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Insights & Perspectives

The CAR group of Ig cell adhesion proteins–Regulators of gap junctions?

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Abstract

Members of the CAR group of Ig-like type I transmembrane proteins mediate homotypic cell adhesion, share a common overall extracellular domain structure and are closely related at the amino acid sequence level. CAR proteins are often found at tight junctions and interact with intracellular scaffolding proteins, suggesting that they might modulate tight junction assembly or function. However, impairment of tight junction integrity has not been reported in mouse knockout models or zebrafish mutants of CAR members. In contrast, in the same knockout models deficits in gap junction communication were detected in several organ systems, including the atrioventricular node of the heart, smooth muscle cells of the intestine and the ureter and in Sertoli cells of the testes. Possible interactions between BT-IgSF and connexin41.8 on the disturbed pattern of pigment stripes found in zebrafish mutants and between ESAM and connexin43 during hematopoiesis in the mouse are also discussed. On the basis of the combined data and phenotypic similarities between CAR member mutants and connexin mutants I hypothesize that they primarily play a role in the organization of gap junction communication. Also see the video abstract here: https://youtu.be/i0yq2KhuDAE.

KEYWORDS
BT-IgSF (IgSF11), CAR, CLMP, connexins, ESAM, gap junctions, IgCAMs

INTRODUCTION TO THE COMMON CHARACTERISTICS OF CAR GROUP MEMBERS

The Coxsackie- and Adenovirus Receptor (CAR) is the founder of a small and evolutionary conserved group of Ig-like cell adhesion proteins[1] (Figure 1a). It was initially characterized as a high-affinity receptor protein for subtypes of coxsackie- and adenovirus in 1997 and therefore, named Coxsackie- and Adenovirus receptor.[2,3] A highly related protein to CAR was cloned in 2002 from mouse and human tissues and named brain and testis specific immunoglobulin superfamily protein (abbreviated BT-IgSF) due to its strong expression in the brain and testis.[4] Independent of this study BT-IgSF was also found to be up-regulated in intestinal-type gastric cancers and designated IgSF11.[5] (also termed V-set and Immunoglobulin domain containing 3, abbreviated VSIG-3).[6,7] Bioinformatics screenings of expressed sequence tags and genomic databases led to the identification of another protein in humans and mice that could be categorized into the group due to its high similarity to CAR; therefore, named CAR-Like Membrane Protein – abbreviated CLMP.[8] This protein was separately detected in a screen for upregulated genes in visceral adipose tissue from the Otsuka Long-Evans model of diabetes and was subsequently designated adipocyte adhesion molecule (ACAM).[9] The

