HMGB1, RAGE, and the Myeloid Response to Pancreatic Cancer.

by

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We have ablated HMGB1 in CD11c+ cells (DCH mice) to evaluate the role of DC HMGB1 loss, immunologically, in vaccination strategies to tumor and in vivo in the setting of pancreatic carcinogenesis. HMGB1 is a chromatin-associated molecule regulating the response to several transcriptional factors, as well as promoting cytosolic autophagy in the setting of stress. When released extracellularly following necrosis or during stress, it serves as a damage associated molecular pattern (DAMP) molecule. DCH mice represent a novel model to answer several questions. First, is HMGB1 necessary for DC function? Second, can KO-DCs confer protection as therapeutic vaccines in anti-tumor models of PDAC? Lastly, how is carcinogenesis affected in a Kras-driven model of pancreatic neoplasia (KC mice) in the presence of KO-DCs? KO-DCs to be less responsive to maturational stimuli, have inhibited T cell stimulatory capacity, and skew naïve T cells towards regulatory phenotypes. KO-DCs fail to initiate anti-tumor immunity in multiple tumor vaccine models compared to controls. Unexpectedly, DCH mice inoculated with transplantable (pancreatic and colorectal cancer) tumor cells were significantly protected from tumor growth. BM transplants from DCH mice into KC mice resulted in significant inhibition of neoplasia and the associated inflammatory infiltrate. As DCs actively secrete HMGB1 during maturation, we next examined the role of the HMGB1 receptor, the receptor for advanced glycation-endproducts (RAGE) in the promotion of PDAC. We back-crossed RAGE-KO mice
into the KC strain (KCR) and evaluated the role of RAGE in modulating the myeloid response to pancreatic carcinogenesis. We observed a significant delay in neoplasia in KCR mice and a correlation with a reduction in the accumulation of myeloid-derived suppressor cells (MDSCs). In the absence of RAGE and the development of malignant precursor lesions, non-immunosuppressive macrophages were detected in both the spleens and pancreata of KCR mice in lieu of MDSCs. These findings suggest a crucial role for myeloid cells in PDAC, including cell types conventionally thought to be inhibitory for tumor (DCs). Further investigation regarding the dynamic relationship between myeloid cells and PDAC is essential to for the success of immunotherapies in this disease.
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The senior author in scientific literature is listed as the last in a list of what can often be many co-authors. They are the most esteemed contributor to the work, and likewise, I will save my gratitude towards my scientific, professional, and life mentor, Dr. Michael Lotze, for the end of this section.

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all leaders should strive to emulate. I have him to thank for my project, I have him to thank for letting me be the academic director of the UPCI Summer Academy, one of the most rewarding things I have had the honor of participating in, and I have him to thank for being in many ways a parent, when my own parents were miles and miles away. Thank you.
ABBREVIATIONS

APC = antigen presenting cell
ASC = the adaptor protein apoptosis associated speck-like protein containing a CARD.
ASK-1 = apoptosis signal kinase-1
ATM = ataxia telangiectasia mutated
ATP = adenosine triphosphate
BAI1 = brain angiogenesis inhibitor 1
BMDC = bone marrow derived dendritic cell
BNIP3 = BCL2/adenovirus E1B 19 kDa protein-interacting protein 3
C1q = complement 1q
C3/C4 = complement 3 and 4
DAMP = damage-associated molecular pattern molecule
DC = Dendritic cell
DCH = CD11.HMGB1−
EGF = epidermal growth factor
FAK = focal adhesion kinase
FC = flow cytometry
FcR = Fc receptor
Gab2 = Grb2-associated binder 2
Gas6 = growth-arrest-specific 6
H2O2 = hydrogen peroxide
HIF-1 = Hypoxia-inducible factor 1
HMGB1 = high-mobility group box 1
HRG = Histidine-rich glycoprotein
Hsc70 = heat shock cognate protein of 70 kDa
HSP = heat shock protein
HSPB1 = heat shock protein β-1
ICAM-1 = intercellular adhesion molecule-1
IL = interleukin
IVIS = in-vivo imaging system
KC = Pdx.1cre x KrasG12D
KCR = Pdx.1cre x KrasG12D;RAGE-KO
LAMP = lysosome-associated membrane proteins
LAP = LC3-associated phagocytosis
LC3 = microtubule-associated protein 1 light chain 3
TSP1=thrombospondin-1
Tulp1=tubby-like protein 1
ULK1= unc-51-like kinase 1
UTP=uridine 5 triphosphate
VEGF=vascular endothelial growth factor
β2-GPI=β2-glycoprotein-I
MISSION STATEMENT FOR THIS DISSERTATION

A diagnosis of pancreatic adenocarcinoma (PDAC) is a death sentence for approximately 94% of patients and for which treatment options are severely limited, including chemotherapeutic drugs, although lately improved, FOLFIRINOX and NAB-PACLITAXOL, with limited effectiveness [1]. Most patients present with advanced disease and are not candidates for surgical resection [2,3]. The host inflammatory response is a critical component within early PanIN (pancreatic intraepithelial) lesions to the progression to frank invasive cancer. Pancreatitis is a major risk factor for PDAC, and regulatory immune infiltrate found in both the stromal and vascular compartments has been identified in both settings [4,5].

Unlike cancers such as melanoma and renal cancer, PDAC patients have yet to benefit substantially from novel immunotherapeutic approaches such as IL-2, alpha interferon, αCTLA4 and αPD1. Recently, there have been some reported responses to αCD40 [5]. This is thought to be mediated by an immunosuppressive pancreatic tumor microenvironment (TME) [6]. The TME consists of transformed ductal epithelium, a fibrotic stromal compartment that is so extensive; it is considered a hallmark of PDAC, disorganized vasculature, and regulatory (particularly myeloid) immune infiltrate. Tumors within a necrotic environment demonstrate increases in DAMPs such as HMGB1 and S100 proteins [7,8] and the expression of their cognate receptors [9]. This study examines the role of both HMGB1 and one of its receptors, RAGE, on two individual myeloid subsets (DCs and MDSCs), thereby evaluating their respective roles in the PDAC TME for clinical evaluation and assessment for development of novel therapies and diagnostics [5,7,9,10].
1.0 PROJECT AIMS

Dendritic cells (DCs) are cells which link innate immunity with adaptive immunity and are critical for initiating and maintaining adaptive responses [11]. Their role in human cancer as indicators of immune reactivity in adult (and not childhood tumors) [12] as well as their modern application as components of vaccines has captured the interest of a generation of tumor immunologist and cancer clinicians [13,14]. DCs have formed the basis of many anti-tumor immunotherapies aimed at directing immune responses to tumor associated antigens (TAA) and tumor specific antigens (TSA) [11,15]. To date, most DC-based anti-tumor vaccines have demonstrated marginal clinical success. This could be due to the difficulties involved in overcoming self-tolerance both at sites of T cell priming and in the peripheral tumor microenvironment, as well as a limited understanding of their role in the efferent phase of the immune response within the tumor [15]. Within the heterogeneous tumor microenvironment several poorly understood heterotypic interactions occur between tumor, stroma, and immune infiltrate [11]. High Mobility Group Box-1 (HMGB1) is a well-characterized chromatin binding protein that facilitates DNA accessibility during transcription. Normally localized in the nucleus, HMGB1 can be translocated to the cytosol via acetylation whereby it enhances autophagic flux[16]. It can also be released outside of the cell either actively during cellular stress and activation or during necrosis as a damage associated molecular pattern (DAMP) molecule [17].
We and others have shown that HMGB1 modulates DC function [18]. We have demonstrated that cytosolic HMGB1 is critical to sustaining autophagy [16]. Because autophagic substrates are an important source of antigen in professional antigen presenting cells (APCs), we hypothesized that HMGB1 plays a vital role in DC autophagic antigen capture and processing. We have also shown that DC maturation is inhibited when HMGB1 is antagonized via drugs such as ethyl pyruvate [19], and soluble HMGB1 receptors (sRAGE) without inducing DC cell death (FIGURE 3-S2-A and 3-S2-B). Additionally, both murine myeloid and human monocyte-derived DC chemotaxis in response to CCL19 and CXCL12 is dependent on extracellular secretion of HMGB1, demonstrated using several small molecule and antibody inhibitors [18,20]. Global murine HMGB1-KO animals die of hypoglycemia late embryonic or early postnatal [21]. To generate DC-specific HMGB1-KO, we crossed global HMGB1loxP/loxP mice with mice expressing yeast Cre-recombinase under the control of the CD11c promoter. We termed the resultant strain DCH mice. We originally hypothesized that DC HMGB1 would be indispensable for mounting an anti-tumor immune responses in the setting of PDAC. The goals of this project were to characterize KO-DCs and to evaluate the role of HMGB1 in DC vaccine-induced adaptive immunity in mice bearing pancreatic tumors. We also suspected KO-DCs would contribute to carcinogenesis in an oncogenic Kras-driven spontaneous murine model of PDAC (KC mice) due to impaired DC-mediated anti-tumor immune surveillance mechanisms and consequently emergence of an adaptive immune response. (FIGURE 1-1)
Figure 1-1. Generation of CD11c.HMGB1−/− (DCH) transgenic mice, project design and proposed implementation

1.1 AIM 1: CHARACTERIZE THE ROLE OF HMGB1 IN DC IMMUNOGENICITY

Original hypothesis: KO-DCs would exhibit diminished immunogenicity compared to WT DCs derived from control littermates. We harvested BMDCs from the femurs and tibias of WT and DCH mice. WT and KO-DC maturation potential was assayed by flow cytometric (FC) analysis of maturation markers. T cell stimulatory capacity in an allogeneic model was evaluated via FC analysis of proliferative CFSE dilution, and T cell polarization was assayed via ELISA. The ability of KO-DCs to elicit anti-tumor responses from bulk splenocytes derived from naïve mice that had been challenged with and subsequently vaccinated against pancreatic tumor was assayed by ELISA.
1.2 AIM 2: EVALUATE THE ROLE OF DC HMGB1 IN MODULATING VACCINE-INDUCED ANTI-TUMOR IMMUNITY

Original Hypothesis: DCH-derived BMDCs co-cultured with irradiated Panc02 cancer cells or TRP2 melanoma tumor antigen would result in diminished pancreatic or melanoma anti-tumor immunity in mice challenged with subcutaneous Panc02 or B16 melanoma cancer cells, respectively, compared to control littermate DCs. We challenged WT mice with Panc02 or B16 murine tumor cells. Post-engraftment, mice were vaccinated subcutaneously with WT or KO-DCs either co-cultured with irradiated Panc02 (2:1 ratio) or pulsed with TRP2. Tumor volume was recorded in a blinded manner. WT or KO-DC capacity to elicit Panc02-specific anti-tumor immunity from effector cells was assayed via ELISA.

Figure 1-2. Therapeutic vaccine protocols used both Panc02 (murine pancreatic cancer cell line) and B16 (murine melanoma cancer cell line) challenge with DC/DCH Panc02/TRP2 antigen, respectively. NOTE: The illustration does not depict TRP2 or B16, however a similar vaccination strategy was employed. See Materials and Methods in Chapter 3 for details regarding the B16 model.
1.3 AIM 3: INVESTIGATE THE ROLE OF DC HMGB1 DURING Pancreatic CARCINOGENESIS

Original Hypothesis: Spontaneous pancreatic carcinogenesis will be enhanced in KC mice with KO-DCS (DCH\(\rightarrow\)KC BM chimeras). Irradiated KC mice (FIGURE1-4) received BM transplants from WT or DCH donors. WT\(\rightarrow\)KC and DCH\(\rightarrow\)KC BM. (FIGURE 1-5) chimeras were sacrificed at 23-25 weeks of age. A pathologist blinded to the groups scored WT\(\rightarrow\)KC and DCH\(\rightarrow\)KC pancreatic tumor lesion frequency and progression and compared to control mouse pancreatic tissue in H&E-stained sections. We assayed immune infiltrate both systemically (within the spleen) and intra-tumorally within the pancreata by flow cytometry.

Figure 1-3 KC mice accurately depict the histopathology of human PDAC patients. (A) Control littermate pancreas tissue stained with H&E depicting normal pancreatic anatomy at both 12 and 24 weeks.
(B) Tissue from KC mice with readily identifiable malignant lesions and severe fibrogenesis. NOTE: This spontaneous Kras-driven model of murine PDAC is explained further detail in Chapter 3.

Figure 1-4 and Figure 1-5. Bone marrow chimeras were generated for the studies completed in Aim 3 of this work.
2.0 BACKGROUND

2.1 DC IMMUNOBIOLOGY

DCs are the quintessential professional antigen presenting cell (APC)[22]. They are found ubiquitously throughout tissues where they facilitate adaptive immune responses by way of peripheral antigen capture, protection from premature degradation, and subsequent processing for presentation to immune effector cells in the context of major histocompatibility complex (MHC) class I and class II molecules [23,24]. In the setting of cancer, DCs in the periphery encounter antigen derived from transformed cancer cell populations (TAA or TSA). Antigen is then processed into small 8-20 amino acid fragments by the proteosome (for MHC-I loading) or the lysosome (for MHC-II presentation). Cross-presentation also occurs when extracellular-acquired antigen is loaded onto MHC-I molecules for presentation to CD8$^+$ T cells. Autophagosomes have now been found to be an appreciable source of antigen and appear to be critical for the phenomenon of antigen cross-presentation [25]. In concert with additional stimulating signals such as TLR ligation within the phagosome [26], a state of DC maturation is
induced in which antigen presentation and co-stimulatory molecule surface expression is enhanced, lymph node (LN) homing chemokine receptors (i.e. CCR7) are upregulated, and a remodeling of the actin cytoskeleton occurs [24]. DCs then migrate via chemokine receptor ligand binding (i.e. CCL19) to the LN by way of the lymphatic system where they provide the three required stimulatory signals of antigen (MHC I/II), co-stimulation (CD80/86), and polarizing cytokines (IL-12, IL-10, etc) to naïve T cell populations residing within the T cell zone of the LN. These primed and polarized, antigen-specific T cells then exit the LN by way of the high endothelial venule (HEV) and enter the periphery where they mediate the TAA or TSA-specific lysis of transformed cell populations [23,24]. Alternatively, soluble tumor antigen can be released from necrotic or apoptotic cancer cells and travel via the lymphatic vasculature directly to a draining lymph node. Here, lymph node-resident APCs including dendritic cells phagocytose this tumor antigen, process it for MHC molecule loading, and present it to resting CD4+ and CD8+ T cell populations. In this way, DCs can capture free TAA or TSA for the successful initiation of an anti-tumor adaptive response. [27,28]

APCs including dendritic cells DCs, macrophages, and B cells possess proteolytic capabilities by which they process antigen and present it in the form of cell surface MHC Class I and II molecules. [14] Immunogenic peptides derived from a pathogenic source (i.e. viral or bacterial proteins), and endogenous tumor-associated and –specific proteins . [14] The immuno-proteosome has been ascribed the function of processing these antigens from their tertiary structures into the relatively small peptide fragments which are cleaved and loaded onto MHC Class I molecules via TAP protein delivery into the endoplasmic reticulum to meet nascent heavy chain and β2 microglobulin. [15, 16]. Along with this specialized organelle, autophagic
substrate is now recognized as a significant source of antigen, making autophagy a highly immunologic process within APCs and critical for cross-presentation [29]

Autophagy is a vital process to MHC class II expression in DCs. In mice lacking the critical autophagy-related gene, ATG5, antigen processing in DCs is substantially impaired. [17] DCs isolated from ATG5<sup>−/−</sup> mice exposed to herpes simplex virus infection showed markedly diminished HSV surface antigen expression and likewise fail to sufficiently stimulate CD4 T cell responses, resulting in rapid disease progression in these animals. [17] Similar results have been reported with siRNA knock-down of ATG12, resulting in significant decreases in the surface expression of the EBV antigen, EBNA1. [18] Likewise, human immunodeficiency virus-1 (HIV-1) targets the autophagic pathway within DCs as an evolved mechanism of evasion. [6] Largely mediated by envelope proteins, HIV induces a rapid suppression of autophagy, resulting in increases in intracellular HIV content and impaired antigen presentation to HIV-specific CD4 T cells. This effect can be reversed following treatment with the autophagy inducing drug, rapamycin. [6] Furthermore, in mouse macrophages and B cell lines treated with the autophagy inhibitors 3-MA and wortmannin, MHC expression on the cell surface is diminished. [19]

Autophagy facilitates the activation of immature DCs. Autophagy enhances maturation of DCs pulsed with Bacillus Calmette Gue'rin (BCG) in an in vitro tuberculosis infection model. Induction of autophagy by both serum and amino acid starvation prior to BCG maturation markedly enhances the expression of surface MHC and the costimulatory molecule, CD86. [20] In addition, these DCs demonstrate significant increases in the production of IL-6, suggesting enhanced ability to promote immunity. [20] In a murine TB model, pre-treatment of mouse DCs and macrophages with rapamycin before pulsing with the immunodominant mycobacterial antigen, Ag85B resulted in 3-fold increases in IL-2 secretion by stimulated T-cells. Moreover,
when DCs taken from mice are stimulated with rapamycin or serum starvation to induce autophagy prior to *ex vivo* maturation, both elicit potent Th1 responses and the overall efficacy of BCG vaccination are enhanced two-fold. [21]

Stimulation of autophagy is an important mechanism in the transduction of signals which facilitate antigen handling within APCs. In fact, it is the bridge which links recognition of pathogens or tissue damage with antigen presentation. [5] In the case of the DC bacterial sensor, NOD2 (nucleotide-binding oligomerization domain-containing-2), stimulation with the bacterial ligand, muramyldeipeptide (MDP) increases autophagic activity within the cell in an Atg5, Atg7, and Atg16L1 dependent manner, resulting in maturation and successful presentation to CD4+ T cells. [5] Autophagy is required for antigen-specific T cell responses following NOD2 stimulation. [5] Interestingly, DCs from individuals with Crohn’s disease, a disorder of chronic inflammation in the gut due to immune responses against commensal bacteria, show diminished autophagy due to the expression of ATG16L1 variants which enhance risk for disease development. [5] Furthermore in B cells, autophagy links B cell receptor (BCR) signaling to the expression of costimulatory molecules such as CD40 and TLR-9. [22, 23] This signal could be abrogated by treatment with monoclonal antibodies which ablate autophagic induction. [22] In stressed stromal and epithelial cells display of antigen in the form of MHC class I and II molecules on the cell surface serve as vehicles for immune interrogation and recognition. Similarly to APCs, autophagy is critically implicated in the immunogenicity of these cells by modulating both antigen presentation and cross-presentation pathways.

Cross-presentation is the pathway in which APCs (primarily DCs) present antigen from exogenous sources (i.e. tumor cells) to cytotoxic T cells. The level of autophagic activity within the antigen donor cell significantly affects the efficiency in which cross-presentation to CD8+ T
cells occurs. [24, 25] When autophagy in melanoma cells serving as antigen donors is abolished by inhibitors or siRNA, cross-presentation is reduced to negligible levels. [25] In contrast, when autophagy is increased in both melanoma cells and HEK cells expressing OVA antigen with drug treatment or serum starvation, cross-presentation is not only restored, but dramatically enhanced. [25] Furthermore, isolated autophagosomes themselves are sufficient vehicles for cross-presentation. DCs pulsed with cellular fractions from lysed tumor cells containing autophagosomes are stimulated better than any other fraction, or recombinant antigen alone. [25]

In addition to tumor cells, mouse fibroblasts infected with influenza demonstrated greater DC cross-priming ability when levels of autophagy were increased, resulting in improved virus-specific CD8+ T cell responses. [24] Both greater availability of MHC class I molecules on the cell surfaces and increased type I interferon responses were measured under conditions of enhanced autophagy. [24] In addition, while the notion of autophagic cell death remains controversial, cells dying under scenarios of higher levels of autophagy demonstrate significantly greater immunogenicity. [24, 26, 27].

