Review

Connexins in leukocytes: shuttling messages?

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Abstract

Gap junctions, formed by the connexin (Cx) protein family, are intercellular channels that permit the cytoplasmic exchange of ions and small metabolites between neighboring cells, a process called gap junction intercellular communication (GJIC). These channels possess unique properties, including distinctive permeabilities for various signaling molecules, which depend on the connexin member(s) that form them. Importantly, GJIC must be properly controlled as its misregulation might contribute to diseases. Morphological and functional studies have revealed 'gap junction-like' structures and cell-to-cell communication involving cells of the immune system. The connexins involved in such contacts have been partially identified in recent years. This review focuses on the potential physiological roles of gap junctions in the development and recruitment of leukocytes as well as in the regulation of the immune response. Furthermore, the importance of GJIC in immuno-inflammatory pathologies is illustrated in atherosclerosis.
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1. Introduction

Inflammation has evolved as a mechanism to defend the body against invading microorganisms and to respond to injuries. In both physiological and inflammatory leukocyte migration, immune cells have to breach the vascular barrier, a process referred to as diapedesis, extravasation or endothelial transmigration. The endothelium changes when there is an injury in the underlying tissue, such that a normally non-permissive surface becomes permissive for the adhesion of circulating leukocytes thus supporting the recruitment of inflammatory cells from the blood stream. A multistep adhesion cascade has been proposed for leukocyte recruitment consisting of four steps [1,2]. In the first step, leukocytes tether then roll on the endothelial cells (ECs). This is followed by a triggering step, via chemokines and their receptors, that leads to the rapid activation of leukocyte integrins, and then a third step at which point the leukocyte

adheres tightly onto the endothelial surface. Finally, diapedesis occurs and the leukocyte crawls across ECs. Recent reviews concerning endothelial junctions and leukocyte transmigration have highlighted how molecules of the adherens and tight junctions regulate leukocyte extravasation [3,4]. Although gap junctions were previously thought not to play a role in leukocyte transmigration, recent data challenges this view.

Gap junctions, formed by the connexin (Cx) protein family, connect adjacent cells together thus providing a direct means of intercellular communication [5]. Six connexins assemble in the plasma membrane to make a connexon or hemichannel. Two hemichannels from neighboring cells join to form an intercellular channel that clusters with other intercellular channels to build a gap junction. There are presently over 20 connexins, each of which can create functional channels with certain isoforms [6,7]. Gap junction channels formed by different connexins have unique properties including distinctive permeabilities for various dyes and signaling molecules [7,8]. Increasing data suggest that connexons expressed on the plasma membrane of a variety of cells might be more than just precursors for the formation of gap junction channels [9,10]. In fact, the

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controlled opening and closing of hemichannels formed by various connexins have been observed. Furthermore, some hemichannels have been shown to be permeable to a number of substances, including important signaling molecules [10–13]. Much evidence, obtained from various cell systems, has shown the regulation of connexin expression and gap junctional intercellular communication (GJIC) by pro-inflammatory mediators, thus uncovering the importance of gap junctions in the modulation of the inflammatory response [14–18]. In this part of the spotlight issue, we focus on gap junctions in leukocytes and the immune response, specifically on their importance in the process of leukocyte maturation and recruitment. In particular, the development of atherosclerosis is presented to illustrate the role played by GJIC in immuno-inflammatory diseases.

2. Evidence for gap junction communication in leukocytes

Leukocytes are principal players in the immune system that undergo a developing process in order to deal with infections and injuries inflicted to the body. To fight off unwanted aggressors, leukocytes must leave their site of production, circulate in the blood, and get recruited to the initial spot of injury. This process requires coordinated communications between leukocytes and other cells. The role of GJIC during these different steps is slowly emerging.

2.1. Leukocyte formation

All blood cells originate from a common hematopoietic stem cell. In the adult, the stem cells are found mainly in the bone marrow and thymus, where they can either divide to produce more pluripotent stem cells or differentiate to various committed progenitor cells, each able to generate only one or a few types of blood cells. This process is strictly regulated by the specialized hematopoietic microenvironment, which includes stromal cells. Based on the gap junction-like structures observed in the bone marrow, it was hypothesized that gap junctions help to coordinate functional networks of stromal cells that support blood cell formation [19–22] and subsequent studies have supported this idea as described below.

