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Review

Function of Connexin-43 in macrophages

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Abstract: Knowledge on the function of Connexin-43 on macrophages is gradually increasing and recent studies show how macrophages utilise Connexin-43. Migration, antigen-presentation and some forms of intercellular communication in macrophages are Connexin-43-dependant. Delicate processes, such as electrochemical support in conduction of the heartbeat in the AV-node, immunomodulatory regulation in the lungs and macrophage-differentiation are performed using Connexin-43 in macrophages. The relevance on pathophysiology becomes evident in inflammatory bowel disease, tumour networks and HIV in which aberrant function of Connexin-43 has been observed. Although many physiological, as well as pathophysiological functions were found to be Connexin-43-dependant, some still remain debated: the involvement of Connexin-43 in phagocytosis and polarisation, as well as its involvement in the mortality in murine sepsis are still unclear. These functions as well as further involvement in increasingly complex functions of the macrophage pose possible fields of research.

Keywords: connexin-43; Cx43; gja1; connexins; macrophage; monocyte

1. Introduction

Macrophages are studied since well over a hundred years [1]. Their function as phagocytic cells and first line defence against pathogens was documented in historic works concerning bacteria [2, 3]. Over the years, macrophages were found to be involved in processes such as regenerative responses [4], inflammation of systemic organs [5, 6], tumour-killing [7], hypercholesterolemia [8], sepsis [9] and many more. These findings increasingly established macrophages as a diverse and heterogenic cell type of the immune system [10]. Subsequently, some of these newly found functions were discovered to be intertwined with a very versatile protein: Connexin-43 (Cx43).

Connexins, in general, are essential gap-junction proteins, which enable intercellular communication, transfer of ions and signalling molecules between cells [11, 12] and may even exert functions, that do not depend on channel formation [13]. Apart from this, mutations in connexins cause disorders ranging from changes in skin, cataracts, hearing loss to complex syndromes [14] and death in mutant embryos [15]. Cx43, also known as GJA1, is one of these connexins. It is an intricate part of intercellular communication, as cellular structures, such as gap junctions, extracellular vesicles and tunnelling nanotubes rely on it [16]. Cx43 is also prominently featured in the immunological synapse [17], thus making it the prime target for investigation in immune cells. In macrophages, Cx43-dependant intercellular signal transfer is involved in a plethora of physiological and pathophysiological processes, such as immunomodulation [18, 19], regulation of the heartbeat [20] and purinergic signalling [9]. Furthermore, Cx43 also acts as gateway to the macrophages outer-world as much as it allows the environment to influence the macrophage and its expression of Cx43 itself [9, 21, 22].

All things considered, this review summarises the current literature concerning the role of Cx43 in the macrophages and its role in physiological as well as pathophysiological processes.

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Lastly, it is important to note, that the investigation of Cx43 on macrophages is limited, since homozygous Cx43 knockout mice are lethal due to heart failure [15, 23]. These circumstances led to studies making use of different circumventions, such as heterozygous mice [24, 25], macrophage-Cx43 specific knockout mice [9, 18] and fetal liver cell transplantation from homozygous Cx43 knockout mice into irradiated recipient-mice [26].

2. Dawdling & devouring: rolling around for initiative

Macrophages are essentially phagocytic cells. They migrate, phagocytose and present antigens to other cells of the immune system.

2.1. Migration

The expression of Cx43 in macrophages was found as early as 1997, as the potential interaction between macrophages and vessel wall endothelium during infiltration was considered [8]. Yet, blocking connexins with connexin mimetic peptides had little influence on macrophage transendothelial migration [27]. The influence of Cx43 in macrophage-migration was further investigated in studies regarding cells from heterozygous Cx43 knockout mice [24, 25]. Interestingly, the newer studies present macrophage-Cx43 not only regulating the migratory ability of macrophages, but also their influencing the migration of other cells.

The expression of Cx43 on macrophages is increased in lipopolysaccharide (LPS) conditioned medium [9, 22, 28]. This consequently enhances their migratory ability. Although, migration can be inhibited by immunosuppressive drugs, such as mTOR inhibitors and steroids, as well as by downregulation of Cx43 expression. [25].

Heterozygous Cx43 macrophages also appear to have an altered secretion of signalling molecules, thus decreasing migration of cells like smooth muscle cells [24] and neutrophils, although macrophages from homozygous fetal livers do not show altered migration for neutrophils. Moreover, heterozygous Cx43 macrophages have also been identified to alter the progression of diseases, such as atherosclerosis, by influencing cell migration. Atherosclerosis progression was found to be less prominent in Cx43 heterozygous mice, as their plaques contain significantly less neutrophils, due to reduced macrophage-induced chemotaxis and subsequent accumulation [29]. Overall, influence over the macrophages-microenvironment is closely tied to Cx43 and will be discussed more thoroughly in chapter three through five.