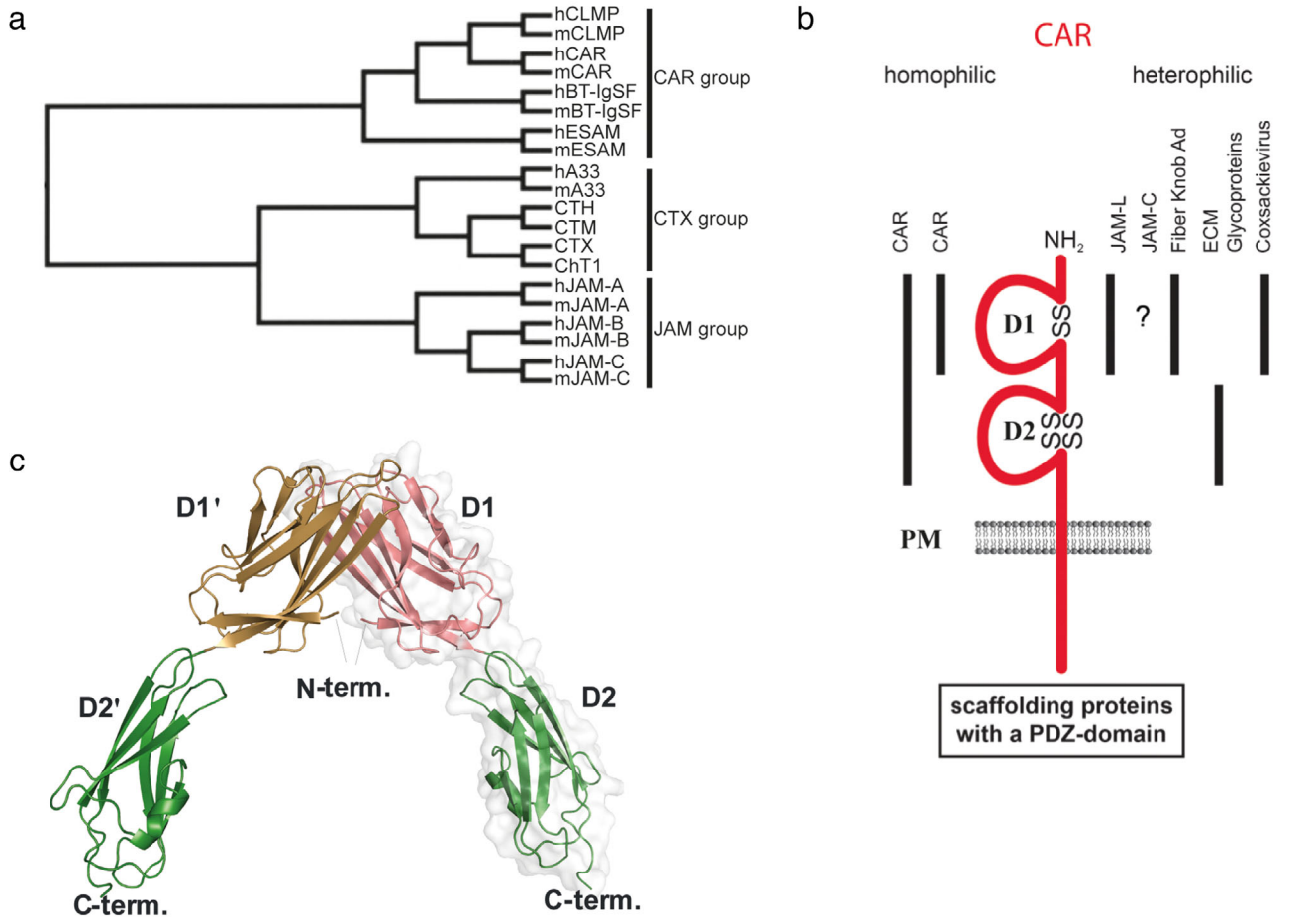

FIGURE 1 The CAR group of IgCAMs. (a) Dendogram showing the relationship between the four members of the CAR group of proteins which is composed of CAR, CLMP, BT-IgSF and ESAM. Comparison of their amino acid sequence with other transmembrane Ig-like proteins, together with their overall organization of their extracellular domains the four CAR group members might be categorized into larger families which also contain the CTX- and JAM-groups. (b) Scheme of the overall domain organization of CAR group members, shown here for CAR itself. The two Ig-like domains are indicated as loops that are linked by disulfide bonds indicated by SS-bridges. In addition homophilic and heterophilic binding regions of the extracellular domains of CAR are indicated by black vertical bars on the left and right side of the scheme, respectively. For example, heterophilic interaction of JAM-L is mediated by the N-terminal D1 domain of CAR. In crystals the D1 domain of CAR self-associates (see below in c); however, binding experiments using single domains shows also binding between D1 and D2 allowing an antiparallel D1-D2 interaction of two CAR molecules.[13] Binding regions of the three other CAR members have not yet been defined. For BT-IgSF it is known that it interacts with VISTA on T-cells.[7] For all members of the CAR group interactions with scaffolding proteins have been described via their C-terminal ends of their cytoplasmic segments. (c) Crystal structure of a homodimer of the complete extracellular region of mouse CAR (D1 and D2 versus D1' and D2') reveals a U-shaped dimer. Parts belonging to D1 and D2 are colored pink and green, respectively. N- and C-termini are indicated. D1, N-terminal domain of the V subtype Ig-like domain; D2, C-terminal domain of the C2 subtype of Ig-like domain (modified from[1,8,13])

fourth member of the CAR group is ESAM (endothelial cell-selective adhesion molecule) which was identified as an endothelial protein located at tight junctions.[10,11]

Protein structure

All members are type I transmembrane proteins sharing an identical overall domain structure in their extracellular part and are highly related at the amino acid level (Figure 1a). The extracellular regions are composed of two Ig-like domains belonging to the V and C2 subsets of IgG domains: the membrane-distal V-domain at the N-terminus and the C2 domain close to the plasma membrane. The latter contains an extra pair of cysteine residues that form an additional intrachain disulfide bond and probably functions to stabilize this domain (Figure 1b). All extracellular parts of CAR group members harbor one or two potential N-glycosylation sites. Among CAR group members BT-IgSF has the longest cytoplasmic segment of 167 amino acid residues (in mice), while that of ESAM is about 120 and those of CAR and CLMP are about 110 residues long.[8] Another hallmark of CAR group members is the presence of PDZ (PSD-95/Disc-larg/ZO-1) recognition motifs at their C-terminal ends (Figure 1b). At least two major isoforms generated by alternative pre-mRNA splicing have been detected for CAR.[12] Furthermore, a similar genomic organization

has been described for mammalian CAR group members suggesting an evolutionary relationship. On the basis of their amino acid sequences and their overall organization of extracellular domains the four CAR group members CAR, BT-IgSF, CLMP and ESAM can be categorized together into a larger family. Other related families are the CTX- and JAM-groups of Ig-like proteins (Figure 1a).[8]

So far, the three dimensional structure of the full extracellular region has only been solved for CAR. In crystals the extracellular region of CAR forms U-shaped dimers through binding of their N-terminally located V-domains[13,14] (Figure 1c). In each monomer V- and C2-domains associate in a head-to-tail manner and form a rod-like, dumbbell-shaped structure. A short junction between the globular Ig domains defines the dumbbell's "grip" that creates flexibility between the Ig domains. A linker stretch of five residues tethers this structure to the plasma membrane. The binding interface between the N-terminally located V-domains that are implicated in homodimer formation has a size of 684 Å^2 per monomer. This region is formed by various side chains of amino acid residues derived from β-strands G, F, C, C', and C'', as well as by the FG-connecting loop. Four salt bridges, two hydrogen bonds and side chain-derived hydrophobic interactions are implicated in binding. A similar interface has also been reported for the single V-domains of human CAR.[15] The U-shaped dimeric complex formed by CAR is similar to the U-like structure described for JAM-A.[16,17] Calculations of CLMP or BT-IgSF tertiary structures on the basis of the mouse CAR template by applying Phyre2.[18] show similar arrangements of β-sheets in their Ig domains [1] and suggests a similar binding mode between two V-type domains.

