The gap junction protein Cx43 regulates B-lymphocyte spreading and adhesion

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Summary

The gap junction protein connexin43 (Cx43) is widely expressed in mammalian cells and forms intercellular channels for the transfer of small molecules between adjacent cells, as well as hemichannels that mediate bidirectional transport of molecules between the cell and the surrounding environment. Cx43 regulates cell adhesion and migration in neurons and glioma cells, and we now show that Cx43 influences BCR-, LFA-1- and CXCL12-mediated activation of the Rap1 GTPase. Using shRNA knockdown of Cx43 in WEHI 231 cells, we show that Cx43 is required for sustained Rap1 activation and BCR-mediated spreading. To determine the domains of Cx43 that are important for this effect, Cx43-null J558 μm3 B cells (which express a wild-type IgM BCR) were transfected with wild-type Cx43–GFP or a C-terminal-truncated Cx43 (Cx43ΔT–GFP). Expression of wild-type Cx43–GFP, but not Cx43ΔT–GFP, was sufficient to restore sustained, BCR-mediated Rap1 activation and cell spreading. Cx43, and specifically the C-terminal domain, was also important for LFA-1- and CXCL12-mediated Rap1 activation, spreading and adhesion to an endothelial cell monolayer. These data show that Cx43 has an important and previously unreported role in B-cell processes that are essential to normal B-cell development and immune responses.

Key words: B lymphocyte, Connexin43, Cytoskeleton

Introduction

The antigen receptor on B lymphocytes (BCR) mediates the uptake of antigen for presentation to T lymphocytes and initiates signals that promote B cell growth and differentiation (Fairfax et al., 2008; Rodríguez-Pinto, 2005). One of the key cellular responses that results from BCR activation by membrane-bound antigen is the initiation of actin-dependent cell spreading, a cellular process that is a key mechanism for B-cell activation (Fleire et al., 2006; Lin et al., 2008). This spreading is thought to promote antigen gathering, to maximize formation of BCR micro-clusters and an immune synapse, which lowers the threshold for B-cell activation (Fleire et al., 2006). The immunological synapse is the cell–cell contact site between the antigen-presenting cell (APC) and the B cell, resulting in B-cell activation and differentiation into antibody-secreting plasma cells (Harwood and Batista, 2008).

Along with the initiation of B-cell spreading, B-cell adhesion is of crucial importance to B-cell development and activation. The binding of leukocyte-function-associated molecule-1 (LFA-1, CD11a/CD18, αLβ2 integrin) and very late antigen-4 (VLA-4, α4β1 integrin) on B cells to intercellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1), respectively, on APCs reduces the threshold for B-cell activation by antigen-containing lipid bilayers. This reduction in threshold, presumably by APCs, occurs by increasing the duration of attachment and promoting cell spreading (Carrasco and Batista, 2006; Carrasco et al., 2004). This results in optimal BCR signaling and subsequent differentiation of B cells into plasma cells and memory B cells (Batista et al., 2001). Integrin-mediated adhesion to vascular endothelial cells and subsequent spreading is also crucial for B cells to cross vascular endothelial layers and extravasate into lymphoid organs and other tissues (Ley et al., 2007).

The action of the GTPase Rap1 is crucial to both spreading and adhesion of B cells (Lin et al., 2008; McLeod et al., 2004). The ubiquitously expressed Rap1 GTPases cycle between an active GTP-bound and an inactive GDP-bound form through the action of a diverse group of guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) (Bos, 2005). After BCR signaling, activated Rap1 can bind to and activate effector proteins, which regulate integrins (Han et al., 2006; Katagiri et al., 2003), cadherin-mediated cell junction formation (Yajnik et al., 2003), cell polarity (Katagiri et al., 2006), Rac-mediated actin dynamics, cell motility (Arthur et al., 2004; Freeman et al., 2010) and lymphocyte trafficking (Katagiri et al., 2004). As a result of the importance of Rap1 in regulating these diverse cellular processes, it has been accurately described as a master regulator of adhesion, polarity and cytoskeletal reorganization (Lin et al., 2010).

The gap junction protein connexin43 (Cx43) is expressed in many cell types including hematopoietic cells (Bermudez-Fajardo et al., 2007; Laird, 2006; Oviedo-Orta et al., 2002). Cx43 is a 43 kDa membrane protein that spans the membrane four times. Its topology includes intracellular N-terminal and an intracellular C-terminal domain, two extracellular loops, and a single cytoplasmic loop (Solan and Lampe, 2009). Cx43 monomers combine to form hexameric hemichannels called connexons that can then associate with connexons on adjacent cells to form gap junctions that allow

the passage of ions and small molecules (generally less than 1 kDa) such as Ca^{2+}, ATP and cAMP (Laird, 2006). Along with a role in cell–cell communication, Cx43 also regulates cell motility and migration in glioma and neuronal cells (Bates et al., 2007; Cina et al., 2009; Elias et al., 2010; Elias et al., 2007). Knockdown of Cx43 protein levels using shRNA constructs or in studies using neural progenitor specific conditional-knockout mice results in reduced migration of neurons in the developing cortex of mouse embryos (Cina et al., 2009; Elias et al., 2010; Elias et al., 2007).

Although Cx43 is widely accepted to be important for neuronal migration, the role of the C-terminal domain of Cx43 in this process remains to be clearly defined. In 2007, Elias and colleagues used in utero co-transfection into the developing rat brain of Cx43 shRNA and a C-terminal-truncated Cx43 expression vector to show that the extracellular domains, and not the C-terminal domain are required for proper neuronal migration (Elias et al., 2007). In contrast to this report, Bates and co-workers (Bates et al., 2007) show, using the Cx43 low-expressing C6 glioma cell line, that overexpression of a C-terminal-truncated Cx43 has a dominant-negative effect on motility compared with that in cells where wild-type Cx43 is overexpressed (Bates et al., 2007). In agreement with these results, Cina and colleagues (Cina et al., 2009) used in utero electroporation of a C-terminal-truncated Cx43 on a neuron-progenitor-specific conditional-knockout mouse background to show that the C-terminal domain is required for neuronal migration to the cortical plate (Cina et al., 2009). Elias and co-workers were able to show, using in utero co-transfection of Cx43 shRNA and a C-terminal-truncated Cx43 into the developing rat brain, that the C-terminal domain of Cx43 is required for the transgenital to radial migration switch of developing interneurons (Elias et al., 2010). Although there is evidence for the C-terminal domain of Cx43 being required for neuronal migration, the molecular mechanism remains unknown.

Cx43 is expressed in B cells (Oviedo-Orta et al., 2000), but its function is not clearly understood. Cx43-knockout mice die perinatally from hypoxia because of malformation of the heart (Reaume et al., 1995), making assessment of the adult immune system difficult. However, heterozygote Cx43 mice do exhibit defects in T-cell and B-cell development (Montecino-Rodriguez and Dorshkind, 2001). It is not clear, however, whether the defect is intrinsic to the B cell or due to defects in other cell types with which B cells interact. Given the role of Cx43 in cell migration in neuronal cells, we hypothesized that Cx43 was involved in cell spreading and adhesion to vascular endothelial cells. By using a combination of loss- and gain-of-function approaches, we found that Cx43 expression is necessary and sufficient for BCR-mediated changes in activation of the Rap1 GTPase, a master regulator of cytoskeletal reorganization, cell adhesion and migration in B cells (Lin et al., 2008; McLeod et al., 2002; McLeod et al., 2004). Cx43 was also important for LFA-1- and CXCL12-mediated Rap1 activation and cell spreading. The increase in LFA-1-mediated spreading correlated with an increase in B-cell adhesion to an endothelial cell monolayer. These results demonstrated that Cx43 is a regulator of B-cell morphology and cytoskeletal responses, as well as BCR signaling.

Results

Cx43 is expressed in immature and mature B cells and is a target of BCR signaling

Cx43 was expressed in the membrane IgM^+ WEHI 231 immature murine B-cell line and in the membrane IgG^+ A20 mature murine B-cell line, but not in J558 μm3, 5TGM1 or MPC11 plasmacytoma cell lines (Fig. 1A). The broad bands observed were consistent with differentially phosphorylated populations of Cx43 (Lampe et al., 2000). Immunofluorescence microscopy with antibodies against Cx43 showed strong cell-surface staining in B-cell lines, as well as in normal splenic B cells (Fig. 1B, white arrows). There was also an accumulation of Cx43 in intracellular aggregates (Fig. 1B, yellow arrows), which was later identified as colocalizing with the endoplasmic reticulum (ER) and endosome markers (supplementary material Fig. S3D).

