Wydawnictwo UR 2023
ISSN 2544-1361 (online)
doi: 10.15584/ejcem.2023.2.20

http://www.ejcem.ur.edu.pl
European Journal of Clinical and Experimental Medicine
Eur J Clin Exp Med 2023; 21 (2): 365–396

REVIEW PAPER

Innate defenses to intestinal cell death in necrotizing enterocolitis – spotlight on macrophage efferocytosis and its efficacy in rescuing inflamed intestinal mucosa

Sri Harsha Kanuri [1], Newly Bagang [2], Ayse Sena Ulucay [1], Popular Pandey [1], Gaaminpreet Singh [1]

[1] Department of Physiology and Biophysics, Case Western Reserve University, School of Medicine Cleveland, Ohio, USA
[2] Department of Pharmacology, Karturba Medical College, Manipal, Manipal Academy of Higher Education, Manipal, Karnataka, India

ABSTRACT

Introduction and aim. Necrotizing enterocolitis (NEC) is a grave gastrointestinal disease of preterm infants which is widely prevalent in the neonatal intensive care units. Current treatment options are very limited with high mortality and morbidity. With no disease specific interventions, understanding nascent cellular events that occur immediately after microbial insult can offer insights for devising novel treatment options for curtailing the disease progression in NEC. In this regard, intestinal cell death in NEC is a primordial cell-signaling event and is regarded as a harbinger of future pathological derangements such as increased intestinal permeability, intestinal dys-homeostasis, and systemic inflammation.

Material and methods. We performed PubMed search of relevant articles that describes the host response to intestinal cell death in NEC by cellular battalion including dendritic cells, lymphocytes, neutrophils and macrophages which are important in containing intestinal inflammation.

Analysis of the literature. We particularly focused this review on enumerating macrophage efferocytosis, and pertinent novel treatment modalities based on this physiological process that has inherent capability for down regulating inflammation and promoting tissue repair in NEC. We highlighted its mechanistic aspect including mediators, receptors and signaling mechanisms and its physiological significance.

Conclusion. Macrophage efferocytosis is an overlooked and undervalued physiological defense mechanism to clear the dying intestinal epithelial cells for facilitating tissue healing and restoring the intestinal homeostasis. Any impairment of this critical defense mechanism can result in rapid clinical progression and systemic complications. Understanding its importance in the pathogenesis of NEC is important for designing novel therapeutic interventions to attenuate disease progression.

Keywords. efferocytosis, inflammation and immune responses, intestinal cell death, macrophage, necrotizing enterocolitis

Corresponding author: Sri Harsha Kanuri, e-mail: harsha9009@gmail.com

Received: 15.01.2023 / Revised: 7.03.2023 / Accepted: 4.04.2023 / Published: 30.06.2023

Kanuri SH, Bagang N, Ulucay AS, Pandey P, Singh G. *Innate defenses to intestinal cell death in necrotizing enterocolitis – spotlight on macrophage efferocytosis and its efficacy in rescuing inflamed intestinal mucosa.* Eur J Clin Exp Med. 2023;21(2):365–396. doi: 10.15584/ejcem.2023.2.20.

Introduction

It has been estimated that 7 out of 100 very low birth weight infants (VLBW) develop necrotizing enterocolitis (NEC) in Neonatal Intensive Care Unit.[1] The pooled estimate of NEC by Quality Effect Models and Random Effect models is approximately 6% and 7% respectively.[1] In a prospective analysis study, examination of 473,895 VLBW infants born between 2006 and 2017 from 820 United States clinical centers revealed that, the incidence of medical and surgical NEC is approximately 58.3% and 41.7% respectively.[2] The risk factors that are responsible for NEC are classified into prenatal factors (maternal cocaine use, pregnancy-induced hypertension, maternal infections and decreased placental blood flow), intrapartum factors (maternal cardiac arrest, umbilical cord prolapse, chorioamnionitis and placental abruption) and clinical course factors (patent ductus arteriosus (PDA), endotracheal intubation, bag mask ventilation, hypothermia, hypoventilation, fortified breast milk, infections, antibiotics, feeding intolerance and H2 receptor antagonists.[3] Due to these risk factors, intestinal tissues of preterm infants are exposed to wide variety of pathological insults such as lipopolysaccaride (LPS), tumor necrosis factor-alpha (TNF-α), nitric oxide, interferon gamma (IFN-γ) and cytokines.[4,5] This makes them vulnerable to assorted types of intestinal cell death ranging from apoptosis, necrosis, pyroptosis, necroptosis to autophagy (Fig. 1).

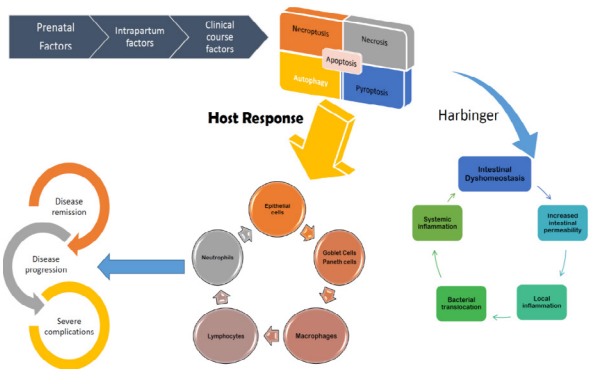

Fig. 1. Assorted cell death and host response in NEC. Preterm and very low birth weight infants are exposed to prenatal, intra-natal and clinical course risk factors. This results in assorted types of intestinal cell death ranging from apoptosis, necrosis, necroptosis autophagy, and pyroptosis. Intestinal cell death can be regarded as a harbinger of future pathological derangements such as changes in intestinal permeability, local inflammation, and translocation of bacterial load inside blood vessels. These changes lead to spread of systemic inflammatory response. Host response to intestinal cell death is mediated by cellular battalion ranging from epithelial cells, goblet cells, neutrophils, lymphocytes, dendritic cells and macrophages.
Depending on the robustness of host immune response, clinical outcomes of NEC can range from complete remission to clinical progression and systemic complications with associated mortality and morbidity

Apoptosis has been widely documented in the postmortem examination of intestinal tissues in the NEC patients.[6] Apoptosis can occur via caspase-8 mediated direct pathway or mitochondrial mediated indirect pathway, with both pathways converging on the secondary caspases (3 and 9) for final execution of the apoptotic cascade.[7] Previous work indicates that, apoptosis of the intestinal epithelial cells provides the first signal for subsequent intestinal necrosis and resulting pathological consequences.[6] Proliferation of pathogenic bacteria within the intestinal lumen releases toxic stimuli causing activation of inflammatory cascade and ultimately resulting in the intestinal necrosis.[4] Necroptosis is another form of cell death requiring the activation of receptor interacting protein kinases, as well as phosphorylation and membrane translocation of mixed lineage kinase domain-like protein due to the presence of damage associated molecular patterns (DAMPs) such as TNF-α.[8] Previous studies indicate that, activation of toll-like receptor 4 (TLR4) signaling is mainly responsible for the occurrence of necroptosis in the NEC animal models which is partially inhibited by the supplementation of breast milk.[9] Pyroptosis is another type of cell death primarily mediated by caspase-1 characterized by cellular swelling, osmotic lysis, release of pro-inflammatory cytokines, interleukin (IL) IL-1β and IL-18, nuclear condensation and DNA cleavage.[10] Pyroptosis is another kind of cell death implicated in the pathogenesis of NEC due to the documentation of increased mRNA levels of IL-1β, IL-18 and nucleotide-binding domain, leucine-rich repeat–containing proteins pyrin domain containing 3 (NLRP3) in the intestinal tissues of premature infants.[11] Although autophagy is generally regarded as a protective mechanism, it can mediate cell death, which can identified by the excess accumulation of large size autophagic vacuoles within the cytoplasm of dying cells.[12] According to a study performed by Yu et al., autophagy was the initial cellular event that heralded the subsequent onset of apoptosis in the intestinal epithelial cells in the rat model of NEC.[13] In the same study, administration of erythropoietin resulted in the protection against autophagy and apoptosis in the intestinal epithelial cells via protein kinase B/mammalian target of rapamycin and mitogen activated protein kinase (MAPK) pathways respectively.[13] The array of pathological findings that can be found on examination of the intestinal tissues on NEC infants can range from ischemia necrosis, inflammation, bacterial overgrowth, epithelial regeneration, fibrosis and granulation tissue formation.[14] Intestinal cell death in NEC is regarded as a nascent cellular event that heralds the onset of future

pathological derangements in the intestinal epithelium and surrounding intestinal milieu which can facilitate the rapid local disease progression and systemic complications. Specifically, it is a harbinger of leaky intestinal barrier, dissonance in intestinal homeostasis, local inflammation, and systemic inflammation (Fig.1). The key to clinical remission and cessation of disease progression from early stages of NEC rests on launching counteractive robust defense mechanisms for neutralizing the bacterial insult and invigorating anti-inflammatory pathways (Fig.1). This will eventually pave the way for tissue healing and restore of physiological intestinal homeostasis so that early clinical remission can occur without further delay (Fig.1). Disjointed, uncoordinated and unabated immune responses can be counterproductive and leads to rapid disease progression with associated local and systemic complications. This review will discuss physiological host defense mechanisms by epithelial cells, neutrophils, lymphocytes, and macrophages upon encountering intestinal cell death during primordial stages of NEC. We particularly discussed the physiological efferocytosis executed by macrophages in a detailed manner and highlighted its crucial role in facilitating tissue healing and restoring mucosal integrity by its inherent anti-inflammatory pathway activation. The step-by-step process of macrophage efferocytosis occurring in the intestinal milieu needs to be comprehended in systematic and methodical manner. We surmise that, this physiological host defense response can be manipulated and exploited for devising macrophage-based cell therapies, which can be potentially utilized for countering the clinical disease progression in the NEC disease models. This will ultimately be beneficial for optimizing the clinical outcomes and attenuating mortality as well as morbidity in NEC.

Aim

To understand and glean the innate immune cellular defense responses kickstarted in responsible to intestinal cell death in NEC

Material and methods

PubMed search of relevant articles describing host response particularly macrophage mediated responses in response to intestinal cell death in NEC were carefully reviewed and analyzed. Furthermore, pertinent treatment modalities based on the signaling mechanisms of macrophage efferocytosis were discussed. Comprehending this unrecognized physiological defense mechanism is crucial in devising future basic science research studies to understand its potential role in disease pathogenesis of NEC. These studies might become a physiological basis for crafting novel therapeutic interventions for derailing clinical progression in NEC.

Analysis of the literature

Host response to cell death in preterm and full infants

As of today, there are few studies documented that ascertain the differences between preterm and full-term infants in their ability to fight the microbial infections. In response to group B streptococcus infection, whole blood and cord blood monocytes from preterm infants responded with impaired cytokine profile as compared to the full-term infants.[15] Additionally, blood lymphocyte production of cytokines in response to group B streptococcus infection was poor in preterm and full-term infants as compared to the adults.[15] In concurrence with the above findings, incubation of cord blood from infants of different gestational ages with LPS revealed decreased secretion of TNF-α, IL-6 and granulocyte-colony stimulating factor (G-CSF) in the very low pre-term infants as compared to more advanced gestational age infants.[16,17] This emphasizes the notion that, pro-inflammatory cytokine secretion profile is directly proportional to the gestational age. Reduced secretion of pro-inflammatory cytokines (IL-1, IL-6, and IL-8) in the pre-term infants can be attributed to the reduced expression of TLR4 receptor signaling ligands (myeloid differentiation gene 88 and IRF5 interferon regulatory factor 5 [IRF5]) as well as impaired activation of the downstream signaling molecules such as mitogen activated protein kinase 14 and extracellular signal regulated kinase (ERK1/2) upon stimulation with bacterial LPS.[18,19] Analysis of the cord blood of preterm infants with gestational age (<28 weeks and 28-32 weeks) revealed decreased innate immune receptors (cluster of differentiation [CD14]), TLR2, TLR4 and myeloid differentiation protein 2 on the leukocytes.[20] As a result, preterm infants had reduced ability to defend against gram-positive and gram-negative infections and attenuated capacity for opsono-phagocytosis of the dead bacteria and these abnormalities were reversed partially by administration of IFN-γ.

Because of these impaired defense mechanisms, preterm infants are more likely to be prone to severe infections and clinical abnormalities as compared to the full-term infants. Accordingly, in a prospective observational cohort study, pre-term infants are more likely to develop late onset sepsis due to their impaired secretion of cytokines and immune hypo-responsiveness in response to staphylococcus epidermidis infection.[21,22] Pre-term infants with bronchopulmonary dysplasia are more likely to progress to chronic lung disease due to the increased presence of non-classical immature $CD14^{+}/CD16^{+}$ macrophages resulting in persistent inflammation in the lung tissues.[23] Increased presence of immature macrophages in the lungs of premature infants is due to failure of maturation of monocytes into mature macrophages, which makes them less prepared as well as inexperienced to handle any pertinent bacterial infections.

