博士論文 (要約)

Effects of damage associated molecular patterns (DAMPs) on immune cells and a search for a novel DAMP in dogs

(イヌにおけるダメージ関連分子パターン(DAMPs)が免疫細胞に与える影響と新規 DAMP の探索)

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Contents

General introduction

Recognition of “non-self” by the immune system

The immune system is responsible for maintaining homeostasis by recognizing and eliminating "non-self", such as foreign substances, pathogens and abnormal body cells. The immune system is typically divided into two categories; innate and adaptive immunity. In adaptive immunity, lymphocytes recognize antigens by their antigen receptors including B cell receptors and T cell receptors. Genetic reconstitution of these antigen receptors produces individual lymphocytes that correspond to a myriad of antigens. These lymphocytes undergo positive and negative selection in the thymus, resulting in the survival of lymphocytes that do not respond to "self" but only recognize "non-self" antigens. Antigen-specific lymphocyte clones proliferate in response to antigen stimulation, and eliminate pathogens (Schwartz, 1989).

Innate immune system is a defense network that is always ready to resist invaders and also plays an important role in bridging to adaptive immunity.

The mechanism of recognition of foreign substances by the innate immune system had not be clear for long time. Discovery of Toll-like receptors (TLRs) in 1990s revealed the mechanism for recognition of pathogens by the innate immune system (Lemaitre *et al*., 1996; Medzhitov *et al*., 1997; Takeda *et al*., 2003). Innate immune cells, such as macrophages, recognize components of pathogens (Pathogen-associated molecular

patterns; PAMPs) by pattern recognition receptors (PRRs) including Toll-like receptors (TLRs), and then eliminate pathogens, antigen presentation, and promotion of adaptive immunity (Janeway *et al*., 2002; Akira *et al*., 2006).

Damage-associated molecular patterns (DAMPs)

Recent studies have shown that PRRs recognize not only PAMPs, but also damage-associated molecular patterns (DAMPs) released from damaged tissues and dying cells and induce sterile inflammation (Bianchi *et al*., 2007; Krysko *et al*., 2012; Gong *et al*., 2020). Namely, the innate immune system recognizes ectopic self-derived molecules as "non-self".

In the human body, ~10^5 cells die every second by programmed cell death (Fuchs *et al*., 2011). Although these apoptotic cells are quickly sensed and eradicated by the macrophages and other phagocytes, necrotic cells caused by trauma and inflammation releases self-derived molecules called DAMPs (Bianchi *et al*., 2007; Krysko *et al*., 2012; Gong *et al*., 2020). DAMPs are also recognized by PRRs and induce inflammation through the production of cytokines such as tumor necrosis factor -α (TNF-α) (Scaffidi *et al*., 2002; Andersson *et al*., 2011). In homeostasis, DAMPs play an important role as "danger signals" that convey tissue danger, however, excessive release of DAMPs induce severe and/or chronic inflammation. Actually, sterile inflammation such as autoimmune

diseases, arthritis, cardiovascular disease, neuroinflammation and cancer are attenuated in TLRs deficient mice, suggesting that DAMPs play an important role in the pathogenesis of these diseases (Yang *et al*., 2016; Marshak., 2006; Klein *et al*., 2017; Caso *et al*., 2007; Pierer *et al*., 2011; Huang *et al*., 2009). Therefore, it is important to clarify how DAMPs induce sterile inflammation.

To date, various types of DAMPs, including high-mobility group box protein 1 (HMGB1), heat shock proteins (HSPs), S100 proteins (calcium-binding cytosolic proteins), interleukin (IL)-1A, IL-33, nucleic acids (NAs), adenosine triphosphate (ATP), and uric acids, have been identified. Some of these DAMPs are recognized by the TLRs and reportedly induce sterile inflammation (Gong *et al*., 2020). For example, HMGB1, which normally regulates gene expression in the nucleus, is recognized by PRRs such as TLR4 and receptor for advanced glycation end product (RAGE) when released into the extracellular space and then induces an inflammatory response by producing cytokines such as TNFα or IL-1β, resulting in migration of neutrophils and monocytes (Scaffidi *et al*., 2002; Andersson *et al*., 2011). Recently, several novel DAMPs have been detected successively, and the regulatory mechanisms of sterile inflammation by DAMPs remain unclear (Shichita *et al*., 2012; Tanaka *et al*., 2020).

DAMPs in dogs

The leading causes of disease related deaths in dogs includes cancer and several inflammatory diseases (Fleming *et al*., 2011). Actually, increased levels of circulating HMGB1 have been reported in dogs with several diseases, such as tumors, hematological disease, hepatobiliary and pancreatic diseases (Ishida *et al.*, 2011; Kim *et al.*, 2019). Therefore, DAMPs may play an important role in the pathogenesis of diseases in dogs as well and could be a new therapeutic and diagnostic target for them. However, studies on DAMPs in dogs are very limited and the mechanism of the inflammatory response by DAMPs released from dying canine cells has not been understood.