Clustering the BCR on WEHI 231 cells caused an apparent increase in the molecular mass of Cx43 on SDS-PAGE gels (Fig. 1C). This change was first seen at 5 minutes after crosslinking, persisted for 15 minutes, and started to decline at 30 minutes (Fig. 1C, top panels). This BCR-induced bandshift was probably due to phosphorylation because treatment of cell lysates with calf intestinal phosphatase before SDS-PAGE reduced the amount of the higher molecular mass bands (Fig. 1C, lower panels). The C-terminal domain of Cx43 contains multiple phosphorylation sites that have been implicated in regulating Cx43 function and internalization (Lampe and Lau, 2004). The Cx43 bandshift in this B-cell line is consistent with the findings that Cx43 is phosphorylated in response to receptor signaling and activation with phorbol esters in other cell types (Lampe et al., 2000; Loo et al., 1995; Sáez et al., 1997; Warn-Cramer et al., 1996).

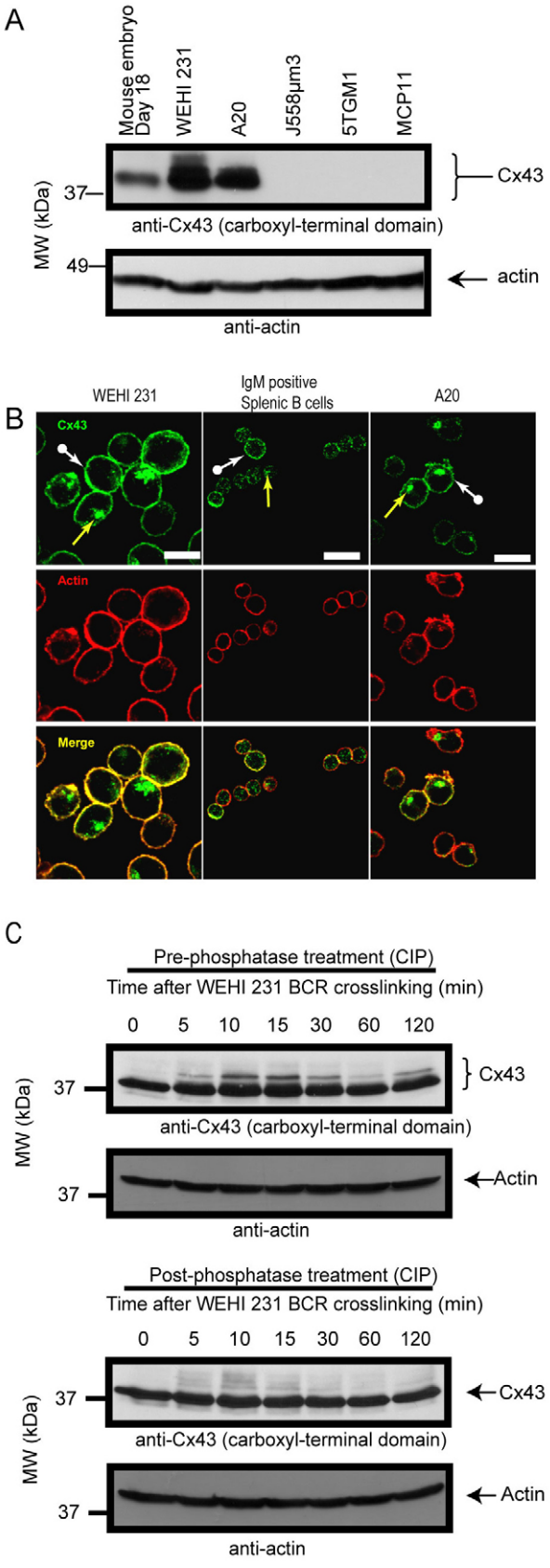
Cx43 is required for sustained activation of the Rap1 GTPase

Activation of the Rap1 GTPase is crucial for BCR-induced cytoskeletal reorganization, cell spreading, integrin activation and immune synapse formation, as well as for chemokine-induced migration (Lin et al., 2008; McLeod et al., 2002; McLeod et al., 2004). Because Cx43 has been implicated in neuronal and glial cell migration and adhesion, all of which are regulated by Rap1, we hypothesized that Cx43 contributes to BCR-induced Rap1 activation. To test this, we used shRNA-encoding vectors (Bates et al., 2007; Shao et al., 2005) to knock down the expression of Cx43 in WEHI 231 cells. Cx43 shRNA construct 2 (specific for the cytoplasmic loop), as well the combination of shRNA constructs 1 (specific for the third transmembrane domain) and 2 reduced Cx43 protein levels (Fig. 2A). Compared with WEHI 231 cells transfected with a scrambled shRNA construct, cells transfected with shRNA 1+2 constructs or shRNA 2 alone were less able to effectively sustain BCR-mediated Rap1 activation over a time course of 0 to 60 minutes (Fig. 2B).

Cx43 expression is necessary for BCR-mediated B-cell spreading

The process of BCR-mediated B-cell spreading is dependent on the activation of Rap1 (Lin et al., 2008; McLeod et al., 1998). Because knocking down the level of Cx43 in WEHI 231 cells resulted in a reduction in sustained activation of Rap1, we hypothesized that Cx43 was important for BCR-induced B-cell spreading. To test this, using real-time imaging, the ability of WEHI 231 cells to spread on coverslips coated with immobilized anti-IgM antibodies that activate the BCR was assessed. Compared with WEHI 231 cells transfected with a scrambled shRNA construct, cells transfected with shRNA 1+2 exhibited significantly reduced cell spreading (Fig. 3A, bottom; Fig. 3B, red line). In addition to undergoing decreased spreading, WEHI 231 cells transfected with Cx43 shRNA 1+2 spread non-uniformly and sent

out multiple, dynamic membrane projections (Fig. 3A, bottom), as opposed to the uniform radial spreading exhibited by the cells transfected with the nonsense vector (Fig. 3A, top). Consistent with the data from knockdown B-cell lines, splenic B cells from Cx43 heterozygous knockout (*Cx43*$^{+/-}$) C57/BL6 mice, which express reduced amounts of Cx43 compared with wild-type splenic B cells (Fig. 3C), exhibited a reduced ability to spread on anti-IgM-coated coverslips when compared with wild-type splenic B cells from a normal littermate control (Fig. 3D; quantified in Fig. 3E). Taken together, these data show that Cx43 is important for BCR-induced B-cell spreading.



The C-terminal tail of Cx43 is required for sustained BCR-mediated Rap1 activation and B-cell spreading

J558 plasmacytoma cells, stably transfected with Igα and Igμ so that they express a functional BCR on the cell surface (J558 µm3), have been used to assay both the biochemical and structural requirements for BCR signaling (Flaswinkel and Reth, 1994; Tolar et al., 2009). J558 µm3 cells do not spread radially like WEHI 231 cells (supplementary material Fig. S1A and S1B, bottom) in response to BCR signaling. When J558 µm3 cells are plated on anti-IgM-coated surfaces to activate the BCR, they extend small, actin-rich protrusions after 30 minutes (supplementary material Fig. S1B,C and Fig. S2C). The formation of membrane protrusions or projections requires a wild-type BCR because cells expressing a mutated BCR with two Igβ tails (J558Cβ) and one Igβ tail (J558αtrunc) showed a decrease in BCR signaling (supplementary material Fig. S2B) (Jang et al., 2010) and a significant decrease in the number of projections (supplementary material Fig. S2C,D). This indicates that J558 µm3 cells have the capacity to reorganize their cytoskeleton into membrane projections in a BCR-dependent manner, but are unable to sustain a BCR-mediated, radial, B-cell spreading response.

Loss-of-function experiments in WEHI 231 cells showed that Cx43 is required for both sustained BCR-mediated Rap1 activation and for spreading (Fig. 2B and Fig. 3). J558 µm3 cells, which do not express Cx43, exhibit low and transient BCR-mediated Rap1 activation (Fig. 4A, leftmost lanes). We therefore hypothesized that expression of Cx43–GFP in J558 µm3 cells would be sufficient to restore their ability to sustain BCR-mediated Rap1 activation and spreading. Transfection and expression of wild-type Cx43–GFP, Cx43ΔT–GFP (a C-terminal truncation of Cx43; supplementary material Fig. S3A,B) or GFP alone did not alter cell surface BCR

Fig. 1. Cx43 is expressed in B cells and is a target of BCR signaling. (**A**) Expression of Cx43 in B lymphoma (WEHI 231, A20) and plasmacytoma (J558 µm3, 5TGM1 and MPC11) cell lines as determined by immunoblotting with an antibody specific for the C-terminal tail of Cx43. Day 18 mouse embryo brain lysate was used as a positive control. Stripping the blot and re-probing for actin indicated equal loading of the lanes. (**B**) Localization of endogenously expressed Cx43 (green) using immunofluorescence. Cx43 expression by WEHI 231 B cells, mature splenic B cells and A20 cells was detected by anti-Cx43 antibody specific for the C-terminal tail, followed by a fluorescently tagged secondary antibody. Actin was detected using Rhodamine–phalloidin (red). Cx43 was localized at the plasma membrane (white arrows) and in an intracellular aggregate (yellow arrows). Scale bars: 10 µm. (**C**) BCR signaling leads to phosphorylation of Cx43. Cell lysates were prepared from WEHI 231 B cells after crosslinking of the BCR with anti-IgM for the indicated times. The lysates were then divided into two equal portions, one was treated with calf intestinal phosphatase (CIP) and one was not. The lysates were separated by SDS-PAGE and Cx43 was detected using an antibody specific for the cytoplasmic tail. Stripping the blots and re-probing for actin indicated equal loading of the lanes. Representative experiment of three similar, independent replicates.