Preterm infants were shown to have lesser total white cell count, neutrophils, lymphocytes, monocytes and basophils at birth as compared to the full term infants.[24] The ability to fight bacterial infections in the preterm infants is usually subpar due to the presence of functional abnormalities such as decreased leukocyte recruitment, attenuated pattern recognition receptor function and diminished bacterial killing capacity.[19] Neutrophils which are the first responders to infections are powerless and incompetent in the preterm infants due to their functional defects such as decreased phagocytosis, slow mobility, and reduced antimicrobial protein secretion thereby predisposing them to severe bacterial and fungal infections.[25-28] Neutrophils in preterm infants are also demonstrated to possess diminished respiratory burst as well as defective neutrophil extraceullar traps (NETs) formation resulting in their reduced potency in executing intracellular and extracellular microbial killing.[29] In fact, the antimicrobial proteins and peptides that are secreted by neutrophils, macrophages and lymphocytes in response to microbial infections were shown to be proportional to the gestational age of the infant.[29]

In response to TLR agonists, preterm monocytes exhibited decreased phagocytosis, impaired activation of ERK1/2 and Extra-cellular signal-regulated kinase and nuclear factor – kappa B (NF-κB), reduced production of TNF-α as well as diminished acidification of bacterial phagosome underscoring paralyzed and futile early immune responses against bacterial infections in them.[30] Furthermore, monocytes and dendritic cells launch perturbed innate immune defenses in response to bacterial LPS in the very low birth weight pre-term infants, a factor associated with increased risk of severe bacterial inflammation with increased morbidity and mortality in them.[31] The ability of the monocytes and dendritic cells to ingest, process and present the foreign antigens to the T-lymphocytes in the neighboring lymph nodes (LNs) through major histocompatibility complex class II (MHC class II) for mounting an immune response is also impaired in the preterm infants.[32-34]

Evaluation of cord blood demonstrated reduced amounts of specific antibodies against Diphtheria, Tetanus, Pertussis, Neisseria meningitidis and Hemophilus influenza in the preterm infants less than 32 weeks of gestation as compared to the term infants due to decreased trans-placental transfer of protective maternal antibodies into their blood circulation.[35] Studies have shown that preterm infants usually have altered thymic 1 and 2 (Th1/Th2) lymphocyte ratio with predominance of anti-inflammatory Th2 phenotype and reduced production of IFN-γ making them susceptible for severe viral infections.[33] Analysis of B-lymphocytes in the preterm infants revealed multitude of abnormalities ranging from decreased expression of TNF-α receptor ligands (Traf interacting receptor for Tall1, B-cell maturation protein, and B-cell activating factor belonging to tumor necrosis factor family), diminished proliferation, lesser production of immunoglobulins such as IgA and IgG, disrupted isotype switching to altered B and T-lymphocyte interactions.[36] Adaptive immune responses are also suboptimal in the pre-term infants where administration of vaccines induced weaker protective B-cell antibody titers thereby offering lesser protection against infectious diseases.[37] In a prospective study studying the lymphocyte populations in the cord blood by flow cytometry, there is a predominance of naïve helper and cytotoxic T-lymphocytes generated by thymus and bone marrow in the preterm and term infants with lower counts of naïve T-lymphocyte populations in the infants with lower gestational age.[38] The classical and alternate complement pathways are also grossly impaired in the preterm infants thus making them vulnerable to microbial threats due to impaired leukocyte recruitment, opsonization and bacteria clearance.[39] Due to haphazard maturation and deficiency of anti-microbial defense mechanisms in the preterm infants, the chances of bacterial clearance in earlier stages of infection are very less thereby leading to rapid disease progression, severe disease pathology, and systemic spread of microbial infections.[40]

Cellular squad defenses to intestinal epithelial cell death in NEC

Gut mucosa cells including intestinal epithelial cells, Paneth cells and goblet cells.

In response to apoptosis and associated impairment of the intestinal barrier, intestinal mucosal cells including intestinal epithelial cells (IECs) try to compensate by mounting on specific defense mechanisms to mitigate the intestinal damage. IECs interact with microorganisms through pattern recognition receptors such as TLRs and nucleotide-binding domain, leucine-rich repeat-containing receptors to mediate secretion of pro-inflammatory mediators to curtail the bacterial induced intestinal damage (Fig. 2).[41]

Lamina propria of the intestinal mucosa along with gut associated lymphoid tissue has many IgA producing plasma cells.[42] As a part of mucosal immune response, IECs secrete polymeric immunoglobulin receptor (pIgR) which binds to the IgA on the basolateral side of IECs. Next IgA-pIgR complex gets internalized and migrates to the luminal or apical side of IECs.[43,44] Once the pIgR-IgA complex enters the apical side of IECs, secretory IgA enters the intestinal lumen and offers protection against bacteria or viruses in the lumen to curb further intestinal apoptosis (Fig. 2).[44] Additionally, anti-bacterial peptides such as stem cell factor, intestinal alkaline phosphatase and acyloxyacyl hydrolase, secreted by IECs offers protection against bacterial proliferation and invasion.[41,44] Furthermore, keratinocyte growth

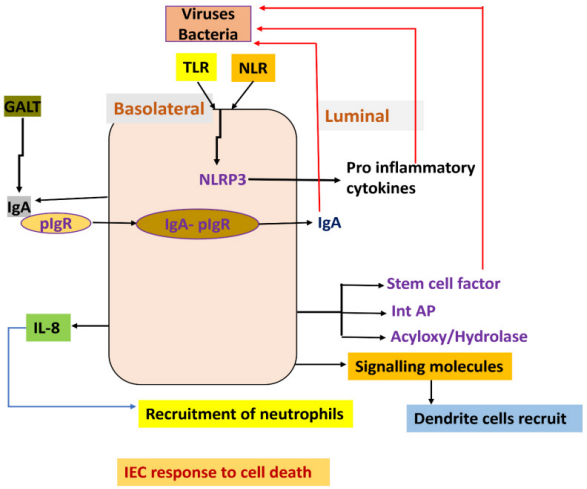

Fig. 2. Intestinal epithelial cell response to cell death. Viruses and bacteria activate Toll like and NOD like receptors on intestinal cells which results in NLRP3 inflammasomes with the resultant production of pro-inflammatory cytokines. This can also result in recruitment of poly IgA-polymeric immunoglobulin receptor complex from basolateral side to luminal side of intestinal epithelial cells. These pathological events produce additional pro-inflammatory cytokines and other tissue factors that results in the recruitment of neutrophils and dendritic cells towards the intestinal milieu that serves to amplify the immune response to neutralize the microbial threat and contain the spread of infection

factor which is secreted by intraepithelial lymphocytes helps to protect the IECs and restore the integrity of intestinal epithelial barrier.[44] During bacterial infections, epithelial cells secreted signaling molecules to promote the recruitment of dendritic cells from the blood stream to the intestinal mucosa. This results in upregulation of occludins locally leading to opening of tight junctions between the epithelial cells thereby permitting the dendritic cells to sample the pathogenic bacteria. Eventually these bacterial peptides are processed and presented to the lymphocytes in the neighboring lymph nodes, resulting in an immune response against the bacterial threats.[45] The basolateral surface of IECs usually secrete IL-8, which is responsible for recruitment of neutrophils to the intestinal lumen for setting in motion a robust antimicrobial response to contain their spread.[46] Moreover, monocyte chemoattractant protein-1 (MCP-1) secreted by Paneth cells and goblet cells has been shown to downregulate TNF-alpha mediated IEC apoptosis via reducing the migration of plasmacytoid dendritic cells.[47] Furthermore, Paneth cells secrete anti-microbial defensin peptides which are instrumental in strengthening the innate immune response by neutralizing the threat imposed by gram positive and gram negative bacterial infections.[48] Goblet cells which are interspersed between IECs also been shown to contribute to the anti-microbial immune response through secretion of mucin as well as trefoil like peptides and resistin-like molecule β molecules.[49] Previous studies demonstrated that, IECs undergoes apoptosis only 12-18 hours after bacterial entry with upregulation of TNF-alpha and nitric oxide.[50] Taking advantage of this delayed onset of apoptosis, IECs and intestinal mucosa through coordinated effort will be able to launch a sturdy mucosal immune response with a cellular battalion comprising of dendritic cells, lymphocytes, neutrophils and macrophages to partially neutralize the bacterial insult, subdue the microbial spread and restore the intestinal mucosal integrity in the initial stages of disease.[50]

Dendritic cells or antigen presenting cells (APC)

During bacterial infections, $CD103^{+}CD11b^{+}$ dendritic cells (DCs) are the primary dendritic cells that are responsible for delivering the ingested cargo to the mesenteric lymph nodes (MLNs) for eliciting a vigorous immune response.[51] During the physiological conditions, overcrowding at the villus tip can result in apoptosis of the IECs through spingosine-1 phosphate (S1P) and rho associated kinase dependent caspase-3 signaling. Once the apoptotic IECs undergo shedding, DCs are most important immune cells that sample the apoptotic IECs and transport them to the MLNs for initiating an immune tolerance or immune response.[52] LPS released during bacterial infections can cause disruption of tight junctions between the epithelial cells through secretion of zonula occluding-1 (ZO-1) and this will permit the DCs to sample the intestinal lumen for bacterial products for orchestrating the immune response.[45] In this regard, $CD4^{+}/OX41^{+}$ DCs can be regarded as strong antigen-presenting cells (APCs) whereas $CD4^{-}/OX41^{-}$ DCs are relatively weak APCs with regard to their ability to sample, process and respond to the foreign antigens (Fig. 3).[53]

The evidence for this finding comes from studies revealing APCs with cytoplasmic epithelial DNA and epithelial cytokeratin extensively localized to lamina propria, Peyer's patches and MLNs.[54] During homeostasis, dendritic cells are responsible for mounting a comprehensive immunosuppression program by downregulating genes associated with inflammasome (NLRP3, caspase-1 and IL-1-β) and mitogen activated protein (MAP) kinase resulting in a non-inflammatory apoptosis.[52] However, during inflammatory bowel conditions, increased TNF-alpha induced excessive and unregulated apoptosis and necroptosis through caspase-8 and RIPK1/RIPK3 respectively results in disruption of this protective immunosuppression program mediated by dendritic cells.[52] Subsequently, dendritic cells can directly ingest the apoptotic IECs in the lamina propria with the help of αvβ5 integrin and CD36 receptors and then migrate to the mesenteric lymph nodes

in chemokine receptor 7 dependent manner to mount an robust immune response.[55,56] Antigen uptake by the dendritic cells can also occur indirectly and be classified as M-cell dependent, neonatal Fc receptor dependent and goblet cell dependent.[57] Once apoptotic IECs or foreign antigens are ingested by dendritic cells, next steps including degradation, processing, transportation and loading of antigen proteins on MHC class II molecules will have to proceed in a systemic manner for presentation to T-lymphocytes in MLNs (Fig. 3).[58,59] After migration to the MLNs, dendritic cells also upregulate the expression of α4β7 integrin and chemokine receptor 9 (CCR9) on the naïve T-lymphocytes and this will promote their homing to the intestinal mucosa for inciting an immune response to future bacterial infections.[60,61] Moreover, dendritic cells can secrete IL-10 and IL-12 by themselves upon exposure with LPS or *Streptococcus faecium* and these cytokines will also be responsible for shaping the immune responses against bacterial infections (Fig. 3).[62] Furthermore, dendritic cells can stimulate intestinal IgA production through secretion of B-cell activating factor belonging to the TNF family, a proliferation-inducing ligand and retinoic acid for amplifying the immune response against bacterial insults (Fig. 3).[57]

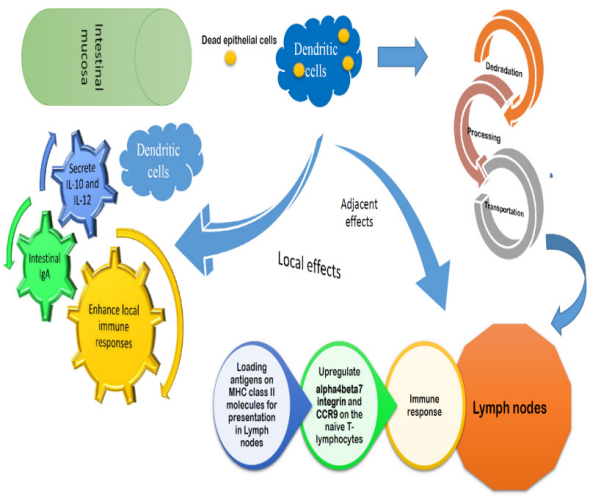

Fig. 3. Schematic representation of dentritic cell responses to dead intestinal epithelial cells. Occurrence of intestinal cell death can initiate dendritic cell recruitment. Ingestion of dead epithelial cells by dendritic cells results in degradation, processing, and presentation of antigens on MHC class II for presentation to T-lymphocytes for initiation of immune response. These events then trigger immune cells response in lymph nodes. Furthermore, involving up-regulation of α4β7 integrin and CCR9 on T cells surface can their activation and migration towards to the site of intestinal injury. The dead epithelial cells are entrapped by dendritic cells that produce local effects at the site by secreting IL-10, IL-12, cytokines, initiating IgA and local immune cell responses

Lymphocytes

During homeostatic apoptosis or contact with commensal bacteria, APCs aided antigen presentation in MLNs results in stimulation of intestinal lymphocytes. Fork head box-3 protein $CD4^+$T and $CD8^+$ regulatory (reg) lymphocytes which expresses higher concentration of IL-9, IL-10 and IL-13 are specifically recruited to the intestinal epithelial cells undergoing apoptosis.[57] CD4 T^+ helper cells and CD8 T^+ reg lymphocytes secreted cytokines such as IL-10, IL-17, IL-22, and transforming growth factor – beta (TGF-β) are responsible for restoring and healing the injured intestinal epithelium during physiological processes such as tissue development, regeneration and healing.[63] It is important to understand that, Th17 lymphocytes are implicated in the pathogenesis of multiple chronic inflammatory conditions whereas T reg lymphocytes are deemed to be protective through suppression of inflammation, immune tolerance and remodeling of tissues.[64] Naïve T-cells with their specific cytokine profile implicated in the pathogenesis of inflammatory bowel disease are usually classified into three groups namely Th1 (IFN-gamma, TNF-alpha), Th2 (IL-4, IL-5 and IL13) and Th17 (IL-17A, IL-17F, IL-21 and IL-22) lymphocytes (Fig. 4).[65,66]