A peculiarity of CAR, which is not found for the related proteins CLMP and BT-IgSF, is that adenovirus fiber knobs can bind to its N-terminal domain. The fiber knob is the homotrimeric protein of the adenovirus capsid which binds CAR on host cell surfaces for infection.[19,20,13,15,14] Interestingly, CAR-CAR homodimer formation and fiber knob binding to CAR in crystals is mediated by overlapping amino acid residues within the N-terminal domain. Due to its trimeric structure the binding affinity of the fiber knob to CAR is 100 to 1000-fold higher compared to the formation of CAR homodimers. This makes the fiber knob an attractive tool for functional interference. Indeed, in cell biological studies disruption of cell-cell contacts has been observed.[13,21]

Cell-cell contacts

An important hallmark of CAR members is that they mediate cell-cell aggregation in cell culture via a homophilic binding mechanism. For example, this was discovered by expressing CAR in heterologous cell systems or by performing antibody perturbation experiments. After a short incubation period CAR expressing cells form large cell clusters which can be perturbed by CAR-specific antibodies.[22,13] Similarly, if transfected in cells ESAM, CLMP and BT-IgSF can mediate homotypic adhesion in a Ca^{2+}/Mg^{2+}-independent manner. However, one study reported a rather moderate aggregation activity for CLMP when compared with cadherin1.[9,23,6,10,11,24] In addition to homophilic binding, heterophilic interactions with JAM-C, JAM-L and extracellular matrix glycoproteins have also been described for CAR. So far heterophilic binding has not been studied for ESAM and CLMP.[25,26,13,14,27,28] Heterophilic interactions between BT-IgSF (VSIG-3) and the B7 family member Vista (V-domain immunoglobulin (Ig) suppressor of T cell activation) in T cells have recently been detected. This interaction inhibits human T-cell proliferation and cytokine production.[7]

Expression pattern

Another characteristic of CAR members is their spatiotemporal pattern of localization primarily during developmental periods. For example, during prenatal development CAR is widely expressed in various tissues and organs such as brain, retina, liver, heart, pancreas, skin and lung. Shortly after birth, CAR expression decreases particularly in the brain. Strikingly, CAR gets re-expressed upon cardiac remodelling in patients suffering from dilated cardiomyopathy,[29,30] during myocarditis[31,32] and after myocardial infarction[33] suggesting a function in tissue regeneration. ESAM is primarily detected in the developing vasculature of the mouse embryo with high levels in the lung and heart and lower levels in the kidney and skin. Besides expression in endothelial cells ESAM is also found in hematopoietic stem cells and in platelets but not in epithelial cells.[34–36] Although expression of CLMP and BT-IgSF has not been extensively studied some information about their localization is known. CLMP has been detected during developmental periods in smooth muscle cells of the gastro-intestinal tract, ureter and in neuronal cells in the brain .[37] The level of expression of BT-IgSF in the hippocampus and cerebellum is maintained at mature stages.[38,39] In zebrafish BT-IgSF was detected in chromatophores and their precursors during migration to the hypodermis to organize into specific horizontal patterns.[23] At the moment current data allow the following provisional summary on the localization of CAR group members. CAR appears to have the most widespread expression whereas ESAM is the most locally restricted among the four group members. In most cases CAR and CLMP are primarily expressed at developmental stages and expressions become reduced at advanced stages. BT-IgSF appears to be expressed at mature stages.

CAR MEMBERS ARE ASSOCIATED WITH TIGHT JUNCTIONS AND THEIR INTRACELLULAR SEGMENTS BIND SCAFFOLDING PROTEINS

Tight junctions are complexes made up of multiple transmembrane proteins including occludin, claudins and scaffolding proteins such as ZO (zonula occludens) and MAGI (membrane-associated guanylate kinase inverted protein). For example, these complexes are concentrated at the apical-lateral borders of epithelial cells where they control paracellular permeability. CAR has been described as an adhesion protein concentrated at contact sites between CAR-positive cells mediating homotypic adhesion.[22] In addition it recruits ZO-1, an established tight

junction protein, to cell-cell contact sites for which the cytoplasmic segment of CAR is required. Co-localization of both ZO-1 and CAR at the apical pole of the lateral membrane of epithelial cells was detected using electron microscopy, indicating that CAR is part of or closely associated with tight junctions. Moreover, an increase in transepithelial resistance in monolayers of MDCK cells overexpressing CAR and reduced diffusion of fluorescent markers across epithelial cell layers supports the notion of CAR playing a role in tight junctions.[40,41] Co-localization of CAR with ZO-1 was also demonstrated in mature cardiac myocytes located in the intercalated discs of the heart.[42] The evidence for physical association between CAR and ZO-1 was revealed by co-immunoprecipitation[40,41] and by yeast-two-hybrid system experiments. The latter also showed that CAR interacts with a number of different PDZ-containing scaffolding proteins.[43–45] Consistent with the above findings, the cytoplasmic tails of CAR isoforms contain class I PDZ binding motifs that bind scaffolding proteins (for details see[1]).