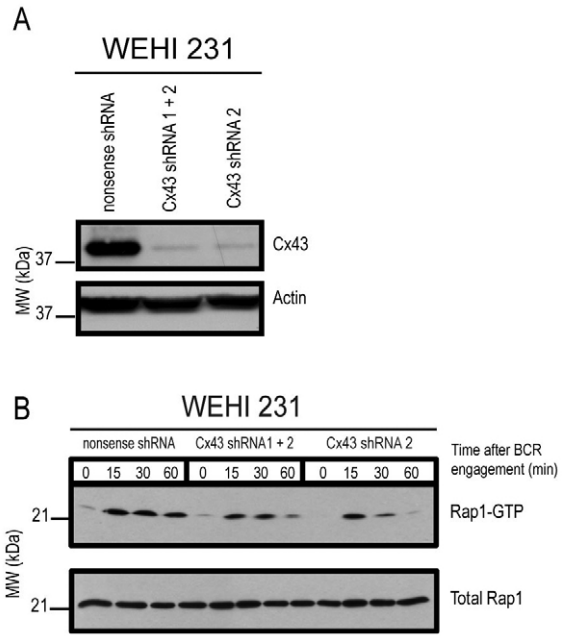


Fig. 2. Reduction of Cx43 expression alters the ability of B cells to sustain activation of the Rap1 GTPase. (**A**) Cx43 protein levels in WEHI 231 cells transfected with a scrambled Cx43 'nonsense' shRNA, Cx43 shRNA construct 1 (targeted to the third transmembrane domain) and Cx43 shRNA construct 2 (targeted to the cytoplasmic loop of Cx43) together or Cx43 shRNA 2 alone, as assessed by immunoblotting. Anti-actin was used as a loading control. (**B**) Rap1 activation in WEHI 231 cells transfected with Cx43 nonsense, Cx43 shRNA 1+2 or Cx43 shRNA 2. Cells were stimulated for the indicated times with anti-IgM and activated Rap1 was precipitated using a GST–RalGDS fusion protein and total Rap1 detected by blotting with anti-Rap1 antibodies. Representative experiment of three similar, independent replicates.

levels as determined by FACS (data not shown). After BCR activation using a soluble anti-BCR antibody, there was a minimal, transient increase in the activation of Rap1 in J558 μm3 GFP cells (Fig. 4A). Expression of Cx43–GFP in J558 μm3 cells resulted in increased Rap1 activation that was sustained for at least 30 minutes (Fig. 4A). Expression of Cx43–GFP in J558 μm3 cells also resulted in an increase in real-time spreading on immobilized anti-IgM (Fig. 4B, middle panels; quantified in Fig. 4C, compare green line with blue line; supplementary material Fig. S4A, SEM). Changes in cell spreading were also seen when cells were fixed after defined times of cell spreading (supplementary material Fig. S4B; quantified in supplementary material Fig. S4C). This increase in spreading was determined to be dependent on a wild-type BCR because transfection of Cx43–GFP into the BCR mutant cell lines J558Cβ and J558αtrunc were unable to initiate a spreading response (Fig. 4D,E). This is consistent with WEHI 231 data showing that Cx43 is important for both sustained Rap1 activation and spreading.

The C-terminal tail of Cx43 contains a proline-rich domain, two tyrosines and ten serine residues, some of which are phosphorylated in other cell types. This domain has been shown to be important for the motility of C6 glioma tumors and neuronal cells (Bates et al., 2007; Cina et al., 2009). To determine whether the C-terminal tail of Cx43 is important for BCR-mediated Rap1 activation, J558 μm3 cells were transfected with a C-terminal tail truncation of Cx43–GFP that had amino acids 244–382 removed (Cx43ΔT–GFP) (supplementary material Fig. S3A,B). Unlike the full-length Cx43–GFP, the mutant form lacking the C-terminal cytoplasmic domain did not confer upon J558 μm3 cells the ability to maintain Rap1 activation. Instead, there was a transient increase in Rap1 activation at 5 minutes that was not sustained (Fig. 4A, rightmost lanes). Similarly, the mutant form of Cx43, lacking the C-terminal cytoplasmic domain, did not result in a sustained real-time spreading response (Fig. 4B, rightmost panels; quantified in Fig. 4C). Interestingly, J558 μm3 Cx43ΔT–GFP cells showed an initial increase in B-cell spreading from 0 to 5 minutes, followed by a decline (Fig. 4C, red line). The brief increase in dynamic, asymmetrical spreading observed coincides with the Rap1 activation at this time point (Fig. 4A). Similar results were obtained when examining J558 μm3 Cx43ΔT–GFP spreading using fixed samples (supplementary material Fig. S4B,C). These data suggest that Cx43 has two important functions in mediating signaling responses crucial for BCR-mediated Rap1 activation and spreading: a C-terminal-domain-independent function, which is required for initiation of Rap1 activation and spreading, and a C-terminal-domain-dependent function, which is required for sustained Rap1 activation and spreading.

Cx43–GFP enhances LFA-1-mediated Rap1 activation and spreading

The process of B-cell spreading is important during interactions with APCs and during the process of transendothelial migration (TEM) through vascular endothelial cells, where it is required for maximal adhesion before extravasation (Batista et al., 2007; Ley et al., 2007). Similarly to BCR-mediated spreading, LFA-1-induced spreading is also dependent on Rap1 activation (Lin et al., 2008). Because sustained BCR-mediated Rap1 activation and spreading were dependent on Cx43 expression, we hypothesized that LFA-1-mediated Rap1 activation and spreading were also influenced by expression of Cx43. LFA-1 signaling was initiated by incubating J558 μm3 cells transfected with GFP, Cx43–GFP and Cx43ΔT–GFP with the TIB213 anti-LFA-1 monoclonal antibodies. LFA-1 clustering led to a minimal increase in Rap1 activation in J558 μm3–GFP cells, but bigger and more sustained activation in J558 μm3 Cx43–GFP cells (Fig. 5A). Truncation of Cx43 in J558 μm3 CxΔT–GFP cells led to an increase in Rap1 activation at 5 minutes; however, consistently with BCR signaling, the increase in Rap1 activation was not sustained (Fig. 5A).

Because LFA-1-induced spreading is dependent on Rap1 activation, we assessed whether Cx43 expression also alters the LFA-1-mediated spreading response. J558 μm3 GFP, Cx43–GFP and Cx43ΔT–GFP cells were incubated on an anti-LFA-1-coated coverslip for 3 hours, after which they were fixed, stained and contact area with the coverslip measured. J558 μm3 GFP cells, similarly to the anti-BCR spreading assay, showed a minimal amount of spreading, whereas the cell–coverslip contact area of Cx43–GFP-transfected J558 μm3 cells was twofold larger (Fig. 5B; quantified in Fig. 5C). There was a slight increase in contact area of J558 μm3 Cx43ΔT–GFP cells compared with that in cells transfected with GFP alone, but the contact area was still less than that seen with full-length Cx43–GFP (Fig. 5B,C). These results indicate that Cx43, and specifically the C-terminal domain, is required for optimal LFA-1-mediated Rap1 activation and spreading.