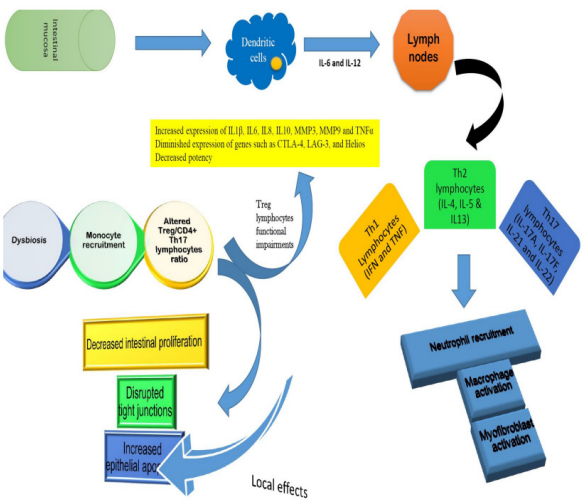

Fig. 4. Lymphocytes responses towards the intestinal cell death. Ingestion of dead intestinal cells by dendritic cells and their subsequent migration towards lymph nodes results in Th1, Th2 and TH17 dependent secretion of IF-γ, TNF-α, IL-4, IL-5, IL-13, IL-17A, IL-17F, IL-21, IL-22 cytokines. These cytokines cause neutrophil recruitment, macrophage and myofibroblast activation leading towards increased epithelial cell apoptosis. Moreover, the intestinal dysbiosis due to infection causes monocyte recruitment, altered Treg/$CD4^+$ lymphocyte ratio finally contributing towards the decreased intestinal proliferation, disruption of tight junctions and intestinal apoptosis

Activated Th1 lymphocytes secreted cytokines (IFN-γ and TNF-α) that execute intestinal epithelial

apoptosis through macrophage activation and via myofibroblast induced production of matrix metalloproteinases (MMPs) that provoke tissue matrix degradation.[65] Th2 lymphocytes induced production of IL-13 is also implicated in instigating increased intestinal epithelial apoptosis and disruption of intestinal epithelial barrier integrity.[65] Th17 lymphocytes induced release of IL-17 can stir up the recruitment of neutrophils from the blood stream into the intestinal mucosa for potentiating the microbial killing activity.[65] In the presence of bacterial or viral infection, CD 103$^+$ APC induced antigen presentation can activate Th17, Th1 and cytotoxic lymphocytes by TLR stimulation via IL-6 and IL-12 p40 subunit secretion.[57] The ratio of Treg/CD4$^+$lymphocytes to Treg/CD8$^+$lymphocytes in the NEC intestinal tissues is substantially lowered as compared to the surgical control intestinal tissues of age-matched gestational age.[67] Alteration of Treg/CD4$^+$ / CD4$^+$ T lymphocyte ratio secondary to dysbiosis of the intestinal bacteria one of important risk factors for developing NEC in premature infants (Fig. 4).[68] The authors in a recently concluded clinical research study observed that, about 60% depletion of Treg lymphocytes in the NEC intestinal tissues has been associated with increased gene expression of IL1β, IL6, IL8, IL10, MMP3, MMP9 and TNFα and accompanying intestinal inflammation as compared to age-matched gestational controls.[67,69] Treg lymphocytes in NEC intestinal tissues also exhibited several functional impairments including decreased potency in suppressing IL17 production and CD4$^+$CD25$^-$ T conventional cell proliferation in addition to diminished expression of genes cytotoxic lymphocyte associated protein 4, ligand lymphocyte activation gene 3, and IKZF2 zinc finger protein.[64] Downregulated expression and functional aberrations of Treg lymphocytes can fuel unabated and exacerbated inflammatory response thereby kindling severe intestinal lesions in NEC. Monocyte activation and recruitment to the intestinal mucosa during bacterial infections is shown to exacerbate altered Treg/CD4$^+$ Th17 lymphocytes ratio locally via upregulation of TNF- α and IL-6 as well as downregulation of IL-10 and TGF-β thereby contributing to the intestinal injury in NEC.[70] During bacterial infections, LPS-TLR4 signaling in the IECs leads to CCL25 dependent recruitment of CD4$^+$ Th17 lymphocytes and associated downregulation of Fork head box-3 protein (Treg lymphocytes) leading to exacerbated inflammatory response in NEC.[71] IL-17 secreted from the CD4+ Th17 lymphocytes was demonstrated to provoke decreased enterocyte proliferation, disruption of tight junctions and increased epithelial apoptosis leading to intestinal injury in NEC (Fig. 4).[71]

Neutrophils

Neutrophils are recruited to the inflamed intestinal epithelium as a first line defense mechanism in response to bacterial infections. Their primary responsibilities include killing of microorganisms, healing of injured mucosa, attenuation of inflammation and restoring the intestinal homeostasis. During chronic inflammation, IL-17 and IL-23 secreted by Th17 lymphocytes and dendritic cells/macrophages respectively are mainly responsible for the increased transcription of granulocyte monocyte colony stimulating factor (GM-CSF) in the bone marrow.[72,73] Increased GM-CSF in the bone marrow results in increased production of neutrophils and their subsequent release into the peripheral blood stream (Fig. 5).[73] To reach the intestinal lumen at the site of inflammation, neutrophils must penetrate through the endothelial, interstitial and epithelial barrier by trans-endothelial and trans-epithelial migration.[73-75] The numerous sequential steps in the trans-endothelial migration of neutrophils include tethering, rolling, arrest, adhesion, crawling and paracellular migration.[73,74] Trans-endothelial migration involves P, E, and L selectins, P-selectin glycoprotein ligand, leukocyte specific integrin molecule (CD11b), CD38, intracellular adhesion molecular and vascular adhesion molecule.[73,74] Trans-epithelial migration of neutrophils encompasses sequential steps such as basolateral adhesion, trans-migration and apical adhesion.[75] This trans-epithelial migration of neutrophils instigates the production of TNF-alpha through upregulation of ADAM metalloproteinase domain 17 or a disintegrin and metalloprotease 17 (ADAM17) thereby triggering the onset of intestinal inflammation.[76] Once the neutrophils reach the intestinal lumen, they remain in close contact with the apical surface of intestinal epithelial cells and form cryptic abscesses for promptly responding to the bacterial stimuli.[75] It is important to understand that, a large number of neutrophils undergoing trans-epithelial migration might be associated with disruption of epithelial junctional proteins such as ZO-1, claudin 1, β-catenin, E-cadherin and junctional adhesion molecule-A leading to impairment of epithelial barrier integrity which can subsequently promote excess neutrophil and bacterial transmigration.[77] Enhanced bacterial entry into the intestinal mucosa can cause macrophage polarization from M2 to M1 phenotype, leading to increased pro-inflammatory cytokine secretion, a phenotype change that can provide a signal for more neutrophil recruitment to the intestinal lumen.[78] Once neutrophils reach the intestinal lumen, they usually exert their anti-inflammatory effect through reactive oxygen species (ROS) production, NET formation, phagocytosis and degranulation.[73,74] The various types of anti-microbial granules secreted by neutrophils can be classified as primary (myeloperoxidase, neutrophil elastase, cathepsin-G, and lysozyme), secondary (lactoferrin and MMP-8) and tertiary (MMP-9), all of which synergistically contribute to strengthen anti-bacterial defenses at the site of mucosal injury (Fig. 5).[73-75]

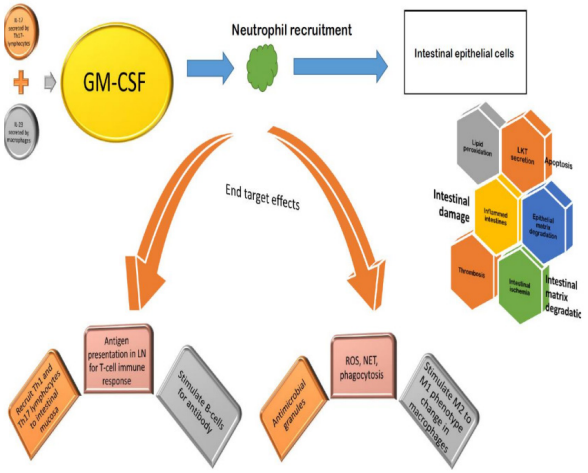

Fig. 5. Neutrophils responses towards intestinal epithelial cell death. The IL-17 and IL23 cytokines by lymphocytes and macrophages causes GM-CSF upregulation which leads to initiate neutrophils recruitment at the site of intestinal injury. This results in end target effects including recruitment of Th1 and Th17 lymphocytes to the intestinal cell mucosa, antigen response mediated by T cells activation, stimulation of B cells. These cellular events further release ROS, induce phagocytosis and conversion of macrophage from M2 to M1 phenotypes. Unfortunately, excess and uncontrolled activation of neutrophils results in multiple pathological derangements including lipid peroxidation, epithelial matrix degradation, intestinal ischemia, leukotriene secretion and intestinal damage

Activated neutrophils might induce secretion of chemokines C-X-C motif chemokine ligand 10 and CCL2, which can promote recruitment of Th1 and Th17 lymphocytes to the site of inflamed mucosa (Fig. 5). Lymphocyte recruitment to the site of intestinal inflammation leads to the production of pro-inflammatory cytokines (TNF-α, IL-12, IL-15, and IL-23) for neutralizing the bacterial threat and curbing the associated intestinal inflammation.[73,79] In addition to their effect in the intestinal lumen, neutrophils also act on the neighboring lymph nodes to initiate an immune response against the pathogens. Neutrophils can also deliver the microbial antigens to APCs (i.e. dendritic cells) which can ultimately promote proliferation of $CD4^+$ T lymphocytes in the lymph nodes to amplify the innate immune response (Fig. 5).[80] Activated neutrophils close to the B-lymphocytes in lymph nodes can also stimulate plasma cell proliferation and subsequent antibody production (Fig. 5).[80] Neutrophil recruitment to the site of intestinal inflammation is not always productive as it can be counter-productive in some instances. NET can result in release of phosphatidylserine positive micro-particles and causes microvascular thrombi through TLR4 signaling on the platelets and endothelial cells leading to microvascular ischemia. This can further exacerbating the intestinal damage during bacterial inflammation of the gut.[81] Unfortunately, excessive, and uncontrolled neutrophil activation at the inflamed mucosa can also aggravate the intestinal injury through lipid peroxidation, leukotriene secretion, amplified inflammation, epithelial matrix degradation, microvascular thrombosis, and intestinal ischemia (Fig. 5).[73,74,78]

Macrophages

Macrophages are one of most important armories of cellular defense that are recruited to the site of tissue injury for neutralizing the bacterial threat and repairing of inflamed intestinal mucosa. In response to bacterial infection, innate lymphoid cells located within the intestinal epithelium secrete (GM-CSF) which is responsible for proliferation of monocyte precursors in the bone marrow.[82] This will ultimately lead to an increase in the monocyte population in the blood stream. These monocytes will eventually migrate to the site of intestinal inflammation and transform into inflammatory macrophages.[83] In contrast to the tissue-resident-macrophages, which are non-inflammatory, these inflammatory macrophages are more responsive to the presence of microbial antigens.[83] These inflammatory macrophages secrete proteases, reactive oxygen species (superoxide anion, hydrogen peroxide and hydroxyl radicals) and pro-inflammatory cytokines (IL-1β, TNF-α, IL-6, IL-8, IL-10, IL-12, and IL-18) which are helpful in promoting resolution of intestinal inflammation, enhancing tissue repair and tissue remodeling.[84] Macrophages can directly engulf the pathogens (bacteria and viruses) for direct microbial killing or presentation to the immune cells in the neighboring lymph nodes through a process called phagocytosis which involves the following steps namely; pathogen recognition, cytoskeleton remodeling, phagosome formation and phagolysosome maturation.[85] Another important function of macrophages is ingestion, degradation, transport and loading of bacterial antigens onto MHC class II molecules for presenting them to T cell receptors for initiating the lymphocyte mediated innate immune response.[84] The specific cytokine profile secreted by the macrophages can determine the appropriate T-cell mediated immune response in the inflamed intestinal tissue; with IL-12 and IL-18 promoting Th1 lymphocyte focused response whereas IL-4 and IL-6 favoring Th2 lymphocyte centered response.[86-88]

Macrophage efferocytosis

The number of intestinal epithelial cells that undergo apoptosis every day in the - human small intestine and colon is approximately $2x10^8$ and $2x10^{11}$ respectively during physiological conditions.[89] In contrast, during acute and chronic intestinal inflammatory conditions apoptosis occurs at a much higher rate, eventually leading to accumulation of dead epithelial cells at the site of intestinal injury. Timely removal of dead epithelial

cells and neutrophils is essential for rehabilitation of inflamed intestinal mucosa, regaining of dead tissue homeostasis, tissue repair and healing.[90] Macrophages are one the important immune cells that engulf dead and apoptotic cells by a process known as efferocytosis.[90,91] Defective repair of these early apoptotic cells by defective macrophages during intestinal inflammation can be detrimental to the host as it can lead to tissue necrosis, chronic inflammatory diseases (chronic obstructive pulmonary disease, asthma or atherosclerosis) and autoimmune diseases (systemic lupus erythematosus and diabetes).[92] The process of efferocytosis requires coordinated effort by a cluster of signaling molecules including find-me signals, eat-me signals, don't eat-me signals, bridging molecules and phagocytic receptors for initial interaction between dying cell and phagocytes.[90] Engagement of apoptotic cell and macrophage leads to cytoskeletal rearrangements, engulfment of the dead intestinal cell body, followed by phagosome maturation and its eventual dismantling.[91] Apart from clearance of apoptotic cells, efferocytosis also helps to dampen the tissue inflammation through secretion of anti-inflammatory mediators (IL-10, TGF-β, and pro-resolving lipid mediators) and promote adaptive immune response through antigen presentation.[90,91] In the following sections, information regarding identifying and processing of the dead intestinal epithelial cells including find-me signals, eat-me signals, bridging receptors, mechanism of macrophage efferocytosis and its anti-inflammatory effects will be presented in a detailed manner.