CLMP also co-localizes with the tight junction markers ZO-1 and occludin in transfected cells, suggesting that CLMP, as well as CAR, might be a tight junction molecule. In line with the cell-cell adhesion function and localization to tight junctions, overexpression of CLMP in MDCK cells also resulted in a significant increase in transepithelial resistance.[8] Interestingly, expression of a mutated variant of human CLMP unable to integrate into the plasma membrane leads to the mislocalization of ZO-1, further supporting the idea of an interaction between both proteins.[46] BT-IgSF was found to be associated with the blood-testes barrier in Sertoli cells and with the scaffolding protein PSD95 in neuronal cells.[39,47] ESAM was found to associate with MAGI-1 in endothelial cells and associates with tight junctions.[48]

LESSONS FROM KNOCKOUT STUDIES: CAR MEMBERS AFFECT THE LOCALIZATION AND PROTEIN LEVEL OF CONNEXIN43 and 45, THE PHOSPHORYLATION STATUS OF CONNEXIN43 AS WELL AS CELL-CELL COUPLING

Overall, the data summarized above indicate that CAR members are present in larger protein complexes at tight junctions of the plasma membrane and strongly argue that they play a role at tight junctions. However, ample evidence from constitutive as well as conditional CAR member knockouts challenges this view. In cases where tight junctions in knockouts of individual CAR members have been analyzed functionally and by electron microscopy no significant deficits in either tight junction function or integrity have been observed.[49,50,47,51] In contrast, changes in the localization, expression intensity and phosphorylation of the gap junction proteins connexins were consistently detected in knockouts of CAR, CLMP or BT-IgSF. Gap junctions are formed by connexins that are transported in vesicles and inserted as hemichannels into the plasma membrane. Here they can rapidly diffuse and aggregate in specific regions of the plasma membrane including tight junctions to form large clusters and dock to hemichannels on opposing cells.[52]

Several examples follow detailing how deletion of CAR members can affect changes in the localization, phosphorylation and expression at the protein level of connexin 43 and 45. Absence of CAR members in the mouse leads to problems in the cardiovascular, digestive, hematopoietic and reproductive organ systems and in teleost fish mutations in CAR members cause disorganization of chromatophores.

CAR-KO

CAR deficient mice die at early embryonic stages due a to malfunction and malformation of the heart.[53–55] While CAR is initially found uniformly on all cells of the embryonic heart, throughout development it becomes restricted to intercalated discs at the atrio-ventricular node in the adult heart where tight, gap and adherens junctions are concentrated.[56] In conditional mouse mutants the absence of CAR in the mature heart resulted in impaired electrical conductance at the atrioventricular node as indicated by a prolonged PR interval. Mislocalization and reduced expression of connexin43 and 45 in cardiomyocytes was detected (Figure 2a,c). Importantly, analysis of cell-cell coupling experiments that used a fluorescent dye that diffused through gap junctions revealed an increase dye spreading (Figure 2b). These results point to an increase in gap junction-mediated cell-cell communication between CAR-deficient cardiomyocytes.[57,49,50] Consequently, the PR interval and also the beating frequency of CAR-deficient hearts increased.[49] However, barrier integrity as tested by fluorescent tracers was not perturbed in CAR conditional mutants. This suggests that tight junctions, despite the apparent reduced expression of some components, are maintained.[49,50] In line with these in vivo data are observations on cultivated embryonic CAR-deficient cardiomyocytes in vitro. A redistribution of connexin43, decreased protein levels of connexin43 and 45, altered phosphorylation and an increased beating frequency was detected in CAR-deficient cardiomyocytes compared to wild type myocytes (Matthäus et al., our unpublished data). More recently, a link between CAR and connexin43 was also detected upon adenovirus infection of HaCaT cells.[58] Taken together, in embryonic cardiomyocytes as well as in the adult heart the absence of CAR induces decreased protein levels, phosphorylations, and/or mislocalizations of connexin43 and 45 resulting in impaired cardiomyocyte coupling. In contrast tight junctions and barrier functions appear normal.