Cx43–GFP expression increases B-cell adhesion to bEND.3 endothelial cells

Transendothelial migration (TEM) is a complex, multi-step process that requires initial arrest and spreading of B cells on top of the

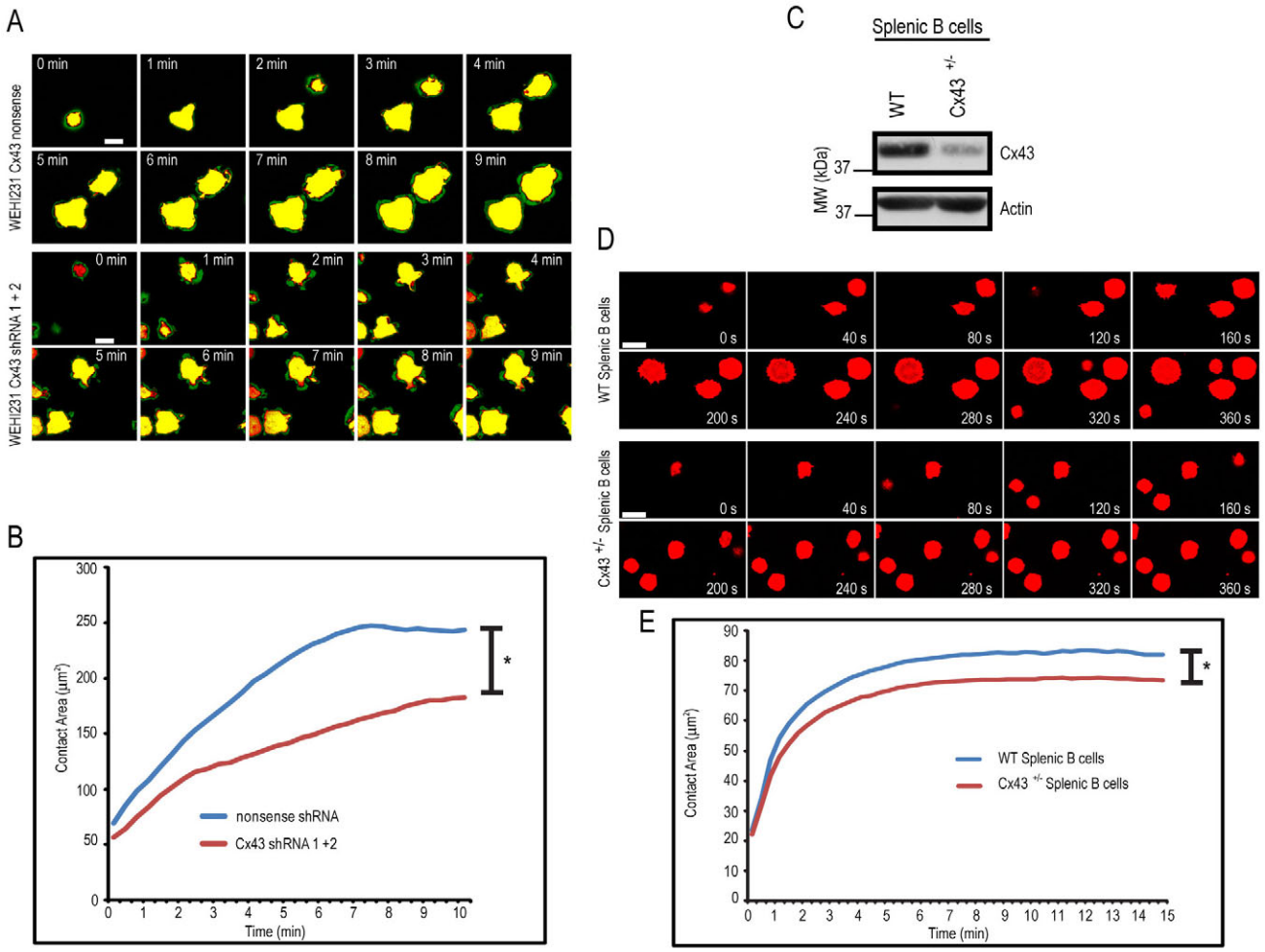


Fig. 3. Decreased Cx43 expression impairs BCR-induced spreading. (**A**,**B**) Real-time spreading of WEHI 231 cells transfected with Cx43 nonsense shRNA or Cx43 shRNA constructs 1+2 as in Fig. 2. Cells were labeled with CellTracker Green CMFDA and plated on anti-IgM-coated coverslips for 20 minutes at 37°C. A single confocal scan was taken at the cell–coverslip interface every 20 seconds. The cells were pseudo-colored using the Olympus software to visualize fine membrane projections. Yellow equates to the highest pixel intensity and green is the lowest pixel intensity. Representative images are shown from 0 to 9 minutes at 1 minute intervals (scale bar: 10 μm). The mean contact area of each time point between the cell and the substrate is shown in B (pooled data from three independent experiments, n=36 individual cells for nonsense shRNA transfected cells; n=49 individual cells for shRNA 1+2 transfected cells). *P<0.05. (**C**) Cx43 levels in splenic B cells from WT and *Cx43*$^{+/-}$ mice. (**D**,**E**) Real-time analysis of the spreading of WT splenic B cells and *Cx43*$^{+/-}$ B cells on immobilized anti-IgM was performed as in A except that the cells were labeled with CellMask Orange. For each time point, the mean contact area of each time point between the cell and the substrate is shown in D (pooled data from three independent experiments, *P<0.05; n=87 individual cells for WT splenic B cells, n=89 individual cells for *Cx43*$^{+/-}$ splenic B cells).

endothelial cells (Ley et al., 2007). Initially, B cells come into contact with endothelial cells by selectin-mediated capture and rolling. During this process, B cells are exposed to integrin ligands and chemokines on the surface of activated endothelial cells, which work in concert, triggering both outside-in and inside-out activation of integrins such as LFA-1, resulting in firm adhesion, arrest and spreading (Ley et al., 2007). This adhesion of B cells to endothelial cells is dependent on both integrins and Rap1 (Lin et al., 2009; McLeod et al., 2004). LFA-1 binding of ICAM-1 induces a conformational change in LFA-1, resulting in outside-in signaling and Rap1 activation, and chemokine signaling (e.g. CXCL12/CXCR4) leads to Rap1 activation, which is required for optimal LFA-1 activation (Freeman et al., 2010; McLeod et al., 2004; Shattil et al., 2010). Because chemokine-mediated Rap1 activation is important for integrin activation and adhesion, the ability of J558 μm3 cells transfected with GFP, Cx43–GFP and Cx43ΔT–GFP to activate Rap1 in response to the chemokine CXCL12 was assessed. CXCL12 induced small, transient Rap1 activation in J558 μm3 GFP cells. This was greatly enhanced by expressing wild-type Cx43–GFP, but not by Cx43ΔT–GFP, which only supports transient Rap activation (Fig. 6A).

Cx43–GFP expression in J558 μm3 cells enhanced cell spreading on immobilized anti-LFA-1, LFA-1-mediated Rap1 activation and CXCL12-mediated Rap1 activation. Both of these processes are involved in B-cell adhesion to endothelial cells. Therefore, we hypothesized that Cx43–GFP, and specifically the C-terminal domain of Cx43, would be required for strong adhesion to an endothelial cell monolayer. To achieve maximal adhesion, the J558 μm3 cells were pre-treated with CXCL12 before being added to a monolayer of TNFα-activated bEND.3 endothelial cells, which normally express Cx43 (Fig. 6B). J558 μm3 GFP cells adhered to the activated bEND.3 cells, but there