Find-me signals

As apoptotic cells and phagocytes are not in proximity, there should be some mechanisms set in place so that they can closely interact with one other. A chemoattractant signal would be the most logical solution to this problem so that neighboring phagocytes would get attracted towards the dying cells. Once the intestinal epithelial cells undergo apoptosis, they tend to release some chemotactic signals for attracting neighboring primary phagocytes for their removal.[93] Lyso-phosphatidylcholine, fractalkine, ATP, UTP and S1P are some examples of the chemotactic signals released by the dying intestinal cells to attract macrophages towards them (Fig. 6).[94-97]

Other find-me signals that have been studied previously include endothelial monocyte activating protein, human tyrosyl t-RNA synthase, thrombospondin-1 (TSP-1) along with its heparin binding domain, ribosomal protein, soluble IL-6 receptor and apoptotic micro-blebs.[98] In some scenarios, apoptotic cells can also secrete a "keep out" signal such as lactoferrin that mainly functions to restrict the recruitment of neutrophils to the site of apoptosis without effecting mononuclear phagocytes; which is a classic feature of non-inflammatory apoptosis.[98,99] In this regard, the presence of anti-lactoferrin antibodies has been documented in few autoimmune diseases such as rheumatoid arthritis, ulcerative colitis, Crohn's disease and systemic lupus erythematosus.[98] Find-me signals may be secreted by the same apoptotic cell and, they tend to act synergistically in the local microenvironment to attract primary phagocytes.[95] The potent chemotherapeutic drug doxorubicin was demonstrated to increase apoptosis as well as SIP expression in jurkat cells.[97] S1P, which is a bioactive lipid secreted by the apoptotic cells, has been shown to attract THP-1 monocytes, U937 leukemia cells (pro-monocytic, human myeloid leukemia cell line),

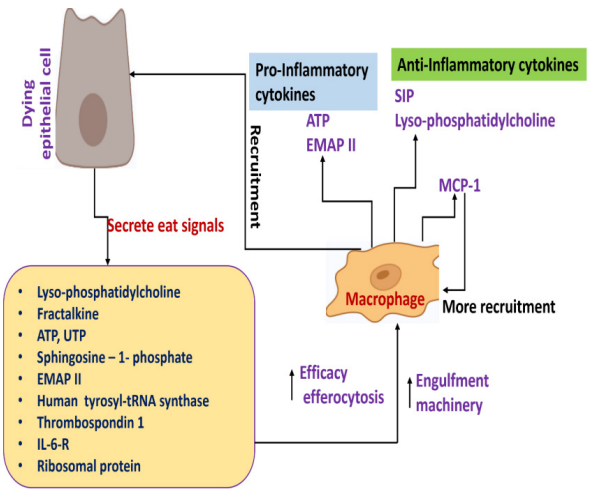

Fig. 6. Find me signals of dying epithelial cells for macrophage recruitment. Apoptotic epithelial cells release "Find me signals" for macrophage recruitment for initiation of macrophage efferocytosis. Find me signals can range from lyso-phosphatidylcholine, fractalkine, ATP, UTP, sphingosine-1-phosphate, endothelial monocyte activating polypeptide II, human tyrosynl-tRNA synthase, thrombospondin, IL-6-R to ribosomal protein. The release of these mediators causes macrophages recruitment towards the dying cells. In parallel presence of various pro-inflammatory cytokines further exaggerate the macrophage recruitment towards damaged epithelial cells resulting in their efferocytosis and heightened engulfment process through increased upregulation of efferocytosis machinery including macrophage receptors and bridging receptors

primary monocytes and macrophages.[97] Once macrophages are attracted to the apoptotic cells by these signals, they can secrete MCP-1which can attract more professional phagocytes to the apoptotic site thereby amplifying the processes of efferocytosis (Fig. 6).[100] It is possible that, the main purpose of these find-me signals is not only to attract the professional phagocytes but also to increase the expression of engulfment machinery so as to increase the efficiency of efferocytosis for enabling effective clearance of apoptotic cells (Fig.

6).[95] Furthermore, find-me signals has also been shown to stimulate pro-inflammatory cytokines (ATP and endothelial monocyte-activating polypeptide II) as well as anti-inflammatory cytokine secretion (S1P and lyso-phosphatidylcholine) from macrophages apart from their primary chemo attractive function (Fig. 6).[98,101,102]

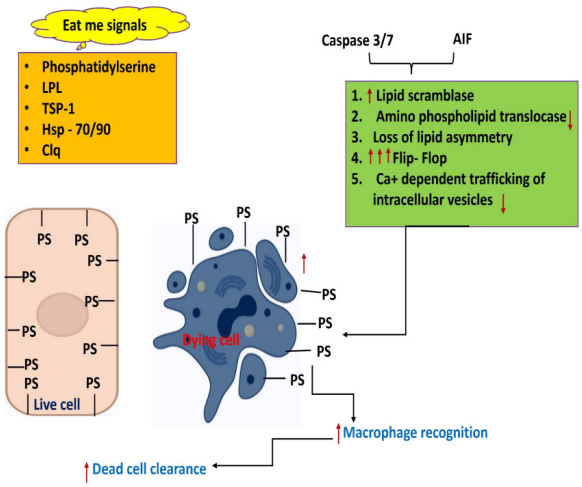

Fig. 7. Eat me signals of apoptotic cells for macrophage efferocytosis. Eat me signals such as Phosphatidyl serine (PS), lipoprotein lipase (LPL), thrombospondin-1 (TSP-1), HSP70&90 and Complement 1q (C1q) are utilized by dead epithelial cells for macrophage interaction for efferocytosis and their subsequent clearance. Primarily the phosphatidylserine expressed on the outer side of the dying cell, and it is recognized site by macrophage for engulfment and clearance. The exposure of PS can be due to number of factors such as increased activity of lipid scramblase, downregulation of amino phospholipid translocase, loss of lipid asymmetry, increased flip-flop and Ca^{2+} dependent trafficking of intracellular vesicles. The activity of these lipid degrading enzymes and calcium dependent trafficking is governed by caspase 3/7 and apoptosis inducing factors release. These events culminate in the dead cell clearance through macrophages

Eat-me signals

Once the professional phagocytes recognize the find-me signal, they migrate to the area where cells are actively undergoing apoptosis. In this scenario, apoptotic cells should display specific eat-me signals so that they can be differentiated from the nearby live cells by the professional phagocytes.[95] Display of eat-me signals indicates that the cells are in the early stage of apoptosis, and they need to be cleared before damage to the plasma membrane. Earlier removal of dying intestinal epithelial cells arrests the release of intracellular contents into the interstitium, and averts the escape of danger signals into systemic circulation and secondary inflammatory consequences.[103] A number of eat-me signals have been described in the literature so far, out of which phosphatidylserine, calreticulin, oxidized phospholipids (oxLDL), TSP-1 and complement binding sites (C1q and Cb/bi), chaperones (HSP70&90) and changes in glycocalyx are important (Fig. 7).[95,103-105]

Phosphatidylserine

One of the most studied eat-me signals is phosphatidylserine. In the resting cell, 70% phosphoryl choline, 20% phosphatidylethanolamine and 100% sphingomyelin are exposed extracellularly whereas 100% of phosphatidylserine is located intracellularly facing inner leaflet of plasma membrane.[105] During cellular activation, even though phosphatidylserine is transiently exposed extracellularly due to physiological flip-flop, it is reverted back to the intracellular location due to preserved action of amino-phospholipid translocase.[105] However, in apoptotic cells the scenario changes so that irreversible inactivated amino-phospholipid translocase, loss of lipid asymmetry and sustained increased flip-flop results in permanent exteriorization of 100% phosphatidylserine on the outer leaflet of plasma membrane (Fig. 7).[104-106] During apoptosis, caspases 3 and7's dependent activation of Xrp8 protein (lipid scramblase) can result in loss of plasma membrane lipid symmetry and subsequent phosphatidylserine exposure (Fig. 7).[106] Alternatively caspase independent mechanism of phosphatidylserine exposure has also been described through activation of scramblase by apoptosis inducing factor in T-lymphocytes undergoing apoptosis (Fig. 7).[107] Calcium dependent trafficking of intracellular vesicles formed during apoptosis from cytoplasm to plasma membrane is also proposed as another mechanism for phosphatidylserine externalization.[108] It is important to note that, deficient calcium dependent trafficking of intracellular vesicles from cytoplasm to plasma membrane in some cancer cell lines such as T98G (human glioblastoma multiforme cell line) and D32, leads to attenuated phosphatidylserine exposure and subsequent escape from efferocytosis.[108] The amount of phosphatidylserine exposed in outer leaflet of plasma membrane quantified by sensitive paramagnetic resonance method in live and apoptotic jurkat cells is <0.5 pico-moles and 240 pico-moles/million cells respectively.[109] It is important to understand that, macrophage receptors do not recognize and engulf the cells with phosphatidylserine exposure <5 pico-moles/million cells in the outer leaflet of plasma membrane.[109] This, in turn shows that apoptotic cells need to have 280-fold more phosphatidylserine exposure in the outer leaflet of the plasma membrane as compared live cells for their specific recognition and clearance by macrophage efferocytosis.[109]

Calreticulin

Calreticulin (CRT) is a protein localized to the endoplasmic reticulum and it participates in calcium homeostasis and intracellular protein folding.[105,110] Although it is mainly intracellular, it can migrate to the cell sur-

face by associating with MHC class I molecules.[111] In viable cells, the CRT present on the cell surface cannot induce phagocyte efferocytosis due to presence of co-existent inhibitory signal regulatory protein-alpha (CD47-SIRP-α) signaling pathway.[110] During apoptosis, the increased expression of CRT in the cell membrane stimulates lipoprotein receptor protein (LRP) on the phagocyte to enhance efferocytosis as the CD47-SIRP-alpha signaling pathway is inhibited.[110] Moreover, CRT has been shown to present in the cell in two forms: namely the cis- and trans-form.[105] In the cis-form, CRT interacts with LRP of the phagocyte through intermediary pattern recognition molecules such as collectins (mannose-binding lectin, complement 1q, surfactant protein D) and mediates efferocytosis.[105] Whereas in trans form, CRT can directly interact with LRP of the phagocyte without the assistance of collectins to mediate efferocytosis.[105]

Changes in glycocalyx and heat shock proteins

During homeostasis, the negative charge of the plasma cell membrane physiologically provides an electrostatic repulsive force to restrict the interaction between viable cells and phagocytes.[112] However, in apoptosis, changes in glycosylation status of the plasma membrane (increased N-acetylglucosamine-, mannose-, and fucose-containing epitopes and decreased in N-sialic acid) results in loss of negative charge and thus allows their interaction with macrophages facilitating efferocytosis.[112,113] In some instances, eat-me signal expression can be induced on tumor cell plasma membrane by chemotherapeutic drugs to promote interaction with antigen presenting cells for subsequent anti-tumor immune response and elimination. In multiple myeloma, bortezomib treatment increases the expression of eat-me signal HSP90 on dying tumor cells to facilitate their interaction with dendritic cells for generation of anti-tumor immune response and immunogenic death.[114,115]

Don't eat-me signals

Don't eat me signals function by limiting the interaction between the dying cells and macrophages and thereby preventing the occurrence of efferocytosis. In the next few paragraphs, we present information regarding four important Don't eat me signals namely CD47, plasminogen activator inhibitor (PAI-1), CD31 and CD24. All these agents function through different signaling mediators to accomplishing their task of curtailing the dead cell removal at the site of tissue injury.

CD47

CD47 is an integrin associated protein and it belongs to immunoglobulin superfamily.[116] It is a ligand for transmembrane receptors such as SIRP-α and thrombospondins and mediates physiological functions including cell motility, adhesion and phagocytosis.[116] The activation of CD47-SIRP-α on the surface of cancer cells leads to downregulation of the eat-me signals such as phosphatidyl serine, antigen-antibody complexes and CRT-LRP. This interaction is shrewdly devised by cancer cells to mask their recognition from macrophages so that it provides an avenue for escaping from innate immune mediated clearance pathways. Furthermore, interaction of CD47-SIRP-α also polarizes the macrophages towards M2 phenotype which have less efferocytosis capacity.[117] This CD47-SIRP-α signaling pathway presents an important target for chemotherapeutic drug development so that cancer cells can be made susceptible to macrophage clearance and immune mediated destruction.[117] CD47 blocking antibodies have been shown to enhance macrophage efferocytosis and prevent the atherosclerosis in animal models providing a novel therapeutic strategy to reduce the incidence of cardiovascular disease.[118]

Plasminogen activator inhibitor-1 (PAI-1)

PAI-1 is a member of serine protease family and it is implicated in microvascular thrombosis, immune cell recruitment and inflammation.[119] In viable neutrophils, PAI-1 acts as a don't eat-me signal by downregulating the CRT-LRP signal system and thereby preventing them from macrophage interaction and clearance.[119] However, in apoptotic neutrophils the levels of PAI-1 are decreased leading to elevated CRT-LRP signaling mediated clearance by macrophages.[119]

CD31

CD31 is another don't eat-me signal mainly expressed in leukocytes and neutrophils.[120] It is a member of immunoglobulin superfamily of membrane adhesion molecules.[120] During interaction between the viable cells and macrophages, activation of CD31 mediated signaling resulted in active temperature dependent disengagement of macrophages from viable cells and prevented their clearance.[120] In the contrary, CD31 signaling in apoptotic cells promoted their taut engagement with macrophages through proteo-liposomes, engulfment and their removal.[120]

CD24

CD24 expressed in cancer cells primarily interacts with Siglec-10 receptor on macrophages and resulting in decreased efferocytosis through immune-receptor tyrosine based inhibitory motif and tyrosine phosphatases 1 and 2 based signaling.[121] Accordingly, administration of CD24 blocking antibody disrupted the immune receptor tyrosine based inhibitory motif (ITIM) based signaling and promoted increased immune based macrophage mediated clearance.[121]