2.2. Phagocytosis and antigen-presentation

After arriving to the inflammation-site by migration or transmigration, macrophages start to phagocyte. By engulfing and digesting pathogens during phagocytosis, macrophages act as first line responder of the innate immune system.

2.2.1. Phagocytosis

Cx43 was initially proposed to be involved in phagocytosis, as various functions, some of which are important for phagocytosis, were found to rely on connexins, based on the conflicting results shown below. This was later shown to not be the case as described in the conflicting results below.

Anand R.J. et al. found significant, although partial inhibition of phagocytosis in heterozygous Cx43 knockout mice and oleamide-inhibited murine macrophages. They obtained heterozygote Cx43 knockout macrophages via peritoneal lavage from heterozygote mice and homozygous Cx43 knockout macrophages from embryonic livers of homozygous knockout mice. The cells were identified as macrophages based upon their surface expression of CD45 [30] using coverslips [31]. The macrophages were given sheep erythrocytes, latex beads, and *Escherichia coli*, they evaluated the phagocytosis by light and confocal microscopy [30].

Glass A.M. et al. on the other hand found no difference in phagocytic capabilities between wild type and Cx43 knockout macrophages. They obtained macrophages through peritoneal lavage and bone marrow harvesting from radiation chimeric mice as well as from fetal livers. The cells were then

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identified in flow cytometry as CD11b and F4/80 double positive. The macrophages were given sheep erythrocytes, zymosan particles and *listeria monocytogenes* and their phagocytic ability was measured using a fluorescence microscope and flow cytometry.

Glass A.M. et al. critiqued the methodology used by *Anand R.J. et al.*, as CD45 is widely accepted as being present on multiple cell types derived from hematopoietic cells – not only on macrophages [26], unlike F4/80, which is specific for murine macrophages [32]. Another recent study by *Dosch M. et al.* found phagocytosis to be independent of Cx43. The macrophages were obtained from conditional macrophage-Cx43-knock-out-mice via peritoneal lavage. The cells were incubated with latex beads and were evaluated with a phagocytosis assay kit. Neither pharmacological blocking (Gap27) nor Cx43 deletion hindered phagocytosis [9]. It is unclear, if the different methodologies, i.e. derivation of macrophages and material used to phagocytose, may have impacted the macrophages ability to properly perform phagocytosis. An observational study covering different methodologies may delve further into target-dependant phagocytosis.

2.2.2. Antigen-presentation

After phagocytosis, macrophages may present pathogens to other immune cells, such as dendritic cells (DCs). Macrophages and DCs were found to cooperate in uptake, transfer and presentation of antigens. Different possibilities for antigen transfer were discussed, until the transfer of loaded MHC class II molecules was proposed to be Cx43-dependant trogocytosis, as molecules from donor cells were detected on the surface of acceptor cells [33]. Trogocytosis is a process whereby lymphocytes extract surface molecules of antigen presenting cells and express them on their own membrane [34-36]. Therefore, trogocytosis was suggested to be Cx43-dependant [33]. MHC class I presentation on the other hand was found to be identical between wild type and homozygous Cx43 knockout macrophages, in the case of *Listeria monocytogenes* infection [26]. Of note, macrophages with deleted Cx43 are more proficient in T cell priming. The mechanism behind this, is postulated to be an increased accumulation of antigens and hence more efficient presentation, as they were unable to transfer the antigens to neighbouring DCs [33].

These results in fact support the emerging opinion, that immune cells transmit molecules to other cells for immunomodulation and communication. However, antigen presentation is not the only Cx43-dependant intercellular communication in macrophages as described in the following chapter.

3. Intercellular communication is a two-way street

Intercellular communication is essential in the function of the immune system, but the established connections between macrophages and parenchymal cells exhibit other Cx43 dependent functions. Cx43 in macrophages also proofs itself essential in non-immune functions, such as the regulation of the heartbeat [20] and diseases, such as HIV [37].