### 2.2 DC AND EFFECTOR CELL INTERACTIONS

DCs are often described as the “link between innate and adaptive immunity”, as they interact and form immunological synapses with innate lymphocytes such as natural killer (NK) cells and are key components promoting adaptive immune selection via their interactions with B-cells, and CD4+ and CD8+ T cells [14,30]. NK cells and DCs reciprocally activate each other via cytokine exchange and release of HMGB1 at the immunologic synapse, presumed to occur within the
tissue. There ‘signal 0’s’ including PAMPs such as LPS and DAMPs such as HMGB1 recruit inflammatory cells such as NK cells and DCs to initiate the immune response. NK cells secrete HMGB1 (which functions to both mature DC via pattern recognition receptor ligation and protects them from NK cell-mediated “quality control” lysis. In return, DCs secrete the NK cell-activating cytokine, IL-18 [30,31].

Although DC:T cell interactions can take place in the periphery, we classically think of them occurring within the T cell zone of the lymph node. Naïve, resting T cells localized to lymph nodes have already undergone the process of T cell thymic selection, and via RAG recombinase-mediated rearrangement, represent a repertoire of T cells capable of being selected by DCs. Each of these lymphocytes expresses a unique T cell receptor (TCR) [22]. In healthy humans these T cells are nonreactive to self-antigen (a process mediated by thymus-resident DCs), and each clone possesses a distinct TCR, a subset of which are capable of recognizing pathogens and tumors [22,32]. If a fully mature DC successfully interacts with a T-cell expressing a TCR that recognizes the antigen-bearing MHC molecule it’s presenting, it can become activated. This activation requires additional signals to initiate a T cell response, each provided by the DC. In addition to MHC-antigen-TCR binding (Signal 1), co-stimulatory molecules such as CD80 and CD86 present in relative abundance on mature DCs must bind their T cell counterpart CD28 (Signal 2). Lastly, the DC will secrete cytokines such as IL-12, IL-23, IL-27, IL-35, and IL-10 which will determine the phenotype of the now activated T cell (Signal 3). Once primed, the T cell will undergo substantial clonal expansion supported by the T cell-proliferative cytokine, IL-2 these clones now secrete. At this point, the DC-activated lymphocytes exit the LN, cross endothelial barriers from the bloodstream, recognize appropriate antigen, and mediate their effector function [13,33].
2.3 DC-BASED ANTI-TUMOR VACCINES

Given the superior capacity of DCs to prime anti-tumoral CD4+ Th1 cells and cytotoxic CD8+ T cells, they have been the target of many immunotherapies [34,35]. This is accomplished by the isolation and differentiation of DCs, followed by \textit{ex-vivo} pulsing with TAA/TSA peptide, protein or autologous tumor lysate, often accompanied by various adjuvants. Activated DCs are then reinfused directly into lymph nodes or into the lymphatics or into the skin so that they may migrate to the lymph node. Although DC-based vaccines represent some of the most exciting cancer treatments, they have only enjoyed modest clinical success [36,37]. This is likely due to cancer cell immune evasive strategies and the presence of various regulatory immune cells during the effector phase of the immune response resident at the tumor site. Included in these are cells which mediate reparative wound healing (particularly of myeloid lineages) and Tregs [38].

2.4 DAMAGE ASSOCIATED MOLECULAR PATTERN MOLECULES

Damage associated molecular pattern (DAMP) molecules broadly function much like their pathogen associated molecular pattern (PAMP) counterparts in that they serve to alert the host to cellular or tissue damage in a nonspecific manner [8,39]. Via conserved signatures, these proteins, lipids, sugars, or nucleic acids ligate pattern recognition receptors (PRRs) expressed on
the cell surfaces of immunologically relevant cells such as dendritic cells and macrophages. This general class of receptors include both immunostimulatory receptors such as the toll-like receptors (TLRs), the receptor for advanced glycation endproducts (RAGE), and NOD-like receptors (NLRs) which signal through pro-inflammatory pathways such as NF-κB, as well as immunosuppressive receptors such as CD24 and TIM-3, the latter of which results in the diminution of nucleic acid sensing in professional antigen presenting cells [8,39-41].

Unlike PAMPs, which are comprised of mostly “foreign” molecules unique to infectious organisms such as bacteria, viruses, and fungi and include signatures present on cell walls, flagella, and non-mammalian nucleic acid sequences or conformations, DAMPs can be broadly described as intracellular molecules which are undetectable in the extracellular milieu under healthy, basal conditions [41]. During cytotoxic insult or immune activation, necrotic or stressed cells release DAMPs both actively or passively where they then facilitate an array of downstream effects that can include the initiation of an inflammatory response, reparative wound-healing and neo-vascularization, and the resolution of tissue damage [7,41].
<table>
<thead>
<tr>
<th><strong>Pattern Recognition Receptor (PRR)</strong></th>
<th><strong>DAMP ligand(s)</strong></th>
<th><strong>Downstream effector(s)</strong></th>
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<tr>
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<td>HMGB1</td>
<td>RAS, MAPK, NF-kB</td>
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</tr>
<tr>
<td>TIM-3</td>
<td>HMGB1</td>
<td>Suppression of TLR9 signaling</td>
</tr>
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</table>

Table 1

2.5 HMGB1 BIOLOGY

HMGB1 is a 25kD protein with distinct cellular compartmental roles [39,42]. Initially identified as a nuclear protein, chromatin-bound HMGB1 facilitates DNA accessibility in a variety of transcriptional activities including p53 transactivation and V(D)J recombination through stabilization of V(D)J recombinases and recombination-activating gene (RAG) 1 and 2 [39,42]. Under conditions of cellular stress, HMGB1 is translocated from the nucleus to the cytosol via what is thought to be acetylation-mediated or Redox modification by ROS (FIGURE 3-1-A and 3-1-B) where it induces a state of heightened autophagy by displacing Bcl-2 bound to the autophagic protein, Beclin1 [43,44]. This method of sustaining autophagy serves in part to mitigate stress-induced organelle damage [45] and may also have a role in enhanced antigen
delivery in response to TLR ligation-induced HMGB1 translocation (**FIGURE 3-1-A** and **FIGURE 3-1-B**) [46,47]. HMGB1 along with various heat shock proteins (HSPs) are among the first proteins to be described as a damage associated molecular pattern (DAMP) molecule. DAMPs function much like their pathogen associated counterparts in that they serve to alert immunologically relevant cells to potential pathology within the host- in this case, cell or tissue damage [17,48]. During cytotoxic insult or necrosis, cellular components normally located within the cell become released either passively or actively into the extracellular milieu (**FIGURE 3-C**) where, with the exception of HSPs, they bind to pattern recognition receptors (PRR) on resident macrophages and DCs such as TLR2/4, and RAGE [9]. This results in the propagation of inflammatory responses to injury or infection [17,48,49] and the recruitment of macrophages, neutrophils, and fibroblasts via NF-κB-dependent chemotaxis [50]. Very recently, it was discovered that HMGB1’s various immunological functions are regulated by its redox state, as HMGB1 contains three redox-susceptible cysteines.

### 2.6 DC HMGB1

HMGB1 plays a critical role in various aspects of DC immunobiology. In response to maturational stimuli, DCs translocate nuclear HMGB1 into the cytosol and releases it into the extracellular milieu (**FIGURE 1-2-C**) [18,20,48,51]. This accompanies a rearrangement of the actin cytoskeleton and upregulation of the chemokine receptors CCR7 and CXCR4, followed by CCL19 and CXCL12 induced migration, respectively [18,52]. In the extracellular milieu HMGB1 binds to these chemokines and induces conformational changes which enhance their
ability to bind to their cognate receptors. In the presence of HMGB1 blocking antibodies or antagonists, both of these processes fail to occur in both human monocyte and murine myeloid-derived DCs (FIGURE 2-1-A and 2-1-B)[18]. Additionally, studies in human DCs have demonstrated the requirement of HMGB1 for maturation-induced upregulation of the co-stimulatory molecules CD80 and CD86, as well as the IL-10 suppressing protein, CD83. We have been able to duplicate these results with various HMGB1 inhibitors (FIGURE 1-1) [53-55]. DC maturation occurs in a RAGE-dependent manner [51]. Furthermore, studies have indicated that human DCs fail to secrete IL-12 when HMGB1 is neutralized. Likewise, HMGB1 is important for DC-mediated Th1 polarization of naïve CD4⁺ T cells, as well as T cell proliferation via RAGE expression on T cells. These findings are contradictory to this work, although considerations must be taken into account regarding discrepancies between human and murine models, disease of interest, and inhibition versus genetic knockouts. [48,54-56] Additionally, HMGB1 itself serves as a chemoattractant for DCs (FIGURE 2-2-C).

2.7 PRE-CLINICAL MODELS OF PANCREATIC CARCINOGENESIS

Currently, there are several pancreatic tumor models used for the evaluation of carcinogenesis and for the pre-clinical identification of novel therapeutic strategies. Traditionally, established murine and human pancreatic cancer cells available from cell line repositories have been transplanted either heterotopically or orthotopically into recipient mice [57]. Although convenient, these methods fail to recapitulate the full evolutionary pathway and histopathology of human adenocarcinomas (i.e. progression from normal ductal epithelium to successive pancreatic
intraepithelial neoplasia or PanIN lesions and eventually ductal adenocarcinoma, [FIGURE 2-3] [57]. Arguably, the most robust model of pancreatic adenocarcinoma is the pdx1-Cre Kras\textsuperscript{G12D} (KC) murine model [58]. A floxed stop codon upstream of mutant Kras is excised under the Cre-expressing pancreatic tissue-specific promoter, pdx1. This genetic model accurately depicts PanIN lesion development consistent with human histopathology and tissue samples are often indistinguishable from human biopsies [58]. Constitutively active oncogenic Kras leads to atypical ductal morphology detectable by 12 weeks of age. Concurrently, mutations in the protein Nestin and telomere shortening result in hyperplastic epithelium. Cuboidal cells take on a columnar morphology as proliferation is enhanced. Following the accrual of genomic changes effecting the p16/CDNK2A, p53, SMAD4, and BRCA2 proteins and their respective signaling pathways, mice develop severe dysplasia with malignant ductal epithelium detectable by 24 weeks and the development of frank invasive cancer by approximately one year [3,59].
Figure 2-I Progression of pancreatic ductal adenocarcinoma from ductal cells.

Pancreatic Tumor Progression


---normal---\-----hyperplasia--------\\-----dysplasia---------------\---frank invasive cancer

Pancreatic Intraepithelial Neoplasia (PanIN)
3.0 FOR SUBMISSION CANCER DISCOVERY THE JANUS FACTOR: HMGB1 IN DENDRITIC CELLS PROMOTES CARCINOGENESIS BUT ENHANCES TUMOR VACCINE EFFICACY


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3.1 ABSTRACT

HMGB1, an abundant and evolutionarily ancient nuclear chromatin associated protein is the prototypical damage associated molecular pattern (DAMP) molecule, promoting autophagy, profound metabolic, and immunologic responses to stressed and damaged tissues. It plays a critical role in many aspects of DC biology, promoting DC maturation, antigen cross-
presentation, and elicitation of a Th1 response. To investigate the role of DC HMGB1 in tumor immunity, DC-specific HMGB1\(^{-/-}\) mice (DCH) were generated. Loss of HMGB1 limited DC maturation and chemotaxis in response to CCL19 \((p = 0.001)\). In both transplantable subcutaneous and liver metastasis models of pancreatic and colorectal cancer, DCH mice had less tumor growth \((p < 0.05\) and 0.01, respectively). Interestingly, DCH bone marrow limited development of pancreatic cancer precursor lesions (PanINs) and associated inflammatory infiltrate in animals expressing mutant Kras within the pancreas \((p = 0.0317)\). Mice inoculated subcutaneously with pancreatic or melanoma cancer cell lines and then vaccinated with DCH DCs pulsed with tumor antigen provided no protection compared to WT controls in both models \((p < 0.05)\). Although both WT and KO DCs stimulate allogeneic lymphocyte proliferation, KO DCs skewed responses away from Th1/Th2 phenotypes, and promoted Treg/Th17 polarization as measured by specific cytokine release. KO DCs also expressed lower levels of both RAGE and TLR4 yet comparable TIM3 expression and were less responsive to stimulation with pathogen associated molecular pattern (PAMP) molecules. Overall, our findings suggest a new and critical role for tumor associated DCs, enhancing tumor growth and persistence through an HMGB1 dependent process.

### 3.2 INTRODUCTION

DCs are the primary vehicles for antigen delivery as well as signals related to tissue damage, tissue location, and nature of pathogen to elicit adaptive immune cells [33] as the ‘professional’
antigen presenting cell (APC) [11,60]. Several identifiable DC subsets exist including CD11b+ myeloid DCs, CD8α+ DCs that are effective antigen cross-presenting cells, and plasmacytoid DCs, the primary producers of type I interferons, distinguishable by expression of BDCA-2 in humans and PDCA in mouse [61]. DCs can be tissue resident (such as those within the thymus), or differentiated from monocytes and recruited to sites of cellular injury or infection [52,62]. DCs in the periphery encounter antigen and other signals promoting maturation before transiting to secondary lymphoid sites. When antigen is derived from tumors, they also undergo a maturational program that either promotes tolerance or limits their ability to exit the tumor site, potentially setting up tumor resident tertiary lymphoid sites [63]. Additional signals including pattern recognition receptor (PRR) ligands accompany antigen within endosomal compartments [64] and promote effective DC activation. DC maturation involves upregulation of co-stimulatory molecules and lymph-node homing chemokine receptor expression [11,15,18]. DCs then migrate to the LN where they encounter resting naïve and memory T cells, selecting those cells with cognate interactions with antigen [65]. Pending a successful DC:T cell interaction (the delivery of the three required T cell activating signals: T cell receptor [TCR] engagement, polarizing cytokine delivery, and ligation by co-stimulatory molecules), antigen-specific lymphocytes are activated and licensed to expand [11,15,36]. The role of HMGB1 in this process is centrally important [8].

HMGB1 is a 25kD protein with distinct cellular compartmental roles. Initially identified as a nuclear protein (FIGURE 3-S-1A), chromatin-bound HMGB1 facilitates DNA accessibility to a variety of transcriptional factors including nuclear hormone/nuclear hormone receptor and p53 and p73 transcriptional complexes [21,42]. Under conditions of cellular stress, HMGB1 is translocated from the nucleus to the cytosol (FIGURE 3-S-1B) where it induces a state of
heightened autophagy by displacing Bcl-2 bound to Beclin1, a critical modulator of autophagic flux [16]. HMGB1 is also secreted into the extracellular milieu (FIGURE 3-S-1C), both actively and passively, where it functions as a DAMP, a cognate ligand for several receptors including the receptor for advanced glycation end-products (RAGE), TLR2/4, TIM-3, and CD24 [9,40,66]. Cell surface HMGB1 plays a role in neurite outgrowth and on activated platelets [67]. In this manner, extracellular HMGB1 recruits immune cells (FIGURE 3-S-3C) and mesangioblasts to promote wound healing [16,17]. The role of redox changes in HMGB1 to promote CXCL12/SDF-1 release in the fully reduced form but exclusively cytokine production (TNFα, IL-6, etc.) in the dithiol form has suggested a ‘Janus-like’ role for this extracellular compartmental role of HMGB1.

HMGB1 plays various intrinsic and extrinsic roles within tissues and secondary lymphoid sites. In response to stress and maturational stimuli, DCs translocate nuclear HMGB1 into the cytosol and actively secretes it into the extracellular milieu [60]. In the presence of HMGB1 blocking antibodies or antagonists, both human monocyte and murine bone marrow-derived DCs [62] fail to mature. Several recent studies suggest a role for endogenous DCs to promote carcinogenesis [22, 23]. Transition to malignant neoplasia within the oncogenic K-rasG12D KC murine pancreatic tumor model requires TLR4 ligation on DCs [4], dependent on Myd88 inhibition and skewing of CD4+ helper T cells towards Th2-restricted immune response. Interestingly TLR7 induced inflammation also potently promotes pancreatic tumor development [68].

We generated CD11c-specific HMGB1 knock-out mice using floxed HMGB1 alleles eliminated in CD11c-Cre-recombinase/loxP expressing cells. Here, we demonstrate that while HMGB1 itself is not required for the differentiation of DC from hematopoietic precursors, its
absence in DCH mice limited tumor growth in transplantable tumor models, as well as delaying ‘spontaneous’ carcinogenesis in KC mice. Interestingly, we also demonstrate that HMGB1-KO bone marrow derived DCs (BMDCs) have impaired function and serve as suboptimal APCs in multiple therapeutic murine vaccine models. This supports the notion that DCs can either promote or limit tumor growth in a contextual manner. This intrinsic ‘Janus-like’ biologic role is unmasked when HMGB1 is deleted in DCs.

3.3 MATERIALS AND METHODS

Transgenic Mice
Wild-type C57BL/6/N/tac mice were purchased from Taconic Farms (Hudson, NY, USA). HMGB1<sup>loxP/loxP</sup> mice were generated as previously described [20]. B6.Cg-Tg(Itgax-cre)1-1Reiz/J (CD11c-cre) mice were obtained from Jackson Laboratories (Farmington, CT, USA). These strains were crossed to generate CD11c-Cre:HMGB1<sup>−/−</sup> mice and termed DCH mice. Pdx1-Cre and Kras<sup>G12D/+</sup> transgenic mice were obtained from the MMHCC/NCI Mouse Repository and crossed to generate the genotype Pdx1-Cre:Kras<sup>G12D/+</sup> (termed KC mice). Genomic and recombination screens were done by polymerase chain reaction.

Generation of bone marrow-derived DCs
CD11c<sup>+</sup> DCs were generated in-vitro using the following methods. Fresh bone marrow flushed from the femurs and tibias of mice was resuspended in RPMI supplemented with GM-CSF (100ng/ml) and cultured for 10 days or alternatively cultured with GM-CSF (100ng/ml) and IL-4
(250U/ml) for 7 days. All cytokines were obtained from eBioscience (San Diego, CA, USA). In both instances, media was replaced on day 4. Harvested cells were routinely 80-90% CD11c positive.

**Confirmation of CD11c-specific HMGB1 knock-out**

BMDCs cultured as described above were harvested and labeled with αCD11c microbeads and magnetically sorted using MACS LS separation columns (from Miltenyi Biotec, Leiden, The Netherlands) and purity assayed by flow cytometry. Cells were then lysed and probed for HMGB1 expression via western blot analysis to confirm loss of HMGB1 protein expression. Additionally, cells were stained with fluorescent antibodies specific for CD11c, HMGB1 and Hoechst to confirm loss of HMGB1 in CD11c+ cells by immunofluorescence staining. Antibodies to HMGB1 were obtained from Abcam (Cambridge, UK), αCD11c from BD Biosciences (San Jose, CA, USA), and Hoechst from Molecular Probes (Eugene, Oregon, USA).

**Transplantable tumor models**

DCH mice, control littermates, or heterozygotes were evaluated in the following murine tumor models. Mice were inoculated subcutaneously with 5x10⁵ Panc02 murine pancreatic cancer cells (NCI repository). Tumors were measured by caliper in a blinded fashion and tumor volume calculated using the formula: volume = (width)² x length/2. The experiments were terminated when the first mouse necessitated sacrifice due to excessive tumor growth. Additionally mice received portal vein injections of 5x10⁵ luciferase-transfected MC38 murine colorectal cancer cells (American Type Culture Collection) culminating in the formation of metastatic lesions in the liver. To assay tumor growth, mice received IP injections of luciferin substrate, and
luminescence was detected by IVIS as photons/sec/cm² (PerkinElmer, Waltham, Massachusetts, USA).

**Bone marrow transplantation into a genetic model of early pancreatic carcinogenesis**

1000rad \( \gamma \)-irradiation was administered to 8 weeks old Pdx1-Cre:Kras\(^{G12D/+}\) (KC mice) to deplete bone marrow progenitors. Mice were then reconstituted via tail vein injection of \( 1 \times 10^6 \) freshly isolated bone marrow cells from either DCH or control littermates. Successful reconstitution of hematopoietic cells was verified by a Beckman Coulter AcT10 Hematology Analyzer (Brea, CA, USA). This model allowed for the evaluation of spontaneous pancreatic carcinogenesis in mice with DCs either containing or lacking genomic HMGB1.