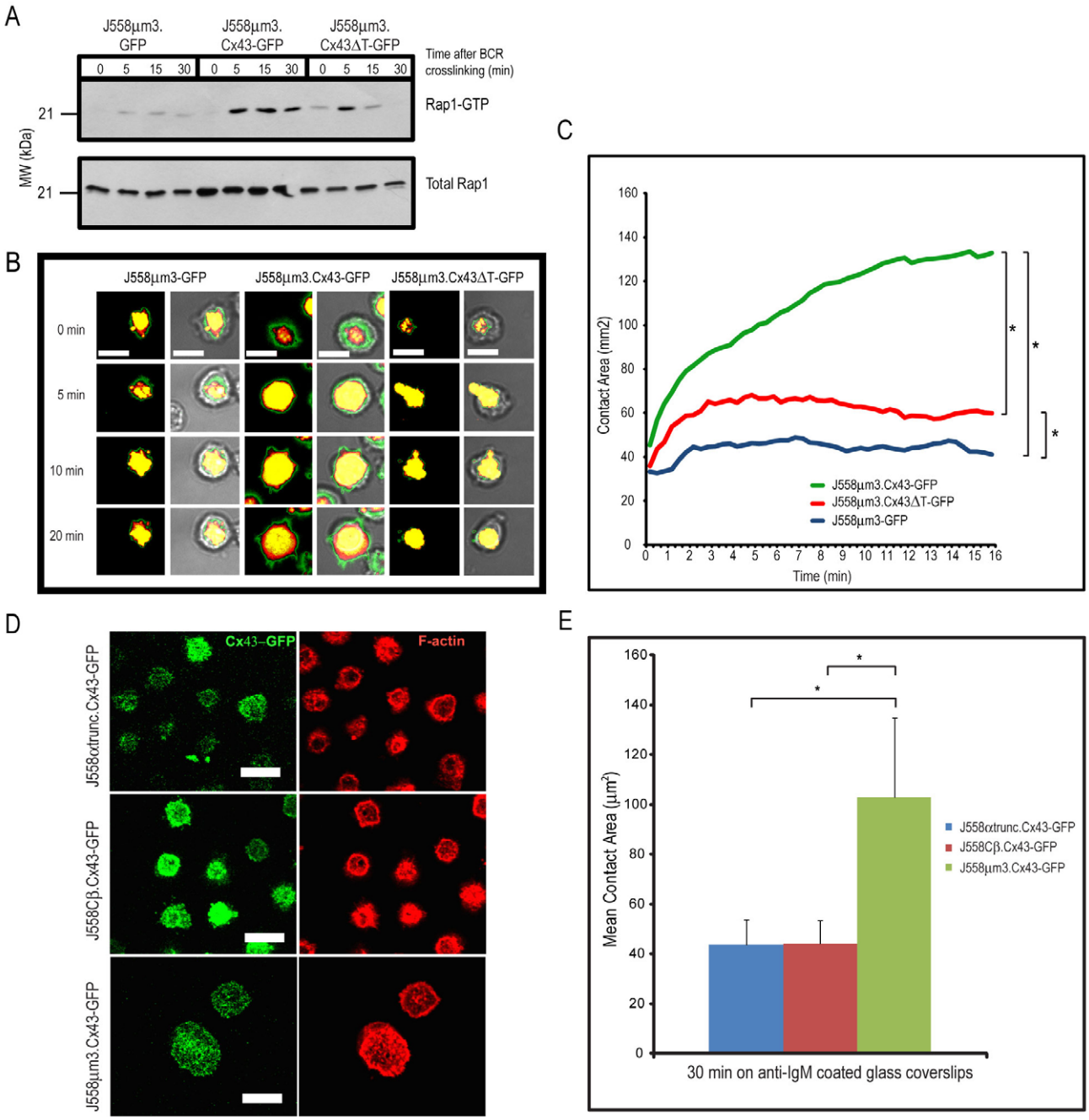


Fig. 4. Wild-type Cx43–GFP but not the C-terminal-truncated Cx43ΔT–GFP supports sustained activation of the Rap1 GTPase and B-cell spreading. (**A**) J558 µm3 B cells transfected with GFP alone, wild-type Cx43–GFP or C-terminal-truncated Cx43ΔT–GFP were stimulated for the indicated times with an anti-IgM antibody. Rap1 activation was assessed as in Fig. 2. (**B**,**C**) Real-time spreading of J558 µm3 GFP, J558 µm3 Cx43–GFP and J558 µm3 Cx43ΔT–GFP cells on immobilized anti-IgM antibody. The cells were labelled with CellTracker Green CMFDA, plated on anti-IgM-coated coverslips and imaged for 20 minutes at 37°C. One single confocal scan was taken at the cell–coverslip interface every 20 seconds for the duration of the time course. The cells were pseudo-coloured using the Olympus software in order to visualize fine membrane projections. Yellow equates to the highest pixel intensity and green equates to the lowest pixel intensity. Representative images are shown at 0, 5, 10 and 20 minutes; left panels represent the pseudo-coloured CMFDA and the right panels include the DIC overlay. Scale bar: 10 µm. (C) Quantification of the mean contact area of each time point of J558 µm3 GFP, J558 µm3 Cx43–GFP and J558 µm3 Cx43ΔT–GFP expressing cell lines spreading on anti-IgM-coated coverslips (mean of four experiments, **P*<0.05, brackets indicate the comparisons being made, *n*=45 individual cells for GFP-transfected cells, *n*=44 individual cells for Cx43–GFP-transfected cells, *n*=44 for Cx43ΔT–GFP-transfected cells). (**D**) Wild-type Cx43–GFP expression is not sufficient to initiate BCR-mediated cell spreading of J558 µm3 cells expressing mutated BCRs. In BCR mutants containing defective α/β integrins (αtrunc and Cβ), Cx43 was not sufficient to restore the BCR-mediated cell-spreading response. Single confocal slices at the cell–coverslip interface of J558αtrunc (top two panels), J558Cβ (middle two panels) and wild-type J558 µm3 cells (bottom two panels) transfected with Cx43–GFP. Cells incubated on anti-IgM-coated coverslips for 30 minutes and the relative size of the contact area compared. (Green, GFP; Red, F-actin). Scale bar: 10 µm. (**E**) Quantification of the mean contact area of J558αtrunc Cx43–GFP, J558Cβ Cx43–GFP and J558 µm3 Cx43–GFP cells (mean of three experiments; **P*<0.05; brackets indicate the comparisons being made; *n*=88 individual cells for J558αtrunc Cx43–GFP cells, *n*=177 individual cells for J558Cβ Cx43–GFP cells, *n*=109 for J558 µm3 Cx43–GFP cells).

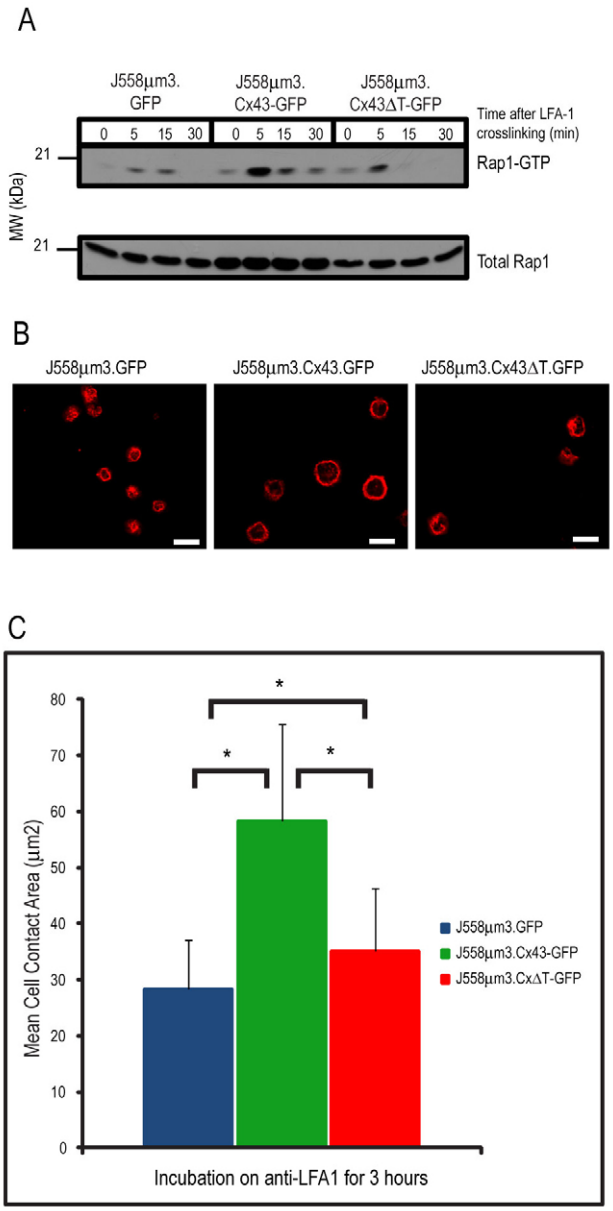


Fig. 5. Cx43–GFP expression enhances LFA-1-induced Rap1 activation and cell spreading. (**A**) J558 µm3 GFP, J558 µm3 Cx43–GFP and J558 µm3 Cx43ΔT–GFP cells were stimulated for the indicated times with soluble anti-LFA-1 and Rap1 activation was assessed as in Fig. 2. Representative western blots of three independent experiments. (**B**) B cell spreading on anti-LFA-1. Single confocal slice taken at the cell–coverslip interface of J558 µm3 B cells spreading on anti-LFA-1-coated glass coverslips (red, F-actin). Scale bar: 10 µm. (**C**) Mean contact area of B-cell spreading on anti-LFA-1 coverslips. The mean contact area of cells from three independent experiments was measured by quantifying the contact area between the cell and coverslip using ImagePro software (error bars represent s.e.m.; **P*<0.05; brackets indicate the comparisons being made; *n*=232 individual cells for GFP transfected cells, *n*=197 individual cells for Cx43–GFP transfected cells, *n*=136 for Cx43ΔT–GFP transfected cells).

was an increase in both the number of cells adhering to the monolayer and the size of the contact area between the B cell and endothelial cell when Cx43–GFP was expressed (Fig. 6C,D). When the transfected cell line expressing Cx43ΔT–GFP was similarly assayed, there was both an increase in adhesion and the size of the contact area between the B cell and endothelial cell compared with that in cells transfected with GFP alone, but not to the same extent as cells expressing wild type Cx43–GFP (Fig. 6C,D). The relative size of the footprint or contact area for cells expressing Cx43–GFP appeared to be larger than in GFP-only cells (Fig. 6D, top panels). To determine whether this increase in adhesion was specific only for the J558 µm3 plasmacytoma cell line, another Cx43-negative plasmacytoma cell line, 5TGM1 (Fig. 1A), was transfected with the same Cx43–GFP constructs. Similarly, 5TGM1 Cx43–GFP cells showed both an increase in adhesion (Fig. 6C, right columns) and in the size of the contacts with bEND.3 cells (Fig. 6D, bottom panels) compared with cells expressing GFP alone. Expression of Cx43ΔT–GFP in 5TGM1 cells failed to increase adhesion compared with wild-type Cx43–GFP (Fig. 6C,D). These results suggest that Cx43, and specifically the C-terminal domain, mediates signaling responses that are important for adhesion of B cells to endothelial cells.