Bridging molecules and macrophage receptors

Intestinal epithelial cells undergoing apoptosis will upregulate eat-me signals and downregulate don't eat-me signals so that recruited macrophages interact and degrade them in a silent immunologically silent manner. Eat-me signals such as phosphatidylserine directly interacts with macrophages and indirectly interacts with phagocytic receptors and bridging molecules for initiating the macrophage induced clearance process. Macrophage phagocyte receptors involved in efferocytosis as described in the literature include brain angiogenesis inhibitor 1 and 3, T-cell immunoglobulin and mucin receptor 1, stabilin-1, 2 and CD300 -A, B and F- (immune receptors), scavenger receptor type F family member 1, tyrosine protein kinase receptor 3, tyrosine protein kinase receptor UFO, αvβ5 integrin, MER protooncogene, and tyrosine kinase (Fig. 8).[122-124]

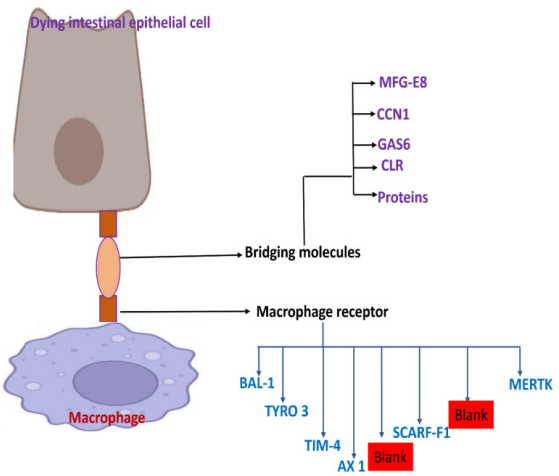

Fig. 8. Macrophage receptors and bridging molecules involved in macrophage efferocytosis. During macrophage efferocytosis, the apoptotic cells are cleared through the recognition of the major "eat-me" signal phosphatidylserine, directly through macrophage. Macrophage receptors and bridging molecules which mediate binding of phosphatidylserine to dying epithelial cells. Schematic shows some examples of macrophage receptor and bridging molecules involved in macrophage efferocytosis

The bridging molecules that mediate interaction between eat me signals on apoptotic cells and macrophage phagocyte receptors include milk fat globule-EGF factor 8 (MFG-E8), cellular communication network factor 1, growth arrest specific 6, complement 1q and protein S (Fig. 8).[122-124] During *Helicobacter pylori* infection of gastric mucosa, apoptotic gastric epithelial cells expressing phosphatidylserine are recognized by brain angiogenesis inhibitor 1 expressed gastric phagocytes resulting in engulfment, degradation, and antigen presentation.[125] Peritoneal macrophages expressing T-cell immunoglobulin and mucin receptor 1 are responsible for engulfing phosphatidylserine expressing apoptotic cells, activation of light chain 3 (LC3)-associated phagocytosis and preventing the development of autoimmunity.[126] TIM3 expressed on macrophages is responsible for mediating immune tolerance by downregulating TLR4-NF-κB mediated pro-inflammatory cytokine production.[127] CD300a receptor expressed on macrophages interacts with phosphatidylserine and phosphatidylethanolamine on apoptotic cells and inhibits engulfment via ITIM dependent manner and its blockade resulted in enhanced efferocytosis and improved neurological outcomes after ischemic stroke.[128-130] Stabilin-1and 2 receptors expressed on macrophages are responsible for clearing PS exposed apoptotic cells along with secretion of anti-inflammatory cytokines and enhancement of tissue healing.[131,132] MFG-E8 secreted by macrophages serves as bridging molecule by binding to phosphatidylserine exposed dead cells and brings them towards αvβ5 receptor expressed macrophages for their engulfment and subsequent removal.[133] Complement-3bi can bind to phosphatidylserine-exposed-apoptotic cells and interacts with CD11b/CD18 receptors on macrophages and assists in their uptake and clearance.[134] Protein C/Gas 6 protein also functions as bridging molecule by binding to phosphatidylserine exposed apoptotic cells and TIM receptors on macrophages causing tyrosine kinase mediated downstream signaling events leading to cytoskeletal rearrangement and efferocytosis.[135,136] Dying fibroblasts release TSP1, which acts as a bridging molecule by recruiting and mediating the interaction between αvβ3 receptors on macrophages and TSP1-CD36 complex on apoptotic fibroblasts for promoting their clearance.[137]

Mechanism of efferocytosis

Spingosine-1-phosphate (SIP) released from the dying intestinal epithelial cells acts on neighboring macrophages through SIP receptors (SIPR).[138] SIP-SIPR interaction on the macrophages can result in increased in erythropoietin (EPO) secretion through nuclear factor activator of T-cells (NFAT) and hypoxia inducing factor (HIF-α) (Fig. 9).[138]

Next, EPO acts through the EPO receptor on the macrophages to stimulate MAPK and PPAR-gamma signaling pathways, resulting in increased nuclear transcription of macrophage receptors and bridging molecules including MFGE8, Gas6, MerTK and CD36.[138] The interaction between the apoptotic cells and macrophages occurs due to binding of eat-me signals, bridging molecules and macrophage receptors. The process of ingesting the dead corpse by active membrane disruption occurs through a process known as micropinocytosis.[139] This is followed by formation of CRK protooncogene adaptor protein (CRKII) -dedicator of

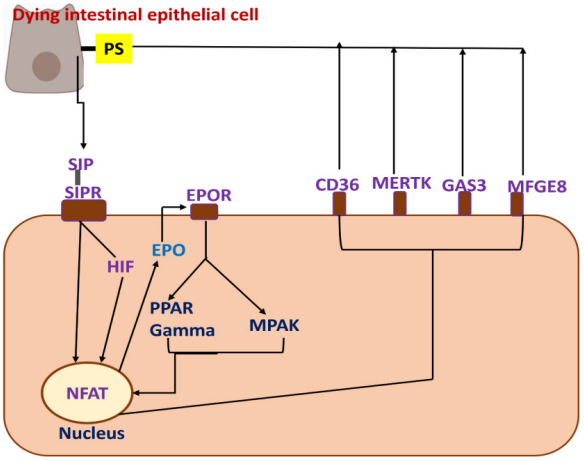

Fig. 9. Exposed PS induced downstream events in macrophages. Apoptotic cells release SIP which bind with neighboring macrophage with SIPR. The SIP-SIPR interaction induces EPO expression through NFAT and HIF. The EPO-EPOR interaction activates the MAPK and PPAR-g pathways resulting in nuclear transcription of macrophage receptors and bridging molecules such as MFGE8, Gas6, MERTK and CD36. (S1P – sphingosine-1-phosphate; NFAT – nuclear factor of activated T cells; HIF-α – hypoxia-inducible factor 1-α; EPO – erythropoietin; EPOR – erythropoietin receptor; Gas 6 – growth arrest-specific 6; MERTK – Mer tyrosine kinase; MFGE8 – milk fat globule-epidermal growth factor 8 protein; PPAR-γ –peroxisome proliferator-activated receptor gamma)

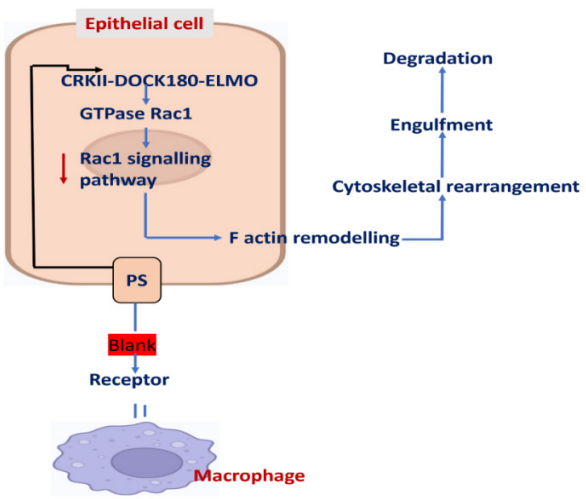

Fig. 10. Mechanism of macrophage efferocytosis. When the dying cell starts to expose PS on their surface, it is recognized by the macrophages directly or indirectly, through bridging molecules. The PS receptors stimulate CRKII-DOCK180-ELMO interaction to activate GTPase Rac1 belonging to rho family which then initiates the Rac1 mediated pathways. Rac1 signaling pathways play a crucial role in regulating the cytoskeleton through F-actin remodeling and cytoskeleton rearrangement, which is necessary for various cellular processes such as engulfment and degradation of cells in phagocytosis

cytokinesis-180 (DOCK180) - engulfment and cell mobility (ELMO) complex which activates GTPase of Rac Family small GTPase (Rac1) belonging to rho family resulting in initiation of Rac1 mediated signaling pathway (Fig. 10).[123,138]

In the final step, F1 acting remodeling leads to cytoskeletal rearrangement, engulfment, and degradation of apoptotic cell within the macrophage.[1123,138] After ingesting the dead cells, LC3 is recruited to the phagosome for ensuring complete and efficient degradation of the dying cell in an immunologically silent manner by a process known as LC3 associated phagocytosis (LAP) (Fig. 11).[140]

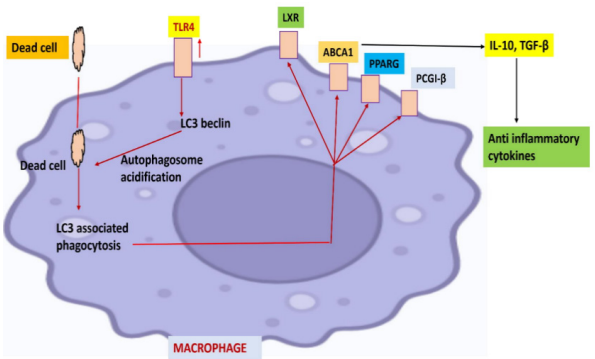

Fig. 11. LC3 associated phagocytosis and its anti-inflammatory effects. LC3-associated phagocytosis (LAP) is a type of phagocytosis that is mediated by the autophagy protein LC3. LC3 is recruited to the phagosome during engulfment, where it acts to increase the size of the phagosome and recruit lysosomal enzymes to degrade the engulfed material. In addition to its role in clearing debris and pathogens, LAP also has anti-inflammatory effects. In LAP, destruction of the phagosome containing the dead cell can result in increased secretion of anti-inflammatory cytokines such as IL-10 and TGF-beta which suppress inflammation. To further promote the degradation of the engulfed material in a non-inflammatory manner, TLR4 signaling assist in recruitment of LC3 and beclin to phagosome, autophago-lysosome formation, acidification

LAP can be differentiated from general autophagy by features such as formation of single membrane vesicles as opposed to double membrane vesicles, occurring few minutes as compared to hours after engulfment of corpse and not dependent on formation of pre-initiation complex (FAK Family interacting protein, ULK1/2 Unc-51 like autophagy activating kinase 1/2, and autophage related protein 13).[139] Destruction of the phagosome containing the dead cell without the associated LAP can result in increased secretion of pro-inflammatory cytokines and decreased secretion of anti-inflammatory cytokines with a propensity for increased occurrence of autoimmune diseases.[140] Liver X receptor (LXR), ATP binding cassette transporter, peroxisome proliferator activated receptor-gamma (PPAR-γ), per-

oxisome proliferator activated receptor gamma co-activator-1 beta and cholesterol are the important nuclear transcription factors implicated in the production of anti-inflammatory cytokines such as IL-10 and TGF-beta after ingesting the dead cells by macrophages.[139] Previous studies have demonstrated the association between deficient autophagy and auto-immune diseases such as Crohn's disease and systemic lupus erythematosus. After macrophage engulfment of apoptotic cells, activation of TLR4 signaling might result in recruitment of LC3 and beclin to phagosome, autophago-lysosome formation, acidification, and destruction in an immunologically silent manner.[141]

Fate of microorganisms after efferocytosis

During apoptosis of infected macrophages with *Mycobacterium tuberculosis* (Mtb), ingestion, and phagosome maturation by neutrophils is associated with killing of pathogens via upregulated host oxidase NADHPH oxidase-2 complex induced production of ROS and free radicals.[142] Effective compartmentalization of bacterium in the dead cells within the auto-phagosome is the key step for subsequent maturation, lysosome fusion and pathogen destruction.[143] Thus, efferocytosis represents an innate anti-bacterial immune response from the host tissues to limit bacterial replication and spread to the neighboring tissues.[143] However in the cases where virulent strains of Mtb causes necrosis of host macrophages, inhibition of phagosome maturation during neutrophil efferocytosis can cause uninhibited bacterial replication and subsequent progression of disease.[142] From the above findings, it is evident that bacterial infections are more likely to be cleared by efferocytosis during apoptosis of infected cells before progression to necrosis where pathogen induced aberrations renders the host clearance mechanisms less effective. Depending on the virulence of the microorganism, T-lymphocytes might assist in activation of efferocytosis and antibacterial defenses of macrophages through secretion of cytokines (TNF-α and IFN-γ).[143] If macrophage efferocytosis is not sufficient to curb the microbial threat, then apoptosis of macrophage laden microorganisms will be executed by T-lymphocytes followed by their removal by primary phagocytes.[143] In case of viral infection induced epithelial apoptosis, engulfment of dead corpses by the macrophages causes complete destruction and blockade of viral replication thereby leading to neutralization of viral threat.[144]

Macrophage phenotypes and immune silencing during efferocytosis

Increased ingestion of apoptotic cells by macrophages results in intracellular accumulation of large amounts of lipids, carbohydrates and proteins which might energize nuclear receptors including PPAR- γ, δ and LXR resulting in their transition into pro-resolving macrophages (Fig. 12).[122]