**Histology**

Harvested pancreatic tissue from DCH and control mice was formalin fixed (Sigma-Aldrich, St. Louis, MO, USA), sectioned, and mounted onto glass slides. Following this, they were stained with hematoxylin and eosin by the University of Pittsburgh Department of Pathology. Images were visualized and captured at a magnification of 10X by Nikon Eclipse E800 fluorescent microscope under bright field settings (Melville, NY, USA). Scoring of tissues for the presence and frequency of pancreatic intraepithelial neoplasia (PanIN) lesions was performed by our pathologist (Dr. Aatur Singhi, University of Pittsburgh, Department of Pathology) blinded to the results.

**Flow Cytometry**
Flow cytometric analysis was performed either on a C6 flow cytometer (Accuri Cytometers, Ann Arbor, MI, USA) instrument or on a Becton-Dickinson BD LSRFortessa™ available within the University of Pittsburgh Cancer Institute Flow and Imaging Cytometry core facility and analyzed using FlowJo software (Tree Star Inc, Ashland, OR, USA). Murine spleens were homogenized through a 70μm nylon filters (BD Biosciences, San Jose, CA, USA) and washed with PBS. Red blood cells were lysed with Red Blood Cell Lysing Buffer (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer’s instructions. Single cell suspensions were derived from the pancreas by mechanical separation and collagenase digestion (Sigma-Aldrich, St. Louis, MO, USA). The resulting single cell suspensions were then stained with the following fluorescently labeled antibodies: B220, CD3α, CD4, CD8, CD11b, CD11c, CD25, CD86, F4/80, Gr1, IAb, NK1.1, RAGE, TLR-4, TIM-3 and corresponding isotype controls (all from BD Biosciences, San Jose, CA, USA except TLR-4 which was obtained from BioLegend, San Diego, CA). During analysis, viable cells were identified via forward and side scatter and gated accordingly.

**DC Vaccination Models**

Wild-type C57BL/6 mice (Taconic Farms, Hudson, NY, USA) were evaluated in the following therapeutic DC vaccination models. On Day -1 bone marrow from DCH and control mice was harvested and placed into GM-CSF/IL-4 culture as described above. On day 0, mice were subcutaneously challenged with 5x10^5 Panc02 murine pancreatic cancer cells. Differentiated WT and KO BMDCs were co-cultured overnight with γ-irradiated Panc02 cells at a ratio of 2:1. 1x10^6 DCs were injected subcutaneously contra-laterally into the tumor on day 7 and again on day 14. Tumor volume was measured by caliper in a blinded fashion. The experiments were terminated when the first mouse necessitated sacrifice due to excessive tumor growth. To assess
Panc02-specific T cells, splenocytes from mice were harvested, and CD4⁺CD8⁺ cells were magnetically sorted using a combination of αCD4 and αCD8 microbeads (Miltenyi Biotec, Leiden, The Netherlands). T-cells were then co-cultured at a ratio of 1:1 with Panc02 cells or an irrelevant colorectal cancer cell line, MC38. Reactivity was assayed by IFNγ detection in the supernatants of the co-cultures as described below. In a separate model, WT mice were challenged with 5x10^5 B16 murine melanoma cells in the same manner as described above. As an alternative to DC:tumor co-culture, DCs were pulsed with 10uM melanoma-specific antigen for 4hrs and then subcutaneously injected on days 5 and 10. Tumor volume was measured by calipers in a blinded fashion.

**ELISA analysis**

Supernatants from co-culture experiments were assayed for detectable levels of IFNγ, IL-4, IL-10, and IL-17 by ELISA Ready-SET-Go! Kits (eBioscience, San Diego, CA, USA) according to the manufacturer’s instructions.

**T cell proliferation and polarization analysis**

WT Balb/C (Taconic Farms, Hudson, NY, USA) splenocytes were harvested as described above, stained with CFSE dye (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions, and co-cultured with WT or KO BMDCs at a ratios of 100:1, 10:1, and 1:1 (splenocyte:DC) for 96 hrs in a mixed leukocyte reaction. Expansion of reactive splenocytes was assayed by proliferative dye dilution via flow cytometry. Supernatants were then collected and assayed for the presence of cytokines indicating T cell polarization by ELISA as described above.
Analysis of TLR-responsiveness

Fully-differentiated BMDCs derived from DCH mice and their control littermates were cultured for 48hrs with a panel of TLR agonists provided by InvivoGen (San Diego, CA, USA) according to the manufacturer’s instructions. DCs were then harvested and stained for the maturational marker, CD86 and analyzed by flow cytometry.

Analysis of Chemotaxis

5x10^5 BMDCs derived from DCH mice and their control littermates were plated in the upper chamber of a transwell plate. 50ng/ml of rCCL19 supplemented media was placed in the lower chamber. Migrated BMDCs present in lower chamber after 5 hrs of incubation time were quantified. Transwell plates were Costar Transwell Permeable Supports (Corning, NY, USA) were used to assay BMDC chemotaxis in response to rCCL19.

Statistical analysis

Data are expressed as means +/- SEM of at least two independent experiments performed in triplicate. Statistical analysis was performed using a two-tailed student’s T test. P values below 0.05 were assigned statistical significance.
3.4 RESULTS

**HMGB1 is dispensable for the differentiation and viability of DCs.**

We crossed HMGB1<sup>loxP/loxP</sup> C57/B6 mouse strain [69] with mice expressing Cre-recombinase under the control of the DC-specific promoter, CD11c. A proportion of the resultant progeny of this cross lacked the HMGB1 gene specifically within their DCs (FIGURE 3-1-A). The frequency of HMGB1-deficient mice followed the pattern of Mendelian inheritance (data not shown). Genomic loss was confirmed by both western blot analysis of CD11c<sup>+</sup> magnetically sorted cells (FIGURE 3-1-B and 3-2-B) and immunofluorescence (FIGURE 3-1-C).

To determine if HMGB1 itself was required for the development and differentiation of DCs from hematopoietic stem cells, bone marrow from both DCH mice and loxP/loxP control littermates were harvested and cultured in media supplemented with GM-CSF to generate DCs. Over the course of 10 days, the proportion of CD11c<sup>+</sup> cells detected by flow cytometry was equivalent regardless of strain, and was comparable to the usual BMDC yields with regard to DC purity (FIGURE 3-2-A). The increase in frequency of CD11c<sup>+</sup> cells over the course of the 10 day culture also correlated with loss of HMGB1 expression (FIGURE. 3-2-B).
Figure 3-1 Generation of CD11c.HMGB1-/- (DCH mice) and confirmation of HMGB1 loss. (A) Graphical representation of HMGB1 gene locus in WT, loxP/loxP, and DCH mouse strains and the breeding scheme used to generate DCH mice. (B) Representative PCR images of HMGB1loxP/loxP, heterozygote, and DCH strains and western blot analysis of HMGB1 expression in magnetically sorted BMDCs from control and DCH mice. (C) Representative immunofluorescent images BMDCs derived from control and HMGB1 mice. Nuclear staining is blue, CD11c staining is red, and HMGB1 staining is green.
Figure 3-2 HMGB1 is dispensable for the differentiation and viability of DCs. (A) BM derived from control and DCH mice was cultured in GM-CSF and harvested daily over a period of 10 days and stained anti-CD11c or isotype control antibodies for flow cytometric analysis of DC yield and purity. (B) Western blot analysis of HMGB1 expression in WT and DCH strains in BM cells as they differentiate into CD11c+ DCs.

**Genomic ablation of HMGB1 in DCs inhibits tumor growth in subcutaneous and metastatic models of pancreatic and colorectal cancer respectively.**

DCs are an integral part of the interplay between tumor and host [11]. To determine if lack of HMGB1 within DCs would influence tumor engraftment or growth kinetics in a subcutaneous model of pancreatic cancer, cohorts of DCH, control littermates, and heterozygotes were challenged with 5x10^5 Panc02 murine pancreatic cancer cells in the right flank. Tumor volume was then calculated by caliper in a blinded fashion. While tumors readily grew in both the WT and HET cohorts, there was a significant inhibition of growth in DCH mice (FIGURE 3-3-A). We then wanted to confirm this finding in another tumor model. Cohorts of WT and DCH mice were challenged with portal vein injections of luciferase-transduced MC38 murine
colorectal cancer cells. These cells go on to establish metastatic lesions in the liver which grow progressively as we have previously described [70,71]. Tumor volume was detected by injecting mice with luciferin and measuring luminescence via IVIS. As expected, WT tumor-bearing mice experienced tumor growth consistent with previous [70]. However, DCH mice were significantly protected from both tumor engraftment and growth (FIGURE 3-3-B). DCH mice were also protected in long term survival studies approaching statistical significance (p = 0.064) (FIGURE 3-S-3B). Interestingly, both DC and DCH mice strains were able to respond successfully to a conventional immunotherapy, high-dose IL-2 administration (FIGURE 3-S-3A).
Figure 3-3 Genomic ablation of HMGB1 in DCs inhibits tumor growth in subcutaneous and metastatic models of pancreatic and colorectal cancer respectively. (A) WT (n=3), heterozygote (n=3), and DCH (n=3) mice were subcutaneously injected with Panc02 cells in two independent experiments, and tumor volume was measured by caliper every 5 days. (B) WT (n=3) and DCH (n=3) mice received portal vein injections of luciferase™ MC38 colorectal cancer cells in three independent experiments to induce metastatic liver lesions which were quantified by IVIS using luciferin substrate. (+/- SEM, *p<0.05, **p<0.01).
Genomic ablation of HMGB1 in DCs protects in a pancreatic spontaneous cancer model.

While transplantable tumor models remain a useful tool for the study of cancer biology and the evaluation of therapeutic strategies, they fail to recapitulate the step-wise process of carcinogenesis in which inflammatory cells play an important role. KC mice develop malignant precursor lesions within the ductal epithelium termed pancreatic intraepithelial neoplasia (PanIN) lesions which are histopathologically indistinguishable from early human pancreatic neoplasia. Importantly, marked fibrogenesis and inflammatory cell infiltrate is observed within the tumor micro-environment of these animals. The stromal compartment plays a significant role in the progression towards invasive neoplasms [5]. Bone marrow from DCH mice and control littermates were harvested and transplanted into irradiated KC mice recipients, along with WT to WT and KC to KC controls.

Mice from each cohort were sacrificed at 24-26 weeks of age. At this point, PanIN lesions are readily detectable in KC mice. As expected WT to WT bone marrow (BM) chimeras exhibited normal pancreata by H&E, while KC to KC BM chimeras exhibited both neoplastic ducts and extensive fibrogenesis. This was also observed in KC mice which had received HMGB1\textsuperscript{loxP/loxP} littermate BM controls (FIGURE 3-4-A). Strikingly, KC mice which had received DCH bone marrow exhibited near normal ductal morphology and a significantly diminished occurrence of both low and high grade PanIN lesions (FIGURE 3-4-A and 3-4-C) when harvested tissue slides were scored by a pathologist in a blinded fashion. Additionally, KC mice both develop quantifiable pancreatic nodules as carcinogenesis progresses, and the pancreata become larger in size. DCH to KC BM chimeras exhibited significantly lower occurrences of nodules and decreased pancreas weight when compared to control to KC BM controls.
chimeras. (FIGURE 3-4-B) Taken together, these findings suggest that DCs cooperate to promote early carcinogenesis in an HMGB1-dependent manner.

![Image of histological sections and bar graphs showing differences in pancreatic tissue and lesion frequency between WT, KC, WT→KC, and DCH→KC BM chimeras.]

Figure 3-4 Genomic ablation of HMGB1 in DCs is protective against spontaneous carcinogenesis in a model of oncogenic K-Ras driven pancreatic cancer. (A) Representative H&E stained pancreatic tissue from 23-25 week old WT (n=3), KC (n=3), WT→KC BM chimeras (n=3), and DCH→KC BM chimeras (n=3). (B) Quantified numbers of pancreatic nodules in WT→KC and DCH→KC BM chimeras. (C) PanIN lesion frequency and grade in WT→KC and DCH→KC BM chimeras assessed by blinded pathologist. All experiments described in this figure were performed twice. (+/- SEM, *p<0.05)
Next, we examined the frequencies of various immune cell subsets within the spleen and the emerging pancreatic tumor microenvironment in both WT→KC and DCH→KC BM chimeras. Single cell isolates from the spleens of mice from both cohorts were analyzed by flow cytometry. DCH→KC chimeras exhibited significantly diminished levels of myeloid-derived suppressor cells (MDSCs), as well as decreased levels of nominal regulatory T cells (Tregs) (FIGURE 3-5-A), while exhibiting comparable levels of other immune cell types. Similarly, single cell isolates from the pancreata of mice from both cohorts were also examined for immune cell frequencies. DCH→KC BM chimeras exhibited uniformly lower levels of immune cell infiltrate, and notably, DCs, MDSCs, and nominal Tregs were significantly diminished (FIGURE 3-5-B). These findings suggest that in addition to delayed carcinogenesis in DCH→KC chimeras, these mice also exhibit less inflammatory infiltrate within the pancreas.
Figure 3-5 Genomic loss of DC HMGB1 results in diminished infiltrate in the emergent pancreatic tumor micro-environment. (A) Spleens from the WT→KC and DCH→KC BM chimeric mice described in Figure 4 were harvested and processed into single cell suspensions for flow cytometric analysis of systemic immune cell frequencies. Cells were stained with antibodies to phenotypically identify DCs, macrophages, myeloid derived suppressor cells (MDSCs), B cells, CD4+ T cells, CD8+ T cells, and NK cells. Depicted are representative flow cytometric plots of immune cell frequencies and quantified percentages compiled from all mice. (B) Pancreata from the same mice described in (A) were harvested and processed into single cell suspensions and analyzed by flow cytometry in an identical manner as the splenocytes to assay immune cell infiltrate within the pancreatic tumor microenvironment. (+/- SEM, *p<0.05)
Genomic ablation of HMGB1 in DCs renders them sub-optimal as therapeutic vaccines in transplantable melanoma and pancreatic tumor models.

DCs represent a unique immunotherapeutic and are often utilized in vaccine strategies [11,15]. This is accomplished by the isolation and differentiation of DCs, followed by the ex-vivo pulsing with tumor associated or specific peptide, protein, or lysate. Activated DCs are then reinfused so that they may migrate directly to the LN and mediate their effects on resting T cell populations there, thereby abrogating the need for intratumoral DC antigen capture and trafficking. Having demonstrated that DC-specific HMGB1 KO resulted in a protective phenotype in both transplantable and spontaneous genetic models of mouse tumor, we sought to determine if HMGB1 KO would affect DC ability to serve as vehicles for antigen delivery in established therapeutic DC vaccination models.

Cohorts of WT C57/Bl6/N/Tac mice were injected subcutaneously with $5 \times 10^5$ Panc02 cells. BMDCs were then derived from DCH mice and control littermates and co-cultured with $\gamma$-irradiated Panc02 cells overnight as a source of antigen. Mice then received subcutaneous injections of the Panc02:DC mixtures from both strains, irradiated Panc02 alone, or control PBS contra-laterally to the tumor on day 7 and day 14. As expected, WT DCs that had been exposed ex-vivo to irradiated Panc02 cells significantly delayed tumor growth as measured by caliper in a blinded fashion. This protective effect was completely abrogated in the cohorts of mice which were vaccinated with DCH:Panc02 mixtures (FIGURE 3-6-A). To verify the lack of HMGB1 KO DCs ability to induce Panc02-specific T cells, bulk splenocytes were sorted from vaccinated animals and then co-cultured at a 1:1 ratio with irradiated Panc02 cells or an irrelevant tumor cell line (MC38). Supernatants from these co-cultures were harvested and assayed for the presence of IFN$\gamma$ as an indicator of anti-tumor reactivity. As expected, significantly elevated levels of IFN$\gamma$
were only detected in cultures derived from WT DC:Panc02 vaccinated mice, indicating that HMGB1 KO had eliminated the ability for DCs to induce tumor-reactive lymphocytes (FIGURE 3-6-C). To confirm that HMGB1 was critical for DC-mediated anti-tumor immunity, we then repeated a therapeutic vaccination experiment in an antigen-specific system using another tumor cell line (B16 melanoma and the tumor antigen TRP2 with vaccinations on days 5 and 10). Once again, WT DCs pulsed with TRP2 significantly inhibited tumor cell growth, and this effect was completely abolished in HMGB1-KO DCs (FIGURE- 3-6-D). Taken together, these findings suggest that HMGB1 is critical for DC induction of anti-tumor immunity in murine therapeutic vaccination models and confirms that DC HMGB1 is important for initiating endogenous adaptive immune responses.
Figure 3-6 DC HMGB1 confers protection in therapeutic anti-tumor vaccination models (A) BMDCs were derived from both WT(DC) and DCH mice and cultured in GM-CSF and IL-4 supplemented media for 7 days. Concurrently, cohorts of WT mice (n=3 per group) were inoculated with 5x10⁵ Panc02 cancer cells subcutaneously (subq) in the right flank. 1x10⁶ Panc02 cells were γ-irradiated and cultured alone or with WT or KO DCs overnight at a ratio of 1:1. These cells were harvested and injected subq contra-laterally to the tumor in each cohort on both days 5 and 10 post tumor challenge. PBS was used as a control. Tumor volume was measured in a blinded fashion. The experiment was performed twice with similar results. (B) Representative images of mice from panel (A) depicting larger and more severely ulcerated tumors in mice which received DCH:Panc02 vaccines. (C) Splenocytes from each cohort of vaccinated mice from panel (A) were harvested at the termination of the experiment. CD4⁺ and CD8⁺ cells were isolated and subsequently co-cultured with Panc02 cells or an irrelevant cancer cell line (MC38) at a ratio of 1:1 for 48 hrs. IFNγ levels in the resultant supernatant were quantified by ELISA analysis. (D) BMDCs were derived from DC and DCH mice as described in panel (A). Mice were challenged with 5x10⁵ B16 melanoma cells at day 0. WT and KO DCs were the pulsed with TRP2 melanoma antigen and vaccinated on days 5 and 10. Tumor volumes were assayed as described in Figure 3 in a blinded fashion. (+/- SEM, *p<0.05, **p<0.01, ***p<0.001)
**HMGB1** DCs exhibit diminished T cell stimulatory capacity and skew T cell polarization away from Th1/Th2 and towards Th17/Treg phenotypes.

In addition to the modulatory role extracellular HMGB1 secreted from APCs, the requirement of endogenous HMGB1 in DCs for their ability to induce *in-vivo* anti-tumor responses in both multiple tumor and therapeutic vaccination models necessitated the assessment of DC T cell stimulatory capacity in a mixed-leukocyte reaction (MLR). Splenocytes from WT BALB/c mice and stained with CFSE were co-cultured with allogeneic BMDCs from both WT and DCH mice at increasing ratios for 96 hours. Both HMGB1 WT and KO BMDCs induced proliferation of CD3+ lymphocytes by MLR, somewhat diminished in KO DCs ([FIGURE 3-7-A](#)). Supernatants from these MLRs were also collected and assayed by ELISA for the presence of cytokines indicative of distinct helper T cell subsets (IFN\(\gamma\), IL-4, IL-10, and IL-17 for Th1, Th2, Treg, and Th17 responses respectively). As expected, splenocytes co-cultured with allogeneic WT DCs secreted large quantities of IFN\(\gamma\), particularly at high DC:splenocyte ratios. IFN\(\gamma\) concentrations measured in the supernatants of KO DC co-cultures were not significantly greater than the control regardless of the culture ratio employed. Cells cultured with DCH BMDCs secreted significantly higher levels of both IL-10 and IL-17 in a dose dependent manner ([FIGURE 3-7-B](#)). Taken together, these findings suggest that DC HMGB1 is important for the induction of lymphocyte proliferation and T cell polarization.
Figure 3-7 HMGB1 KO BMDCs demonstrate diminished lymphocyte stimulatory capacity and polarize T cells towards regulatory phenotypes. (A) Bulk WT BALB/c splenocytes were stained with CFSE dye and cultured for 96 hrs alone or at ratios of 100:1, 10:1, and 1:1 with BMDCs derived from both WT and DCH mice. Proliferative dilution of the dye was assayed by flow cytometry. Depicted are representative histograms from 3 independent experiments. (B) A mixed leukocyte reaction (MLR) was conducted by coculturing bulk BALB/c splenocytes with allogeneic BMDCs derived from both WT and DCH mice at the same ratios as described in panel (A). After 96 hours, the cell culture supernatant was assayed for the presence of IFNγ, IL-4, IL-10, and IL-17 by ELISA analysis.
**HMGB1** DCs are less responsive to PRR ligation and express lower levels of the HMGB1 receptors TLR4 and RAGE.