Discussion

In this study, by using both loss- and gain-of-function approaches, we show that the gap junction protein Cx43 has a previously unappreciated role in B-cell spreading and adhesion. Cx43 becomes phosphorylated after BCR signaling, enhances signals required for sustained BCR-mediated activation of the Rap1 GTPase and cell spreading, as well as LFA-1-mediated Rap1 activation and spreading. Our results also show that the C-terminal tail of Cx43 is required for BCR-, LFA-1- and CXCL12-mediated Rap1 activation, spreading and adhesion to endothelial cells.

Immature and mature B lymphoma cell lines and normal splenic B cells, but not plasmacytoma tumor cell lines, express Cx43 (Fig. 1A). This differing expression pattern over a developmental time course is similar to that seen in neurons. In neuronal progenitors, which migrate during development, Cx43 levels are high. By contrast, Cx43 levels are low in later stages of differentiation where migration is no longer necessary (Rozental et al., 2000). In support of this, developing neurons require Cx43 expression to correctly migrate along radial glial cells in the cortex (Cina et al., 2009; Elias et al., 2010; Elias et al., 2007), indicating that Cx43 is required for normal neuronal development. Interestingly, Cx43 heterozygote mice show a decrease in the number of IgM-positive B cells, suggesting that Cx43 expression has a role in normal B-cell developmental processes (Montecino-Rodriguez and Dorshkind, 2001). This is consistent with the idea that a developing B cell interacts with stromal and endothelial cells during development, possibly with the assistance of connexin proteins. However, when B cells undergo differentiation into the plasma cell stage, Cx43 expression is reduced. The reported reduction in B-cell numbers seen in $Cx43^{+/-}$ mice, however, could be due to the role of Cx43 levels in the bone marrow stromal cells or other cells that affect B-cell development. A B-cell-specific knockout mouse would be required to resolve this issue.

In B cells, both cell–cell contact and cytoskeletal rearrangements are required for optimal adhesion, spreading, immune synapse formation and migration. Although these processes are regulated by different receptors, they have the same fundamental requirements of being facilitated by changes in the actin cytoskeletal architecture. BCR-mediated spreading results from the activation of cytosolic signaling proteins, many of which are also involved in Cx43 regulation. After BCR crosslinking, the ITAMs (phosphorylated immunoreceptor signaling motifs) of Igα and Igβ become

phosphorylated by Src-family kinases, leading to: (1) recruitment of the Syk tyrosine kinase; (2) recruitment and activation of the enzymes PLCγ2 and PI3K; and (3) activation of the Ras–Rac–Rap pathways (Burkhardt et al., 1991; Engels et al., 2001; Gold et al., 1992; Gold et al., 1990; Ishiai et al., 1999; Lin et al., 2008; McLeod et al., 1998; Rowley et al., 1995; Yamanashi et al., 1992). The cytoplasmic domain of Cx43 is also modified by phosphorylation. It has been shown to be phosphorylated by Src, MAP kinases and PKC, resulting in changes to the state of the pore and life cycle or turnover (Lampe et al., 2000; Loo et al., 1995; Sáez et al., 1997; Warn-Cramer et al., 1996). BCR activation does alter the apparent molecular mass of Cx43 in B cells (Fig. 1B); however, the residues that are modified in lymphocytes, as well as any functional consequences of the individual residues, have yet to be determined.

A likely possibility for how Cx43 is involved in the regulation of the B-cell cytoskeleton is that it is being used as an adaptor or scaffold protein, providing sites for protein interactions with regions of its cytoplasmic tail that are required for BCR signaling. In J558 µm3 cells, expression of Cx43 might allow these cells to activate normal B-cell signaling pathways, which are no longer required because of their developmental stage, resulting in their ability to activate Rap1 and initiate B-cell spreading. When a truncated form of Cx43 (Cx43ΔT–GFP) was expressed, there was less BCR-mediated cell spreading, suggesting that this region of Cx43 might be the site of interaction with proteins that regulate the arrangements of the actin cytoskeleton. We have shown that Cx43 expression does have a dramatic effect on the activation of Rap1, which is essential for B-cell spreading and immune synapse formation (Lin et al., 2008; McLeod et al., 2004). Because Cx43 has no intrinsic enzymatic activity, it is likely that it is acting as an adaptor or scaffolding protein, recruiting proteins involved in the regulation of Rap1. This function is partially abrogated when the C-terminal domain is deleted, resulting in the inability to maintain Rap1 activation and spreading in J558 cells. This also suggests that there is another domain within Cx43 that can act to enhance Rap1 activation, although the C-terminal domain is required for sustained Rap1 activation and spreading.

Cx43 might also act to stabilize the actin cytoskeleton by directly connecting to it, providing a stable platform from where spreading can be initiated. In astrocytes, Cx43 interacts with the scaffolding protein drebrin (Butkevich et al., 2004), which interacts with the cytoplasmic tail of Cx43 and stabilizes gap junctions by binding to the F-actin cytoskeleton. Although B cells have not been shown to express drebrin, they do express other members of the actin-binding protein (ABP) family drebrin-like proteins, including