CLMP-KO

The absence of the CAR related protein CLMP resulted in disturbed contractions of smooth muscle cells of the intestine and ureter in constitutive knockout mice. This caused a high rate of mortality and provoked a severe bilateral hydronephrosis in these mutants due to insufficient transport of chyme and urine. Furthermore, uncoordinated calcium signaling was detected in smooth muscle layers of both tissues, which is known to be based on gap junction activity (Figure 3e). Similarly to the CAR knockout phenotype in the heart, in CLMP mutants

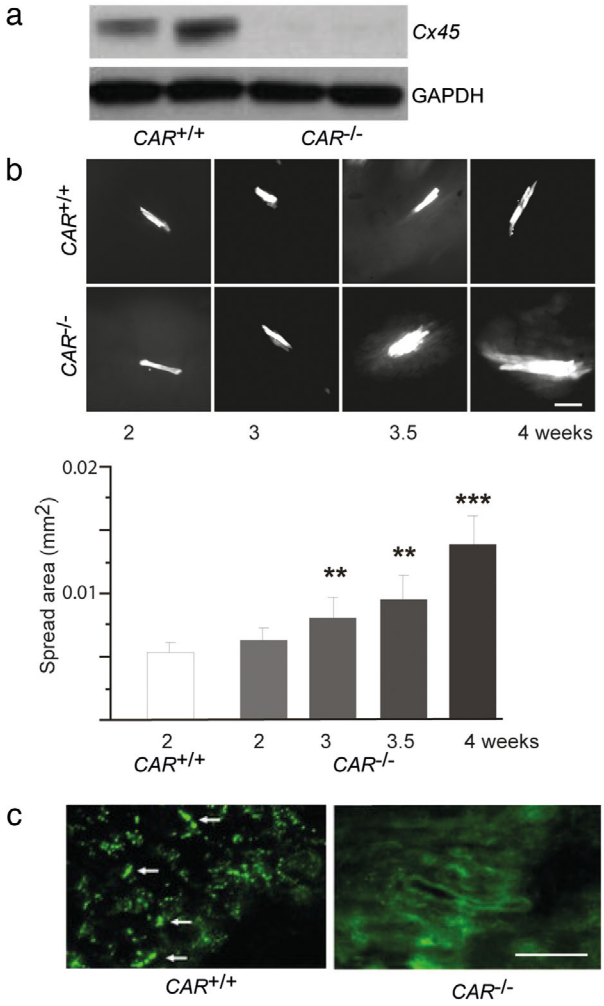

FIGURE 2 Regulation of connexin 45 in the heart by the cell adhesion molecule CAR. (a) Western blot analysis of tissue extracts of the mouse mature heart shows a severely reduced level of connexin45 in the absence of CAR. Two independent samples for each genotype are shown. GAPDH indicates protein loading. (b) Altered cell-cell coupling in the absence of CAR in the mature heart. A fluorescent dye that passes through gap junctions was injected into individual cardiomyocytes in slices after CAR ablation by tamoxifen administration. CAR was removed by a cre recombinase (MerCreMer) selectively expressed in the heart. CAR gradually disappears from heart tissues. The increased lateral and longitudinal dye coupling is most prominent in cardiac slices at the 4-week point which is quantified in the lower panel. Scale bar, 100 µm. (c) Reduction and mislocalization of connexin45 in the mature heart in the absence of CAR as shown by immunofluorescence staining of sections using an antibody to connexin45. Figure was modified and reproduced from.[57,49] Arrows indicate cell junctions. Scale bar, 30 µm. The text "* Indicates the *P*-values of significance, ** $P < 0.01$, *** $P < 0.001$; Mann-Whitney test. This Figure is reproduced from Lisewski et al., 2008 and explained in more detail