In vitro studies of cultured bone marrow or thymic stromal cells revealed Cx43 as the principal gap junction protein expressed by these primary cell types [23–25]. In addition, Cx31 and Cx45 have been detected in some stromal cell lines [26]. Moreover, functional studies have demonstrated the transfer of dye or electrical current between stromal cells [23,25]. The consistent observations of high levels of Cx43 expression in neonatal bone marrow and low expression of this protein in adult bone marrow has lead to the hypothesis that GJIC is required during periods of active hematopoiesis, as observed in the growing neonate [24,27]. Consistent with this idea is the finding that drug-induced elimination of committed hematopoietic cells led to a dramatic increase in Cx43 expression in the bone marrow that returned to normal when hematopoietic progenitors had replenished the hematopoietic compartments [24]. Conversely, differentiation of stromal cells into adipocytes, a cell type found in the yellow non-hematopoitic bone marrow, was associated with reduced Cx43 expression levels and decreased GJIC [28]. Evidence that the expression level of Cx43 is critical for normal hematopoiesis in vivo was obtained recently using Cx43-deficient mice [29]. Indeed, lack of Cx43 expression during embryogenesis compromised the terminal development of primary B and T lymphocytes. In addition, the authors observed similar defects in heterozygous Cx43 (Cx43$^{+/-}$) embryos that express reduced levels of Cx43. However, the hematopoietic system was returned to normal at 4 weeks of age in the Cx43$^{+/-}$ mice. Interestingly, the regeneration of lymphoid and myeloid cells in Cx43$^{+/-}$ mice was severely impaired after drug-induced elimination of hematopoietic cells, thus providing further support to the hypothesis that GJIC is mostly required during periods of active hematopoiesis.

Gap junctions between stromal and hematopoietic cells have also been observed in the bone marrow in situ and long-term cultures of bone marrow [21]. Subsequent dye transfer studies demonstrated functional coupling between these cell types. It is thus tempting to hypothesize that GJIC between stromal cells and hematopoietic cells may allow for direct transfer of stromal cell-derived signals into developing hematopoietic cells, thus regulating blood cell formation. However, the actual existence of such a pathway remains controversial. It has been reported that chemical blockade of GJIC between stromal and hematopoietic cells decreased blood cell formation, an effect that was more pronounced in primitive than in committed progenitors [30]. In contrast, other researchers have been unable to observe dye transfer between stromal and hematopoietic cells or to detect connexin expression in hematopoietic progenitors by reverse transcription-polymerase chain reaction (RT-PCR) [23,26,29]. Taken together, it appears that gap junctions may play a role in hematopoiesis, particularly during active periods when blood cell formation is initiating or regenerating. However, the question as to whether GJIC between stromal cells, between hematopoietic cells or between both cell types is most critical for active hematopoiesis remains to be answered.

2.2. Connexin expression in leukocytes

The presence of gap junctions between leukocytes (homocellular contacts) as well as between leukocytes and other cells (heterocellular contacts) have been extensively reported (Table 1). Furthermore, increasing information is becoming available on the connexin isoforms expressed in the different leukocytes as well as on the regulation/induction of GJIC by pro-inflammatory mediators in these cells (Table 2). Nevertheless, most of these reports result from in vitro studies and remain to be proven in vivo.

Table 1
Gap junction formation by leukocytes

Leukocytes	Interactors	Species	Conditions	Evidences	References
Neutrophils	Neutrophils	Human	LPS or TNF-α, unidentified EC derived factor(s)	Dye coupling	[33]
Neutrophils	Neutrophils	Hamster	Ischemia reperfusion injury	EM	[31]
Neutrophils	EC line	Human	Untreated	Dye coupling	[34]
Neutrophils	Epithelial cell line	Human	Untreated	Dye coupling	[34]
Neutrophils	ECs	Hamster	Ischemia reperfusion injury	EM	[31]
Monocytes	Monocytes	Human	LPS or TNF-α and INF-γ	Dye coupling	[39]
Monocytes	Monocytes	Hamster	Ischemia reperfusion injury	EM	[31]
Monocytes	Neutrophils	Hamster	Ischemia reperfusion injury	EM	[31]
Macrophages	Macrophages	Murine	Arranged in chains	Electrical coupling	[35]
Macrophages	Macrophages	Murine	Peritonitis	EM	[37]
Macrophages	Macrophages	Canine	GM-CFC	EM	[36]
Macrophages	Neutrophils	Trout	Bacterial agents	EM	[32]
Macrophages	Epithelial cell line	Murine	Peritonitis	Dye coupling, EM	[37]
Microglial cells	Microglial cells	Murine	LPS and INF-γ or TNF-α and INF-γ or unstimulated	Dye coupling	[38]
Lymphocytes	Lymphocytes	Human	PHA	Electrical coupling	[46]
Lymphocytes	Lymphocytes	Human	PHA	Electrical coupling, EM	[48]
Lymphocytes	Lymphocytes	Bovine	PHA	Electrical coupling	[44,45]
Lymphocytes	Lymphocytes	Rabbit	PHA	EM	[49]
Lymphocytes	EC	Murine	Lymphnode in situ	EM	[50]
Lymphocytes	Epidermal Langerhans cells	Murine	Antigen activated	EM	[51,52]
Lymphocytes	Dermal Langerhans cells	Human	Dermatitis in situ	EM	[60]
Lymphocytes	ECs	Human	HTLV-1 +	Dye coupling	[80]
Lymphocytes	ECs	Human		Dye coupling	[53]

EC, endothelial cell; LPS, lipopolysaccharide; TNF-α, tumor necrosis factor-α; INF-γ, interferon-γ; GM-CFC, granulocyte-macrophage colony-forming cell; PHA, phytohemagglutinin; HTLV-1+, human T-cell leukemia virus type 1+; IF, immunofluorescence; WB, Western blot; NB, Northern blot; RT-PCR, reverse transcriptase-polymerase chain reaction; FACS, fluorescence-activated cell sorter; EM, electron microscopy.