3.1. Physiological communication in heart, lung and intestine

3.1.1. Electrochemical communication of the heart

Macrophages are well known to influence cardiac disease and repair [38]. They only recently have been implicated in nonimmune context [39]. Macrophages were found to couple to cardiomyocytes using Cx43 containing gap junctions, they do so primarily in the distal part of the AV node. These macrophages regulate the heartbeat, by reducing the action potential and aiding in repolarisation, thus allowing for higher conduction rates. Macrophage specific Cx43 homozygous knockout mice not only show impaired AV-node-conduction, but also additional abnormalities in the atria and ventricles. In summary, macrophages may play a role in conduction abnormalities in and beyond the AV node, such as AV-block, atrial fibrillation and ischemia-induced ventricular arrhythmias [20].

3.1.2. Immunomodulatory communication in the lungs

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The involvement of Cx43 in alveolar macrophages was also only described recently [18, 19]. By using real-time imaging in situ, alveolar macrophage-epithelium gap junction channels containing Cx43 were identified by *Westphalen K. et al*.. After inducing inflammation with LPS, alveolar $CD11c^{cre/cre}$ $Cx43^{floxed/floxed}$ macrophages remained sessile and attached themselves to the alveoli. To investigate, if Cx43 may be responsible for the macrophage-immobility, bacteria and PBS were microinjected and the cells observed. The study found neutrophils freely entering and migrating, yet macrophages remained sessile – ruling out non-specific physical factors. Thus, they concluded, that Cx43 was not responsible for macrophage-immobility. Cx43 was found to play another role in these sessile macrophages. They were found to utilise the epithelium as a conducting pathway, communicating with synchronised Ca^{2+} waves [18]. This intercellular communication was found to be immunosuppressive: activating Akt $Ca2^{+}$-dependently, a serine/threonine kinase which influences cell survival, growth, proliferation, angiogenesis, metabolism, and migration [40]. Cx43 knockout macrophages were also identified to increase secretion of proinflammatory cytokines themselves (MIP-1α) and of the epithelium (CXCL1,5), indicating mutual cytokine suppression. Additionally, Cx43 knockout macrophages enhanced the alveolar recruitment of neutrophils [18].

Similar findings were also seen in human cells. *Beckmann A.* investigated the communication between a bilayer of human macrophages and human alveolar epithelial cells. The goal was to identify, if macrophage-epithelial gap junctions exist in humans. Interestingly, co-cultures were found to express Cx43, whereas isolated macrophages did not. The study did not investigate intercellular communication [19]. These results lay a cornerstone for future research, concerning macrophage immunomodulation in the lungs, as well as present potential targets for medical treatment.

3.1.3. Intercellular communication in the intestine

Aside from cardiomyocytes [20] and alveolar epithelium [18, 19], macrophages were also found to communicate with the epithelium of the intestine. Macrophages use Cx43 to form functional gap junctions with the epithelial cells and use paracrine and hetero-cellular signalling to communicate. The involvement of this communication is in direct relation to the inflammatory bowel disease (IBD) (see below) [41].

3.2. Pathological communication in IBD, tumours and HIV

The use of Cx43 in intercellular communication is not exclusive to physiological processes: tumours, HIV and inflammatory bowel disease are some of the diseases, which misuse macrophage Cx43 to their advantage.

3.2.1. IBD

Macrophages establish communication to epithelial cells using Cx43. This communication contributes to the dysregulation of the intestinal epithelial barrier in IBD. Interestingly, Connexin expression in Inflammatory bowel disease tissues is relocated more basolateral in epithelial cells, compared to normal tissue. This phenomenon may facilitate the interaction of intestinal epithelial cells with infiltrated macrophages, allowing for the progression of the disease. Intestinal epithelial cells were therefore seeded in six-well plates on top of activated macrophages to mimic the observed architecture in the intestinal tissue. After inducing optimal conditions, calcein dye transfer could be recognised by a shift in fluorescence, which indicates the transfer from epithelial cells to macrophages [41]. Nevertheless, such remodulation of cellular structures, as seen in IBD, is just a mild example of cellular reorganisation, a more extreme example can be found in tumours.

3.2.2. Tumour networks

By immunostaining for macrophage markers, tumour-associated macrophages (TAMs) were found in anaplastic thyroid. They are evenly intermingled with cancer cells, but were also found in long chains, which derive from perivascular clusters across the tumour. TAMs are in direct contact

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with other macrophages, cancer cells and blood vessels and may allow communication and molecular transfer between them and cancer cells. These contacts are established using long, irregular, thin, moniliform cytoplasmic processes, which express Cx43. Networks made by TAMs are robust and resilient structures, which allow for their advantage on the neighbouring non-tumour tissues [42].