We performed flow cytometric analysis of both DCH and control BMDCs for expression levels of the immunostimulatory HMGB1 receptors TLR4 and RAGE, and the immunosuppressive receptor TIM-3. Interestingly, we observed lower levels of cell surface expression of both RAGE and TLR-4, while TIM-3 expression remained unchanged in the absence of HMGB1 (FIGURE 3-8-A). This observation suggests that DC maturation may be inhibited both in vivo in DCH mice and in vitro in BMDCs cultured from them. Of note, the down-regulation of at least TLR4 and RAGE is not DC-specific as our group has observed this in other cell-specific HMGB1 KO mice such as HMGB1 KO NK or NKH mice (data unpublished, Guanqiao Li and Xiaoyan Liang).

Next, we assessed responsiveness to a panel of TLR ligands. HMGB1-expressing and KO BMDCs were cultured for 48 hours in media supplemented with the agonists for TLRs 1-9 or an untreated control and then harvested for flow cytometric analysis for the presence of DCs which expressed very high levels of the DC maturation marker, CD86 as an indicator of PRR responsiveness. As expected, we noted a diminution of CD86\(^{hi}\) DCs derived from DCH mice in comparison to control littermates for a host of TLR ligands (FIGURE 3-8-B). Interestingly, KO DCs were actually more responsive to TLR7 and TLR9 agonists, which both serve as nucleic acid sensors [72].
Figure 3-8 HMGB1 KO DCs express lower levels of stimulatory HMGB1 receptors and are less responsive to TLR agonists. (A) BMDCs derived from DCH and control littermate strains were harvested for flow cytometric analysis of the HMGB1 receptors TLR4 and RAGE, as well as the immunosuppressive receptor, TIM-3 and accompanying isotype controls. (B) BMDCs derived from DCH and control littermate strains were harvested and treated for 48 hrs with a panel of TLR ligands. These cells were then analyzed by flow cytometry for the expression of the DC maturation marker CD86.
**HMGB1 is required for CCL19-mediated chemotaxis in-vitro.**

Preliminary data from our group (FIGURE 3-S-3A and 3-S-3B) suggested that both the sequestration of HMGB1 in the nucleus with ethyl pyruvate or inhibition of secretion with sRAGE blocked CCL19 chemotaxis *in-vitro* and this inhibition could be rescued with the addition of recombinant HMGB1 (FIGURE 3-S-3C). To determine if this result could be recapitulated in our DCH-derived BMDCs, we plated 5x10^5 KO and control DCs in transwell plates for 5 hrs in media supplemented for 5 hours with CCL19 (or not) and quantified the number of migrated DCs by harvesting cells in the lower chamber. We found that chemotaxis in response to CCL19 was abolished in DCH-derived BMDCs (FIGURE 3-9).

![Figure 3-9](image)

**Figure 3-9 HMGB1-/− BMDCs fail to undergo chemotaxis in response to CCL19.** 5x10^5 BMDCs were plated in the upper chamber of a transwell plate. 50ng/ml of rCCL19 supplemented media was placed in the lower chamber. Migrated BMDCs present in lower chamber after 5 hrs of incubation time were quantified (n=3).
3.5 DISCUSSION

Dendritic cells are the “link between innate and adaptive immunity, as they interact and form immunological synapses with innate lymphocytes including natural killer (NK) cells and promote selection of adaptive immune B-cells, and CD4+ and CD8+ T cells [14,30]. DCs also tolerize and therefore limit autoimmunity [73,74]. DCs act as both passive and active participants in the maintenance of both cancer cell proliferation and immune evasion [4,75].

We demonstrate that HMGB1 is not necessary for differentiation of DCs from hematopoietic progenitors as CD11c+ cells can be successfully derived from DCH mouse bone marrow cells when cultured with either GM-CSF alone, or GM-CSF/IL-4-supplemented media. When HMGB1 is genomically ablated in these cells, they are rendered dysfunctional in a variety of ways. Interestingly, disrupting DC function in this way supports their role in limiting the antitumor adaptive responses. DCH mice were protected in several tumor models, including the oncogenic KRas model of pancreatic neoplasia [59,76]. Variations of the models including genomic ablation of p53, high-fat feed diets, and induction of pancreatitis were not examined in this work and therefore represent a possible area of exploration for additional studies [4,77].

Endogenous DCs may play a role in promoting tumor growth, and that this is dependent on their expression of HMGB1 [4]. An alternative explanation is that DCHs could elicit more effective anti-tumor immunity during the progression of pancreatic neoplasia, thereby reducing PanIN lesion occurrence and frequency in the DCH to KC BM chimera cohorts, and tumor burden in the transplantable pancreatic and metastatic models, however this remains a hypothesis which necessitates further study.
Given that DCs prime CD4+ Th1 cells and cytotoxic CD8+ T cells, they have frequently been the target of immunotherapies aimed at eliciting potent anti-tumor adaptive immune responses [34,35]. This is accomplished by the isolation and differentiation of DCs, followed by ex-vivo pulsing with TAA/TSA peptide, purified protein or encoded in genetic constructs, or autologous tumor lysate, often accompanied by various adjuvants [11]. Although DC-based vaccines represent some of the most exciting cancer treatments, they have only enjoyed marginal clinical success [36,37]. This is likely due to cancer cell immune evasive strategies and the various regulatory immune cells during the effector phase of the immune response at the tumor site[38]. We demonstrated that DCs derived from the bone marrow of DCH mice and cultured ex-vivo for use as a therapeutic vaccine fail to confer significant protection from tumor challenge in both antigen-specific (B16, TRP2) and non-specific (irradiated Panc02) models. These DC vaccination models have been consistently evaluated by a variety studies are are particularly useful in the evaluation of DC-mediated anti-tumor responses in murine systems [78,79].

DC maturation, much like T cell activation and polarization, is a process which involves multiple signals acting in concert to successfully facilitate the initiation of an adaptive immune response [22]. In addition to antigen capture and presentation via both lysosomal and proteosomal degradation pathways, ligation of PRRs by PAMPs and DAMPs is critical for DC maturation [41]. HMGB1 is a transcriptional regulator [21]. We found that DCH were inhibited in their ability to stimulate allogeneic lymphocyte proliferation. Heightened production of IL-10 and IL-17 from DCH stimulated lymphocytes compared to WT controls, which secreted significantly more IFNγ and IL-4 into the cell culture supernatant suggests that induced lymphocyte proliferation in the absence of DC HMGB1 is skewed towards more regulatory phenotypes [80,81].
In response to maturational stimuli, DCs translocate nuclear HMGB1 into the cytosol and release it into the extracellular milieu \[18,20,48,51\]. This accompanies a rearrangement of the actin cytoskeleton and upregulation of the chemokine receptors CCR7 and CXCR4, followed by CCL19 and CXCL12 mediated migration, respectively \[18,52\]. In the extracellular milieu HMGB1 binds to these chemokines and induces conformational changes which enhance their ability to bind to their cognate receptors \[82\]. We demonstrated an inability to migrate in response to the LN-homing chemokine, CCL19 and reduced expression of stimulatory HMGB1 receptors and responsiveness to PRR ligation, suggesting these signaling pathways may be disrupted and providing a possible explanation of our observations.

Taken together, these data suggest that DCs play a dichotomous role in cancer biology and treatment, and that HMGB1 is a critical protein in both contexts (FIGURE 3-10). Endogenous DCs in cancer patients are subject to immune dysregulation both systemically and in the tumor micro-environment \[33,59\]. Additional studies are needed to fully elucidate the mechanism behind the tumor-resistant phenotype observed within DCH mice. For instance, our group has established that HMGB1 also plays a role in mitochondrial quality control by promoting mitophagy of effete mitochondria \[83\]. We have performed preliminary studies demonstrating DCH-derived BMDCs have significantly lower basal levels of oxygen consumption in which reconciles with previously published studies involving MEFs and various cancer cell lines (FIGURE 3-S-4). Combinatorial therapeutic strategies aimed at inhibiting cells within the tumor microenvironment are critical to successful immunostimulatory approaches such as APC vaccines, cytokine and cell-based therapies, and tumor targeting antibodies \[84\]. Just as critical, however, is acquiring a deep understanding of what roles individual myeloid cells such as DCs and MDSCs play within a tumor bearing mouse or patient \[7,59,85,86\]. DCs are
crucial for early immune surveillance and the subsequent destruction of nascent transformed
tumor cells [11]. Additionally, as has been observed repeatedly in animal models and human
clinical trials), DC vaccination could effectively induce anti-tumor responses when differentiated
from hematopoietic cells or peripheral blood mononuclear cells [87]. The benefits of this therapy
were it to be “optimized” (antigen, adjuvant, etc.) would be invaluable, as it could elicit an
effective immune response that could control cancer cell nests. However, endogenously-derived
CD11c+ DCs differentiating from the hematopoietic stem cells of cancer patients enter a
physiological environment that is systemically broken from an immunological perspective [75].
Circulating peripheral cytokines that promote autophagy and wound healing responses are
elevated. DAMPs such as HMGB1, ATP, and S100 proteins exist in unusually high levels within
the extracellular space within tumors [81,88-90]. Successful DC:T cell interactions involving
tolerogenic antigen and subsequent regulatory responses could propagate tumor growth and
invasiveness. Given that HMGB1 is critical to DC functionality, as we understand it, rendering
DCs dysfunctional unmask the dichotomous role DCs in the context of cancer biology and
therapy.
Figure 3-10 HMGB1 is critical for both DC vaccination and DC-induced pro-tumor inflammation. Endogenous DCs cooperate to promote pro-tumor inflammation via DAMP/PAMP receptor ligation and thereby exacerbate carcinogenesis. DCs derived from murine bone marrow are not subject to tumor-induced immune dysregulation. Because HMGB1 is important for DC functionality, its genomic absence both inhibits DC anti-tumor therapy and unmasks a role for DCs in facilitating a pro-tumor microenvironment.
3.6 SUPPLEMENTAL DATA

Figure 3-11. Cytosolic translocation and extracellular release of DC HMGB1 in response to maturational stimuli. Murine BMDCs were cultured for 7 days in RPMI supplemented with mGM-CSF and treated for 24 or 48 hours with 100ng/ml LPS. (A) IBs of nuclear and cytosolic fractions of BMDCs show the translocation of nuclear HMGB1 to the cytosol in response to maturation signals. This is recapitulated by IF (B). (C) At 48 hrs, a significant quantity of DC HMGB1 is detectable in the cell-culture supernatant by ELISA after LPS stimulation.
Figure 3-12 Secretion of HMGB1 by DCs facilitates CCL19-mediated chemotaxis, and recombinant HMGB1 is a DC chemoattractant. 5x10^5 BMDCs were plated in the upper chamber of a transwell plate. 50ng/ml of rCCL19 supplemented media was placed in the lower chamber. Migrated BMDCs present in lower chamber after 5 hrs of incubation time were analyzed with the CCK8 cell counting kit (abs. at 450nm). (A) Antagonism of HMGB1 by both nuclear sequestration (10mM EP) and autocrine/paracrine blockade (3μg/ml sRAGE-Fc) resulted in a failure of BMDC migration in response to CCL19. (B) The effects of EP on chemotaxis could be entirely reversed with the addition of 1ug/ml rHMGB1, suggesting that extracellular HMGB1 is both required and sufficient for CCL19-mediated chemotaxis. (C) rHMGB1 alone is a DC chemo-attractant.
Secretion of HMGB1 by DCs facilitates CCL19-mediated chemotaxis, and recombinant HMGB1 is a DC chemoattractant. $5 \times 10^5$ BMDCs were plated in the upper chamber of a transwell plate. 50ng/ml of rCCL19 supplemented media was placed in the lower chamber. Migrated BMDCs present in lower chamber after 5 hrs of incubation time were analyzed with the CCK8 cell counting kit (abs. at 450nm). (A) Antagonism of HMGB1 by both nuclear sequestration (10mM EP) and autocrine/paracrine blockade (3μg/ml sRAGE-Fc) resulted in a failure of BMDC migration in response to CCL19. (B) The effects of EP on chemotaxis could be entirely reversed with the addition of 1μg/ml rHMGB1, suggesting that extracellular HMGB1 is both required and sufficient for CCL19-mediated chemotaxis. (C) rHMGB1 alone is a DC chemo-attractant.
Figure 3-14. HMGB1-KO in DCs lowers basal levels of mitochondrial oxygen consumption. Real-time analysis of oxidative phosphorylation measured by the Seahorse instrument following the addition of (a) oligomycin, (b) FCCP, (c) 2-DG, (d) rotenone in the manner previously described [83]
4.0 RAGE PROMOTES PANCREATIC CARCINOGENESIS AND ACCUMULATION OF MDSCS.


NOTE: All data contained in this chapter was generated by Philip Vernon except qPCR data (Pawel Kalinski, Ravi Muthuswamy) and the generation of the KCR triple-transgenic strain (Tara J Loux).

4.1 ABSTRACT

Pancreatic ductal adenocarcinoma (PDAC) has an aggressive natural history and is resistant to therapy. The receptor for advanced glycation end-products (RAGE) is a pattern recognition receptor for many damage associated molecular pattern (DAMP) molecules. RAGE is overexpressed in both human and murine models of PDA as well as most advanced epithelial
neoplasms. The immunosuppressive nature of the PDAC microenvironment is facilitated, in part, by the accumulation of regulatory immune cell infiltrates such as myeloid-derived suppressor cells (MDSCs). To study the role of RAGE expression in the setting of mutant Ras-promoted pancreatic carcinogenesis (KC), a triple transgenic model of spontaneous murine PDA in a RAGE-null background (KCR) was generated. KCR mice had markedly delayed pancreatic carcinogenesis and a significant diminution of MDSCs compared to KC mice at comparable time points post weaning. While RAGE was not required for the development or suppressor activity of MDSCs, its absence was associated with temporally limited pancreatic neoplasia and altered phenotype and function of the myeloid cells. In lieu of MDSCs, KCR animals at comparable time points exhibited mature CD11b^+Gr1^-F4/80^+ cells which were not immunosuppressive in vitro. KCR mice also maintained a significantly less suppressive milieu evidenced by marked decreases in CCL22 in relation to CXCL10 and diminished serum levels of IL-6.

4.2 INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is a largely lethal disease. The incidence and mortality rates in the United States are nearly identical, and patients who achieve a 5 year survival from time of diagnosis are rare (6%) [2,91]. Histopathologically, PDA progresses in a conserved fashion beginning with the emergence of malignant precursor lesions termed pancreatic intraepithelial neoplasia or PanIN lesions which progressively become more severely hyper- and dysplastic and finally culminate in frank invasive cancer [3,6]. While
immunotherapies such as cellular and peptide-based vaccines, monoclonal antibodies against tumor signaling molecules, and recombinant cytokines have exhibited moderate successes in many tumor types, pancreatic cancer patients have yet to benefit substantially from these strategies [10]. A significant obstacle to immunotherapeutic intervention is the immunosuppressive pancreatic tumor microenvironment [5]. Immune suppression is facilitated by soluble factors such as TGFβ, IL-10, VEGF, and FasL derived from both the tumor and stromal compartments, the downregulation of NK cell surface ligands such as MICA/B in tumor tissue, and a marked increase in regulatory immune cell infiltrate consisting of regulatory T cells (Tregs), tumor associated macrophages (TAMs), and myeloid derived suppressor cells or MDSCs [5,76,92].

MDSCs are a recently identified heterogeneous population of hematopoietic cells of myeloid lineage [86,93]. While normally present in relatively low quantities in the peripheral blood of healthy individuals, PDAC patients exhibit high frequencies of these cells in the peripheral blood [94]. Furthermore, MDSCs traffic to tumor tissue where they directly inhibit anti-tumor immune effector cells through a variety of mechanisms including depriving T cells of nutrients via arginase-I production and tryptophan and cysteine depletion, interfering with trafficking by inducing the downregulation of CD62L and L-selectin, and upregulating reactive nitrogen and oxygen species (NOS and ROS) [86,93,95,96]. MDSCs further support immune suppression in the tumor micro-environment by promoting reparative wound healing and angiogenesis and facilitating the recruitment of regulatory T cells [97].

Identifying MDSCs based on cell surface marker expression in cancer patients has been difficult given the phenotypic heterogeneity. Several subsets have been characterized as CD11b+CD14+, CD11b+CD33+, and CD11b+CD15+, with the latter primarily being recruiting to
primary tumor tissue in PDA patients [93,98]. In mice, however, these cells are readily and reliably identified by the co-surface expression of CD11b and Gr1 and can be further characterized based on the expression of IL-4Rα and limited expression of co-stimulatory molecules such as CD80 [86,99]. They accumulate in the spleen, blood, and tumor in a variety of murine tumor models [59,89]. In a spontaneous Kras^{G12D}-driven transgenic model of PDA that recapitulates the histopathology of human PDA with high fidelity (termed “KC” mice), MDSC accumulate both systemically (measured in the spleen) and locally in pancreas tissue [59].

The receptor for advanced glycation end-products (RAGE) is an MHC class III encoded protein, characterized as a damage associated molecular pattern (DAMP) molecule receptor. It serves as the cognate receptor for the prototypical DAMP, high-mobility group box 1 (HMGB1) and several S100 proteins including S100A8 and S100A9 [49,88]. RAGE-mediated signaling plays a role in the pathogenesis of epithelial derived cancers such as PDA by activating key survival pathways such as autophagy in cancer cells as well as propagating and sustaining pro-tumor host inflammatory responses [9,49,71,100]. While normal pancreatic ductal epithelial cells do not routinely stain positively for RAGE, with the emergence and progression of pancreatic neoplasia, RAGE is markedly upregulated and overexpressed [9]. The contribution of RAGE to intratumoral MDSC accumulation was first demonstrated in RAGE^{−/−} mice in an inducible skin cancer model [101]. In addition, mice deficient for the RAGE ligand, S100A9, exhibit a significant reduction in the incidence and burden of colitis-associated colorectal tumors and show demonstrable decreases in intratumoral and splenic MDSC frequency [89,90,102]. To determine if RAGE plays a role in the accumulation of MDSCs during pancreatic carcinogenesis, we have backcrossed RAGE-null mice into the KC strain. The resultant Pdx1-Cre:Kras^{G12D/+ :}

Rage^{−/−} mice are termed “KCR”. We demonstrate here that the targeted ablation of RAGE in the
emerging Kras-driven tumor microenvironment limits development of PanIN lesions and the associated accumulation of MDSCs.

4.3 MATERIALS AND METHODS

Mouse strains
Wild-type C57BL/6 mice were purchased from Taconic (Hudson, NY, USA). RAGE knockout (Rage\(^{-/-}\).GFP) mice (SVEV129xC57BL/6) were obtained from Dr. Angelika Bierhaus [103] as a kind gift. Pdx-1-Cre and Kras\(^{G12D/+}\) transgenic mice were obtained from the MMHCC/NCI Mouse Repository. The genotypes Pdx1-Cre:Kras\(^{G12D/+}\) (termed KC) and Rage\(^{-/-}\) were crossed to generate Pdx1-Cre:Kras\(^{G12D/+}\) : Rage\(^{-/-}\) mice (termed KCR). Genomic and recombination screens were done by polymerase chain reaction and analysis of GFP expression (data not shown).