2.2.1. Neutrophils

Gap junction-like structures were initially observed by electron microscopy (EM) between hamster neutrophils as well as between neutrophils and ECs [31] and then detected between trout neutrophils and macrophages [32]. After initial failure to detect connexin mRNA or protein in peripheral blood cells, early studies indicated that connexin expression in neutrophils is inducible. The presence of Cx43 was detected by immunofluorescent staining (IF), only after stimulation with lipopolysaccharide (LPS), in hamster and

Table 2
Connexin expression in leukocytes

Leukocytes	Connexins	Species	Conditions	Evidences	References
Neutrophils	Cx43	Human	LPS or TNF-α	IF, WB	[33]
		Human	Untreated	IF, WB	[34]
		Hamster	LPS	IF, WB, EM	[31]
	Cx40	Human	LPS or TNF-α	IF, WB	[33]
		Human	Untreated	IF, WB	[34]
	Cx37	Human	Untreated	IF, WB	[34]
Monocytes/ macrophages	Cx43	Human	Atherosclerotic macrophage foam cells	NB	[40]
		Human	LPS and INFγ or TNF-α and INFγ	WB, NB	[39]
		Mouse	J774 macrophage cell line	WB, NB	[42]
		Mouse	LPS and INF-γ	IF, WB, EM	[41]
		Hamster	LPS	IF, WB, EM	[31]
		Murine	Brain stab wounds or primary cultures of microglias	WB, EM	[38]
	Cx37	Human and mouse	Early atheromas	IF	[43]
Lymphocytes	Cx43	Human	Peripheral blood or tonsil-derived cell	WB, RT-PCR, FACS	[54]
		Mouse	Lymph node-derived cells	IF	[81,82]
	Cx40	Human	Tonsil-derived-cells	WB, RT-PCR, FACS	[54]
	Cx37	Human	Peripheral blood	IF	[58]
		Mouse	Lymph node derived cells	IF	[81,82]; Discrepancy with [54]

IF, immunofluorescence; WB, Western blot; NB, Northern blot; RT-PCR, reverse transcriptase-polymerase chain reaction; FACS, fluorescence-activated cell sorter; EM, electron microscopy; LPS, lipopolysaccharide; TNF-α, tumor necrosis factor-α; INF-γ, interferon-γ.

human neutrophils [31,33]. Subsequent studies revealed the expression of both Cx40 and Cx43, but not Cx32, in human neutrophils after stimulation with LPS or TNF-α [33]. Although these activated human neutrophils were able to form homocellular gap junctions in vitro, they were not dye-coupled unless treated with EC derived factor(s) [33]. More recently, it was demonstrated that unstimulated human neutrophils express Cx37, Cx40 and Cx43 [34]. Protein expression at the cell surface was confirmed by Western blots that revealed the presence of all three connexins in the membrane fractions. Cx37 was mainly localized in the pseudopodia of neutrophils whereas Cx40 and Cx43 showed a more granular organization. Importantly, expression of these connexins permitted GJIC between neutrophils and ECs in a rapid bidirectional and adhesion-dependent manner, which was decreased after treatment with TNF-α [34]. Taken together, there is various support for homocellular and heterocellular gap junctions in neutrophils. However, additional evidence at the level of messenger RNA is needed to verify the connexin isoform expressed under the different conditions.

2.2.2. Monocytes/macrophages

The establishment of intercellular communication between macrophages, based on electrical coupling of adherent murine macrophages, was first reported by Levy et al. [35]. Subsequently, gap junctions were morphologically detected between progeny of canine macrophages by freeze fracture EM [36]. Gap junction structures have since been described by EM between murine macrophages and an intestinal epithelial cell line [37]; between hamster monocytes as well as monocytes and neutrophils [31]; and between rainbow trout macrophages and neutrophils [32]. Further support for GJIC between monocytes/macrophages and other cells has come from dye transfer assays. Dye coupling was observed between murine peritoneal macrophages as well as between murine macrophages and intestinal epithelial cells [37]. At brain stab wounds and in primary culture of murine microglias, a low dye coupling was observed. This coupling was dramatically increased with the treatment of IFN-γ and LPS or IFN-γ and TNF-α as well as inhibited by a gap junction blocker [38]. In addition, freshly isolated human monocytes treated with LPS or TNF-α and IFN-γ were dye-coupled [39]. However, these studies are in conflict with other reports that demonstrate the lack of GJIC between monocytes/macrophages and other cells. For example, the transfer of dye was not observed in untreated human or mouse monocytes/macrophages [40,41], between human monocytes/macrophages and ECs or between human monocytes/macrophages and SMCs [39,40]. To date, the expression of two connexin isoforms in monocytes/macrophages has been reported. The presence of Cx43 was found in the mouse macrophage cell line J774 [42], activated peritoneal macrophages from hamsters and mice [31,41]; brain stab wound and primary cultures of murine microglia [38]; and human monocytes/macrophages stimulated with TNF-α and INF-γ or LPS and