3.2.3. Tunnelling-nanotubes in HIV

HIV exploits Cx43-containing tunneling-nanotubes (TNTs). In HIV-infected macrophages, Cx43 expression is induced three days post infection and remains high. The gap-junctions formed between infected and uninfected macrophages were proven functional, by identifying Lucifer Yellow diffusion in-between these cells. No dye uptake was observed when extracellular dye was presented to infected and uninfected macrophages, suggesting that Cx43 hemichannels are only present at the tip of the TNT. The study concludes, that TNTs are required for efficient intercellular communication and viral spread, hence selective Cx43 blocking may present itself as future therapeutic target against HIV [37].

4. A microenvironment with macro-consequences

The macrophage's interaction with the microenvironment provides an alternative, much broader form of communication. Physiological and pathophysiological processes alter the macrophages environment, thus changing its expression of Cx43 and reaction to the environment [22, 25, 28]. The change in Cx43 expression furthermore influences the macrophages gene expression for chemokine secretion or activation of the complement pathway [29]. Moreover, Cx43 deletion also alters the release of molecules, such as adenosine triphosphate (ATP) [9].

4.1. The environment changes the macrophage: LPS, acute peritonitis and sepsis

4.1.1. LPS and Cx-43

Inflammatory sites, in which macrophages aggregate, are proposed to influence connexin channels [21]. Although, LPS was once found to inhibit lymphocyte-macrophage communication via an unclear mechanism [43], some recent studies found LPS-induced Cx43 expression [22] while others argue an increase in intercellular communication, measured by dye transfer [44]. Cx43 expression in bone marrow-derived macrophages increased in a dose- and time-dependent manner during contact with LPS [22]. The same reaction was found for Kupffer cells of the rat liver. Cx43 was found to be predominantly localised at the cell to cell interfaces, thus indicating gap junction formation and probable intercellular communication between Kupffer cells in vivo. In vitro, Kupffer cells were in fact found to increase dye transfer after the administration of LPS and IFN-gamma, which correlates with increased Cx43 expression [44]. This may be interpreted as a higher communication rate [37, 41, 44]. It was also found that the induction of Cx43 in macrophages via LPS can be significantly decreased by tacrolimus and methylprednisolone, two commonly administered immunosuppressive drugs used after solid organ [25].

Certain infectious diseases, such as the infection with *mycobacterium tuberculosis*, can also increase expression of Cx43, thus enhancing intercellular communication, which leads to an increase in apoptosis rate and expression of inflammatory factors [45]. It is also important to note, that murine peritoneal macrophages, which are in contact with LPS and ATP also induce other inflammatory processes, such as the activation of the NLRP3 inflammasome. this model, NLRP3 inflammasome activation was found to change the intracellular redox modulation, progressing renal inflammatory cell injury [28].

4.1.2. Acute peritonitis, sepsis and Cx-43

But what happens when the pathological burden of an infection overthrows the immune system? Murine models were also assessing the relation of Cx43-functionaility and mortality in sepsis and acute peritonitis. Macrophage Cx43 was found to be inversely correlated with mortality, as

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heterozygous Cx43 mice and pharmacologically Cx43-blocked mice show increased mortality in sepsis [30] and acute peritonitis [22]. Intriguingly, the opposite was also reported in a murine sepsis model with macrophage-specific depletion of Cx43. Furthermore, Cx43 positive macrophages were found in the human peritoneal cavity of patients with peritonitis, but not in control patients, suggesting the involvement of macrophage Cx43 in septic processes in humans [9].

4.2. The Macrophage changes the environment: Adenosine triphosphate release

Peritonitis was also found to increase extracellular ATP levels.These findings hint at the involvement of macrophages in systematic ATP release during sepsis [9]. Extracellular ATP is utilised as autocrine regulation and paracrine communication by immune cells [46]. Active ATP-release from inflammatory cells can occur both via vesicular exocytosis or via hemichannels, including Cx43 [47, 48]. The ATP-release allows for multifunctional modulation of the cell, tissue and organism [49].

Cx43 and P2X7 purinergic receptors have been found to be co-localised on peritoneal macrophages [50]. ATP-release from macrophages occurs in response to TLR-2 and TLR-4 activation in a Cx43-dependant manner [9]. After the release, ATP typically activates P2 receptors [51-54]. *Dosch et al.* therefore support the findings already made for the P2Y1 receptor [55, 56], and other immune cells [51, 57]. These findings further establish the involvement of Cx43-dependant ATP-release in a variety of situations, including certain inflammatory diseases [58] as well as physiological situations i.e. intercellular immunomodulation in the lungs as established by *Westphalen K. et al.* [18], as the regulation and propagation of calcium signals by macrophages is also ATP-dependant [46].