Flow Cytometry
Flow cytometric analysis was performed on the C6 flow cytometer (Accuri Cytometers, Ann Arbor, MI, USA) instrument provided by the University of Pittsburgh Cancer Institute Flow and Imaging Cytometry core facility and analyzed using FlowJo software (Tree Star Inc, Ashland, OR, USA). Murine spleens were homogenized through a 70\(\mu\)m nylon filter (BD Biosciences, San Jose, CA, USA) and washed with PBS. Red blood cells were lysed with Red Blood Cell Lysing Buffer (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer’s instructions. Single cell suspensions were derived from the pancreas by mechanical separation and collagenase digestion (Sigma-Aldrich, St. Louis, MO, USA). The resulting single cell
suspensions were then stained with the following fluorescently labeled antibodies: CD11b, CD11c, F4/80, Gr1, IL-4Rα and corresponding isotype controls (all from BD Biosciences, San Jose, CA, USA). In cases, the cells were then permeabilized with 0.2% Triton-X and stained with antibodies to intracellular iNOS/NOS Type II and Arginase-I (BD Biosciences, San Jose, CA, USA). During analysis viable cells were identified via forward and side scatter and gated accordingly. Phenotyping of MDSC cell populations in KC and KCR mice (Figure 3) were based on a CD11c^+Gr1^+ gate.

Histogram
Harvested pancreatic tissue was formalin fixed (Sigma-Aldrich, St. Louis, MO, USA) and stained with hematoxylin and eosin and mounted onto glass slides by the University of Pittsburgh Department of Pathology. Images were visualized and captured at a magnification of 10X by Nikon Eclipse E800 fluorescent microscope under bright field settings (Melville, NY, USA).

Co-culture assays
4x10^5 Bulk wild-type splenocytes were obtained as previously mentioned from C57/BL6 mice. To activate effector splenocytes, cells were incubated with T-activator CD3/CD28 Dynabeads (Dynal AS, Oslo, Norway) at a ratio of 1:1 according to the manufacturer’s instructions. For co-culture conditions, splenocytes from KC and KCR mice were incubated with αGr1 or αCD11b microbeads and magnetically separated using MACS LS separation columns (all from Miltenyi Biotec, Leiden, The Netherlands) according to the manufacturer’s instructions and purity assayed by flow cytometry. 4x10^5 (termed “low”) or 8x10^5 (termed “hi”) Gr1^+ or CD11b^+ cells were
incubated with bulk WT splenocytes for 96 hours. The resulting supernatants were harvested for analysis.

**In-vitro differentiation of MDSCs from murine bone marrow**

MDSCs were generated *in-vitro* using the following method. Fresh bone marrow flushed from the femurs and tibias of mice was resuspended in media supplemented with G-CSF (100ng/ml) and GM-CSF (250 U/ml) and cultured for 3 days. On day 4, 80 ng/ml of IL-13 was added to the culture for an additional 24 hours [104]. All cytokines were from eBioscience, San Diego, CA, USA. Harvested cells routinely were 50% CD11b⁺Gr1⁺ and were magnetically sorted as previously mentioned for functional analysis.

**ELISA analysis**

Supernatants from co-culture experiments or serum from murine peripheral blood (obtained via direct cardiac puncture) was assayed for detectable levels of IL-2, IFNγ, and IL-6 by ELISA Ready-SET-Go! Kits (eBioscience, San Diego, CA, USA) according the manufacturer’s instructions.

**Quantitative PCR**

Pancreatic tissue from 35 week old mice was harvested and snap frozen in liquid nitrogen. Messenger RNA was then isolated and analyzed for the relative quantity (RQ) of CCL22, CXCL10 mRNA and then normalized to a control housekeeping gene, HPRT1 and displayed as a ratio. Analysis was performed with StepOne software (Applied Biosystems, Foster City, CA, USA)
Statistical analysis

Data are expressed as means +/- SEM of at least two independent experiments performed in triplicate. Statistical analysis was performed using a two-tailed student’s T test. P values below 0.05 were assigned statistical significance.

4.4 RESULTS

Targeted ablation of RAGE attenuates development of high grade PanIN lesions in KC mice.

To determine if RAGE contributes to pancreatic carcinogenesis, 20 week and 35 week old WT, RAGE-null, KC, and KCR mice were sacrificed and their pancreata harvested and stained with H & E for visualization of PanIN lesion formation. At 20 weeks, both the KC and KCR strains show the occurrence of atypical ducts. However, glandular and islet cells remain largely intact, and both strains exhibit a paucity of fibrotic and inflammatory tissue (FIGURE 4-1-A). At 35 weeks of age KC mice exhibit extensive atypical ductal morphology within pancreatic tissue, the emergence of low and high grade PanIN lesions, and severe fibrogenesis indicative of emergent pancreatic carcinogenesis. In KC mice in which RAGE has been chromosomally ablated (KCR mice), the frequency of hyperplastic and dysplastic ductal epithelium is substantially decreased and the integrity of glands and beta cells in the islets is largely retained (FIGURE 4-1-B). Furthermore, we have previously reported that these mice have a significantly enhanced survival rate [9]. These findings suggest that RAGE directly or indirectly contributes to the pathogenesis of early pancreatic carcinogenesis.
Figure 4-1 KCR mice exhibit mark decreases in PanIN lesion development and progression. (A and B) H & E staining of pancreatic tissue sections from wild-type, RAGE-null, KC, and KCR mice harvested at (A) 20 weeks of age and (B) 35 weeks of age. PanIN lesions are designated with arrows.
**RAGE promotes splenic MDSC accumulation during Kras-mediated pancreatic carcinogenesis.**

MDSCs accumulate within the spleens of KC mice as they progress towards ductal adenocarcinoma [59]. To determine if the integrity of the RAGE signaling pathway is an important factor in this observation, WT, RAGE-null, KC, and KCR mice were sacrificed at 35 weeks of age at which point animals expressing mutant Kras display evidence of pancreatic carcinogenesis manifested by the presence of low and high grade PanIN lesions within the pancreatic ducts (FIGURE 4-1-B). Spleens from these mice were processed into single-cell suspensions for flow cytometric analysis of immune cell frequencies. Both WT and RAGE-null animals exhibit comparable basal levels of CD11b\(^+\)Gr1\(^+\) MDSCs ranging from 3-4% (FIGURE 4-2-A) in the absence of apparent pathology. As has been previously documented, Kras-driven pancreatic carcinogenesis resulted in a significant increase in splenic MDSC frequency to 20-35\% [59]. Interestingly, KCR mice fail to accumulate MDSCs with a relative paucity of CD11b\(^+\)Gr1\(^+\) cells (FIGURE 4-2-A and FIGURE 4-1-B). This is significantly less than the KC strain (p=0.0225) at comparable times. However other cells of myeloid-lineage such as myeloid dendritic cells (DCs) identified by the co-expression of CD11b and CD11c are elevated in both KC and KCR mice to similar extents, suggesting that the reduction in splenic MDSCs in the KCR strain is restricted to CD11b\(^+\)Gr1\(^+\) cells.

To observe how the kinetics of MDSC accumulation are affected by the presence or absence of RAGE, mice from all strains were sacked at 20, 24, 26, and 35 weeks of age and their splenocytes assayed for MDSC frequency. As precursor lesions develop in the pancreas, the KC mice begin to accumulate high levels of MDSCs at 26 weeks of age. This is significantly elevated when compared with KCR mice (p=0.049) at the same time point. This becomes further
accentuated by 35 weeks of age (p=0.023) (FIGURE 4-2-C). The absence of RAGE is associated with a significant lack of MDSC accumulation over time in these animals. These findings suggest that RAGE is critical for in vivo MDSC accumulation during pancreatic carcinogenesis. Of note, a study by Connolly et al using the Kras-driven pancreatic neoplasia model with the additional p48 mutation found that splenic CD11b^+Gr1^+ expansion was not evident until 9 months of age and that MDSCs isolated from non-tumor tissue did not exhibit a suppressive phenotype [105].
Figure 4-2 RAGE ablation is associated with limited splenic MDSC accumulation during Ras-mediated pancreatic carcinogenesis. (A) Representative flow cytometry diagrams of splenocytes stained for co-surface expression of MDSC markers CD11b and Gr1 and myeloid DC markers CD11b and CD11c from 35 week old wild-type (n=2), RAGE-null (n=2), KC (n=8), and KCR (n=5) mice. Note the boxes denoted MDSCs are distinct from the boxes denoting DC populations. (B) Scatter plots depicting the frequencies of splenic MDSCs identified by expression of CD11b, Gr1, and IL-4Rα and frequencies of splenic DCs of myeloid origin (CD11b+CD11c+) in mice from panel A. (C) Wild-type, RAGE-null, KC, and KCR mice were sacked at 20 (n=2 per strain), 24 (n=2 per strain), 26 (n=2 per strain), and 35 weeks (n=2 WT and RAGE-null,
n=8 KC, n=5 KCR). The kinetics of splenic MDSC accumulation with the progression of pancreatic neoplasia (KC and KCR mice) is depicted.

**RAGE is dispensable for the differentiation and suppressor activity of MDSC.**

The failure of MDSCs to accumulate within the spleens of KCR mice could be due to a requirement for RAGE to develop MDSCs within the bone marrow compartment. To determine if RAGE is critical for either the differentiation of MDSCs from hematopoietic progenitor cells or their immune inhibitory activity, both KC and KCR mice were sacrificed at 20 weeks of age and their spleens harvested for MDSC phenotyping and functional analysis. At 20 weeks of age, both KC and KCR mice exhibit small but measurable CD11b+Gr1+ MDSC populations as assessed by flow cytometry. These cells are present at levels comparable to WT and RAGE−/− animals (2-3%) (FIGURE 4-3-A). These cells were stained for cell surface expression of the MDSC marker, IL-4Rα and the mature macrophage marker, F4/80 which is absent or expressed at low levels on MDSCs, which typically display an immature myeloid phenotype [89,90]. These cells were also stained for intracellular expression of iNOS/NOS Type II and arginase-I which facilitate the suppressive function of MDSCs on effector and helper T cells [106,107]. CD11b+Gr1+ cells from both the KC and KCR strains expressed IL-4Rα, iNOS/NOS Type II, and arginase-I (FIGURE 4-3-B) Interestingly, MDSCs derived from the spleens of KCR mice exhibited higher expression of both IL-4Rα and arginase-I than those derived from KC mice (FIGURE 4-3-A and 4-3-B). As expected, MDSCs from neither strain expressed F4/80 (FIGURE 4-3-B).

To confirm that these cells which phenotypically resembled MDSCs were capable of suppressing T cell activity and to determine if RAGE was required for suppressor function, they
were isolated by magnetically activated cell sorting based on their positivity of Gr1 and evaluated in an in vitro co-culture assay. Bulk WT splenocytes were stimulated with αCD3;αCD28 activator beads alone or in a co-culture with the isolated MDSCs from both the KC and KCR strains at a ratio of 1:1 (“MDSC [low]”) or 1:2 (“MDSC [hi]”). After 96 hours of co-culture, the resultant supernatants were harvested and analyzed by ELISA for levels of the proliferative T cell survival cytokine, IL-2 and the Th1-polarizing cytokine IFNγ as indicators of relative T cell proliferation and activity. As expected, stimulation with αCD3;αCD28 activator beads induced significant secretion of both IL-2 (p<0.01) and IFNγ (p<0.05) from WT splenocytes (FIGURE 4-3-C). When co-cultured with MDSCs at either ratio isolated from KC or KCR mice, the secretion of these cytokines was inhibited and remained at non-stimulated levels. Importantly, there was no significant difference between MDSCs derived from either strain in terms of their suppressive capacity (FIGURE 4-3-C). To further validate the lack of a requirement for RAGE in MDSC hematopoiesis, MDSCs were generated in vitro from both non-tumor bearing WT and RAGE−/− mice. CD11b+Gr1+ cells were generated to equal extents (50%) in marrow from both strains (FIGURE 4-S1-A). Furthermore, these cells were sorted and evaluated for suppressor function and previously described. Cells from both strains were equally suppressive (FIGURE 4-S1-B). These findings suggest that RAGE is not critical for the development of MDSCs in vivo or their ability to exert suppressor function in vitro. Moreover, the failure of KCR mice to accumulate splenic MDSCs during pancreatic carcinogenesis is not due to an impaired ability to generate functioning MDSCs.
Figure 4-3 RAGE is dispensable for the differentiation and suppressor activity of MDSC. (A) Representative flow cytometry diagrams of splenocytes from 20 week old KC (n=2) and KCR (n=2) mice stained for co-surface expression of MDSC markers CD11b and Gr1. (A, B) Flow diagrams of splenocytes gated for CD11b and Gr1 positivity and stained for surface expression of IL-4Rα and F4/80, and intracellular expression of iNOS/NOS type II and arginase-I from KC and KCR mice. (A) Graphical representation of the MFIs of each marker (+/- SD). (B) MFI values illustrated on diagrams. Demarcations based on isotype controls (data not shown). (C) MDSCs from mice in panels A and B were magnetically separated and co-cultured with bulk wild-type splenocytes stimulated with CD3/CD28 activator beads at rations of 1:1 (MDSC [low]) and 2:1 (MDSC [hi]). Graphs depict IL-2 and IFNγ levels detected in the resultant supernatants by ELISA (+/- SEM, *p<0.05, **p<0.01, NS = not significant).
Figure 4-4 RAGE is not required for the hematopoietic differentiation of MDSCs or for suppressor activity. (A) Representative flow cytometry diagrams of bone marrow-derived MDSCs obtained from 8 week old wild-type and RAGE-/- mice. Cells were stained for the co-expression of CD11b and Gr1, as well as the dendritic cell marker, CD11c as a control. (B) In vitro cultured MDSCs from panel A were magnetically separated and co-cultured with bulk wild-type splenocytes stimulated with αCD3/αCD28 activator beads at ratios of 1:1 and 2:1. Graph depicts IL-2 and IFNγ levels detected in the resultant supernatants by ELISA (+/- SEM).
Splenic myeloid cells derived from KCR mice exhibit a mature phenotype and do not exhibit suppressor function.

A lack of MDSC accumulation during pancreatic carcinogenesis in KCR mice could be due to a failure in MDSC recruitment. Alternatively, the absence of RAGE could result in the induction of the differentiation of MDSCs from an immature (Gr1⁺) to a mature (Gr1⁻) phenotype or a failure to prevent such a differentiation from occurring. To investigate the latter hypothesis, 35 week old WT, RAGE-null, KC, and KCR mice were sacked and their splenocytes analyzed for expression of CD11b and the mature macrophage marker, F4/80 by flow cytometry. In both WT and RAGE-null mice, populations of CD11b⁺F4/80⁺ and CD11b⁺F4/80⁻ cells are discernible (FIGURE 4-4-A). In the KC strain, as expected, there is a significant increase in the frequencies of CD11b⁺F4/80⁻ cells which are mostly Gr1⁺ MDSCs (FIGURE 4-4-A and 4-4-B). Interestingly, compared with the KC strain, the majority of cells in the KCR spleen which stained positive for CD11b now expressed F4/80, suggesting these cells now exhibited a mature myeloid phenotype (FIGURE 4-4-A and 4-4-B) [108].

To determine if these CD11b⁺F4/80⁺ cells which comprised the majority of CD11b⁺ cells in the KCR spleen had lost their capacity to suppress effector immune cells, they were positively selected based on the expression of CD11b by MACS. CD11b⁺ cells from KC spleens were also isolated to compare with the F4/80⁺-enriched cells derived from the KCR mice. Again these cells were co-cultured in vitro with bulk WT splenocytes that were stimulated with αCD3;αCD28 activator beads at 2 ratios for 96 hours as described previously for FIGURE 4-3. Resultant supernatants were then harvested and analyzed by ELISA for secreted IL-2 and IFNγ. As expected, CD11b⁺ myeloid cells derived from the KC strain which were enriched for Gr1⁺F4/80⁻ cells inhibited the secretion of both IL-2 and IFNγ (FIGURE 4-4-C). In contrast, myeloid cells
isolated from the KCR strain at the same time point and enriched for Gr1 F4/80+ cells failed to suppress activated splenocyte cytokine secretion significantly (p<0.05). This suggests that in lieu of the MDSCs that accumulate in KC mice with the progression of pancreatic neoplasia, in the absence of RAGE, myeloid cells are either recruited or differentiated into mature F4/80+ cells which lack suppressor activity and could contribute to both the paucity of “classical” MDSCs and the inhibited occurrence of malignant pancreatic lesions.
Figure 4-5 Splenic myeloid cells derived from KCR mice exhibit a mature phenotype and do not exhibit suppressor function. (A) Representative flow cytometry diagrams of splenocytes from 35 week old KC (n=6) and KCR (n=5) mice stained for the cell surface expression of the myeloid-lineage marker CD11b and the mature macrophage marker F4/80. (B) Scatter plot depicting the frequencies of splenic myeloid cells exhibiting immature phenotypes identified by the expression of CD11b and the lack of F4/80 expression in mice from panel A. (C) CD11b+ splenocytes from mice in panels A and B were magnetically separated and co-cultured with bulk wild-type splenocytes stimulated with αCD3/αCD28 activator beads at ratios of 1:1 (CD11b+ [low]) and 2:1 (CD11b+ [hi]). Graphs depict IL-2 and IFNγ levels detected in the resultant supernatants by ELISA (+/− SEM, *p<0.05).
RAGE promotes a regulatory milieu within the emerging pancreatic tumor micro-environment.

The presence or absence of host regulatory immune cell infiltrate in the pancreatic tumor micro-environment is in part regulated by factors present both within tumor tissue and circulating in the peripheral blood [109,110]. To investigate whether there were differences in the tumor micro-environments of both KC and KCR mice that might contribute to both the failure to accumulate MDSCs in the absence of RAGE and the influx of mature, non-suppressive myeloid cells in KCR mice (FIGURE 4-4), pancreatic tissue was harvested from 35 week old WT, RAGE-null, KC, and KCR mice and analyzed for mRNA levels of chemokines known to attract either immunoregulatory (CCL22) or immunostimulatory (CXCL10) immune cells by quantitative PCR [111,112]. To determine what the relative levels of mRNA of these chemokines are in relation to each other, the ratio of CCL22 to CXCL10 was obtained. As expected, KC mice demonstrate a significant increase in CCL22:CXCL10 indicating a pronounced regulatory chemokine milieu in the pancreata of these mice. In the absence of RAGE, this ratio is significantly diminished (p=0.037), suggested that the emerging tumor micro-environments of these animals is substantially less likely to recruit regulatory infiltrate (FIGURE 4-5-A).