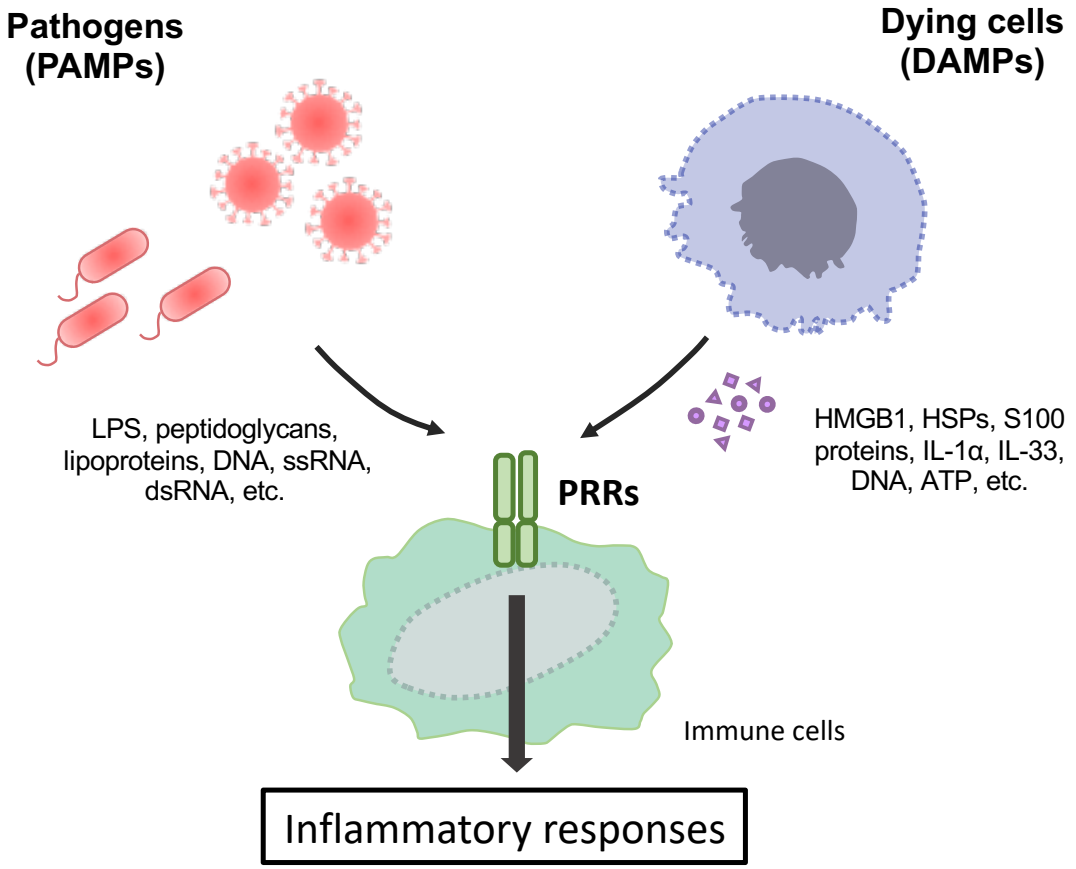
Furthermore, recently, dogs have been highlighted as a promising model for human diseases such as cancer and genetic diseases, as many naturally occurring diseases in dogs closely resemble those in humans (LeBlanc *et al*., 2020; Switonski., 2014; Shearin *et al*., 2010). Research on DAMPs in dogs may provide an ideal solution to the gap between laboratory rodents and humans as a translational research.

The purposes of this study

Accumulated evidence has highlighted the importance of recognition of DAMPs by innate immune cells in cancer and inflammatory diseases. However, the detail

mechanism underlying regulation of sterile inflammation by DAMPs has not been fully elucidated. In addition, there have been no studies on DAMPs using canine cells.

The aim of this thesis was to determine the impact of self-derived DAMPs from canine cells on immune cells and to search for novel DAMPs. In Chapter 1, I analyzed the effects of supernatants prepared from 13 different canine cells on the gene expression of pro-inflammatory cytokines (Tnf) in macrophage cell lines. Furthermore, I performed a comprehensive analysis by RNA sequencing to search for genes prominently induced by the supernatant from necrotic canine cells. In Chapter 2, I focused on CCL24 induced by necrotic cell-derived supernatants and next clarified the role of CCL24 in cancer using a syngeneic mouse model. Finally, in Chapter 3, I searched for the novel DAMP which induces CCL24 and its signaling pathway.