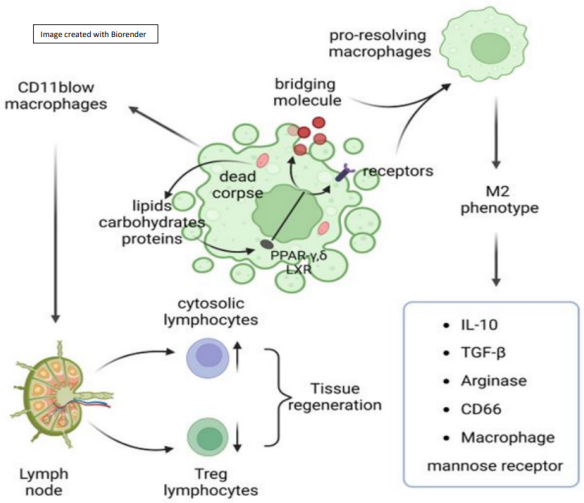

Fig. 12. Anti-inflammatory effects secondary to macrophage efferocytosis. Accumulation of large amounts of protein, lipids, and carbohydrates due to macrophage ingestion of dead cell activates the PPAR- γ, δ and LXR nuclear receptors. Activation of PPAR-γ and of LXR results in increased production of macrophage receptors and bridging molecules for efferocytosis. As efferocytosis continues, pro-resolving macrophages with M2 phenotype produce anti-inflammatory cytokines (e.g., IL-10 and TGF-beta) and express increased amounts of arginase, macrophage mannose receptor and CD36. Once macrophage engulfment reaches its capacity, macrophage transforms into CD11b low phenotype with deprived efferocytosis function. The CD11b low macrophage then migrate to neighboring lymph nodes to interact with cytotoxic and regulatory T-lymphocytes to suppress the activity of other immune cells, preventing overreaction, auto-immunity and chronic inflammation

Alternatively, these pro-resolving macrophages can be generated by cytokines such as IL-4 and IL-13.[145] Pro-resolving macrophages are tuned to perform increased efferocytosis with increased transcription of bridging molecules & receptors for binding more apoptotic cells for clearing the cellular debris from the inflammatory milieu (Fig. 12).[145] Furthermore, these pro-resolving macrophages are polarized to M2 phenotype with enhanced secretion of anti-inflammatory cytokines (IL-10 and TGF-β) and express increased amounts of arginase, macrophage mannose receptor and CD36 (Fig. 12).[145] Treg cells can also induce the production of alternative activated macrophages which are characterized by enhanced phagocytic capacity with increased expression of CD206 (macrophage mannose receptor) and CD163 (hemoglobin scavenger receptor) as well as reduced potency to secrete LPS induced pro-inflammatory markers.[146]

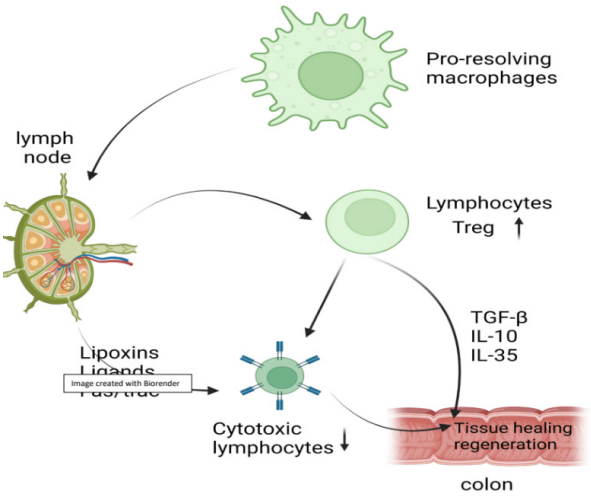

Fig. 13. Tissue healing secondary to macrophage efferocytosis. Pro-resolving macrophages migrate to the site of injury and to the lymph nodes where they interact with T cells to control inflammation. Macrophages are known to express ligands for FAS ligand/TNF-related apoptosis-inducing ligand death receptors on cytotoxic T-lymphocytes whose activation can initiate caspase dependent apoptotic signaling leading to their decreased survival. Pro-resolving macrophages can promote the recruitment of Treg cells from the lymph nodes towards the site of intestinal injury resulting in secretion of anti-inflammatory cytokines such as TGF-beta, IL-10 and IL-35. Overall, pro-resolving macrophages play a crucial role in the healing process by migrating to the lymph nodes by controlling inflammation, promoting tissue repair, modulating the immune response, and facilitating the removal of damaged cells

These resolution-phase macrophages regulated by cAMP, are mainly responsible for tissue homeostasis and repopulation of innate lymphocyte population in the resolution phase of acute inflammation.[147] Once the macrophage engulfment of apoptotic corpses reaches a saturation threshold, they transform into $CD11b^{low}$ phenotype characterized by their attenuated efferocytosis function. It is important to note that, these $CD11b^{low}$ phenotype can also be induced by pro-resolving mediators such as resolvin D1, resolvin E1 and dexamethasone.[148] As soon as these pro-resolving macrophages lose their engulfment capacity, they immediately migrate to the neighboring lymph nodes and secrete 12-lipo-oxygenase metabolites to interact with T-lymphocytes to mediate silencing of the innate immune response, which is instrumental for tissue regeneration, and healing (Fig. 13).[148] Moreover, macrophages can directly migrate and interact with T-lymphocytes in the lymph nodes to cause negative regulation of immune system via secretion of macro-molecular mediators such as interferons and complement components.[149] Activation of macrophage ligand receptors such as programmed cell death 1, cytotoxic lymphocyte associated protein 1, histocompatibility leukocyte antigen-G and histocompatibility leukocyte antigen-E) can directly suppress the cytotoxic action of T-cells, natural killer cells in the lymph nodes.[150] Macrophages are known to express ligands for FAS ligand and TNF-related apoptosis-inducing ligand death receptors on T-lymphocytes whose activation can potentiate caspase dependent apoptotic signaling in them and thereby leading to their decreased survival (Fig. 13).[150] Furthermore, macrophage induced secretion of chemokines such as CCL5, CCL20, CCL12 and CCL22 might cause Treg recruitment to the inflammatory milieu thus paving the way for tissue regeneration (Fig. 13).[150-152] Enhanced Treg recruitment might favor downregulation of immune response by inhibition of $CD4^+$ and $CD8^+$ lymphocytes through secretion of IL-10, IL-35 and TGF-β (Fig. 13).[152] Thus, macrophage engulfment of apoptotic cells along with suppression of immune response results in clearance of corpses from the inflammatory milieu in an immunologically silent manner that promotes tissue healing, regeneration, and repair.

Impaired efferocytosis and its consequences resulting in NEC intestinal injury

Any wreckage in this protective physiological phenomenon of macrophage efferocytosis can derail the clearance mechanisms of dead corpses from the inflamed tissues leading to defective tissue regeneration with subsequent progression to tissue necrosis, chronic inflammation, and autoimmunity. The physiological phenomenon of macrophage efferocytosis will be flawed when macrophages are dysfunctional or apoptotic corpuses become poor meal for engulfment of macrophages. This can occur due to variety of reasons ranging from decrease in the eat-me signals, increase in the don't eat-me signals, decreased production of bridging molecules, downregulated expression of macrophage receptors, presence of autoantibodies, NET formation, TLR4 signaling to accumulation of oxidized phospholipids Each of these abnormalities that can potentially impair macrophage efferocytosis in NEC models will be discussed individually in the next few paragraphs.

Lipid peroxidation due to accumulation of free radicals and peroxy-nitrates is implicated in the pathogenesis for transmural necrosis in the NEC models.[153,154] These free radicals can potentially oxidize the membrane phospholipids of macrophages resulting in accumulation of oxidized phospholipids in NEC.[155] These oxidized phospholipids can bind and saturate efferocytosis receptors and thereby binding capacity of macrophages for the apoptotic epithelial cells is attentuated due to competitive inhibition at the site of intestinal injury in the NEC models.[156] Moreover, oxidized phospholipids generated via lipid peroxidation might harbor

some neo-epitopes which might stimulate the production of autoantibodies by B-lymphocytes.[157] Due to their cross reactivity, these autoantibodies circulating in the blood might bind and mask the eat-me signals on the apoptotic epithelial cells.[158] This prevents their recognition by macrophages and thereby leads to their decreased clearance.

Aberrant activation of TLR4 signaling and its role in the pathogenesis of intestinal necrosis in NEC models has been extensively studied.[159,160] Enhanced TLR4 signaling in response to microbial associated molecular patterns such as LPS stimulates NF-κB mediated gene transcription of pro-inflammatory cytokines leading to epithelial apoptosis and mucosal injury in NEC. With increased presence of inflammatory markers in the intestinal tissue, macrophages are more polarized toward M1 phenotype which are more pro-inflammatory but possess less efferocytic capacity for clearing dead epithelial cells in the intestinal milieu.[161] Upregulated TLR4 signaling along with increased secretion of pro-inflammatory cytokines leads to decreased expression of lipoprotein receptor protein 1 and MerTK as well as reduced activation of LXR resulting in decreased clearance of apoptotic cells and reduced anti-inflammatory defenses.[162]

LPS stimulation of the macrophages can alter the gene expression of the transcription factors such as PPAR-γ (decreased) and IRF5 (increased) leading to attenuated expression of macrophage receptors (CD36 and CD14) and bridging molecules (Gas-6 and MFG-E8) leading to decreased efferocytosis potency in macrophages.[145,163-165] Inflammatory mediators such as LPS and TNFα can impair efferocytosis by altering the balance between RhoA and Rac in the macrophages.[145] High mobility group box1 (HMBG1) can bind and mask the macrophage receptor (αvβ3) thereby limiting its binding to MFG-E8 and subsequently to the PS exposed on apoptotic epithelial cells.[145] Receptor for advanced glycosylation end products (RAGE) and annexin V can directly bind to the phosphatidylserine exposed on apoptotic epithelial cells thereby blocking its recognition and removal by macrophages.[145]

MMPs such as streptolysin-1, implicated in the degradation of mucosal extracellular matrix, has been demonstrated in the NEC models.[166] These MMPs were also shown to degrade the eat-me signals (LRP-1) and efferocytosis receptors (MerTK) from the apoptotic cells and macrophages respectively leading to decreased efferocytosis in the intestinal tissues of NEC.[166] Furthermore, increased TNF-α might lead to NF-κB mediated increased transcription of CD47 (Don't eat-me signal) in the apoptotic epithelial cells thus concealing them from recognizing by macrophages for their timely removal.[167] The presence of pro-inflammatory cytokines such as TNF -α along with other toxins in the inflamed intestine in NEC can inhibit the binding of macrophages with apoptotic cells at the site injury through cytosolic phospholipase A2 (cPLA2) and Rho-GTPase (Rho Family of Guanosine-5'-tri-phosphatases) thereby impairing the dead cell clearance.[168]

Interferon regulatory factor 5 (IR5) levels are elevated in the macrophages and promotes M1 pro-inflammatory phenotype polarization in the murine model of NEC.[169] This increased expression of IR5 in macrophages can potentiate the disintegration of bridging molecule MFGE8 and its receptor αvβ3 ultimately leading to decreased macrophage engulfment capacity of apoptotic cells.[161]

Recruitment of neutrophils to the site of inflammation is an important part of innate immune response for combating against microbial threats in NEC.[170] Formation of NET is one of the most important mechanisms by which neutrophils neutralize the microorganisms in the inflamed gut.[170] NET is characterized by formation of web like structures, and these complexes accommodate anti-microbial proteins and DNA- like structures with histones.[170] It has been demonstrated that, NET formation impedes the process of efferocytosis through degradation of macrophage receptors, $\alpha_v\beta_3/\alpha_v\beta_5$ integrins via secretion of neutrophil elastases.[171] Moreover, histones present in the NETs can inhibit efferocytosis by competitive inhibition of macrophage receptors (MerTK and $\alpha_v\beta_5$) thereby preventing the interaction between bridging molecules Gas6 and MFG-E8 and apoptotic cells.[172]

It is important to understand that the underlying causes of decreased efferocytosis in NEC is the result of combined interplay of factors caused by imbalance of inducers and inhibitors of macrophages efferocytosis of apoptotic cells. As a result, there is an increased chance of progression from apoptosis to necrosis of intestinal tissues, which is the hallmark of NEC. Eventually, the increased lysis of necrotic intestinal cells results in spilling of intracellular contents into the extracellular milieu. The epitopes present in the extracellular nuclear DNA from the lysed cells might initiate the production of autoantibodies resulting in autoimmunity related clinical disorders. HMGB1 is nuclear protein which when released into the extracellular environment from necrotic intestinal cells can provoke inflammation leading to production of pro-inflammatory cytokines and predominance of M1 low-efferocytosis index macrophages.[173] Since there is a low population of pro-resolving high efferocytosis index macrophages, there is a lesser chance of healing and tissue repair after intestinal injury. This might lead to progression of disease with systemic complications, which is associated with high mortality and morbidity in NEC.