We have recently reported that ablation of RAGE in the emerging tumor micro-environment attenuates STAT-3/IL-6 pathway activation in the KC model [9]. IL-6, a cytokine often associated with epithelial-derived cancers and general tissue damage, has also been implicated in MDSC recruitment and persistence through a STAT3-dependent signaling pathway [85,113,114]. To compare serum IL-6 levels between the KC and KCR strains, peripheral blood was obtained via direct cardiac puncture of 35 week old mice and assayed for IL-6 concentration by ELISA. While serum IL-6 levels were relatively low in WT and RAGE-null mice, KC mice
exhibited a significant increase in the concentration of the cytokine in their peripheral blood. This was not the case for the KCR strain, which exhibited significantly diminished levels of IL-6 (p=0.038) which were comparable with both WT and RAGE-null mice (FIGURE 4-5-B left). Further illustrating the role of IL-6 in the recruitment and survival of MDSCs, mice were divided into two groups, regardless of strain: those with <100pg/ml serum IL-6 and those with >100pg/ml IL-6. The MDSC frequencies in the spleens from mice from these two groups were then assayed by flow cytometry. Mice with >100pg/ml of serum IL-6 were significantly more likely to have greater splenic MDSC accumulation (p=0.022) (FIGURE 4-5-B right). Single cell suspensions derived from the pancreata of 35 week old mice from both strains were examined for MDSC frequencies as well as expression of F4/80 (Figure 4-5-C). A failure to accumulate MDSCs in response to endogenous carcinogenesis was observed in the KCR strain. This was in contrast with the KC strain, which showed a demonstrable expansion of these cells as has been previously reported [59]. Interestingly, nearly uniform F4/80 expression was observed in CD11b+ cells in the KCR strain, but not in KC mice, where the vast majority of CD11b+ cells remained negative for F4/80. Taken together, these findings suggest that RAGE has a critical role in the promotion of an immunoregulatory milieu within the emergent tumor microenvironment during pancreatic carcinogenesis. The lack of the regulatory chemokine CCL22 in relation to the immunostimulatory chemokine CXCL10, coupled with the normal serum IL-6 levels in response to the diminished incidence and timing of Kras-mediated pancreatic neoplasia could contribute to inhibited MDSC accumulation in the absence of RAGE expression.
Figure 4-6 RAGE is associated with a regulatory milieu within the emerging pancreatic tumor microenvironment. (A) Scatter plot depicting the ratio of the relative quantity (RQ) of CCL22 to CXCL10 mRNA derived from the pancreatic tissue of wild-type (n=1), RAGE-null (n=1), KC (n=6), and KCR (n=4) mice at 35 weeks of age as measured by quantitative PCR. (B) Left: Scatter plot depicting IL-6 levels detected in the serum of the peripheral blood in mice from panel A by ELISA (+/- SEM). Right: Scatter plot comparing frequencies of splenic MDSCs in mice from panel A (all strains) with <100pg/ml detectable serum IL-6 with those with >100pg/ml serum IL-6. (C) Representative flow diagrams of cells expressing CD11b, Gr1, and F4/80 in the pancreata of KC and KCR mice at 35 weeks.
4.5 DISCUSSION

In this study, we demonstrate that RAGE plays a critical role in promoting pancreatic neoplasia and the resultant immunosuppressive tumor micro-environment. When RAGE is ablated in mice which spontaneously form PanIN lesions due to mutant Kras expression, the emergence and progression of these malignant lesions are substantially attenuated. Concurrently, the accumulation of splenic MDSCs that occurs over time in KC mice is limited in the absence of RAGE. Our findings suggest that the integrity of RAGE is critical for pancreatic carcinogenesis and responding MDSC accumulation. Importantly, RAGE is not required for the development of MDSCs from myelopoietic progenitor cells or their specific inhibitory activity, as MDSCs are found in both RAGE-null and KCR mouse strains and are phenotypically and functionally intact. Interestingly, during pancreatic neoplasia in the absence of RAGE, a substantial majority of myeloid cells (CD11b+) exhibit a more mature phenotype manifested by the expression of the mature macrophage marker F4/80 and a loss of Gr1 expression [89,108]. When isolated and evaluated in an in vitro suppression assay, these cells had notably lost their inhibitory activity. This suggested that RAGE is a factor in either myeloid cell plasticity or determining which myeloid subsets respond to emergent pancreatic carcinogenesis. However, alternative explanations for the maturation of myeloid cells towards a more mature F4/80+ phenotype exist. Inability to recruit more suppressive cells types or apoptosis of other myeloid subsets could explain this observation. Additionally, the chemokine milieu in the pancreatic tumor micro-environment was markedly less regulatory in KCR mice illustrated by the relative abundance of CXCL10 in relation to CCL22. The levels of IL-6, which have been implicated in
the STAT3-dependent recruitment and retention of MDSCs [85], were also significantly decreased in the KCR strain, and these levels directly correlated with measured MDSC frequency within the emergent pancreatic tumor micro-environment.

The proinflammatory proteins S100A8/A9 induce MDSCs by interacting with RAGE and other glycoproteins on the surface of MDSCs and promote their migration via NF-κB-dependent signaling [89]. In addition, RAGE overexpressed within the tumor and stromal compartments is concurrently ligated by S100A8/A9 synthesized by MDSCs which induces a regulatory chemokine tumor gene profile and serves as a positive feed-back loop for the further recruitment of MDSCs [89,90]. Our findings suggest that RAGE ablation in the emerging tumor micro-environment leads to diminished signaling through the IL-6/STAT3 pathway. It is likely that the failure to accumulate MDSCs in KCR mice is due to a combined effect of RAGE deletion in both the tumor and bone marrow compartments in the global RAGE knockout strain. Future studies will examine targeted ablation in each exclusive compartment to further elucidate these mechanisms. Other cells of myeloid lineage (particularly dendritic cells and macrophages) have been shown by our group and others to secrete another RAGE ligand, HMGB1, in response to various maturational stimuli, therefore HMGB1 secretion by MDSCs represents an additional mechanism for MDSC recruitment to be explored [19,20].

Our observations suggest an important mechanism driving an immunosuppressive environment during early pancreatic carcinogenesis. However, several questions remain concerning the relationship between cancer-related inflammation and the accrual of MDSCs. First it is not clear if the pancreatic carcinogenesis that promotes the accumulation of MDSCs is an epiphenomenon or if the recruitment of MDSCs contributes to carcinogenesis by limiting effective immune editing. Secondly, the failure of various immunotherapies, particularly within
the context of pancreatic cancer, is attributed to the presence of host regulatory immune cells like MDSCs and Tregs [5]. It is not clear if targeting MDSC function in these settings may render these therapies more effective. Our results demonstrating a critical role for RAGE in MDSC accumulation in the context of pancreatic carcinogenesis identifies a novel pathway that can be explored to address these questions possibly by assessing established strategies such as IL-2 administration in this model [115]. Lastly, given the role of RAGE in promoting autophagy within tumor cells and evidence of immune cell-mediated autophagy (particularly T and NK cells), it would be interesting to explore whether interactions with MDSCs modulate these observations in KCR mice [71].

4.6 ACKNOWLEDGEMENTS

We dedicated this chapter to the memory of Angelika Bierhaus, PhD, our friend and benefactor. She was a pioneering RAGE biologist, generous and kind in her dealings with colleagues, who provided us the mice with which we completed this work and who sadly passed away on April 15, 2012 after a long and courageous battle with cancer. Angelika had a great love of life; she was generous, kind, and warm hearted. She was always full of plans for scientific endeavors, even when her disease began to take its toll. She remained steadfast that she would not be defeated, but despite recently celebrating her 50th birthday, she was aware her remaining time was short. She dedicated herself to research, but despite her incredible strength, she was not able to overcome the disease which tragically took her life.
5.0 SIGNIFICANCE OF THIS NEW PERSPECTIVE OF HMGB1, DAMPS, MYELOID CELLS AND CANCER

The etymology of the word "cancer" is crab, just like the zodiac sign. It's from the Latin, originally meaning "a crab," and later, "malignant tumor." The Greek physician Caludius Galen, among others, working in Rome in the 2nd century A.D. noted the similarity of crabs to some tumors with swollen veins. Earlier, Hippocrates and associated writers (fifth to fourth century B.C.) utilized the terms carcinos and carcinoma, for all chronic ulcerations. Celsus (first century A.D.) used the term cancer to refer to a deep ulcer [116].

Since that time, like the word, our understanding of ‘cancer’ has undergone many additional changes. In contemporary biomedicine, the focus was initially directly on the cancer cell itself, its genomic mutations, and its immortality (and reasonably so). However, with the sequencing of the human genome in its entirety and the slow trickle of useful therapeutics targeting cancer cells directly, cancer biologists and clinicians began to examine epithelial-derived cancers such as pancreatic cancer as a microenvironment of heterotypic interactions between tumor cells, stromal fibroblasts, vasculature, and inflammatory infiltrate and not solely the cancer cell themselves. In fact, the presence of actual cancer cells in a resected human solid tumor can be as low as 10%. Cancer is a pathology, but it is also a microcosm of evolution within an organism reflecting Darwinian selection of both the tumor and associated
The DAMP Hypothesis. Most thoughtful individuals would now agree that cancer is fundamentally a genetic disorder of the genes with acquisition of genomic instability and changes in genes that regulate apoptosis, senescence, metabolic pathways, and cell cycle progression. Multiple molecular alterations in human tumors have been catalogued and captured in the setting of the wholesale sequencing of now over a thousand tumors[91]. The development of cancer is thus a result of genetic alterations within the tumor cell itself but is also associated with the extensive host response noted above. We would ask whether a singular, unifying hypothesis would explain the many developmental stages of cancer. Cancer in adults arises as the end stage of chronic inflammation with some exceptions (sarcoma and glioma for example). A more integrated approach is required to understand this integrated biology of adult (and not childhood) cancer. My sense, after my period of training in the Center for DAMP Biology is that the fundamental defect that explains changes that we associate with development of invasive cancer is the loss of apoptotic cell death and emergence of pronounced autophagy, associated with what we call the ‘DAMP Hypothesis’.

Chronic Inflammation and Cancer. As our understanding of how each of these compartments relate to each other and the dynamic between them that led to carcinogenesis broadened, we began to understand how crucial inflammation was to this process [33,42]. Additionally, we examined ways to harness immune cells and their cellular products therapeutically. Anti-tumor immunotherapy could very well become the major success of personalized medicine. Unfortunately, our understanding of the relationship between the immune system and cancer was
and still is limited. As both cancer and immuno-biological research become more integrated, we began to realize the many ways cancer was co-opting immunity via the exploitation of reparative wound healing. This process involves the recruitment of innate and adaptive immune cells as well as neo-vascularization and stromagenesis. Cancer growth requires myeloid cells, so-called tumor associated macrophages (TAMS), which remove effete cells and cellular fragments, release factors such as matrix metalloproteinases (MMPs) that break down the basement membrane enabling cancer cell migration and metastasis. They also release angiogenic and mitogenic factors including VEGF which promote growth of the primary tumor[7,38,99].

**Targeting Myeloid Cells in Tumor Immunotherapies.** Combinatorial therapeutic strategies aimed at inhibiting cells within the tumor microenvironment are critical to successful immunostimulatory approaches such as APC vaccines, cytokine and cell-based therapies, and tumor targeting antibodies. Just as critical, however, is acquiring a deep understanding of what roles individual myeloid cells such as DCs and MDSCs play within a tumor bearing mouse or patient. DCs are crucial for early immune surveillance and the subsequent destruction of nascent transformed tumor cells. Additionally, as has been observed repeatedly in animal models (including in this work itself [See FIGURE 3-6]) and human clinical trials), DC vaccination could effectively induce anti-tumor responses when differentiated from hematopoietic cells or peripheral blood mononuclear cells. The benefits to this therapy, were it to be “optimized” (antigen, adjuvant, etc.) would be invaluable, as it could elicit an effective immune response that could control residual cancer cell nests. However, endogenously-derived CD11c+ DCs differentiating from the hematopoietic stem cells of cancer patients enter a physiological environment that is systemically broken from an immunological perspective. Circulating
peripheral cytokines that promote autophagy and wound healing responses are elevated. DAMPs such as HMGB1, ATP, and S100 proteins exist in unusually high levels within the extracellular space within tumors [7,86]. Successful DC:T cell interactions involving tolerogenic antigen and subsequent regulatory responses could propagate tumor growth and invasiveness. Given that HMGB1 is critical to DC functionality, as we understand it, rendering DCs dysfunctional unmasks the dichotomous role DCs in the context of PDAC biology and therapy.

**Myeloid Derived Suppressor Cells.** The apparent pro-tumor effects of MDSCs have been widely studied and well-characterized. In our KC pancreatic cancer model, nearly 25% of all splenocytes are double positive for CD11b and Gr1. In murine models, significantly lowering the frequencies of these cells can result in enhanced tumor protection (see Chapter 4). This makes them attractive therapeutic targets for epithelial cancers including PDAC. These are heterogeneous populations of cells in human cancer patients, making targeting them directly with a biological such as neutralizing antibodies or small molecule strategies very difficult (CD11b⁺CD14⁺, CD11b⁺CD33⁺, and CD11b⁺CD15⁺) [7]. However, this work suggests an alternative, receptor-based strategy in which blockade of a protein such as RAGE may reduce tumor development and subsequent MDSC accumulation, perhaps making PDAC more susceptible to immunotherapies. Alternatively, based on the findings described in Chapter 3, concerns about collaterally depleting DCs in novel anti-tumor therapies targeting MDSCs or TAMs, may be unnecessary. In fact, they could even represent a mechanism of action for these approaches, or at the very least, contribute to an anti-tumor response. This work demonstrates that myeloid cells, possibly promoting wound healing by cancer-derived DAMPs (HMGB1) and
the receptors they over express (RAGE) are potential obstacles to the treatment of patients with PDAC.

FUTURE DIRECTIONS
With the generation of Cre-recombinase/LoxP technology and the availability of a global HMGB1loxP/loxP mouse, our group and others have been able to circumvent the lethality found in the HMGB1−/− mouse. In our case, we exploited this to derive our DCH transgenic strain. As my mentor, Dr. Michael Lotze says: “You know you’ve asked a good question, when answering it leads to ten more good questions”. There are many questions that remain to be addressed.

PANCREATIC TISSUE MICROARRAY
Crucial to any study is its capacity to provide insights that can translate from a laboratory to a clinical setting. As other studies have shown and this work demonstrates (FIGURE 4-1-2), DCs play an early and important role in spontaneous genetic models of murine pancreatic cancer. Therefore a tissue microarray of biopsies from PDAC patients at various stages should be evaluated with human αCD11c antibody to determine if our observations are recapitulated in patients with pancreatic cancer. Previous studies have suggested, like pediatric cancers that advanced pancreatic cancers from patients harbor few dendritic cells [75]

DCH GENE MICROARRAY
HMGB1’s primary roles are that of a transcriptional regulator and promoter of autophagy and mitochondrial quality control as previously discussed. We have concluded from this work that HMGB1 is critical for the functionality of DCs, thereby inhibiting them in a variety of ways.
However, we cannot conclude from this work whether our observations are directly mediated by HMGB1 itself, any of its transcriptional targets, or a combination of the two. Therefore a gene microarray would be very useful as a hypothesis-generating experiment. We have performed preliminary studies to this end.

**DCH METABOLISM STUDIES**

Our group has established that HMGB1 also plays a role in mitochondrial quality control by promoting mitophagy of effete mitochondria. We have performed preliminary studies demonstrating DCH-derived BMDCs have significantly lower basal levels of oxygen consumption. These studies need to be repeated, and additional experiments such as ATP production should be conducted, as metabolic dysregulation may be linked to much of the findings in this work.

**DCH VACCINIA STUDIES**

In a collaborative effort with Dr. Steven Thorne of the University of Pittsburgh Cancer Institute, we are evaluating the viral clearance in DCH mice challenged with a luciferase$^+$ strain vaccinia virus strain and in IL-18BP deleted virus as measured by IVIS. Interestingly, DCH animals clear the virus more rapidly.

**KCR BONE MARROW CHIMERA STUDIES**

RAGE expression is critical for the development of pancreatic neoplasia and the subsequent accumulation of systemic and intra-tumoral MDSCs [7]. However, we cannot attribute these findings to a specific RAGE-expressing tissue or cell. Although differentiating between tumor
and stroma is experimentally challenging, bone marrow transplants of KCR BM into KC mice would allow us to assess the role RAGE expression in BM-derived cells for our observations.

RAGE AS A CLINICAL TARGET

Given the overexpression of RAGE in PDAC and our studies detailed in Chapter 4, RAGE is an attractive therapeutic target for patients with pancreatic cancer. Examples of potential therapies include RAGE-specific neutralizing or blocking antibodies, small molecule inhibitors, or treatment with a soluble RAGE protein to prevent ligand binding. In addition, with collaborators, we have initiated studies designed to develop and evaluate the role of PET/CT RAGE imaging agents.
APPENDIX A

THE MYELOID RESPONSE TO PANCREATIC CARCINOGENESIS IS REGULATED BY RAGE


A1.1 ABSTRACT

We identified RAGE as an important mediator in the accumulation of regulatory myeloid infiltrate during pancreatic carcinogenesis. The absence of RAGE markedly delayed neoplasia and limited MDSC accumulation in mice expressing oncogenic Kras. In lieu of MDSCs, these mice accumulated non-immunosuppressive macrophages. Thus, RAGE regulates carcinogenesis and subsequent myeloid responses.
A1.2 AUTHOR’S COMMENTARY

Pancreatic cancer is a notoriously difficult-to-treat malignancy with many of the hallmarks of immunosuppression. Prevention of successful anti-tumor immune responses to pancreatic neoplasia is accomplished in part by the recruitment and retention of myeloid-derived suppressor cells (MDSCs) [59]. This heterogeneous population of cells works to directly inhibit the activities of immune effector cells through several mechanisms including nutrient deprivation and the production of reactive nitrogen and oxygen species. While difficult to phenotypically characterize in humans, they can readily and reliably be identified in mice by the co-expression of CD11b and Gr1 [90].

Damage associated molecular pattern molecules (DAMPs) serve as inflammatory signals which alert the host to cellular and tissue injury [17]. DAMPs and their cognate receptors shape the nature of both the innate and adaptive immune responses, in some cases possessing potent adjuvant qualities, while promoting reparative wound-healing in others [17]. The receptor for advanced glycation end-products (RAGE) is a pattern recognition receptor that is ligated by several DAMPs including high-mobility group box 1 (HMGB1) and S100A8/A9 [9]. Aside from lung tissue, expression of RAGE is typically confined to the immune compartment, but becomes overexpressed in many carcinomas, including pancreatic ductal adenocarcinoma [9,49,117]. Studies from our group and others have demonstrated a role for RAGE in sustaining tumor growth via the promotion of a pro-tumor inflammatory milieu, and it has been associated with the release of IL-6 [9].

Previous studies have implicated S100A8/A9 in the recruitment and retention of MDSCs in murine models of colorectal cancer, and peripheral levels of these proteins correlate with MDSC frequencies in the serum of patients with gastric cancers [118]. We sought to determine if RAGE
regulated the myeloid response to early pancreatic carcinogenesis using a murine model of oncogenic Kras-driven pancreatic cancer (KC mice) [119]. These mice were backcrossed into a RAGE-null background to generate mice which would develop malignancy in the absence of the receptor (KCR mice).

We found that in the absence of RAGE, pancreatic carcinogenesis proceeded at a slower rate, and this observation was accompanied by a failure to accumulate MDSCs both systemically and within the tumor micro-environment (TME) which occurs in the KC model (FIGURE 5-1). While RAGE itself was not required for MDSC development, the phenotype and function of the myeloid cells (CD11b⁺) in the KCR strain were distinct from those assayed in KC mice. At comparable ages, in lieu of Gr1 positivity, CD11b⁺ cells within the spleen and pancreas expressed the mature macrophage marker, F4/80 and were indeed non-immunosuppressive in vitro [7]. It is currently unclear whether the apparent lack of MDSCs in the KCR strain is directly contributing to retarded carcinogenesis, or if their absence is a consequence of inhibited neoplasia observed when RAGE is chromosomally ablated. While several explanations exist for the failure to accumulate MDSCs, including diminished longevity/enhanced apoptosis, given the lower levels of CCL22 in the pancreatic TME of KCR mice and studies demonstrating increased tumor expression of CXCL1, CCL5, and CCL7 in response to activation with RAGE ligands, it is likely that MDSC recruitment is disrupted when RAGE is no longer expressed [90,117]. Secondly, the precise role of the CD11b⁺F4/80⁻Gr1⁻ cells which can be observed accumulating in the spleens and pancreata of KCR mice is unknown (FIGURE A1). Although they are not immunosuppressive, future studies will determine whether they have any direct anti-tumor effects, as macrophages have been demonstrated to be critical for anti-tumor immunity mediated by CD40 agonism [5].
Lastly, it is unknown which compartment RAGE signaling needs to be retained in order to accumulate MDSCs. Three distinct possibilities include the tumor itself, the stroma, or the bone marrow compartment. While it is experimentally difficult to distinguish between tumor and stroma, the immune compartment can readily be implicated with the transplantation of WT or RAGE-null bone marrow into KC recipients, and these studies are ongoing.