Schematic diagram of recognition of PAMPs and DAMPs by the innate immunity

Chapter 1

Effects of DAMPs released from canine cells on gene expression in macrophages

Introduction

Dying and damaged cells release self-derived molecules (DAMPs) into the extracellular space. DAMPs induce activation of immune responses through PRRs such as the TLRs. Several molecules have been identified as DAMPs and some of these induce pro-inflammatory cytokines, such as TNF-α via TLR-signaling (Bianchi *et al*., 2007; Krysko *et al*., 2012; Gong *et al*., 2020; Janeway *et al*., 2002).

In a previous study supernatants from necrotic dead cells did not induce TNF-α, a potent pro-inflammatory cytokine induced by the TLR activation in peritoneal macrophages. In addition, the supernatant contained a substantial amount of prostaglandin E2 (PGE2), which is synthesized by the cyclooxygenase (COX) enzymes, COX-1 and COX-2 COX-1 も作る？ (Hangai *et al*., 2016). PGE2 released from the dying or dead cells exerts immunosuppressive effects on macrophages via its receptors, EP2/EP4 (Sugimoto et al., 2007; Rodríguez et al., 2014; Xu *et al*., 2008). Indeed, Tnf mRNA induction by lipopolysaccharide (LPS) which is a TLR4 agonist was suppressed by treatment with the necrotic supernatant. Furthermore, treatment of supernatants from necrotic tumor cells with indomethacin which is a COX inhibitor reduced the production of PGE2 and enhanced the expression of Tnf mRNA in macrophages (Hangai *et al*., 2016). Therefore, both immunoactive and immunosuppressive molecules released from dying or

dead necrotic tumor cells may affect subsequential sterile inflammation.

There are very few studies on DAMPs using canine cells. A previous study revealed that canine hepatocyte-derived mitochondrial DAMPs induce TNF-α production in canine splenocytes and macrophage cell lines (Friedenber *et al.*, 2016). However, it is not clear whether immunosuppressive PGE2 is released from canine cells and how PGE2 interacts with DAMPs on immune cells.

The purpose of this chapter was to determine the effects of canine cell-derived DAMPs on immune cells. For this purpose, I generated supernatants from 12 necrotic canine cell lines and analyzed *Tnf* mRNA expression in macrophage cell lines stimulated with the supernatants. In addition, the amount of PGE2 in the supernatant was measured by ELISA and the immunosuppressive effects of the supernatant on macrophage cell lines was also examined. Furthermore, the effects of DAMPs on gene expression in a macrophage cell line in the presence and absence of PGE2 were comprehensively analyzed using RNA-seq.

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Chapter 2

The roles of CCL24 in tumor immune microenvironment and its association with prognosis

Introduction

In Chapter 1, I found that CCL24 was markedly induced by PGE2-depleted supernatants from necrotic cells. Since the relationship between CCL24 and known DAMPs or TLR signaling was not reported, it is possible that novel DAMPs are involved in the induction of CCL24 expression.

CCL24, also called eotaxin-2, attracts eosinophils and other immune cells via CCR3 receptors expressed on those cells (Menzies-Gow *et al*., 2002). CCL24 is thought to play an important role in allergic diseases. Actually, CCL24 concentration was elevated in the airways and blood of asthmatics (Min *et al*., 2005). and, In animal models of asthma, CCL24 was reported to induces accumulation of eosinophils after allergen exposure (Rådinger *et al*., 2004; Dai *et al*., 2005). Furthermore, CCL24 has been recently reported to play important roles in non-allergic disease such as non-alcoholic steatohepatitis (NASH), systemic sclerosis (SSc), atherosclerosis, rheumatoid arthritis, and also in experimental autoimmune encephalomyelitis (EAE) (Segal-Salto *et al*., 2020; Mor *et al*., 2019; Mor *et al*., 2013; Ablin *et al*., 2010; Mausner-Fainberg *et al*., 2013). Therefore, CCL24 is suspected to be deeply involved in the pathogenesis of various inflammatory diseases.

On the other hand, research on the role of CCL24 in cancer is very limited.

Recently, the tumor immune microenvironment (TIME) constructed by immune cells has been highlighted (Binnewies et al., 2018). Despite the potent effects of CCL24 on immune cells, the role of CCL24 in TIME has not been studied. It is necessary to clarify how CCL24 acts in cancer tissues in order to consider the clinical implications of DAMPs that induce CCL24 expression.

Therefore, in this chapter, I generated murine tumor cell lines overexpressing CCL24. Using syngeneic mice with fully competent immunity, I analyzed the effects of CCL24 on tumor growth and TIME. Finally, I analyzed prognostic data in human cancer patients using the TCGA data.