INF-γ [39]. Moreover, Cx43 mRNA was detected in macrophage foam cells of human atherosclerotic carotid arteries [40]. Interestingly, we observed Cx37 but not Cx43 in macrophages of early atheromas [43]. The induced expression of other connexins in monocytes/macrophages has been examined and neither Cx32 nor Cx40 were detected after treatment with LPS or TNF-α and IFN-γ [39,40]. Until these discrepancies become resolved, it remains unclear whether monocytes/macrophages communicate via GJIC.

2.2.3. Lymphocytes

The initial observations that lymphocytes can establish intercellular communication were reported in the early 1970s. Two separate groups detected electrical coupling between lymphocytes isolated from bovine lymph nodes or human peripheral blood after stimulation with phytohaemagglutinin (PHA) [44–47]. Subsequently, the ultrastructural detection of gap junctions between PHA stimulated lymphocytes was observed [48,49]. This was followed by ultrastructural reports of heterocellular gap junctions between lymphocytes and ECs or Langerhans cells [50–52]. Moreover, bidirectional transfer of cytoplasmic fluorescent dyes between lymphocytes and the endothelium has been reported [53]. Connexin distribution and GJIC has been studied in human and mouse lymphocyte subpopulations. Human peripheral blood-derived T, B and natural killer (NK) lymphocytes express solely Cx43, whereas tonsil-derived T and B lymphocytes express both Cx40 and Cx43 [54]. It is worth mentioning that Cx26, Cx32, Cx37 and Cx45 were not detected in these cells by RT-PCR [54]. However, the expression of Cx37 protein in human peripheral blood lymphocytes was previously reported by another group [55]. Both human and mouse lymphocytes display functional GJIC, as assayed by dye transfer, which can be reduced by pharmacological agents or synthetic peptides known to block gap junctional communication. Interestingly, lymphocytes increase connexin expression or translocate connexins towards cell–cell interfaces upon activation with either PHA or concanavalin A [56–58].

3. A role for gap junction communication in the immune response

Cell-to-cell interactions are of major importance for expanding the competency of cells in the immune system to control infections and maintain tolerance. Activation of the adaptive immune response includes the interaction between T cell antigen receptors and major histocompatibility complex (MHC) molecule–peptide complexes. This nanometer scale gap between the T lymphocyte and the antigen-presenting cell (APC) is referred to as the immunological synapse [59]. Specificity of these recognitions is critical, as reactions to microbial peptides are required for clearance of many infections and responses to self-derived peptides on APCs can give rise to autoimmunity. Despite

the early observation of gap junctions in lymphocytes and the extensive characterization of connexin in primary and secondary lymphoid organs, knowledge on the potential role of gap junctions in the immune system is limited. However, as specific connexin blocking peptides have become more accessible, research on this topic pushes forward quickly.

Gap junctions, composed of at least Cx43, between antigen-presenting Langerhans cells and T lymphocytes were observed both in vitro and in vivo [51,52,55,60]. Saez et al. [58] demonstrated first that synthetic peptides homologous to the extracellular loop of Cx43 drastically reduced proliferation of mitogen-activated T cells, indicating that GJIC may play a role in the adaptive immune response. Subsequently, Oviedo-Orta et al. [57] elegantly showed that disruption of GJIC influenced fundamental aspects of lymphocyte function, including immunoglobulin (Ig) secretion and cytokine production. Indeed, inhibition of GJIC by synthetic peptides homologous to the first and second extracellular loop of Cx43 markedly reduced secretion of IgM, IgG and IgA in mixed cultures of activated purified human B and T lymphocytes. In addition, they observed in these cultures complex temporal inhibitory effects on cytokine synthesis, especially on interleukin-10. Taken together, these results open up towards the novel hypothesis that connexins, and likely GJIC itself, may be an important component of the molecular mechanism underlying lymphocyte activation and function in the immune response. Clearly, further studies are needed to identify which molecules are passing through gap junction channels between APCs and T cells as well as T and B lymphocytes. Considering the narrow space of the immunological synapse, involvement of gap junction hemichannels as passageway for metabolites to the extracellular space and leading to paracrine cell–cell signaling should also be considered [61].

4. Gap junction communication in leukocyte recruitment

Three connexins, namely Cx37, Cx40 and Cx43, have been detected in the vascular endothelium in situ. The precise distribution of these connexins within the vessel wall is known to be species and vessel specific [62]. Cultured human umbilical vein endothelial cells (HUVECs) also express the three vascular connexins and they mainly locate at cell–cell contacts [63]. Interestingly, TNF-α altered the connexin expression pattern in HUVECs and reduced GJIC between these ECs [63]. This reduction in GJIC within the endothelium might serve two important purposes. First, it might protect the endothelium by restricting the spread of injurious signals via EC gap junctions, thus limiting the area of inflammation. Second, as more connexons from the ECs become available for docking, they might form heterocellular gap junctions with leukocytes to control leukocyte migration across the endothelium. As discussed below, there are recent indications of gap junctions between ECs and leukocytes and that GJIC might play a role in leukocyte extravasation.