Pathological effects on brain and lung in NEC – relevance to perturbed efferocytosis

Brain

It is important to understand that, in order to prevent inflammation, timely clearance of apoptotic intestinal cells needs to happen so that intracellular contents containing danger signals are not spilled into the extracellular environment.[174] Apoptosis of intestinal epithelial cells occurring due to the bacterial LPS induced TLR4 signaling will gradually progress to secondary necrosis if macrophage efferocytosis mediated tissue repair is perturbed. Once secondary necrosis of intestinal epithelial cells happens, HMGB1 (danger signal) gets released into the systemic circulation leading to hyper-inflammation and sepsis with an increased production of pro-inflammatory cytokines.[174] Next, pro-inflammatory cytokines can increase the expression of MMPs which can potentially degrade the tight junctions and extracellular matrix of endothelial basement membrane of the blood brain barrier (BBB) resulting in matrix degradation and its subsequent leakiness.[175] In response with BBB injury, brain-resident microglia migrates to the site of BBB injury and produces pro-inflammatory cytokines such as IL-1 β and IL-6, further exacerbating the BBB dysfunction.[175] Moreover, in parallel to these cellular events hypoxia and ischemia which are important risk factors in the pathogenesis of NEC are associated with upregulation of inducible nitric oxide synthase leading to increase in nitric oxide and peroxy-nitrate free radicals, which are known to further aggravate the BBB dysfunction.[175-177] Furthermore, HMBG1 released into the systemic circulation can easily cross the leaky blood brain barrier and enter the brain. Once inside the brain, it can act on the TLR4 receptors of brain microglia and result in activation of NF-κB and IRF3 pathways. This results in secretion of cytokines chemokines, adhesion molecules and ROS species upregulation in the brain leading to pathological neuro-inflammation and neurological sequelae such neurodevelopmental delay.[174,178]

The pathological signs of pro-inflammatory cytokine storm inside the brain can range from impaired oligodendrocyte maturation, loss of hippocampal volume, disordered neuronal development, demyelination to axonal injury.[178-181] These pathological deficits ultimately manifest as clinical consequences ranging from cognitive impairment, psychomotor delay to neurodevelopmental delay in the NEC infants.[178]

Brain microglia engulf dead neuronal cells with the help of receptors such as C-X-3 motif chemokine receptor 3 (CX3CR1), purino receptor 6, purino receptor 12, stabilin 1, signal regulatory protein alpha, triggering receptor expressed on myeloid cells 2, MerTK, and CD11b (Integrin αM).[182] Microglia regularly perform surveillance of the brain parenchyma to remove senescent and dead neuronal cells for aiding in neuronal development.[182] Any failure in this physiological clearance mechanism in the brain parenchyma of preterm infants either due to downregulation of microglial receptors or failure to recognize apoptotic neuronal cells leads to accumulation of dead neuronal cells, secondary necrosis and amplification of inflammatory response. Allerdorf et al. reported that, addition of bacterial LPS to microglial-neuronal cultures resulted in excessive efferocytosis and neuronal loss via microglial surface de-sialylation and upregulation of engulfment complement receptor 3.[183,184] Cytokines are shown to have varying effects on the microglial efferocytosis in the brain. Increase in TNF-α in the brain causes massive upregulation of the microglial efferocytosis in both live and apoptotic neuronal cells resulting in disproportionate neuronal loss.[184,185] In the contrary, the anti-inflammatory cytokine TGF-beta was responsible for physiological pruning and neuronal development through increasing the expression of complement 1q on the neuronal synapses.[184,186] Myelin sheath and synapses are protected from excessive microglial phagocytosis through SIRP-α-CD47 interaction.[187,188] Any alteration in this regulation of microglial efferocytosis in preterm infants might result in excess microglial pruning of myelin sheath and synapses, leading to demyelination of neurons and disruption of synaptic transmission, ultimately resulting in neurodevelopmental delay. Engulfment of apoptotic neuronal cells by microglial cells can also elicit secretion of cytokines and chemokines, both of which can lead to restricted neurogenesis and cognitive decline.[189] Microglial efferocytosis primarily controls the regulation of the number of neuro-progenitor cells in the embryonic cerebral cortex, an essential structure for earlier brain development.[190] As NEC is frequently associated with presence of bacterial LPS, there might be an aggressive reduction in the number of neuro-progenitor cells, primarily through enhanced and uncontrolled microglial efferocytosis, resulting in defective cortical development in preterm infants.[190] Therefore, fine-tuning, and appropriate regulation of microglial efferocytosis is very much essential for physiological pruning of synapses as well as neurogenesis in the preterm infants. Accordingly, any downregulation and upregulation of this protective physiological mechanism and alteration of this delicate balance by inflammatory insults has been shown to be associated with increased risk of neuro-developmental delay in Necrotizing Enterocolitis as well as neurodegenerative diseases such as Parkinson disease and motor neuron disease.[191,192]

Lung

TLR4 receptor is expressed in the pulmonary epithelial cells and vascular endothelial cells.[193,194] Bacterial LPS and DAMPs has been shown to act on TLR4 receptors expressed in the lungs to stimulate pro-inflam-

matory gene expression through energizing NF-κB and MAPK signaling pathways.[195] As discussed above once the escape of danger signal HMGB1 from the necrotic intestinal epithelial cells into the systemic circulation happens, it can find its way towards pulmonary epithelial cells and act on TLR4 receptors. Activation of TLR4 receptors in the lungs by HMGB1 can cause enhanced neutrophil infiltration through production of chemoattractant factor namely CXCL5.[178] Neutrophil accumulation in the lungs can initiate lung inflammation through the release of neutrophil elastases and formation of NETs.[196].As the neutrophils try to eradicate the infection by engulfment of microorganisms by binding to their PAMPs and DAMPs, they can simultaneously undergo a change in gene expression leading to their senescence through apoptosis differentiation program.[197] In other instances, neutrophil phagocytosis of microorganisms such as *Staphylococcus aureus* can inhibit macrophage efferocytosis by upregulation of don't eat-me signals (CD47) and precipitate neutrophil death through necroptosis RIPK1 dependent manner. Moreover, apoptosis and lysis of neutrophils releases serine proteases, which can potentially stimulate the neighboring macrophages to produce pro-inflammatory cytokines such as TNF-α, IL-8 and IL-10 thereby amplifying the lung inflammation.[198]

Therefore, neutrophils that undergo apoptosis should be efficiently removed by alveolar macrophages in the pulmonary tissues before progression to necrosis for preventing the release of their toxic intracellular contents to the extracellular environment for preventing disastrous consequences. Clearance of apoptotic cells in the lung is usually performed primarily by alveolar macrophages and secondarily by dendritic cells & bronchial epithelial cells.[199] Compared to systemic tissue macrophages, lung alveolar macrophages specifically are known to possess reduced efferocytosis capability due to varied reasons such as reduced adhesion, very low expression of protein kinase CβII and inhibition of surfactant protein A and D.[199,200] Defective or lowered efferocytosis has been implicated in the pathogenesis of lung diseases such as asthma, acute lung injury and chronic obstructive pulmonary disease.[199] Engulfment of pathogenic bacteria by M2 phenotype alveolar macrophages might facilitate bacterial persistence due to their intrinsic properties ranging from enhanced oxidative metabolism, decreased antimicrobial activity to increased PGE2 production.[201]

TLR4 receptor is involved in the alveolar macrophage efferocytosis through increased expression of MerTK receptor. Moreover, analysis of blood from preterm and term infants revealed that there is decreased expression of TLR4 expression in the granulocytes and monocytes.[202] Macrophages in the preterm infants might have reduced capacity for efferocytosis due to decreased adherence receptor expression due to very low TLR4 receptor expression along with defective antigen presentation.[202-204] Pathogen-mediated TLR4 signaling has also been to implicated to alter the ratio of Th17/Treg ratio in the lung epithelium leading to increased production of cytokines along with immune cell recruitment further exacerbating the lung injury in NEC.[178]

So, defective alveolar macrophage efferocytosis along with low TLR4 receptor expression are the critical cellular abnormalities that might impair the clearance of dead apoptotic cells from the injured alveolar epithelium leading to persistent and chronic lung inflammation in preterm infants.

Novel therapeutic options for increasing efferocytosis and enhancing tissue repair in NEC

The novel therapeutic interventions that can be useful in increasing the efferocytosis and ultimately facilitating tissue repair and regeneration in NEC will be discussed in the following subheadings: anti-CD47 antibodies, blocking ADAM17 cleavage, tilting SPM:leukotriene ratio, phosphatidylserine liposomes, PPAR gamma agonists, LXR agonists, glucocorticoids and annexins (Fig. 14).[156]

Anti-CD47 antibodies

Tumor cells in multiple cancers including breast, lung, colon, and ovary are known to upregulate CD47 (don't eat me signal) and interact with SIRP-alpha receptor on macrophages to elude efferocytosis and subsequent immune mediated destruction.[205] Interaction of CD47-SIRP-alpha leads to ITIM based activation of tyrosine phosphatases (SHP-1 and 2) and down regulation of actin cytoskeleton which ultimately causes inhibition of efferocytosis by tumor associated macrophages.[206] Clinical research has shown that blocking this interaction with anti-CD47 antibodies yielded therapeutic benefit by promoting efferocytosis and, thereby allowing tumor clearance.[207] Additionally, anti-CD-47 antibody administration also resulted in the activation of dendritic cells by release of tumor cell nuclear and mitochondrial DNA into the tumor microenvironment. This dendritic cell activation results in subsequent priming of cytotoxic T-lymphocytes through release of IFN-γ. Activated cytotoxic T-lymphocytes will set in motion robust anti-tumor innate immune responses leading to enhanced tumor clearance and improved survival rates.[208]

Blocking ADAM17 induced cleavage

Studies have shown that activation of MerTK receptor signaling on macrophages leads to increased synthesis of cytoplasmic 5-lipooxygenase (5-LOX) through suppressed activity of calcium dependent protein kinase II (CaMKII).[209] 5-LOX is the key synthetic enzyme implicated in the synthesis of SPMs (specialized pro-resolving mediators) such as resolvins and lipoxins from long

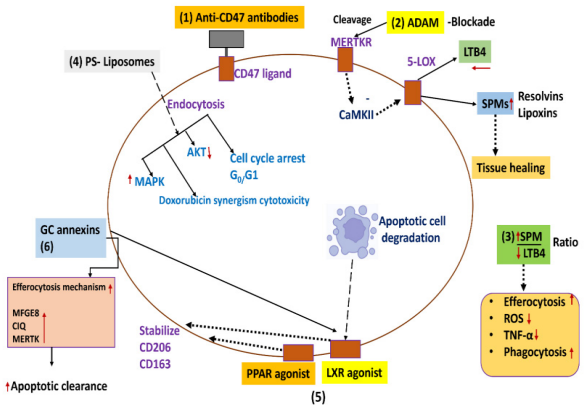

Fig. 14. Treatment modalities for alleviating cell death in NEC based on macrophage efferocytosis. (1) Anti-CD47 antibodies: Anti-CD47 antibodies are designed to bind to the CD47 protein, thus blocking its ability to act as an "Don't eat me" signal. By binding to CD47, the antibodies reduce the expression of CD47 on the surface of the dying cells, allowing macrophages to recognize and engulf them, thereby reducing inflammation and cell death in NEC. (2) Blocking ADAM17 cleavage: ADAM17 is a protease enzyme that is responsible for cleaving certain membrane-bound proteins into their active forms. MerTK (Mer protooncogene tyrosine kinase) is a receptor that is found on the surface of macrophages. MerTK activation leads to increased synthesis of 5-lipooxygenase (5-LOX). 5-LOX is an enzyme that plays a key role in the synthesis of specialized pro-resolving mediators (SPMs) such as resolvins and lipoxins which are known to have anti-inflammatory and pro-resolving properties, and their production by 5-LOX is tightly regulated. Targeting the production of SPMs by modulating the activity of enzymes such as 5-LOX by blocking ADAM17 cleavage could be a potential therapeutic approach to resolving NEC inflammation. (3) Tilting SPM: leukotriene ratio: The clearance of apoptotic cells is dependent on the delicate balance between SPMs and pro-inflammatory LTB4 (Leukotriene B4) in the inflamed tissue. By tilting the ratio of SPMs to leukotrienes, it means to increase the production of SPMs and reduce the production of leukotrienes. This can be achieved by targeting the activity of enzymes such as 5-lipooxygenase (5-LOX), which is responsible for the synthesis of SPMs and leukotrienes. This shift in the balance of pro-inflammatory and anti-inflammatory molecules would reduce inflammation and cell death in NEC. (4) Phosphatidylserine liposomes: phosphatidylserine liposomes are a potential treatment modality for alleviating cell death in NEC by attracting macrophages to the gut, promoting efferocytosis, and reducing inflammation and oxidative stress. PS-liposomes tend to enter cancer cells expressing phosphatidylserine via endocytosis and mediate malignant cell death through activation of MAPK, downregulation of AKT, and cell-cycle arrest at sub-G0/G1 phase. These mechanisms lead to cancer cell death and can be used as a potential treatment modality. This is achieved by binding to PS on the surface of the infected cells and recruiting immune cells resulting in destruction of infected cells. (5) PPAR gamma LXRs agonists: Peroxisome proliferator-activated receptor-gamma (PPAR-gamma) is a nuclear receptor that is involved in the regulation of inflammation and cell death. PPAR-gamma agonists are drugs that can bind to and activate PPAR-gamma, leading to an anti-inflammatory response and the inhibition of cell death. Liver X receptors (LXRs) are nuclear receptors that are activated by oxysterols, which are derivatives of cholesterol. Activation of LXRs has been shown to promote the resolution of inflammation by promoting the activation of anti-inflammatory macrophages. Activation of LXRs by LXR agonists can lead to the stabilization of CD206 and CD163, which are markers of anti-inflammatory macrophages. This stabilization promotes the resolution of inflammation by promoting the activation of anti-inflammatory macrophages. (6) Glucocorticoids and annexins: Glucocorticoids (GCs) have anti-inflammatory and immunosuppressive properties and may help to reduce inflammation and cell death in necrotizing enterocolitis (NEC) by decreasing the production of inflammatory mediators, upregulation of efferocytosis machinery and gene transcription (such as LXR, PPAR-gamma and RXR (Retinoid X receptor) and promoting the long-term clearance of apoptotic cells. GCs also promote macrophage and monocyte efferocytosis through increased upregulation of bridging molecules and efferocytosis receptors such as MFG-E8, C1q, stabilin, CD206, CD163 and MerTK. Annexins, a family of proteins, also play a role in cell death and inflammation in NEC by acting on the lipoxin A4 (LXA4) receptor and causing F-actin reorganization in macrophages, leading to increased engulfment of apoptotic neutrophils, and enhancing their own removal via professional phagocytes through efferocytosis

chain fatty acids.[209,210] Upregulation of SPMs has been shown to be associated with resolution of inflammation in various conditions including sterile peritonitis, ischemia-reperfusion (I/R) injury and remote organ inflammation.[210] Increase in SPMs in the tissues can be beneficial for better resolution of tissue injury as they prevent the decrease of MerTK expression on macrophages and increase efferocytosis.[211] The presence of severe hypoxia and ER stress during inflammation can precipitate the synthesis of metalloproteinase ADAM17 in the intestinal tissues.[212] This enzyme can cause proteolytic cleavage of MerTK receptor from macrophages and thereby impeding the synthesis of SPMs in the intestinal tissues required for resolution of inflammation.[210] In this regard, MerTK deficiency in cardiac macrophages after myocardial I/R injury can result in impaired car-

diomyocyte wound healing, increased infarct size and depressed cardiac pump function.[213] Accordingly, presence of cleavage resistant MerTK receptor on tissue macrophages was associated with better tissue healing and improved clinical outcomes.[209,210,213] Moreover, deletion of ADAM17 on professional phagocytes also yielded better clinical outcomes through enhancing efferocytosis, inducing anti-inflammatory cytokine profile as well as resolution of inflammation.[214] These above findings add credence to the concept that therapeutic agents aimed at cleavage and inactivation of enhanced ADAM17 generated during hypoxia and oxidative stress might be beneficial in enhancing efferocytosis as well as subsiding tissue inflammation via MerTK induced production of pro-resolving mediators.