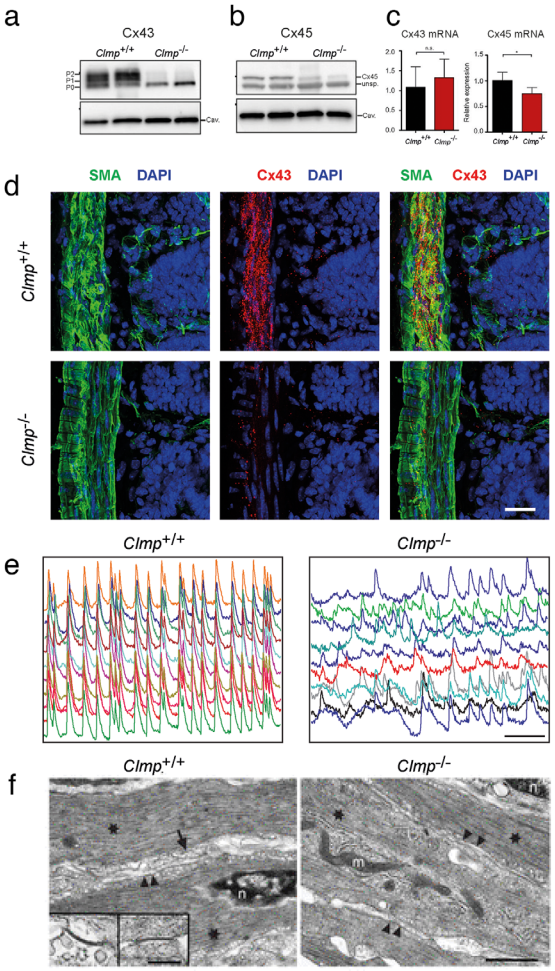

FIGURE 3 Reduced expression of connexin43 and 45 in the smooth muscle layer of the intestine. (a and b) Western blots showing reduced expression of connexin43 and 45 in the smooth muscle layer in the absence of CLMP. Two independent samples are shown. Equal protein loading is indicated by caveolin (Cav.). P0, unphosphorylated; P1 and P2, phosphorylated connexin43. An unspecific (unspec.) band is detected in the connexin45 blot (b). c) Connexin43 mRNA is not whereas connexin45 mRNA is only weakly reduced in intestinal smooth muscle tissue in the absence of CLMP. Studies showed that connexin43 or 45 mRNA levels were not or only weakly reduced, while the protein level was severely diminished, suggesting that the regulation of connexin protein level within CLMP-deficient smooth muscle cells is under posttranslational control. (d) Reduction of connexin43 clusters in the circular smooth muscle layer of the intestine in the absence of CLMP as demonstrated in transversal sections of the duodenum using antibodies to connexin43. SMA denotes the smooth muscle layer. Scale bar, 20 µm. (e) Absence of CLMP caused uncoordinated calcium waves in the smooth muscle layer that are mediated by gap junctions. Single traces of 10 selected regions of interest are shown. Time scale bar, 100 s. (f) Severe reduction of gap junctions in the absence of CLMP as demonstrated by electron microscopy. The arrow denotes gap junctions in the panel of the wild type smooth muscle layer which are enlarged in the two inserts. No differences were observed in the arrangement of actin-myosin filaments between wild type and CLMP knockout. Scale bar, 1 µm and 200 nm (insert). Arrow heads, plasma membrane; m, mitochondria; n, nucleus; stars, actin-myosin filaments (modified and reproduced from[37])

the level of connexin43 and 45 as well as the phosphorylation status of connexin43 was severely reduced in the smooth muscle layer of the intestine and connexin43 in the ureter[37] (Figure 3a-d). Gap junctions were not detected in electron microscopy images (Figure 3f). In both cell layers tight junctions have not been described, but scaffolding

proteins such as ZO-1 are present. Importantly, mutations in the human CLMP gene have been described and are associated with a rare gastrointestinal disorder.[59–61,46] The clinical presentation of these human patients is similar to the phenotype of the mouse CLMP knockout except for the length of the intestine. Patients with CLMP mutations have a very short small intestine, while in the mouse CLMP knockout small intestine length is unchanged. Similarly to mouse mutants CLMP-deficient human patients suffer from an insufficient transport of chyme and urine that caused a fatal delay to thrive, a high rate of mortality, and provoked a severe hydronephrosis. Overall, CLMP mutations caused functional, but not physical obstructions in the intestinal tract and ureter. This resulted in reduced peristaltic contractions due to a lack of gap-junction communication between smooth muscle cells. A focus on the regulation of gap junction activity in the smooth muscle layer might be advisable in order to eventually establish a therapy for this group of patients. It remains to be determined whether the absence of CLMP causes a lack of gap junction formation, connexin stability or a mislocalization at the cellular plasma membrane. The mRNA level encoding connexin43 is not reduced in the intestine of knockout mice compared with wild type (Figure 3c), suggesting that the turnover rate of connexin43 and 45 might be higher leading to an increase in degradation or that translational interferences might lower their expression in CLMP-deficient smooth muscle cells.

BT-IgSF-KO in mice

Changes in the localization and/or activity of connexin43 have also been detected in the absence of BT-IgSF, the third CAR group member considered in this Hypothesis article.[47] In the testis BT-IgSF is found in Sertoli cells at the blood-testis barrier, a highly regulated structure that opens and closes to allow the passage of germ cells. At this structure adherens, tight and gap junctions are closely intermingled. Ablation of BT-IgSF in the mouse causes male infertility due to a deficiency in the blood-testis barrier. However, electron microscopy images did not reveal any changes in the ultrastructure of the blood-testes barrier in the absence of BT-IgSF, but instead a mislocalization of connexin43 was detected. Connexin43 was found throughout the seminiferous epithelium and instead of being restricted to the blood-testes barrier as it is in wild type tissues[47] (Figure 4a). Connexin43 is known to play an essential role in tight junction reassembly at the blood-testes barrier during its restructuring processes.[62] Due to the mislocalization of connexin43 in the absence of BT-IgSF in the testis, connexin43 is no longer able to support the process of resealing the blood-testes barrier leading to infertility.