5. Macrophages dress for the job they want

5.1. Adapting to their environment: polarisation

Macrophages can adapt according to changes in their microenvironment by polarisation [59]. The subtypes are commonly summarised and simplified in M1 and M2 macrophages, even though polarisation is rather a spectrum than a switch [59-61]. The types differ in their functions:

- M1 macrophages accumulate at the inflammation site by strong adhesion, which promotes cell retention and the progression of inflammation [62].
- M2 macrophages possess stronger migratory ability compared to M1 macrophages, have increased phagocytic properties and are in general anti-inflammatory and considered to help tissue repair [62, 63].

Morel S. et al. found that Cx43-heterozygous mice macrophages do not differ from homozygous knockout mice or wild type macrophages in terms of M1 and M2 polarisation. These macrophages were derived from haematopoietic fetal liver cells, which were transferred to lethally irradiated mice [29]. The absent change of polarisation was recently confirmed for peritoneal M2 macrophages under Cx43 blocking or deletion. Markers for M1 differentiation, such as iNOS and Il12rb, on the other hand, were found to be decreased under Cx43 blocking or deletion [9]. The diminished M1 macrophage polarisation may be due to the Cx43-dependant transformation via Angiotensin-2 (Ang-2) [64]. It is important to note, that Ang-2 was also identified with decisive roles in inflammation [65]. This may therefore influence macrophage polarisation in experiments, where mice undergo inflammation, as it is the case in the latter mentioned study [9].

The discrepancy on the influence of Cx43 in macrophage polarisation may alternatively also be due to the inherent difficulties in identification, as markers used for identification of polarisation in vitro, were found to not work for in vivo. For instance, genes modulated inside the iNOS signalling pathway were found to differ slightly compared to in vitro macrophages. Moreover, valid surface markers for M1/M2 macrophages may have yet to be discovered [66].

5.2. Adapting to their environment: differentiation

Macrophages also adapt to their environment by differentiation. Two types of differentiation where found to involve the use Cx43 in either formation or function.

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5.2.1. Foam cells

Lipid-laden foam cells form, when macrophages retain too much cholesterol, thus becoming the prototypical cells associated with atherosclerotic plaque [67]. Foam cells in hypercholesterolemia-induced atherosclerosis were found to express Cx43, while normocholesterolemic precursor monocytes were not. Macrophages similarly increase their expression of Cx43 between groups which were mechanically injured and had hypercholesterolemia, as well as those, which only had hypercholesterolemia. Moreover, neither alveolar macrophages, Kupffer cells nor macrophages from peritoneal or bronchial lavage from normo- and hypercholesterolemic mice, were identified to increase Cx43 expression [8].

5.2.2. Foreign body giant cells

In response to biomaterials in bones, foreign body giant cells (FBGCs) may form, which derive from precursors of the monocyte/macrophage lineage [68, 69]. Implanted biomaterial in minipig femura yields Cx43 positive macrophages and FBGCs. These cells were found in the granulation tissue and on the surface of the implanted biomaterial. Cx43 was identified between macrophages, between FBGCs and between FBGCs and macrophages. Thus, Cx43 seems to play a role in the formation of osteoclast-like FBGCs. Cx43 was also found within the border of FBGCs and in gap junctions formed between fusing macrophages [68]. Additionally, Cx43 may also have a role in biomaterial degradation utilising Cx43 [69].

6. Conclusion

Cx43 and macrophage function are closely intertwined. Cx43 is critical in various physiological and pathophysiological processes, including interaction with neighboring cells, the microenvironment, migration to inflammation sites, antigen-presentation, immunomodulation, as well as supporting macro physiological functions such as heartbeat-regulation. Diseases like IBD, HIV, sepsis and other infections all depend on and are modulated by Cx43. Therefore, it is likely that Cx43 is also of importance in macrophage function in other types of diseases that remain to be explored in future studies. The research on the intricate and versatile functions of Cx43 in macrophages would allow for the establishment of specific drug targeting and modulating of the innate and adaptive immune system.

Abbreviations

Ang-2	Angiotensin-2
ATP	Adenosine triphosphate
Cx43	Connexin-43
DC	Dendritic cell
FBGC	Foreign body giant cell
IBD	Inflammatory bowel disease
LPS	Lipopolysaccharide
TAM	Tumour-associated-macrophage
TNT	Tunnelling-nanotube

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