Tilting SPM-leukotriene ratio

The clearance of apoptotic cells is dependent on the delicate balance between SPMs and pro-inflammatory leukotriene B4 (LTB4) in the inflamed tissue. In atherosclerosis, downregulation of SPMs and predominance of LTB4 can be detrimental and leads to enhanced atherosclerotic plaque instability[215]. Accordingly, supplementation of SPMs had shown clinical benefit in atherosclerosis and hepatic diseases by rectifying this abnormal SPM: LTB4 ratio.[215,216] SPMs that can be used to enhance efferocytosis and promote resolution of inflammation can range from lipoxins, resolvins, protectins, maresins cysteinyl-conjugated SPMs (CTRs) to 13-series resolvins.[217] Resolvin D1 acts through its receptor ALX/FRP2 (Formyl peptide receptors 2) and increases efferocytosis by making the MerTK receptor resistant to cleavage due to senescence, increased M2 polarization and enhanced production of pro-resolving mediators through feed forward mechanism.[216-218] Other mechanisms proposed for action of RD1 in resolution of inflammation include downregulation of ROS, decreased production of TNF-α, ER mediated phagocytosis, release of calreticulin from macrophages, epigenetic mechanisms, and increased metabolism of macrophages (fatty acid oxidation and oxidative phosphorylation).[217] Lipoxin A4 (LXA4) is another SPM that has been studied extensively and it has been shown to increase efferocytosis by promoting cytoskeletal rearrangement and engulfment through signaling molecules such as MYH9 and CDC42.[219]

Phosphatidylserine liposomes

Phosphatidylserine dependent recognition and signaling is a key event in recognition and clearance of apoptotic cells by macrophages as well as resolution of inflammation.[220] In chronic granulomatous disease (CGD), impairment of macrophage efferocytosis was normalized by injection of phosphatidylserine through IL-4 dependent macrophage reprogramming and increase in apoptotic cell uptake.[221] Cancer cells are known to exhibit unusually high amount of phosphatidylserine on their surface (approximately 3-7-fold more than phosphatidylserine expressed by non-tumor cells).[222] Research studies utilizing phosphatidylserine -liposomes have yielded encouraging results by enhanced killing as well as inhibiting the growth of phosphatidylserine expressing cancer cells in animal models of cancer.[223,224] Phosphatidylserine -liposomes tends to enter the cancer cells expressing phosphatidylserine via endocytosis and mediate malignant cell death through activation of MAPK, downregulation of AKT and cell-cycle arrest at sub-G0/G1 phase.[224] Additionally, incorporation of anti-cancer drugs like doxorubicin and gemcitabine into the phosphatidylserine -liposomes have demonstrated synergism in killing effects of the cancer cells as along with inhibiting metastasis in mice models of cancer.[223-225] Furthermore, administration of chimeric antibodies (bavituximab) which are developed to recognize the phosphatidylserine expressed on virus infected cells are known to promote viral destruction and clearance through antibody dependent cytotoxicity in guinea pigs infected with *Pichinde* virus.[226]

LXR agonists and PPAR ɣ agonists

Both PPAR and LXR regulate apoptotic cell uptake as well as anti-inflammatory cytokine profile of macrophage thereby playing a central role on tissue repair and resolution of inflammation.[227-229] Accumulation of fatty acids and oxysterols released from the nuclear membrane of dead cells stimulate PPAR and LXR nuclear receptors respectively in the macrophages and bolsters the process of apoptotic cell removal.[230] Upregulated PPAR and LXR genes are known to further promote the clearance of apoptotic cells by macrophages via increased production of MFG-E8/C1q and MerTK respectively in feed-forward manner.[230] Application of PPAR and LXR agonists had yielded encouraging results in the clinical diseases by increasing macrophage efferocytosis of dead cells. In CGD, neutrophils treated with pioglitazone (PPAR agonists) displayed abundant eat-me signals upon apoptosis via oxidant dependent manner resulting in enhanced efferocytosis and resolution of sterile inflammation.[228] In CGD, macrophages exhibited significant impairment in the efferocytosis of carboxylated beads which was reversed by overnight incubation with pioglitazone (PPARγ receptor agonist).[231] Treatment of macrophages isolated from *Trypanosoma cruzi* infected mice with PPAR ligands caused predominance of M2 polarization with increased expression of arginase-1, TGF-β and mannose receptors which are primarily responsible for efferocytosis, tissue repair as well as resolution of inflammation.[232,233] On the contrary, administration of PPAR antagonists resulted in downregulation of genes responsible for macrophage-induced

uptake of apoptotic cells: namely CD36 (membrane glycoprotein), TG2 (tissue transglutaminase), AXL receptor tyrosine kinase and pentraxin related protein 3.[234] It has been documented that TLR4 receptor activation might inhibit the LXR mediated signaling pathways leading to decreased M2 macrophage phenotype and impaired resolution of inflammation.[235] So, usage of LXR agonists might overcome this inhibition along with prompt resolution of inflammatory response after intestinal tissue injury.[235]

Glucocorticoids and annexins

Glucocorticoids (GCs) and annexins have previously been shown to be efficacious in resolving inflammation and enhancing efferocytosis in some research studies.[156] GCs enhance macrophage efferocytosis by acting through various modes of action including secretion of anti-inflammatory mediators, upregulation of efferocytosis machinery and gene transcription.

GC-treatment-induced increased expression of annexin D-1 in macrophages and monocytes is one of the important underlying mechanisms for increased uptake of apoptotic cells.[236] Moreover, GCs promote macrophage and monocyte efferocytosis due to increased upregulation of bridging molecules & receptors such as MFG-E8, C1q, stabilin, CD206, CD163 and MerTK receptors.[236-238] In lungs of chronic obstructive pulmonary disease (COPD) patients, GCs treatment resulted in increased efferocytosis of apoptotic neutrophils due to upregulation of CD163, CD64 and MerTK receptors on the alveolar macrophages.[239] Furthermore, GCs act at the nuclear level and increase the transcription of lipid sensing receptor genes such as LXR, PPAR-γ and retinoid X receptor thereby promoting the long-term clearance of apoptotic cells.[240]

According to a report by Scannell M et al., apoptotic neutrophils, lymphocytes and thymocytes secrete annexin and related peptide derivates into the conditioned medium which can potentially enhance their own removal by professional phagocytes such as macrophages through efferocytosis.[241] Mechanistically, annexin acts on LXA_4 receptor and causes F-actin reorganization in macrophages leading to increased engulfment of apoptotic neutrophils.[242] In mycobacterial infections, the presence of annexins on the apoptotic cells promotes their engulfment by professional phagocytes such as dendritic cells (DCs) leading to MHC-class I antigen presentation, CD8+T cell activation, immune response and control of bacterial infection.[243]

Necrotizing enterocolitis is a multifactorial disease accounting for approximately 1-5% of NICU admissions.[244] Specifically, it occurs in the preterm newborns who are born at less than 32 weeks pregnancy and weigh < 1500g. Some of the important predisposing factors implicated include sepsis, asphyxia, meconium aspiration syndrome, prolonged parenteral feeding, and immature intestinal immunity. NEC infants usually present with non-specific signs and symptoms. Most of the NEC infants are referred to surgical management due to late detection and for management of complications such as pneumo-peritoneum, ascites, portal venous gas and fixed persistent intestinal loop. Unfortunately, the case fatality rate of NEC infants referred to surgical management is very high (30%-50%).[245] Therefore, earlier detection and prompt management is the key for improving clinical outcomes as well as for preventing mortality and morbidity. As there are no specific therapeutic interventions to arrest the disease progression after its instigation by microbial insult, disease pathogenic mechanisms surrounding intestinal injury in NEC need to be comprehended and fathomed in a meticulous manner. As a result, clinical researchers have started to characterize and grasp the underlying downstream signaling events that are responsible for NEC tissue injury.

Conclusion

Intestinal cell death is one of the critical cellular events that occurs in the acute phase of NEC. Its occurrence is perceived as a forewarning for subsequent derangements of disease progression. It is regarded as a harbinger of pathological events such as increased intestinal permeability, local inflammation, and systemic inflammation. Bacterial LPS-TLR4 signaling with the resultant release of pro-inflammatory cytokines, chemokines and free radicals is postulated to be an important mechanism for inciting intestinal cell death in NEC. The different types of intestinal cell death that are encountered in NEC include apoptosis, necrosis, necroptosis, pyroptosis and autophagy. Upon intestinal epithelial cell death, there will be compensatory response provoked by cellular battalion comprising epithelial cells, Paneth cells, neutrophils, T-lymphocytes, B-lymphocytes, and macrophages for neutralizing the infectious threat, limiting the spread of inflammation as well as for regenerating the injured intestinal epithelium. This review mainly discusses innate cellular defense mechanisms that occur post intestinal cell death in NEC in a scrupulous manner. Particularly, macrophage efferocytosis is explained in an efficient and structured manner with a special emphasis on find-me signals, eat-me signals, macrophage receptors, bridging molecules, mechanism, and anti-inflammatory responses. Macrophage efferocytosis is a principal mechanism of ingesting dead intestinal epithelial cells. This physiological process is of paramount importance as its successful materialization in the intestinal milieu results in efficient dead cell clearance and facilitates mounting of robust anti-inflammatory cytokine responses for promoting tissue healing and regeneration.

Epithelial cells dying in the inflamed gut should be cleared in a timely manner so that tissue repair and healing can occur by replacement of dead cells with new cells. Engulfment of dead cells occurs by a process known as efferocytosis performed primarily by professional phagocytes (macrophages). This physiological mechanism is very important to resolution of intestinal inflammation and any impairment of this phenomenon can cause the dying epithelial cells to undergo necrosis. The release of inflammatory mediators by necrotic intestinal epithelial cells into systemic circulation leads to further cell death, inflammation, and autoimmune diseases.

Dying intestinal epithelial cells secrete chemotactic signals known as find-me signals which facilitates the migration of macrophages towards them. In some instances, cancer cells express specific don't eat-me signals such as CD-47 that prevents their recognition, removal, and subsequent immune response. Epithelial cells in earlier phase of apoptosis express specific eat-me signals such as phosphatidylserine which facilitates their recognition by migrating macrophages. Macrophage phagocyte receptors (BAI-1, TIM-4, CD300, αvβ5 and Mer-TK) as well as bridging molecules (MFG-E8, GAS6 and protein S) compromise efferocytosis machinery which are utilized for engulfment and removal of dying epithelial cells. The interaction of dead cells and macrophages causes formation of CRKII-DOCK180-ELMO complex, Rac1 activation and cytoskeletal rearrangement leading engulfment and destruction of dead cells. Successful elimination of dead epithelial cells by macrophage efferocytosis is associated with tissue healing, repair, and resolution of inflammation due to secretion of anti-inflammatory cytokines such as IL-4 and IL-13.

Decreased efferocytosis in NEC can result from combined interplay of factors such as increased oxidized phospholipids, enhanced TLR4 signaling, increased pro-inflammation cytokines, M1 macrophage phenotype predominance, NET formation, and increased matrix metalloproteinases. Macrophages in the preterm infants might have reduced capacity for efferocytosis due to decreased adherence receptor expression, attenuated antigen presentation as well as very low membrane bound TLR4 receptor. As a result, decreased efferocytosis capability in the preterm infants predisposes them to develop lung and brain complications in NEC due to disrupted removal of dead cells and resulting pathological consequences. Novel therapeutic interventions for increasing efferocytosis and preventing disease progression in NEC can be classified into following categories such as a) anti-CD-47 antibodies, b) blocking ADAM-17 cleavage, c) phosphatidylserine liposomes, d) PPAR γ agonists, e) correcting SPM-leukotriene ratio and f) annexins. Basic science and clinical research studies are warranted for probing the mechanisms of defective efferocytosis in preterm infants susceptible to NEC. Such studies might unveil new molecular targets that can counteract deficiencies for facilitating macrophage efferocytosis. Development of disease specific therapeutic interventions based on these uncovered molecular targets might be beneficial in facilitating dead cell removal and improving tissue repair in earlier stages of NEC. Rectifying the decreased efferocytosis during intestinal inflammation in NEC models might be valuable for reversing disease progression, optimizing clinical outcomes as well as decreasing mortality and morbidity.

Declarations

Funding

The authors received no financial support for the research, authorship, and/or publication of this article.

Author contributions

Conceptualization, S.H.K.; Methodology, N.A.; Software, N.A.; Validation, N.A; Formal Analysis, N.A.; Investigation, N.A.; Resources, N.A.; Data Curation, N.A.; Writing – Original Draft Preparation, S.H.K. and N.B.; Writing – Review & Editing, S.H.K., N.B., A.S.U., P.P. and G.S.; Visualization, S.H.K., N.B., A.S.U., P.P. and G.S.; Supervision, S.H.K., N.B., A.S.U., P.P. and G.S.; Project Administration, S.H.K.; Funding Acquisition, N.A.

Conflicts of interest

None of the authors has any conflict of interest.

Data availability

No data is available. Data sharing is not relevant because no datasets were created and/or analyzed for this study.

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