As we appreciate more deeply the significant role regulatory immune cell infiltrate such as MDSCs plays in facilitating carcinogenesis, it becomes very important to understand which receptors expressed on both these and cancer cells mediate their recruitment and retention. Importantly, the identification of immune-regulatory roles of proteins such as RAGE in various cancers render them appreciable targets for inhibiting pro-tumor myeloid responses in cancer patients.
Figure 5-1 RAGE Signaling Recruits Myeloid Derived Suppressor Cells and DC. A schematic depicting the role of the DAMP receptor, RAGE, in the recruitment of myeloid-derived suppressor cells during early pancreatic carcinogenesis. RAGE ligands such as HMGB1 and S100A8/A9 are present in the tumor microenvironment. They are actively and passively secreted from stressed and dying tumor cells, as well as secreted by certain immune cells. These ligate RAGE, which is overexpressed on transformed cells, and cause the expression of chemokines implicated in angiogenesis and reparative wound-healing. In the absence of RAGE, these MDSCs fail to accumulate, instead resulting in mature macrophage-like cells and the inhibition of carcinogenesis.
APPENDIX B

EAT ME: AUTOPHAGY, PHAGOCYTOSIS, AND ROS SIGNALING


A2.1 ABSTRACT

Phagocytosis has long been a recognized process required for the resolution of dying cells and the subsequent regulation of inflammatory responses mediated by phagocytic cells such as macrophages, monocytes, dendritic cells, and neutrophils. Autophagy, an evolutionarily ancient process of lysosomal self-digestion of organelles, protein aggregates, apoptotic corpse and cytosolic pathogens, has only recently become appreciated for its dynamic relationship with phagocytosis such as microtubule-associated protein 1 light chain 3 (LC3)-associated phagocytosis (LAP). Signal transduction by reactive oxygen species (ROS) plays a critical role in the modulation of autophagy, phagocytosis and LAP, and serves as both a link and an additional layer of regulation between these processes. Furthermore, specific targets for oxidation by ROS molecules have recently begun to become identified in both processes, as have “shared” proteins which facilitate the successful completion of both autophagy and phagocytosis.
High-mobility group box 1 (HMGB1) protein, a DNA-binding nuclear protein and prototypic damage-associated molecular pattern molecule (DAMP), is at the crossroads of autophagy, phagocytosis, and oxidative stress. Given the broad implications autophagy, phagocytosis, and ROS signaling have in human health, disease, and the maintenance of cellular and organismal homeostatic balance, it has become increasingly important to continue to delineate intersections between these pathways and uncover targets for potential therapeutic interventions against autoimmune and inflammatory diseases.

A2.2 INTRODUCTION

Like all living things, most cell types have a limited life span, which ends physiologically or pathophysiologically through the process of cell death. Although it had long been recognized that cell death can be an important part of normal animal development and tissue homeostasis, the mechanism of cell death and how to remove dead cells by healthy neighboring cells remains largely unknown. [120] Two main cell deaths are apoptosis and necrosis. Apoptosis or “programmed cell death” is a process which is indispensable for the maintenance of tissue development and homeostasis and in these regards, organismal survival. Likewise, mechanisms for both the facilitation and resolution of apoptosis are equally indispensible. Biochemical events lead to characteristic cell changes in apoptosis include blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation. Necrotic cell death resulting from cell or tissue injury in many cases represents an event requiring an immune responses to promote pathogen clearance and reparative wound healing. Clearance of dying cells is important in preventing autoimmunity, [121] as animals deficient of mechanisms responsible for the phagocytosis of dying cells often become autoimmune. [122,123] Moreover, failure to
properly clear dying cells has been linked to non-resolving inflammation and developmental abnormalities. [124] Phagocytosis, or the internalization of large particles, is crucial for both the clearance of the apoptotic bodies that are the remnants of completed programmed cell death and for the clearance of necrotic cell debris and antibody coated-foreign pathogens and the initiation of subsequent inflammatory responses. [125-127] Unlike the latter which are recognized by phagocytic cells such as macrophages by pattern recognition and Fc receptors for degradation and presentation to adaptive immune cells, phagocytosis of apoptotic cells is immunologically silent. [128] Typically accompanied by the secretion of regulatory cytokines such as interleukin-10 (IL-10) and transforming growth factor beta (TGF\(\beta\)), disruption of this silence has drastic consequences for the host, including the development of auto-immunity and pathological inflammatory responses [129]. This distinction necessitates the utilization of unique signaling pathways once cargo has become engulfed to ensure proper intracellular processing for either presentation to immune cells, or the silent resolution of successful apoptosis.

Clearance of dying cells is a complex process in which many surface molecules, adaptors, and chemotactic molecules are involved, and it is controlled at multiple levels. Despite these differences in outcomes (pro-versus anti-inflammatory), there is a certain degree of crosstalk between intracellular compartments once cargo has been internalized. Autophagy, literally “self-eating”, is the process by which cells clear protein aggregates, whole organelles, and other components for the concomitant purposes of nutrient derivation and the mitigation of cell stress mediated by potentially cytotoxic aggregates and organelles (e.g. mitochondria) which have persisted past their usefulness and are also an important source of antigen when they degrade invasive pathogens and abnormal self-proteins [130-132]. As a constitutive process involving the formation of double-membranous vesicles which both receive input from
endosomal compartments and other membrane source and ultimately fuse with lysosomes, [133,134] this process has a dynamic relationship with both immuno-stimulatory and silent phagocytosis. This relationship is made further complex by the active role redox-dependent signal transduction plays in modulating both phagocytosis and autophagy (FIGURE A-1). [135-137]

Given the commonalities between the phagocytic and autophagic pathways including shared molecules and their sensitivity to redox signaling, this article reviews the most recent findings which link elements of both pathways together, specifically through redox-dependent signal transduction. Furthermore, these interconnected cellular processes are placed in the context of cell death and immunity in both health and disease.
Fig. A2-1. Schematic representation of the recognition of target cells and pathogen by phagocytosis and autophagy. Reactive oxygen species (ROS) have been identified as signaling molecules in various pathways regulating assessment of both phagosome and autophagosome formation and maturation. Eventually phagosome and autophagosome fuses with the lysosome as phagolysosome and autolysosome so that the pathogens, dying cells, or cell debris can be degraded.
A2.3 EAT-ME, FIND-ME, AND DAMP SIGNALS

In order for a dying cell to become phagocytosed it must be rendered recognizable to phagocytes (tissue-resident macrophages/monocytes and dendritic cells). Generally speaking, the initiation of successful phagocytosis relies on four steps: [126,138] 1) the release of “find-me” signals which recruit phagocytes; 2) the receptor ligation-dependent recognition of “eat-me” signals which facilitate engulfment; 3) the processing and degradation of the corpse mediated through a series of phagosome maturation steps, and 4) suppression or initiation of inflammatory responses depending on additional innate immune stimuli. Recently, potential find-me signals released by apoptotic cells were reported, namely lysophosphatidylcholine (LPC), [139] CX3CL1/fractalkine, [140] sphingosine 1-phosphate (S1P), [141] and nucleotides adenosine triphosphate (ATP) and uridine 5 triphosphate (UTP), [142] which binds to G-protein-coupled receptors including P2Y2, CX3CR1, S1P1-5 and G2A in macrophages and monocytes (FIGURE A-2). Unfortunately, current understanding of “find-me” signals and in what contexts they become more or less important is still limited. Interestingly, the iron-binding protein lactoferrin could serve as an anti-attraction (“keep-out”) signal by different apoptotic cell lines as it inhibits the migration of granulocytes. [143] Although macrophages and dendritic cells are highly motile under basal conditions, certain factors associated with cell death are known to possess chemotactic properties. Many damage-associated molecular pattern molecules (DAMP) which are released actively during cell stress or passively during necrosis (FIGURE A2-3) or late stage apoptosis are associated with chemotaxis. [144] High mobility group box-1 (HMGB1), the prototypic DAMP, when released into the extracellular milieu both attracts macrophages and dendritic cells by itself, and also binds to chemokine such as CCL19 and CXCL12, [145] thereby promoting more efficient gradient-dependent migration towards these chemokines. Other
DAMPs, including nucleotides, ATP, and UDP have also been ascribed chemoattractant properties and are known to be passively released from necrotic cells when the integrity of the plasma membrane is compromised and actively released from apoptotic cells via pannexin 1 channels.[146] Given the fluidity of the plasma membrane during both forms of cell death, it is possible additional, unidentified intracellular proteins may also escape into the extracellular space and recruit phagocytes to sites of injury and apoptosis.

By contrast, “eat-me” signals which facilitate recognition by phagocytes are much more understood. [126,138] Eat-me signals or phagocytosis ligands can be classified into two major categories, membrane-anchored eat-me signals [e.g., phosphatidylinerse (PtdSer), intercellular adhesion molecule-1 (ICAM-1), calreticulin] and soluble bridging molecules [e.g., growth-arrest-specific 6 (Gas6), protein S, globule EGF factor 8 protein (MFG-E8), β2-glycoprotein-I (β2-GPI) and annexin I]. These signals can be trafficked to the cell surface by canonical and non-canonical secretory pathways, as well as “revealed” by both conformational and enzymatic modification of the plasma membrane. [147] PtdSer (which localizes to the inner leaflet of the plasma membrane) becomes displayed on the cell surface when portions of the lipid bilayer flip during apoptosis. [148] This allows interaction with receptors and bridging molecules such as T-cell immunoglobulin domain and mucin domain 4 (TIM4), MFG-E8, brain angiogenesis inhibitor 1 (BAI1) and Mer tyrosine kinase (MerTK) expressed on phagocytes and mediates phagocytosis. [122,123] Proteins, such as calreticulin, can also be upregulated on the surfaces of cells during apoptosis and interact with and activate distinct phagocytic receptors such as low density lipoprotein receptor-related protein (LRP, also called CD91). [149]

Unlike apoptosis, cells which burst during necrosis or are opsonized with antibodies due to the expression of abnormal self or pathogen-derived antigen are recognized by phagocytes
such as macrophage through macropinocytosis mechanisms independent of apoptotic cells (FIGURE A2-3). [150] Macropinocytosis is a form of bulk uptake of fluid and solid cargo into cytoplasmic vacuoles, called macropinosomes, and has been studied mostly in relation to antigen presentation. In contrast, necrotic cells can be phagocytosed by dendritic cells. [151] In addition to the activation of complement cascades, an antibody coated cell or pathogen becomes immediately recognizable to phagocytes which express cell surface receptors that bind to the Fc region of an antibody termed Fc receptor (FcR). [152] Activation of these receptors initiates the phagocytosis of the target cell, and in concert with pattern recognition receptor (PRR) ligation by pathogen associated molecular pattern (PAMP) molecules or DAMPs, initiate maturational programs within the phagocyte which are capable of stimulating adaptive immune responses via presentation of antigenic peptides in class II major histocompatability complexes (MHC-II) derived from the phagocytosed target cell. [153]
Fig. A2-2. Eat me signals, find me signals and phagocytic receptors for the engulfment of apoptotic cells. The apoptotic cells express or release various eat me and find me signals, which bind to phagocyte-cell-surface receptors either directly or indirectly through bridging molecules. Multiple eat-me signals and their receptors can form a cluster within the phagocytic cup as the engulfment synapse to facilitate the clearance of apoptotic cells. LPC: lysophosphatidylcholine (LPC); S1P: sphingosine 1-phosphate; ATP: adenosine triphosphate; UTP: uridine 5 triphosphate; MEG-E8: globule EGF factor 8 protein; Gas6: growth-arrest-specific 6; BAI1, brain angiogenesis inhibitor 1; PtdSer: Phosphatidylserine; TIM4, T-cell immunoglobulin domain and mucin domain 4; Tulp1: tubby-like protein 1; β2-GPI :β2-glycoprotein-I; TREM2: Triggering receptor expressed on myeloid cells 2; MerTK: Mer tyrosine kinase; LRP: low density lipoprotein receptor-related protein.
Damage associated molecular pattern molecules (DAMPs) are molecules that can initiate and perpetuate immune response in the noninfectious inflammatory response. Many DAMPs are nuclear or cytosolic proteins, and released following necrosis. Once released into the extracellular space, DAMPs bind DAMP receptors in macrophages or other phagocytes, and mediate inflammation and/or remove necrotic cells by macropinocytosis and phagocytosis. HMGB1: high mobility group box-1; ATP: adenosine triphosphate; HSP: heat shock protein; TSP1: thrombospondin-1; HRG: Histidine-rich glycoprotein; C1q: complement 1q; C3/C4: complement 3 and 4; MBL: mannose-binding lectin; TREM1: Triggering receptor expressed on myeloid cells 1; RAGE: the receptor for advanced glycation end products; TLR: Toll-like receptor.
A2.4 PHAGOCYTOSIS: A PROCESS OF KILLING A DEAD CELL OR MICROBE

Unlike autophagy, phagocytosis has been a recognized cellular process for over a century [154]. Whereas macropinocytosis involves the constitutive “imbibing” of extracellular fluid in a non-specific fashion, phagocytosis is a highly regulated process dependent on the aforementioned receptor-ligand interactions. The most well characterized form of phagocytosis is the internalization of antibody-coated particles (opsonized) by the FcR, and is therefore discussed as an example of how phagocytosis proceeds at the molecular level.[155,156] It should be noted however, that although there are numerous forms of phagocytosis which operate under both parallel and distinct initial molecular regulation, the downstream mechanisms of vesicular formation and fusion are largely conserved.

Ligation of a singular FcR by an opsonized particle is not sufficient to initiate phagocytosis.[157] In order to propagate a successful signal to initiate phagocytosis, engagement of multiple receptors is required. This is accomplished by the clustering of receptors that occurs immediately following initial engagement. In turn, this clustering facilitates the ligation of more unoccupied FcR sites and so forth. Following this, a tyrosine phosphorylation-dependent signaling cascade [156,158] is transduced within the phagocyte which causes the recruitment of specialized adaptor proteins such as Grb2-associated binder 2 (Gab2) [159] and CrkII [160] which both mediate the recruitment of additional scaffolding proteins and initiate the formation of the cellular machinery required for the reorganization of both the actin cytoskeleton [161] and the plasma membrane [162] necessary for successful phagocytosis. Importantly, phagocytosis is significantly obstructed when these adaptor proteins are chromosomally deleted in mice. [159] Rapid and efficient removal of dying cells and foreign microbes by phagocytes is important during development, tissue homeostasis and in immune responses (FIGURE A2-4).
Initially, the plasma membrane serves as the source of lipid membrane for a newly forming phagosome, [162] although there is evidence suggesting additional organelles such as endoplasmic reticulum contribute membrane as the phagosome matures. [163] The early phagophore forms after scission from the plasma membrane and begins to mature as it traffics towards the lysosome in a GTPase dependent fashion. [164] The late phagosome acquires a much more acidic Ph [165] and fuses with the lysosome via interactions with lysosome-associated membrane proteins (LAMP)-1 and LAMP-2 [166]. This newly formed vesicle is termed the phagolysosome and is a degradative organelle in which lytic proteins process the phagocytic cargo and ultimately derive both nutrients and antigenic peptides for MHC-II presentation to immune effector cells. [153] Toll like receptor (TLR) signaling has been reported to regulate phagosome maturation in macrophages and dendritic cells.[167,168] Moreover, engaging the autophagy pathway via TLR signaling enhances phagosome maturation by microtubule-associated protein 1 light chain 3 (LC3)-associated phagocytosis (LAP). [169] However, other studies demonstrate that TLR stimulation does not impact on phagosome maturation by defined particles and quantitative methodology. [170-172]
Fig A2.4 Discriminating between necrosis, apoptosis and autophagy. Cells can respond to stress in a variety of ways ranging from the activation of survival pathways such as autophagy to the initiation of cell death such as apoptosis that eventually eliminates damaged cells. If apoptotic cells are then rapidly cleared by phagocytes, the dead cells don’t release their intracellular DAMPs and the immune system is not stimulated. Whereas the necrotic cells release of DAMPs then stimulates proinflammatory response. In contrast, autophagy play a dual role in regulation of inflammation depends on context. HMGB1: high mobility group box-1; RAGE: the receptor for advanced glycation end products; TLR4: Toll-like receptor-4.
A2.6 AUTOPHAGY: A LYSOSOMAL DEGRADATION PATHWAY

Much like phagocytosis, autophagy is a process which impinges on the formation, maturation, and fusion of vesicles encapsulated by lipid membrane and facilitates the degradation of selective cargo derived from intracellular components including whole organelles such as mitochondria and ribosomes, as well as cytotoxic protein aggregates. Additionally, foreign pathogens acquired by endosomal fusion of cytoplasmic sequestration are also degraded by a specialized form of autophagy termed xenophagy [130,131]. There are at least three recognized types of autophagy: macroautophagy, microautophagy, and chaperone-mediated autophagy (FIGURE A2-5). [132] This review focuses on macroautophagy and will hereafter be referring to macroautophagy as simply “autophagy”.

The initial steps of autophagy involve the formation of a specialized, double-membranous vesicle termed the isolation membrane, namely phagophore. Recent studies have found that the sources of membrane for these nascent autophagic vesicles can be from the plasma membrane itself, the golgi complex, and even mitochondrial membrane. [134] Additionally, under conditions of differing autophagy-initiating events, the primary membrane source may also be different. For example, during cellular starvation-induced autophagy, studies suggest that mitochondria are the primary source from which membrane is derived. [173] As the isolation membrane matures the protein LC3-I (called Atg8 in yeast) becomes covalently lipidated into LC3-II and incorporated into the membrane as a crucial scaffolding protein. [174] Given this role, the conversion of LC3-I to LC3-II serves as a marker for heightened autophagic flux and not surprisingly, cells deficient for LC3 are unable to successfully initiate autophagy. [175] Prior to fusion of the isolation membrane and the formation of the closed vesicle termed the autophagosome, autophagic cargo is recruited via adaptor molecules such as p62, [176] nuclear
dot protein 52 kDa (NDP52) [177], and neighbor of BRCA1 gene 1 (NBR1) [178]. These molecules contain ubiquitin-binding domains which recognize poly-ubiquitinated protein aggregates, organelles and bacteria. The autophagosome then traffics to and fuses with the lysosome forming the autolysosome wherein the cargo is proteolytically degraded. Importantly, autophagosomes continuously receive input from endosomes and have been demonstrated to fuse with MHC-II loading compartments, thereby making the autophagosome an essential source of antigen for presentation to CD4\(^+\) helper T cells. [179] Furthermore, antigens specifically targeted to the autophagosome by fusion with an LC3 construct are much more effectively presented to adaptive immune cells and illicit functionally superior responses.[180] Notably, autophagy contributes to dying cell clearance during apoptosis [181]. Dying cells in autophagy gene-null embryoid bodies fail to express eat-me signals (e.g., PtdSer) and secrete lower levels of find-me signals (e.g., LPC). [181]

The molecular mechanisms governing the initiation of autophagy in response to various stimuli are complex and not fully delineated. In a general sense, autophagy can be classified as being either mammalian target of rapamycin (mTOR)-dependent or independent. [182] mTOR is a nutrient sensor associated with the lysosome which, when inhibited, initiates signaling events which lead to enhanced autophagy. [183] This is due to mTOR’s function of inhibiting Atg1 which is required during the initiation of autophagy. [184] As such, many pharmacological agents which induce autophagy operate through this pathway (mTOR inhibitors) and include the drug rapamycin.[183] The protein Beclin 1 (called Atg8 in yeast) appears to be central to pre-autophagic signaling. [185] Normally Beclin 1 bound is to the anti-apoptotic protein Bcl-2, this binding is abrogated during autophagic signaling, [186] and Beclin 1 interacts with class III phosphatidylinositol 3-kinase (PI3KC3) to further transduce the message
Fig A2-5. Types of autophagy. Microautophagy refers to the sequestration of cytosolic components directly by lysosomes through invaginations within their limiting membrane. Chaperone-mediated autophagy involves direct translocation of unfolded substrate proteins (KFERQ-like motif) across the lysosome membrane through the action of a cytosolic and lysosomal chaperone heat shock cognate protein of 70 kDa (Hsc70), and the integral membrane receptor lysosome-associated membrane protein type 2A (LAMP-2A). In the case of macroautophagy, the cargo is sequestered within a unique double membrane cytosolic vesicle, an autophagosome. The autophagosome, itself, is formed by expansion of the phagophore. The autophagosome undergoes fusion with lysosome, to form an autolysosome, in which the sequestered material is degraded. Autophagy, an intrinsically nonselective process, can also target selective cargo for degradation such as mitophagy, lipophagy, ribophagy and xenophagy.
A2.7 SIGNAL TRANSDUCTION BY REACTIVE OXYGEN SPECIES

Reactive oxygen species (ROS) are formed as a metabolic by-product of electron transfer to molecular oxygen. This generation can occur in a number of ways which are generally associated with mitochondrial function and respiration. Given the drastic physiological and pathological outcomes which are observed through the modulation of ROS, it can be concluded that ROS signal transduction plays a critical role for maintaining eukaryotic homeostasis.