Cell communication via gap junctions during transmigration was first described by Oviedi-Orta et al. [64]. In this study, they demonstrated by using dye transfer experiments that lymphocytes and ECs generate functional heterocellular gap junction channels during extravasation in vitro. Interestingly, blocking GJIC with pharmacological agents or connexin mimetic peptides caused only a modest reduction in transmigration of lymphocytes across an EC monolayer [61]. Neutrophils and HUVECs also form functional gap junction channels in vitro, as demonstrated by dye transfer experiments [34]. Moreover, this bidirectional coupling was reduced when HUVECs were stimulated with TNF-α but not when stimulated with IFN-γ or thrombin. Therefore, coupling between neutrophils and HUVECs is selectively modulated during an inflammatory reaction, suggesting that this process might be of physiological relevance. Importantly, neutrophil transmigration was enhanced when GJIC was inhibited, suggesting a negative regulatory role for this coupling during the transmigration process. It was also shown in this study that strongly adherent neutrophils were more coupled than weakly adherent ones and that the adhesive properties between connexons played no role in this strengthened cell adhesion process. This prompts a novel hypothesis that the tight adhesion, mediated by integrins and their ligands, between leukocytes and ECs might be modulated by signaling through gap junctions (Fig. 1). Finally, human monocytes were shown to form gap junctions with ECs in a blood brain barrier (BBB) model during the process of transmigration [39]. In addition, blockade of GJIC reduced the number of monocytes that transmigrated, suggesting that cell-to-cell signaling through gap junction channels might even affect the efficiency of the transmigration process across a tight endothelium. Transendothelial migration (TEM) of the different leukocytes appears to be differentially regulated by GJIC, such that inhibition of GJIC increased TEM of neutrophils but decreased TEM of monocytes and had modest effects on lymphocyte TEM. Of major concern in the aforementioned studies is the specificity of the GJIC blocking reagents, pharmaceutical agents are plainly unspecific and the specificity of the mimetic peptides remains to be proven. Clearly, more work is required before definitive proof demonstrates that gap junctions do play a role in leukocyte TEM. Interestingly, regulation of leukocyte recruitment via GJIC might occur at different points of the multi-step adhesion cascade namely tight adhesion and diapedesis (Fig. 1). Based on current data, we hypothesize that there may be a cross talk between gap junctions (formed between leukocytes and ECs) and the integrin–IgSF CAM adhesion complex (also formed between leukocytes and ECs). This form of communication might then modulate the tight adhesion between leukocytes and ECs, controlling whether a leukocyte returns to the blood flow (the case for weak adhesion) or continues to transmigrate into extravascular tissues (the case for firm adhesion). Likewise, we suggest that there may be cross talks between gap junctions (formed between leukocytes and ECs) with the EC tight and adherens junctions. The signals transmitted
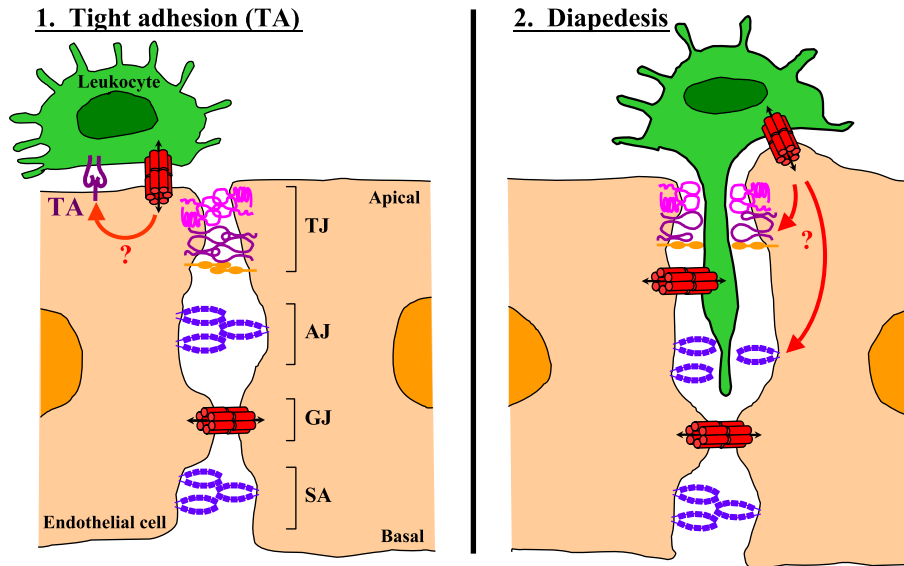
Fig. 1. The role of gap junctions in leukocyte recruitment to tissues. Sequential steps in leukocyte emigration, including tethering/rolling, activation, tight adhesion, and diapedesis (via inter-endothelial junctions TJ, AJ, GJ and SA), are controlled by specific cell adhesion molecules (CAMs). (1) Tight adhesion (TA), mediated by leukocyte integrins and endothelial IgSF CAMs (molecules depicted in dark purple), is triggered by signals such as chemokines. An alternative means to regulate this step might occur via the signaling molecules that pass through GJs formed between the leukocyte and EC (red arrow). This cross talk with the IgSF CAM−integrin complex may modulate the tightness of adhesion, consequently determining whether the leukocyte returns to the blood stream or continues to transmigrate. (2) Diapedesis, the passage of leukocytes across inter-endothelial junctions, appears to be controlled by both TJ and AJ; and recent reports suggest that GJ also participates in this process. The blockage of GJIC increases TEM of neutrophils but reduces TEM of monocytes/macrophages and has a minor effect on lymphocyte TEM. In this model, it is hypothesized that GJIC between the leukocyte and EC cross talks with the EC TJ and AJ, signaling them to "open up" for the leukocytes to pass through (red arrows). IgSF, immunoglobulin supergene family; ECs, endothelial cells; TJ, tight junction; AJ, adherens junction; GJ, gap junctional channel; GJIC, gap junctional intercellular communication; SA, syndesmos/complexus adherens; TEM, transendothelial migration.