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Chapter 3

Search for novel DAMPs inducing CCL24 expression

Introduction

Various molecules have been identified as self-derived DAMPs which are recognized by TLRs and other PRRs. These include proteins (e.g. HMGB1, HSPs, Histone, etc.), nucleic acids (e.g. genomic DNA, mitochondrial DNA and mRNA), extracellular matrix (e.g. versican and biglycan), metabolites (e.g. ATP and uric acid), peptides (e.g. N-formylated peptides) and potassium ion. Although many types of DAMPs have been identified , recent studies have revealed the presence of novel DAMPs involved in several diseases. For example, in a model of stroke, the peroxiredoxin family was found to be released with HMGB1 from necrotic brain cells to exacerbate inflammation after stroke. In addition, during the process of chronicity of acute kidney injury, Mincle (macrophage-inducible C-type lectin), one of the PRRs, deficient mice recovered early from acute kidney injury and prevented the progression to chronic kidney disease. Macrophage-expressed Mincle recognizes β-glucosylceramide and free cholesterol derived from necrotic tubular cells and triggers an inflammatory response. The identification of these novel DAMPs indicates that the mechanism of sterile inflammation by DAMPs is still not fully understood.

Although *Ccl24* mRNA expression is mainly induced by T helper cell type 2 (Th2) cytokines such as IL-4 and IL-13, the induction of CCL24 expression by DAMPs

has not been previously reported. Therefore, it is expected that new DAMPs may be involved in the induction of *Ccl24* mRNA expression. In this chapter, I searched for novel DAMPs that regulate CCL24 expression upstream.

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General discussion and conclusion

Self-derived molecules (DAMPs) released from dying or damaged cells are recognized by the PRRs on innate immune cells, as well as pathogens, triggering an inflammatory response. To date, a variety of molecules have been identified as DAMPs and how they are involved in the pathogenesis of human diseases has been investigated. However, recent studies have found novel DAMPs involved in several diseases, indicating that the mechanism of sterile inflammation by DAMPs is not fully understood. In addition, there was lack of research on DAMPs in veterinary medicine, and it was unclear how canine cell-derived DAMPs act on immune cells to induce an inflammatory response. Therefore, in this thesis, I revealed how canine cell-derived DAMPs induce sterile inflammation, and searched for novel DAMPs based on these findings.

In Chapter 1, I found that the necrotic canine cell-derived supernatant does not induce *Tnf* mRNA expression in macrophages by immunosuppressive PGE2 in the supernatants. Furthermore, I found that depletion of PGE2 in the supernatant by indomethacin enhanced *Tnf* mRNA expression by the necrotic canine cell-derived supernatants. Thus, PGE2 simultaneously released with DAMPs may act as a "brake" to prevent an excessive immune response during acute inflammation, and that DAMP and PGE2 may cooperatively exacerbate chronic inflammation and cancer. Furthermore, comprehensive gene expression analysis using RNA-seq revealed that several

chemokines are induced by DAMPs in macrophages. These chemokines may recruit immune cells and mesenchymal stem cells and may be involved in secondary inflammatory responses and tissue repair. Among these chemokines, the expression of CCL24, which had not been previously reported to be induced by DAMPs, was enhanced by the removal of PGE2.

While CCL24 has been reported to be involved in the pathogenesis of several inflammatory diseases, the roles of CCL24 in tumor tissues have been poorly understood. Therefore, in Chapter 2, I generated a synergistic mouse models using tumor cells overexpressing CCL24 and found that CCL24 expression did not affect tumor growth, but increased the infiltration of eosinophils into the tumor. Further analysis of the TCGA data showed that high expression of CCL24 and eosinophil gene signature correlated with a favorable prognosis for patients. These data suggested that CCL24 may act as a tumor suppressor by recruiting eosinophils. The discrepancy between mouse models and clinical data may be due to a lack of molecules that degranulate eosinophils, such as IL-33, in mouse tumor cell lines.

In Chapter 3, based on the involvement of CCL24 in several inflammatory diseases and cancers, I explored the novel DAMPs inducing CCL24. I found that a novel DAMP inducing CCL24 was a 1-6 kDa peptide by size-exclusion chromatography and

peptidase treatment. Furthermore, the peptide was found to induce CCL24 in a TLR2-MyD88-dependent manner using intraperitoneal macrophages isolated from TLR2, TLR4 or MyD88-deficient mice. Since this peptide is recognized by TLR2, it may be a membrane-derived lipopeptide, but further purification and identification by mass spectrometry should be performed.

In conclusion, I found that dying canine cells released both DAMPs and PGE2, and that removal of PGE2 enhanced CCL24 expression induced by DAMPs. CCL24 was found to act as a tumor suppressor by the recruitment of eosinophils into tumor tissues. Furthermore, a novel DAMP inducing CCL24 was thought to be a peptide recognized by TLR2-Myd88. Identification of the dying cell-derived peptide and the roles of interaction with PGE2 in pathogenesis should lead to further understanding of the mechanism by which DAMPs induce sterile inflammation.

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