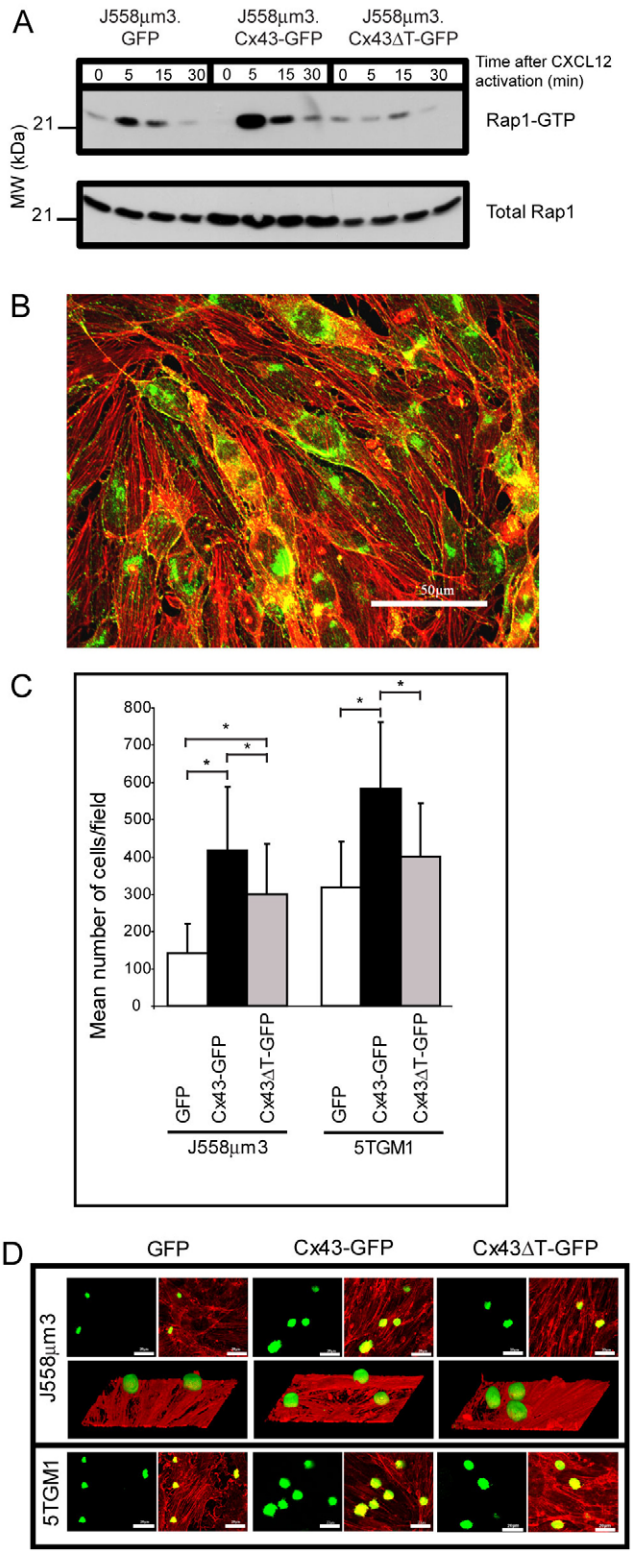


Fig. 6. Cx43–GFP expression enhances B cell adhesion to bEND.3 endothelial cells. (**A**) J558 µm3 GFP, J558 µm3 Cx43–GFP and J558 µm3 Cx43ΔT–GFP cells were stimulated for the indicated times with 100 nM CXCL12 before assaying for Rap1 activation. Representative western blots of three independent experiments. (**B**) Immunofluorescence of Cx43 expression in bEND.3 endothelial cells (3D reconstruction; green, Cx43; red, F-actin). Scale bar: 50 µm. (**C**) Quantification of adhesion to the bEND.3 monolayer (representative quantification of a single triplicate experiment, error bars indicate s.d.; *P<0.05; brackets indicate the comparisons being made). (**D**) Top panel shows transfected J558 plasmacytoma cells: single confocal images of the contact points between CellTracker Green CMFDA labeled cells and TNFα-activated bEND.3 endothelial cells. Below this are 3D reconstructions of the J558 cell lines adhering to the bEND3 monolayer (green, CMFDA-labeled B cells; red, F-actin). Scale bars: 20 µm. Lower panel shows transfected 5TGM1 plasmacytoma cells expressing the same Cx43 constructs as the J558 cells. Shown are single confocal images of the contact points between CellTracker Green CMFDA labeled cells and TNFα-activated bEND.3 endothelial cells showing that expression of only wild-type Cx43 leads to a large contact area between the plasmacytoma cells and the endothelial cell layer.

mABP-1. These adaptors might link Cx43 to the actin cytoskeleton in B cells.

It is also possible that Cx43 facilitates changes in the cytoskeleton by passing molecules through its pore through a hemichannel or gap junction. It has previously been reported that B cells can form functional Cx43 gap junctions with follicular dendritic cells within germinal centers (Krenacs et al., 1997). In our GOF system, Cx43–GFP and Cx43ΔT–GFP were probably present at the surface as a hexamer, as opposed to a monomer, where they are able to mediate their effects on Rap1 activation, B-cell spreading and adhesion. The assembly of Cx43 into a hexamer (or connexon) has been reported to occur in the *trans*-Golgi network before trafficking to the surface (Musil and Goodenough, 1993). Both Cx43–GFP and the C-terminal-truncated Cx43–GFP expressed in C6 glioma cells, were able to traffic to the membrane and were able to communicate, as determined by dye-preloading techniques (Bates et al., 2007). These results suggest that there is no gross defect in hexamer formation and trafficking using these GFP-tagged Cx43 constructs. Because our in vitro spreading assays test B-cell spreading independently of gap junction communication, this is probably not the mechanism by which Cx43 is involved in regulation of the B-cell cytoskeleton. We cannot rule out the possibility that Cx43 mediates these effects by a hemichannel-mediated mechanism; however, our results clearly indicate that the main contribution of Cx43 to cytoskeletal rearrangement is mediated though its C-terminal domain.

We also show that both LFA-1- and CXCL12-mediated Rap1 activation, spreading and adhesion to endothelial cells is enhanced by Cx43. B cells bind to the endothelium by engaging two major integrins, VLA-4 and LFA-1 (Ley et al., 2007). VLA-4 is required for slow rolling of B cells on the endothelium and LFA-1 is required for firm adhesion in preparation for transendothelial migration; however, a possible role for Cx43 in B-cell adhesion has not been investigated. In developing neurons in the brain, Cx43 has been shown to be required for migration from the ventricular zone to the cortical plate, where it was proposed that gap junction coupling mediates adhesion of neurons to the radial glial cells, facilitating normal migration; however, there are conflicting results for the role of the C-terminal domain (Cina et al., 2009; Elias et al., 2010; Elias et al., 2007). Our results show that the C-terminal domain of Cx43 enhances both LFA-1- and CXCL12-mediated Rap1 activation. We did, however, observe a transient increase in Rap1 activation, spreading and adhesion when the C-terminal domain was truncated. Because the small increase in LFA-1-mediated Rap1 activation and spreading was independent of gap junctional coupling, it is possible that there is another domain within Cx43 besides the C-terminal tail that is responsible for mediating this effect. One possible interpretation of the observed increase in adhesion of B cells expressing Cx43ΔT–GFP compared with GFP alone is that it is mediated by gap junction coupling. Truncation of Cx43 in *Xenopus* oocytes has previously been show to form gap junctions that are resistant to uncoupling (Homma et al., 1998), and coupling has been proposed as the mechanism of the effect of Cx43 on neuronal migration (Elias et al., 2007). Although this effect cannot be completely ruled out, the effect of Cx43 coupling on lymphocyte adhesion is probably negligible compared with integrin-mediated adhesion because blockage of integrin activity results in gross defects in adhesion and homing (Abram and Lowell, 2009; Berlin-Rufenach et al., 1999; Lo et al., 2003). Because of this, the more likely possibility in our system is that Cx43 regulates integrin-mediated functions, resulting in the observed changes in adhesion to endothelial cells.

In conclusion, we have shown that Cx43 is important for BCR-, LFA-1- and CXCL12-mediated Rap1 activation, resulting in enhancement of B-cell spreading and adhesion. This is the first report linking Cx43 to Rap1 activation, a key regulator of spreading, adhesion and migration. This link might explain the role of Cx43 in other cell types, and its emerging role as a regulator of neuronal and glial migration. The role of Cx43 in modulating BCR-, integrin- and chemokine-induced Rap1 activation and cytoskeletal rearrangement also makes it likely that Cx43 will have a key role in B-cell development, trafficking and activation.

Materials and Methods

Plasmids

Expression vectors encoding cDNAs for Rat wild-type Cx43 (NAP2-Cx43GFP) and Cx43 lacking the C-terminal domain (NAP2-Cx43Δ244-382GFP) have been described previously (Bates et al., 2007; Mao et al., 2000) (supplementary material Fig. S3A). Both vectors contained genes for GFP fused in-frame to the C-terminal end. The empty vector encoding only GFP (NAP2) has been described elsewhere (Mao et al., 2000). The expression vectors encoding the truncated Igα protein (αtrunc) and the Ig-α/β chimeric protein (Cβ) have also been described previously (Dylke et al., 2007; Jang et al., 2010).

Antibodies

Polyclonal goat anti-mouse IgM (μ-chain specific) and polyclonal anti-IgG (γ-chain specific) were from Jackson ImmunoResearch Labs (West Grove, PA). Polyclonal rabbit anti-Cx43, which recognizes an epitope in the C-terminal domain (amino acids 363–382), was from Sigma (Saint Louis, MO). The monoclonal antibody (mAb) Cx43NT1, which recognizes the Cx43 N-terminal region (amino acids 1–20) was purchased from the Fred Hutchinson Cancer Research Institute (Seattle, WA). Monoclonal mouse anti-human transferrin receptor was from Invitrogen Life Technologies (Carlsbad, CA). The rat-anti-mouse LAMP1 mAb (1D4B) was from the Developmental Studies Hybridoma Bank (Iowa City, IA). The mouse anti-phosphotyrosine mAb was from Upstate (Charlottesville, VA). Mouse anti-β-actin was from Sigma. The rabbit anti-Rap1A/1B was from Cell Signaling Technology (Danvers, MA). Goat anti-mouse IgG conjugated to horseradish peroxidase (HRP) was from Invitrogen (Burlington, Ontario), goat anti-rabbit IgG-HRP was from Jackson ImmunoResearch Labs and the Alexa-Fluor-647-conjugated goat anti-mouse IgG and Alexa-Fluor-568-conjugated goat anti-rat IgG were from Invitrogen Molecular Probes. Rhodamine-coupled phalloidin was from Invitrogen Life Technologies. ER-tracker dye and CellTrackerGreen (CMFDA) were from Invitrogen. The rat anti-LFA-1 mAb TIB213 was from ATCC (Manassas, VA).

Cells and cell growth

The J558 μm3 and J558 15-25 murine plasmacytoma cell lines (Justement et al., 1990) were obtained from Louis Justement (University of Alabama, Birmingham, AL). The WEHI 231 and A20 mouse B lymphomas, the MPC11 murine plasmacytoma and the mouse bEND.3 endothelial cell lines were obtained from ATCC. The murine plasmacytoma 5TGM1 (Oyajobi et al., 2003) was a gift from Babatunde O. Oyajobi (University of Texas, San Antonio, TX). Murine splenic B cells were isolated using a B cell selection kit (StemCell Technologies, Vancouver, Canada) as per the manufacturer's instructions. Cells were cultured in high glucose (4.5 g/l) RPMI-1640 (all B cell lines) or DMEM (bEND.3) supplemented with 2 mM L-glutamine, 110 mg/l sodium pyruvate, 10% heat-inactivated FBS, 50 U/ml of penicillin-streptavidin.