would instruct the EC junctions to "open up" for leukocytes to pass through. As this line of research continues, one challenge will be to identify the signals that are being exchanged through gap junctions between leukocytes and ECs during different physiological states. This will certainly further our understanding of leukocyte migration during immune surveillance and inflammatory reactions that can cause diseases when improperly controlled.

5. Multiple roles for gap junction communication in atherosclerosis

Atherosclerosis is a progressive disease characterized in part by the accumulation of lipids, leukocytes, and smooth muscle cells (SMCs) in the intima of medium and large arteries [65]. This disease is presently the leading cause of illness and death in developed countries. The current view believes that inflammation is a major contributor to atherogenesis [66]. Moreover, evidence is growing that dysfunctional GJIC plays a role in the development of atherosclerosis. Initially, Polacek et al. [40] reported the strong expression of Cx43 mRNA by macrophage foam cells in human atherosclerotic carotid arteries. They extended this finding in a rabbit model of atherosclerosis, demonstrating that the expression of Cx43 is upregulated in macrophage foam cells and downregulated in medial SMCs [67]. In another study,

Cx43 expression in intimal SMCs was shown to increase at early stages of human coronary atherosclerosis and to decrease at later stages of the disease [68]. A genetic polymorphism in the human Cx37 gene was reported as a potential prognostic marker for atherosclerotic plaque development [69]. Furthermore, this Cx37 gene polymorphism was shown to possibly play a role in the manifestation of coronary atherosclerosis in Taiwan and Japan [70,71]. More recently, we demonstrated that expression of the three vascular connexins is altered in mouse and human atherosclerotic plaques [43] and that the reduction of Cx43 expression inhibits the formation of atherosclerotic lesions in low-density lipoprotein receptor-deficient (LDLR$^{-/-}$) mice [72,73]. These studies have provided valuable clues as to how gap junction communication might play a role in the initiation as well as the progression of atherosclerotic plaque development.

