoimmune diseases. Increasingly accumulating evidence has indicated that defensins are involved in autoimmune disorders which makes them an interesting target for drug or biomarker development. However, direct mechanistic proof of their actions in the pathogenesis of autoimmune disorders is largely lacking. Future challenges in this area involve the exploration of defensin-actions in animal models of autoimmune disease, including targeted interventions and gene-silencing experiments.

Acknowledgements

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