BT-IgSF in fish

In teleost fish such as zebrafish a frequent motif is the organization of pigment cells into stripes along dorso-ventral or anterior-posterior body axes and fins.[63] This characteristic pattern of horizontal and light stripes is regulated by cellular interactions between

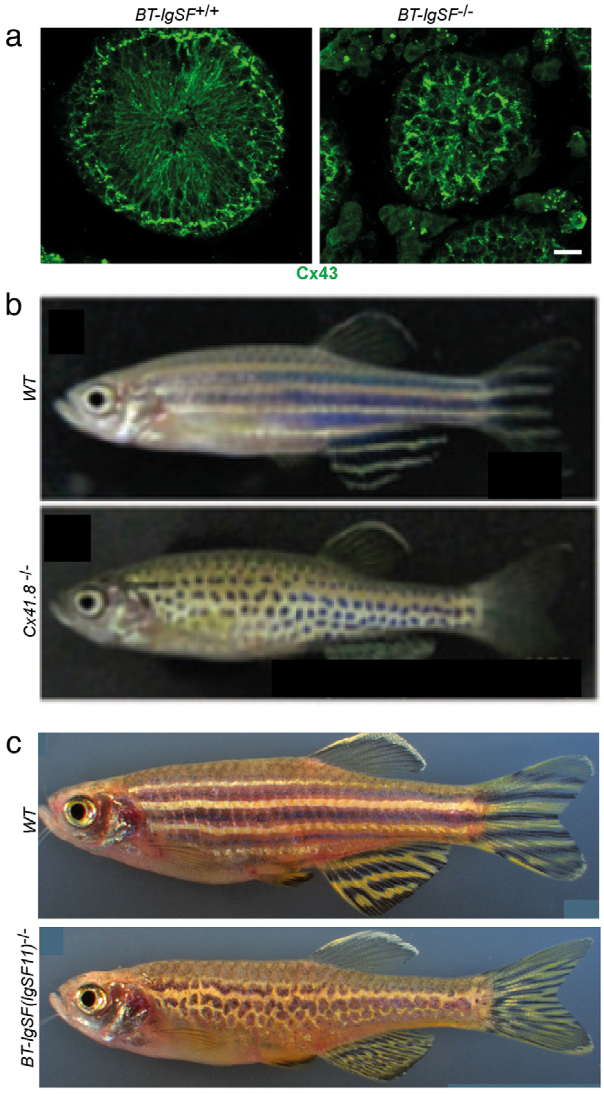

FIGURE 4 Mislocalization of connexin43 in the testes in the mouse knockout of BT-IgSF and impaired pigment stripe formation in BT-IgSF or connexin41.8 zebrafish mutants. (a) Mislocalization of connexin43 (in Green) in mouse testes in the absence of BT-IgSF. Note the diffuse localization of connexin43 in the seminiferi tubuli in the knockout testes whereas in the wild type connexin43 is primarily concentrated at the blood-testes barrier. Scale bar, 20 µm. Assembled and reproduced from[47]. (b and c) Similarities in disturbed pigment stripes in zebrafish mutants of BT-IgSF (IgSF11) or connexin 41.8. Wild types are shown for comparison. (modified and reproduced from[23,66,68])

chromatophores (melanophores, xanthophores and iridophores). A number of genes have been shown to be involved in stripe formation. Among these are the cell adhesion protein BT-IgSF (IgSF11) and connexins which have been both shown to be crucial molecules for cell-cell interaction between pigment cells. In zebrafish and in the cichlid fish specific mutations in the BT-IgSF gene perturbs the pattern of melanophores with irregularly located spots in contrast to the wild type where typical horizontal patterns are formed.[64,23] Interestingly, similar irregular patterns of chromatophores have been described in zebrafish with mutations in the genes of connexin41.8

and connexin39.4 (Figure 4b and c).[65–68] Localization studies of connexin43 in Sertoli cells in the absence of BT-IgSF indicate that BT-IgSF might also affect the localization of connexin41.8 and/or 39.4 in zebrafish xanthophores or melanophores, respectively, resulting in impaired cell-cell communication. Coordinated cross-talk between chromatophores via gap junctions appear to be of critical importance for development and for the correct arrangement of stripes along the body axes of fish.[69,70] In this context it is noteworthy that zebrafish with ZO-1 (Tjp1a, "schachbrett"), which is selectively expressed in a subtype of chromatophores (iridophores), mutations display interrupted stripes[71] and that connexin41.8 and BT-IgSF (IgSF11) both have PDZ-binding motifs at their extreme C-termini.

ESAM-KO

Initial investigations showed that the absence of ESAM in the mouse does not result in obvious developmental defects[72] and endothelial barrier function in the skin and peritoneum was not disturbed.[73] However, if an anti-vascular endothelial (VE)-cadherin blocking antibody was intravenously administered in mature ESAM-deficient mice vascular permeability in the lung but not in the heart, skin or brain became impaired.[74] Importantly, recent data indicated that ESAM appears to be essential for hematopoiesis since approximately half of ESAM knockout fetuses died after mid-gestation due to anemia.[75] In addition after bone marrow injury induced by the cytotoxic drug 5-fluorouracil ESAM knockout mice showed reduced numbers of erythroid progenitors.[76] Whether the absence of ESAM affects the localization or expression of connexins in this system has not yet been investigated. It is, therefore, unclear whether ESAM like the other CAR members interferes with connexin expression, localization or function. However, cross-talk between hematopoietic and bone marrow cells via gap junctions appear to be important during hematopoietic stem cell differentiation.[77] Again, here it is noteworthy to mention that connexin43 – like ESAM – is critical for hematopoietic reconstitution under 5-fluorouracil induced stress hematopoiesis which resulted in an attenuated recovery of blood cells.[78,79] Future studies are needed to test whether mutation of ESAM has an effect on connexin expression and or function during hematopoiesis.