5.1. The "initiation" and progression of an atherosclerotic plaque

The many risk factors that are implicated in atherogenesis are linked by their common ability to promote inflammatory reactions and injury to the endothelium. As a response to injury, the endothelium becomes dysfunctional leading to its increased expression of various cell adhesion molecules and secretion of chemoattractants to recruit specific leukocytes [74]. Leukocyte recruitment in the early
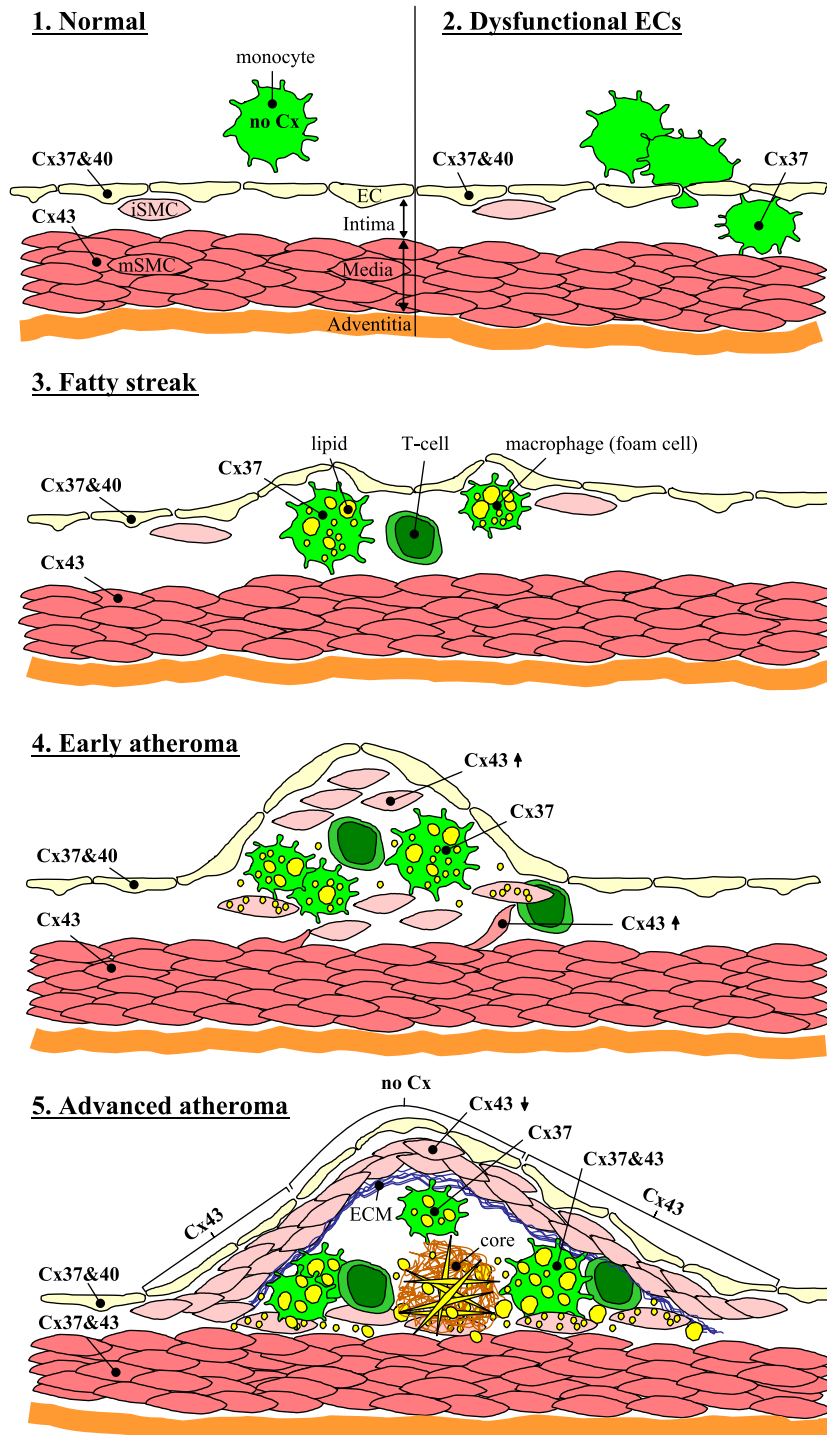
Fig. 2. Altered connexin expression during atherosclerotic plaque development. (1) Normal artery. Cx37 and Cx40 are expressed in ECs; Cx43 is expressed in mSMCs; connexins are not detected in circulating monocytes. (2) Dysfunctional ECs. As a response to injury, ECs become dysfunctional and recruit leukocytes, mainly monocytes/macrophages but also T lymphocytes, into the intima. Note the induced expression of Cx37 in intimal monocytes/macrophages. (3) Fatty streak. As leukocytes accumulate in the intima, monocytes mature into macrophages that take up lipid into their cytoplasm and become macrophage foam cells. Note the continued expression of Cx37 in the intima macrophages. (4) Early atheroma. Some mSMCs migrate into the intima, where the release of pro-inflammatory molecules by themselves and leukocytes induce iSMC proliferation. Lipids start to accumate in the extracellular space and in iSMCs. Note the increased expression of Cx43 in iSMCs compared to mSMCs, however the expression of connexins in ECs and mSMCs has not changed at this stage. (5) Advanced atheroma. A fibrous cap is formed by iSMCs and ECM that covers the lesion area. The central core of this lesion contains necrotic debris, extracellular lipids including cholesterol crystals. Note the disappearance of Cx37 and Cx40 in the diseased ECs, and the induced expression of Cx43 in ECs covering the shoulder regions of the lesion. In addition to Cx37, Cx43 is also detected in macrophage foam cells located in the shoulder regions. Another notable change at this stage is the reduced Cx43 expression in the iSMCs and the induced Cx37 expression in mSMCs. EC, endothelial cell; iSMC, intimal smooth muscle cell; mSMC, medial smooth muscle cell; Cx, connexin; ECM, extracellular matrix; core, lipid and/or necrotic core.

phases of atherosclerosis involves mainly monocytes [75]. However, T lymphocytes are also implicated in the early development of the disease [76]. After adhering to the dysfunctional endothelium, the monocyte transmigrates between intact ECs to penetrate into the arterial intima. In the intima, monocytes proliferate and mature under the influence of cytokines, chemokines and growth factors secreted by themselves and other atheroma-associated cells. Furthermore, the induced expression of scavenger receptors permit macrophages to accumulate lipids within their cytoplasm and eventually progress to the arterial foam cells, a hallmark of the arterial lesion. These foam cells along with the T cells constitute the fatty streak known as the earliest form of atherosclerotic plaques.