ROS is generated by both complexes within the electron transport chain (Complex I and III) as well as by nicotinamide adenine dinucleotide phosphate reduced (NADPH) oxidase (FIGURE A2-6) and additional enzymes which are tightly associated with the mitochondria, including the monoamine oxidases critical for the metabolism of monoamine signaling proteins such as dopamine and serotonin. [187] In the presence of mitochondrial superoxide dismutase (SOD), $O_2^-$ can be converted to hydrogen peroxide ($H_2O_2$), which can then diffuse out of the mitochondria into the cytoplasm. In the presence of high iron concentrations, $H_2O_2$ can form the highly reactive $O_2$ via the Fenton reaction. Catalase is responsible for converting $H_2O_2$ to water and oxygen. $O_2^-$ can also react with nitric oxide to form the highly reactive peroxynitrite ($ONOO^-$). Other sources of ROS include the endoplasmic reticulum and peroxisome.[188,189]

Interestingly, many signaling events result in dramatic elevations in ROS levels. Ligation of receptors by growth factors such as epidermal growth factor (EGF) induces this response [190], but given the reactivity of ROS molecules, it is difficult to imagine how specific ROS-mediated signaling can be. However, evidence is beginning to emerge regarding the trafficking of ROS across the plasma membrane. ROS, specifically hydrogen peroxide, appears to cross the membrane in a specific manner via aquaporin channels, representing a method of potential
regulation. [191] Regardless, there are certainly many identifiable targets of ROS signaling. Studies have demonstrated that phosphotases become transiently inactivated following increases in ROS levels, and many cellular processes including regulation of responses to hypoxia, inflammatory responses (specifically formation of the NLRP3 or NOD-like receptor pyrin domain-containing 3 inflammasome), phagocytosis, and autophagy are all responsive to ROS signaling. [135,136,192]
Fig. A2-6. ROS and the regulation of autophagy. Mitochondria and NADPH oxidase associated with phagocytosis are major source of reactive oxygen species (ROS). ROS act as signaling molecules in regulation of autophagy by targeting autophagy genes (e.g., ATG4), transcription factor [e.g., hypoxia-inducible factor (HIF)-1α] and signal transduction systems [e.g., mammalian target of rapamycin (mTOR) and mitogen-activated protein kinases (MAPKs)]. In contrast, upregulated autophagy inhibits ROS production by mitophagy-mediated impaired mitochondria remove, or increases ROS production by degeradating catalase. LC3: microtubule-associated protein 1 light chain 3; ATM: ataxia telangiectasia mutated; TSC2: tuberous sclerosis protein 2; BNIP3:BCL2/adenovirus E1B 19 kDa protein-interacting protein 3; PKB: protein kinase B; NADPH: nicotinamide adenine dinucleotide phosphate.
A2.8 REDOX SIGNALING AND PHAGOCYTOSIS

ROS signal transduction plays a critical role in the cellular physiology of phagocytic cells. [136] Oxidant bursts observed after growth receptor ligation also occur in macrophages, neutrophils, etc. both during phagocytosis and in response to various stimulatory signals. Specific ROS-sensitive targets in these cells have also been identified. Nuclear factor (NF)-κB is a dimeric transcription factor that is involved in the regulation of a large number of genes that control various aspects of the immune and inflammatory response. NF-κB which is normally localized to the cytosol is translocated to the nucleus in its active form in response to oxidative stress.[193,194] In addition, mitogen-activated protein kinases (MAPK) including p38 and JNK which require phosphorylation for activation are responsive to the kinase apoptosis-signal kinase-1 (ASK-1) which is maintained in an inactive state until ROS-mediated oxidation of its binding partner thioredoxin liberates it. [195]

The most obvious and therefore well-studied role for ROS within phagocytes such as macrophages is for their bactericidal properties. NADPH oxidase is a multicomponent enzyme localized in the plasma membrane of phagocytic leukocytes. The generation of ROS in these cells is mediated by NADPH oxidase which, when absent, results in an inhibited capacity to clear pathogens. [196] It is possible that the ROS generated by NADPH oxidase contribute to host defenses not only through their microbicidal action but also through modulation of redox-sensitive pathways in phagocytes. Recent studies suggest that NADPH oxidase (NOX2) activity decreases proteolytic efficiency of the phagosome through prolonged modification of the lumenal redox environment and oxidation of cysteine cathepsins. [197] Anti-oxidant enzymes are required to neutralize phagocytosis and stimulant-induced oxidant bursts and return ROS levels to basal concentrations in these cells. Additionally, as previously mentioned ROS
signaling particularly through NF-κB may play an important role in the transcription and translation of NF-κB target genes which include pro-inflammatory cytokines and chemokines which both recruit and stimulate phagocytes. [194,198]
A2.9 REDOX SIGNALING AND AUTOPHAGY

Like phagocytosis, autophagy is also responsive to ROS signaling. [135,137] This is beginning to be characterized rather extensively given the often observed concurrent ROS generation and heightened autophagic flux (FIGURE A2-6). Initially, given the consequences of ROS accumulation on organelles and genomic integrity, autophagy was thought to be enhanced purely as a means to mitigate oxidative stress. [199] While this remains true, and has drastic implications in many pathological states such as cancer and neurodegenerative diseases when the integrity of the autophagic pathway is disrupted, there is increasing evidence of direct regulation of this process by ROS molecules modulating specific autophagy proteins. When LC3 becomes lipidated via direct conjugation to phosphoethanolamine, a deconjugation event must occur in order for the molecule to be properly recycled. This deconjugation is mediated by the protease, Atg4. Studies have demonstrated that in the setting of heightened autophagy, particularly starvation-induced, Atg4 is a target for oxidation by hydrogen peroxide. [200] This mechanism for regulation is a contributing factor towards the heightened autophagic state observed in a cell treated directly with exogenous hydrogen peroxide. Hypoxia-inducible factor 1 (HIF-1) plays a key role in the regulation of oxygen homeostasis. ROS induces HIF-1α-dependent expression of BNIP3 which promotes the dissociation of Beclin 1 from its Bcl-2 inhibitors. [201] In addition, ROS regulates autophagic signaling transduction pathways such as mTOR and MAPK to induce autophagy in several cells.

Negative regulation of ROS-mediated autophagy is facilitated by anti-oxidant enzymes which prevent ROS from being elevated for prolonged measures of time. Interestingly, we know autophagy is induced in the setting oxidative stress and that autophagy can serve as its own negative regulator by removing sources of ROS such as mitochondria (mitophagy) and clearing
oxidized proteins from the cytosol (a process in which p62 may be required). [202] However, in some conditions, autophagy can cause abnormal ROS accumulation by selectively promoting the enzymatic ROS scavenger catalase degradation. [203]

**A2.10 HMGB1 AT THE CROSSROADS OF AUTOPHAGY, PHAGOCYTOSIS, AND OXIDATIVE STRESS**

HMGB1, which firstly was thought to function only as a DNA chaperone that enhances replication, repair, and recombination, was then discovered to be a crucial DAMP that mediates the response to infection, injury, inflammation, tissue generation and cell migration.[204-206] To perform its role as a DAMP, HMGB1 must transit from the nucleus, through the cytoplasm, to the extracellular environment [207,208]. This process can occur during cell activation as well as cell death including necrosis, autophagy and late stage apoptosis.[209-212] HMGB1, composed of the A box, B box, and C tail domains, is a redox protein. There are three cysteines at positions 23, 45 and 106 (C23, C45 and C106 respectfully). C23 and C45 readily form an intra-molecular disulfide bridge, whereas the C106 remains in a reduced form [213]. C23 and C45 is required for HMGB1 binding to Beclin 1 during autophagy [212], whereas C106 is required for HMGB1 binding to TLR4 in macrophages. [214]

HMGB1 is a stress sensor and it is overexpressed or released in response to several forms of stress including oxidative stress (FIGURE 7-A) [215]. Oxidative stress such as H$_2$O$_2$ or knockdown of SOD1 induces HMGB1 release in many cells [216-218], whereas antioxidants such as N-Acetyl Cysteine and quercetin inhibit HMGB1 release.[212,218,219] The oxidation of Cys106 (and not Cys23 or Cys45) destroyed the ability of HMGB1 to function as an immunostimulatory in dendritic cells. [209] NADPH oxidase is a highly regulated membrane-
bound enzyme complex that catalyzes the production of superoxide by the single electron reduction of oxygen, using NADPH as the electron donor. Exogenous HMGB1 stimulate neutrophil NADPH oxidase activation and ROS generation in a TLR4 dependent manner, [220] suggesting a feedback loop in HMGB1 release and oxidative stress.

HMGB1 is an important regulator for autophagy (FIGURE A2-7). Nuclear HMGB1 regulates small heat shock protein HSPB1 expression [221]. Phosphorylation of HSPB1 is necessary for the regulation of the actin cytoskeleton, which affects the cellular transport required for autophagy in response to mitochondrial injury. Thus, HMGB1-HSPB1 pathway controls mitochondrial quality by autophagy/mitophagy. Cytosolic HMGB1 is a novel Beclin 1 binding protein which dissociates its inhibitory partner, Bcl-2 [212]. Extracellular reduced HMGB1 binds the receptor for advanced glycation end products (RAGE), but not TLR4, which inhibits mTOR and promotes the formation of the Beclin 1-PI3KC3 complex [222]. In contrast, oxidized HMGB1 induce mitochondrial apoptotic pathway by unknown receptors.[222] The induction of autophagy by both intracellular and extracellular HMGB1 is important for tumor development and a novel target for cancer therapy [206,223,224]. In addition, its receptor RAGE also regulates autophagy in pancreatic cancer cells. [225,226]

HMGB1 is also a regulator for phagocytosis (FIGURE A2-7). Extracellular HMGB1 inhibits phagocytosis by binding PtdSer or αvβ3 in apoptotic neutrophils or phagocytic macrophages, respectively [227,228]. These findings provides another mechanism by which exogenous HMGB1 enhances inflammatory responses by targeting phagocytosis-mediated inflammation reduction during apoptosis. It is unknown whether redox regulates HMGB1’s function in phagocytosis. Moreover, intracellular HMGB1 is also a negative regulator of phagocytosis by associating with Src kinase and inhibiting interactions between Src and focal adhesion kinase.
(FAK) in macrophage. [229] However, RAGE has been shown to enhance phagocytosis in macrophage by binding to PtdSer. [230,231] Future studies are needed to explore why HMGB1 and RAGE binding PtdSer have opposite outcome for phagocytosis.
Fig. A2-7. HMGB1 at the crossroads of autophagy, phagocytosis, and oxidative stress. High mobility group box-1 (HMGB1) is a DNA-binding nuclear protein, released actively following cytokine stimulation as well as passively during cell death. As a DNA chaaperone and damage-associated molecular pattern molecules (DAMP), redox properties of HMGB1 plays a role in many processes including DNA nuclear events, inflammation and immunity. There is a complicated relationship between autophagy, phagocytosis and oxidative stress. Recent studies suggest that HMGB1 is a central player in regulation of crosstalk between autophagy, phagocytosis, and oxidative stress (detail in text). NADPH: nicotinamide adenine dinucleotide phosphate; RAGE: the receptor for advanced glycation end products; TLR4: Toll-like receptor-4; HSPB1: heat shock protein β-1; PtdSer: phosphatidylserine.
A2.11 AUTOPHAGY AND PHAGOCYTOSIS IN CELL DEATH, IMMUNITY, AND INFLAMMATION

Given the nature of both autophagy and phagocytosis and the respective roles they play in maintaining the homeostatic balance at the level of both the cell and the organism, it becomes easy to accept that a dynamic relationship between both exists, particularly within the setting of cell death. This is so much so the case, that molecules previously thought to be specific to one process, such as LC3 in the case of autophagy, are actually playing similar roles in the other. In macrophages phagocytosing pathogens and cellular debris from apoptotic, necrotic, or the newly described “necroptotic” cells which display PtdSer, LC3 is rapidly recruited to phagosomes which lack the typical double-membrane coat that classical autophagosomes exhibit despite the requirement for Beclin 1, PI3KC3, Atg5, and Atg7, but not unc-51-like kinase 1 (ULK1) for this recruitment to occur [169,232,233]. This process has been termed LC3-associated phagocytosis or LAP (FIGURE A2-8). [232] As the up streaming signal, the receptors of TLR, TIM4, FcγR and NADPH oxidase-mediated ROS signal are required for LAP. [232,233] LAP is required for efficient dying cell corpse clearance and phagosomal maturation.

Abnormalities in cell death control can contribute to a variety of diseases, including cancer, autoimmunity, and neurodegenerative disorders. There is now a substantial body of literature which works to elucidate the relationship between autophagy and apoptosis.[234] In fact autophagic proteins are often directly inhibited by apoptotic proteins. Furthermore, evidence that apoptotic caspases can serve as autophagic substrate suggests another layer of direct crosstalk between apoptosis and autophagy. [235] This has compelled many in the field to orient autophagy opposite to apoptosis and term the process as a form of “programmed cell survival”. [185,226] It has also been suggested that autophagy itself can serve as an alternative
form of cell death although this has not been described in a consistent manner. [236] Similarly with phagocytosis, autophagy has been demonstrated to clear apoptotic cell in embryogenesis which is essential for development. [181] They are also linked to nutrient acquisition and energy generation. [237] [238] which is important for cell survival during stress.

The primary function of most phagocytes is to destroy pathogens. Phagocytosis has an essential role in innate immune sensing and degraded products of internalized pathogens may traffic and be modified as for nutrients [237]. In addition, phagocytosis is essential to antigen presentation and adaptive immunity in mammalian cells such as dendritic cells. [237] Several studies reveal a crucial role for autophagy in adaptive and innate immunity such as pathogen elimination, virus replication, T and B cell homeostasis, and antigen processing and presentation, with the term “immunophagy” [239,240] referring to all such processes collectively.

Apoptosis of inflammatory cells and their subsequent clearance by phagocytosis (also called efferocytosis) are key mechanisms orchestrating successful resolution of inflammation and suppress autoimmune responses through the release of anti-inflammatory cytokines IL-10, TGF-β, platelet activating factor (PAF), and prostaglandin E2 (PGE2), [129] and inhibition of pro-inflammatory cytokines such as TNFα and IL-1β. [241] In the absence of LAP, engulfment of dead cells results in increased production of proinflammatory cytokines (e.g., IL-1β) and decreased production of anti-inflammatory cytokines. [232] A key mechanism of inflammation is the activation of the “inflammasome,” which leads to caspase-1 activation and maturation and release of IL-1 family cytokines and other inflammatory mediators (FIGURE A2-10). Interestingly, autophagy was previously considered as an anti-inflammatory mechanism due to loss of the autophagy protein Atg16L1 enhances IL-1β release in sepsis. [242] However, recent study suggests that induction of autophagy by starvation promotes inflammasome-dependent IL-
Iβ secretion. [243] These findings suggest that autophagy play dual roles in regulation of inflammation depending upon the timing and type of autophagic activation.
The process of LC3-associated phagocytosis (LAP). LAP is the process of degradation of phagocytosed cellular corpse from dying cells (e.g., apoptotic, necrotic and necroptotic cells) and pathogens in phagosomes by integrating with autophagy machinery. This process is dependent on some members of the classical autophagy pathway, including Beclin1, PI3KC3, ATG5, and ATG7, but not unc-51-like kinase 1 (ULK1). PtdSer is the eat-me signaling in dying cells, and the receptors including TLR, TIM4 and FcγR in phagocytes (e.g., macrophages and neutrophils) are required for recognizing PtdSer and recruiting LC3 to phagosome to form single-membrane structure. NADPH oxidase (e.g., NOX2) is one of the major sources of ROS to regulate LAP. TLR: Toll-like receptor; PtdSer: phosphatidylserine; NADPH: nicotinamide adenine dinucleotide phosphate; TIM4, T-cell immunoglobulin domain and mucin domain 4; PI3KC3: class III phosphatidylinositol 3-kinase; LC3: microtubule-associated protein 1 light chain 3; RIPK3: receptor-interacting serine-threonine kinase 3; NOX2: NADPH oxidase 2.
Fig. A2-9 Function of phagocytosis, autophagy and LAP in immune cells. Reactive oxygen species (ROS) are emerging as regulators of “eat me” mechanisms such as autophagy, phagocytosis and LC3-associated phagocytosis in various cellular contexts. These eat me mechanisms have been linked to the removal of dying cells, immune regulation and inflammation in immune cells such as macrophages, monocytes, dendritic cells and neutrophils.
Fig. A2-10 Signaling to regulate IL-1β production and release. The nucleotide-binding oligomerization domain-like receptors (NLRs) family, pyrin domain-containing 3 (NLRP3) inflammasome is a multiprotein complex including NLRP3, ASC and the effector cysteine protease caspase 1, which activates caspase 1, leading to the processing and secretion of the pro-inflammatory cytokines interleukin-1β (IL-1β). Phagocytosis and LAP have been demonstrated to contribute to this process. In contrast, autophagy plays a dual role in regulation of IL-1β production and release. ASC: also known as PYCARD, the adaptor protein apoptosis associated speck-like protein containing a CARD.
A2.12 CONCLUSION

The crucial role of both phagocytosis and autophagy in maintaining homeostatic balance and both inciting and regulating host responses to cellular injury makes identification of cross-talk between the two pathways within stressed tissues important and interesting. Given the wide range of pathologic conditions in which autophagy has been implicated as a contributor, identification of novel autophagy-related therapeutic targets is an important task. Cancer biologists and clinicians have begun to identify autophagy modulators (specifically inhibitors) with promising.

Making the relationship between phagocytosis and autophagy more complex, is the role ROS signal transduction plays in both processes. Although direct targets for ROS modulation of both pathways such as NF-κB and Atg4 are beginning to become identified and characterized at the molecular level, much remains unknown regarding the specificity and directionality of ROS signaling. Signal transduction by ROS molecules occurs in a much more regulated and orchestrated manner than previously thought, and ROS messengers directly modulate cell death pathway, phagocytosis and autophagy.
A2.13 APPENDIX ACKNOWLEDGEMENT

As previously mentioned the appendix was modified from an invited review article written with Dr. Daolin Tang. While we worked on this text together, I would be remiss if I did not mention that each of the beautifully depicted illustrations in this appendix was created by Dr. Daolin Tang. Dr. Tang has an exceptional ability to translate complicated biology into extraordinarily aesthetic graphical depictions. For that, I thank him.


197. Rybicka JM, Balce DR, Khan MF, Krohn RM, Yates RM (2010) NADPH oxidase activity controls phagosomal proteolysis in macrophages through modulation of the lumenal