Transfection and retroviral transduction

To express BCR mutants, J558 cell lines were resuspended with 2 μg of the DNA construct (αtrunc or Cβ) and transfected using the Amaxa Nucleofection Kit T (Amaxa Biosystems, Gaithersburg, MD) and the G-016 settings for the Amaxa Nucleofector Device (Amaxa Biosystems). Drug-resistant populations were enriched for BCR-expressing cells by FACS using Alexa-Fluor-633-conjugated goat anti-mouse IgM. Retroviral transduction of WEHI 231 cells was performed as described (Krebs et al., 1999). To express Cx43 in B cell lines, supernatants of 293-GPG cells containing the retrovirally packaged NAP2–Cx43GFP, NAP2–Cx43Δ244-382GFP or NAP2–GFP plasmids (Bates et al., 2007) were incubated with 5×10^5 WEHI 231 or J558 μm3 cells overnight at 37°C. GFP-positive cells were isolated by FACS. Retroviral vectors encoding Cx43 shRNA constructs 1+2, as well as the corresponding scrambled sequence, have been described previously (Shao et al., 2005) and were used for retroviral transduction of WEHI 231 cells.

B-cell stimulation and preparation of cell extracts

Cells were washed with PBS, resuspended at 5×10^6 cells/ml in modified HEPES-buffered saline (25 mM sodium HEPES, pH 7.4, 125 mM NaCl, 5 mM KCl, 1 mM

$CaCl_2$, 1 mM Na_2HPO_4, 0.5 mM, 1 g/l glucose, 2 mM glutamine, 1 mM sodium pyruvate, 50 μM 2-ME), and stimulated with 20 μg/ml of goat anti-mouse IgM, 5 μg/ml anti LFA-1 or 100 nM CXCL12 (R&D systems, Minneapolis, MN). Reactions were stopped by adding 1 ml cold PBS. Cells were then lysed in cold lysis buffer [PBS, 1% Triton X-100, 1% IGEPAL (Sigma), 50 mM $CaCl_2$] (Troxell et al., 1999) containing protease inhibitors (10 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mM pepstatin A, 1 mM Na_3VO_4, 1 mM PMSF). To extract Cx43, cells were lysed using 200 μl of lysis buffer, sonicated for 10 seconds, and incubated at 37°C for 30 minutes before analysis by SDS-PAGE.

Dephosphorylation of Cx43 was performed as follows. WEHI 231 cells were resuspended to 1×10^7 cells in 500 μl quinsaline per time point. Cells were stimulated for 0, 5, 10, 15, 30, 60 and 120 minutes with 20 μg/ml of goat anti-mouse IgM at 37°C. Reactions were stopped using 500 μl cold PBS. Cells were centrifuged at 1500 rpm at 4°C for 2 minutes, then supernatants were removed and were lysed as previously described. Protein concentration of the cell lysates was determined using a BCA assay (Thermo Scientific Pierce, Rockford, IL). For each time point, lysates were split into two sets containing 30 μg of protein each, one subjected to dephosphorylation and the other used as a control. Lysates used as a control were suspended in 1× NEBuffer (New England BioLabs, Ipswich, MA). Dephosphorylation of the remaining lysates was carried out using 1× NEBuffer and 30 units of calf intestinal alkaline phosphatase (CIP) (New England BioLabs Inc, Ipswich, MA) as per the manufacturer's instructions. Lysates were incubated for 60 minutes at 37°C and subsequently suspended in 1× SDS-PAGE reducing sample buffer and incubated for 30 minutes at 37°C.

Intracellular localization of Cx43

Endoplasmic reticulum

J558 μm3 and WEHI 231 cells were incubated for 20 minutes in 1:1000 dilution of ER-Tracker as per the manufacturer's instructions (Invitrogen Life Technologies). Cells were then centrifuged at 1500 r.p.m. for 5 minutes, the supernatant removed and the cells resuspended in RPMI without Phenol Red. The cells were placed onto 35 mm glass-bottom microwell dishes (MatTek, Ashland, MA) for imaging on the Olympus FV1000 confocal microscope.

Lysosomes and early endosomes

Cells were fixed for 15 minutes with 8% paraformaldehyde, and then rinsed once with PBS. Incubation with a solution of PBS containing 0.5% Triton X-100 for 15 minutes on ice was performed to permeabilize cells, followed by two washes with a solution of PBS containing 3% BSA. Cells were incubated in a blocking solution of PBS with 3% BSA for 45 minutes, and then with appropriate dilutions made in PBS with 3% BSA of primary antibody against the intracellular compartments (anti-transferrin receptor or anti-LAMP1). The samples were incubated for 1.5 hours at 22°C or overnight at 4°C. Cells were washed three times with PBS with 3% BSA then incubated for 45 minutes with the appropriate secondary antibody diluted 1:500. This was followed by three more washes and then samples were resuspended in a solution of PBS with 5 mM EDTA in 35 mm glass-bottom microwell dishes (MatTek). All staining was performed with the cells in suspension. Images were acquired using the 60× or 100× planApochromat objectives (NA 1.35) of the Olympus FV1000 confocal microscope.

Rap1 activation assay

Rap1 activation assays were performed as described previously (McLeod et al., 1998). Briefly, a RalGDS–GST fusion protein bound to Gluthathione–Sepharose-4B beads was used to pull down the active form of Rap1, which was detected using an anti-Rap1A/B antibody (Cell Signaling Technologies). Total Rap1 was detected by separating 30 μl of lysate per time point by SDS-PAGE, before addition of RalGDS.

Cell-spreading assays

Cell-spreading assays were performed as described (Lin et al., 2008; Santos-Argumendo et al., 1994). Briefly, 12 mm glass coverslips were coated with 40 μg/ml goat anti-mouse IgM or 10 μg/ml anti-LFA-1 for 2 hours at 37°C or overnight at 4°C. J558 μm3 and WEHI 231 cells were incubated on these coverslips at 37°C and then fixed with 4% paraformaldehyde for 20 minutes at room temperature (RT). The cells were then permeabilized in PBS containing 0.5% Triton X-100. The samples were blocked with PBS containing 3% BSA for 30 minutes at RT then incubated with Rhodamine–phalloidin (1:40 in PBS plus 3% BSA) for 20 minutes at RT, rinsed three times in PBS, mounted onto a coverslip using ProLong Gold anti-fade reagent (Invitrogen). Cell contact sites at the cell–coverslip interface were imaged on an Olympus FlowView1000 confocal microscope. The contact area between the cell and coverslip of spread cells were quantified using ImagePro version 6.2 and the mean contact area was calculated. Spread cells were defined as having a contact area that extended as wide as the cell diameter and a radial ring of F-actin (~30% of cells by 30 minutes in fixed samples). In the event that no cells fitted this criterion, all cell contact areas were measured. Membrane projections were counted manually from compiled Z-Stacks of 0.5 μm step size images of 5–50 cells per time point (60× objective) using Olympus FV-Viewer.

To assess cell spreading in real time, J558 μm3 or WEHI 231 cells were labelled with CellTracker Green CMFDA and splenic B cells were labeled with CellMask Orange (Invitrogen Life Technologies) as per the manufacturer's instructions. The cells were deposited into glass-bottom microwell dishes (MatTek, Ashland, MA) that were coated with goat anti-mouse IgM, and placed in a 37°C incubation chamber mounted on the microscope stage. The cell–coverslip interface was imaged every 20 seconds for 20 minutes. Cell contact sites of individual spreading cells were quantified at each time point using Image Pro version 6.2. In this assay, spreading cells were defined as remaining in one stationary position and having a contact area that changes in shape and size.

Scanning electron microscopy

The cell spreading assay was performed as previously described (Lin et al., 2008). Samples were prepared for SEM using standard procedures at the UBC Bioimaging Facility.

B-cell adhesion to bEND.3 cells

Glass coverslips were coated with 1 μg/ml bovine fibronectin in PBS for 1 hour at RT before plating bEND.3 cells. After 4–7 days, the bEND.3 cells were activated overnight with 10 ng/ml TNF-α (eBioscience, San Diego, CA). J558 μm3 and 5TGM1 cells (4×10^5) were pre-activated for 1 hour with 100 ng/ml CXCL12, labeled with CellTracker Green CMFDA, and then allowed to adhere to the endothelial cell layers. After 1 hour at 37°C, non-adherent cells were gently removed by washing and the remaining cells were fixed for 10 minutes with 4% paraformaldehyde and stained as described for B-cell spreading assays. The cells were imaged using a 10× objective and the number of CMFDA-positive cells per field was determined using Image Pro version 6.2 software. Three-dimensional reconstructions were generated using Olympus 3D FluoView Version 1.7 reconstruction software.

Statistics

A comparison of means was assessed using a Student's unpaired, two-tailed *t*-test.

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