Evidently, diapedesis of leukocytes is a prerequisite for the formation of atherosclerotic plaques. Thus, accelerating or decelerating monocyte/macrophage diapedesis might speed up or slow down atherosclerosis. In fact, transmigration of monocytes/macrophages but not T lymphocytes is significantly reduced by inhibiting GJIC [39,64]. Moreover, reducing the expression of Cx43 in LDLR$^{-/-}$ mice decreased the number of macrophages and T cells in the atheroma as well as the progression of atherosclerosis [72]. Taken together, it appears that GJIC is somehow modified in atherosclerosis leading to the enhanced leukocyte recruitment. A possible mechanism for this transformation may be via altered connexin expression resulting in the improper exchange of signaling molecules that cause miscommunication. In fact, such alterations in connexin expression in leukocytes and the endothelium are known to occur during the development of atherosclerosis [43] (Fig. 2). Knowing that the properties of individual gap junction channels are distinct, it seems likely that the Cx37/Cx40 to Cx43 switch in the endothelium and the Cx37 to Cx37/Cx43 switch in macrophage foam cells will drastically change the messages exchanged between these cells. It is worth mentioning that since monocytes/macrophages can form gap junctions with adjacent monocytes/macrophages, and perhaps even with neighboring T lymphocytes and SMCs, miscommunication among these cells might play an additional role in plaque formation. For instance, lipid uptake by mature macrophages might rely on GJIC.

5.2. The stability and rupture of an atherosclerotic plaque

The continued inflammatory response and accumulation of lipids work together with other events to promote atherosclerotic plaque growth and eventually rupture [66]. During the growing phase, medial SMCs migrate to the top of the intima where they multiply and produce components of the extracellular matrix (ECM). The SMCs and matrix molecules coalesce to form a strong fibrous cap that covers the original atherosclerotic site. Although this adds to the size of the plaque, it also seals the plaque off safely from the blood and reduces the chance of rupture. As this cap matures, some of the cells underneath die and lipids are

released. Therefore, this region is referred to as the lipid or necrotic core of the atherosclerotic lesion. Eventually, the fibrous cap of a plaque might break open, triggering a blood clot to develop over the rupture. Plaques that are most likely to break possess a thinned cap, a large lipid pool and many macrophages. This plaque phenotype is partially dependent on the activities of macrophages. Macrophage foam cells secrete pro-inflammatory cytokines that amplify the local inflammatory response in the lesion as well as reactive oxygen species that further induce macrophage proliferation and lipid uptake. In addition, the activated macrophages produce matrix metalloproteinases (MMPs) that can degrade the ECM thus weakening the plaque's fibrous cap.

In addition to the initiation phase, GJIC might play a role in the progression of atherosclerotic plaques. For example, reducing Cx43 expression in LDLR$^{-/-}$ mice led to the development of atherosclerotic lesions that exhibited thicker fibrous caps with more collagen and SMCs, a phenotype associated with plaque stability [72]. Thus, it seems beneficial to reduce Cx43 mediated GJIC in atherosclerosis. Clearly, it will be interesting to see how changes in expression of other connexins might affect this disease. More recently, Eugenin et al. [39] showed that the increased GJIC in monocytes enhanced the release of MMP-2,3 but not MMP-9 by these cells. This amplified release of MMPs in atherosclerotic lesions could be deleterious since it might promote plaque rupture and induce thrombosis. Although it remains to be proven, we envision that hemichannels on the macrophages in the lesions may also play a role in plaque development. For instance, the hemichannels might become misregulated such that they convert from their normally closed state to an open state leading to intracellular leakage and macrophage death. Taken together, altered GJIC may affect several processes required to promote atherosclerosis.

6. Perspectives and future directions

Are connexins in leukocytes forming gap junction (hemi-) channels to "shuttle messages"? There is substantial evidence in support of leukocyte homocellular and heterocellular gap junction assembly that allows for intercellular communication. On the contrary, it remains only speculative that hemichannels serve as bidirectional gateways between the intra- and extracellular space possibly leading to paracrine cell–cell signalling under particular circumstances. In vitro studies show that altered GJIC affects the migration and development of leukocytes, thus influencing the recruitment of leukocyte subtypes to sites of inflammation as well as the activation state of the immune system. Importantly, these observations are corroborated by recent in vivo studies on atherosclerosis. The dysregulation of GJIC is also implicated in other inflammatory diseases and reactions such as acute pancreatitis [18], cystic fibrosis [77], ischemia–reperfusion injury in liver [78] and heart [79], as well as wound repair in

skin [17]. We are far from identifying all the signals that go through gap junction (hemi-) channels and we know even less about when and how those molecules might cross talk with other molecules in any given situation. Perhaps, the answers to some of our key questions might not be so far away as we bridge 'the gap' between inter-disciplinary sciences.

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