MODULATION OF BARRIER FUNCTION BY CONNEXIN INTERCELLULAR SIGNALING

As described above overexpression of CAR or CLMP in cells increased the transepithelial resistance and resulted in a reduced diffusion of fluorescent markers across epithelial cell layers.[40,8,41] If physiologically relevant these experiments support the view that CAR and CLMP are implicated in barrier function most likely by strengthening tight junction proteins. It also raises the question whether the measured increased barrier activity caused by overexpression of CAR group proteins can be linked to connexins. A number of studies indicated that connexins co-exist in junctional complexes that seal off the intercellular cleft such as at the blood-brain-barrier,[80] blood-testis-barrier[81] as well as during epidermal barrier acquisition.[52,82] For example connexin40 and 43 are associated with tight junction proteins occludin and claudin5 in endothelial cells.[80] In addition to their channel function at the plasma membrane connexins might function in alternative processes such as modulation of cell-cell adhesion or gene expression of N-cadherin.[83–85] However, in the absence of CAR or CLMP a severe reduction in connexins was observed.[37,57,49] Interestingly, there is accumulating evidence suggesting that gap junction signaling is of great importance in regulating barrier integrity. Tight junction barriers should not be considered as simple, static barriers, but instead should be regarded as modulatory, dynamic interfaces controlled by intercellular signaling processes. This is best exemplified by the blood-testes-barrier that must transiently "open" and "close" to allow immature germ cells to cross into the immune-privileged environment to differentiate in spermatids. In this system connexin43 is essential in regulating this opening and closing and maintains barrier integrity.[62] As already pointed out above, absence of BT-IgSF results in the mislocalization of connexin43 in Sertoli cells which in turn might affect the regulated closing or opening of the barrier.

OUTLOOK AND PERSPECTIVES

Taken together, the data from CAR group member knockout mice and the phenotypic similarities to connexin knockouts in the hematopoietic system and in the organization of pigment cells in teleost fish led to the hypothesis of a functional link between connexins and CAR group members. CAR-related cell adhesion molecules appear to play an important role in the expression, phosphorylation and/or localization of connexin43, 45 and 41.8. It remains to be seen whether ESAM plays a similar role and whether the activity of other members of the connexin gene family (21 members in mammals[86]) are also modulated by this group of adhesion proteins. Changes in connexin expression and localization in the absence of CAR members has so far only been observed on cardiomyocytes, Sertoli cells, smooth muscle cells and zebrafish chromatophores. It would be of interest to know whether similar changes also occur in other cell types, in particular in neurons which express high levels of CAR during development.[22,13]

The question also remains as to how CAR members act on connexin43 and 45. ZO-1 or other scaffolding proteins might act as an intermediary since ZO-1 binds to CAR as well as to connexin43 in the intercalated disc in the heart.[57] ZO-1 has been also shown to control gap junction assembly, localization and interferes with plaque size in cell cultures.[87–89] ZO-1 is also known to interact with several other connexins and might serve to recruit signaling molecules implicated in controlling intercellular communication.[90–93] Additional studies with mice expressing mutant versions of genes affecting the interactions between CAR and ZO-1 and between ZO-1 and connexin43 might further this hypothesis. The latter interaction is essential for proper connexin43 function since a frameshift mutation in the gene of connexin43 in human patients deletes the connexin43-ZO-1 interaction and causes a form of oculodentodigital dysplasia.[94] So far, a direct interaction between CAR group members and connexins has been not detected as described for the cell adhesion protein HepaCAM.[95] Further stud-

ies are needed to thoroughly understand the role of intracellular signal transduction pathways downstream of CAR group members modulating the de novo incorporation of connexins into the plasma membrane and the molecular mechanisms by which these signaling pathways regulate expression and trafficking of connexins. For example, the interaction between CAR in keratinocytes and JAM-L on $\gamma\delta$ T cells in the skin controls signals for neutrophil transepithelial migration. This interaction also induces co-stimulation, cytokine production and activation of the MAP kinase pathway via recruitment of the phosphoinositide-3-kinase to a JAM-L intracellular sequence motif.[14,27] In another cellular system, mouse mammary tumor cells, experimental deletion of CAR affected the activity of AKT signaling[96] which is known to regulate the clustering of connexin43.[97]

The localization and size of gap junction plaques in membranes are variables that control cell-cell coupling in general and not only at barriers. Processes such as signalling events and calcium spreading are regulated by connexins not only in the heart and in smooth muscle cells, but also in other organ systems. For example, in the mature brain, gap junction coupling enables the coordination of intercellular calcium waves and action potential spread that leads to electrical synchronization of neurons and synaptic plasticity.[98–100] Therefore, a deeper understanding of connexins regulation by CAR members at the plasma membrane would be of great interest.

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CONFLICT OF INTEREST

No competing interests are declared.

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