Langerhans Cells and Lymph Node Dendritic Cells Express the Tight Junction Component Claudin-1

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Claudin-1 is a critical structural component of tight junctions that have an important role in adhesive properties, barrier function, and paracellular transport of epithelia and other nonhematopoietic tissues. We found claudin-1 in murine CD207+ Langerhans cells (LC) residing in epidermis. Claudin-1 was not detected in other skin dendritic cells (DC). LC expressed claudin-1 in steady state and inflamed skin. Claudin-1 was demonstrated further in lymph node LC under steady state and inflammatory conditions, including after direct tracking with tetramethylrhodamine-isothiocyanate (TRITC). All subsets of skin draining lymph node DC defined by CD205, CD11b, CD11c, and CD8, including a presumably blood-borne lymph node resident CD8+CD207+ LC population, were claudin-1+. TRITC tracking demonstrated claudin-1 in CD207− skin migrant DC in the lymph node, suggesting upregulation of this molecule during migration or once arrived in the lymph node. Claudin-1 expression in CD207+ cells was confirmed at the protein and mRNA levels. Transforming growth factor-β, a factor critical for the induction of LC *in vitro* and *in vivo*, stimulated the accumulation of claudin-1 mRNA and protein when added to bone marrow cells cultured with GM-CSF and IL-4. Claudin-1 may thus have an important function in adhesion and/or migration of LC.

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INTRODUCTION

Langerhans cells (LC) are regarded as a subpopulation of dendritic cells (DC) that are critical for the activation of naïve and resting memory T cells *in vivo* (Romani *et al.*, 2003; Valladeau and Saeland, 2005). LC differ from other DC subsets by a number of surface markers notably CD207. CD207 is a lectin-type receptor expressed on the surface of LC that can internalize ligands into characteristic intracellular tubulovesicular compartments termed ''Birbeck granules,'' the ultrastructural hallmark of LC (Valladeau *et al.*, 1999, 2000, 2002; Hunger *et al.*, 2004). CD207+ cells are found in stratified epithelia such as epidermis, in secondary lymphoid organs including lymph nodes and spleen, and in the lung. LC may also differ from other DC subpopulations in function.

To understand divergent functions in LC and other DC subsets, we performed a microarray analysis with LC-like cells and myeloid DC-like cells induced *in vitro* from human monocytes with a method described by Arrighi *et al.* (2003). Claudin-1 scored higher in LC than DC, a result confirmed by quantitative reverse transcriptase-PCR (RT-PCR) (unpublished results).

Claudin-1 is a structural component of tight junctions (Tsukita *et al.*, 2000; Anderson *et al.*, 2004; Turksen and Troy, 2004). It is a 23-kDa membrane protein with four transmembrane spanning regions, two extracellular loops, and intracellular N and C terminus. It belongs to a large family of claudin molecules with at least 24 members. Intracellular C terminus vary among the various claudin members. The extracellular domains of the claudins interact differentially with each other. Cellular claudin-1, for example, can associate with itself and claudin-3 but not with claudin-2 in neighbouring cells (Furuse *et al.*, 1999). Claudin-1 is critical in the formation of tight junctions. Fibroblasts transfected with its gene adhere to each other by forming tight junction structures (Furuse *et al.*, 1998; Kubota *et al.*, 1999). Claudins are not only involved in cellular adhesion but may also regulate other processes such as paracellular transport. Claudin-1 is expressed in keratinocytes forming the living layers of murine epidermis and is responsible for the physical barrier function that prevents the transcutaneous loss of water and desiccation, at least in newborn mice (Furuse *et al.*, 2002).

We decided to investigate the expression of claudin-1 in murine LC. To this end, we characterized claudin-1 in cutaneous and lymph node LC and DC from skin-draining lymph nodes under steady-state and inflammatory conditions. We investigated claudin-1 in cutaneous LC and DC

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Abbreviations: Ab, antibody; DC, dendritic cells; DNCB, dinitrofluorobenzene; EGFP, enhanced green fluorescent protein; Flt3-ligand, FMS-like tyrosine kinase 3 ligand; LC, Langerhans cells; m, monoclonal; MHC, major histocompatibility complex; p, polyclonal; RT-PCR, reverse transcriptase-PCR; TRITC, tetramethylrhodamine-isothiocyanate

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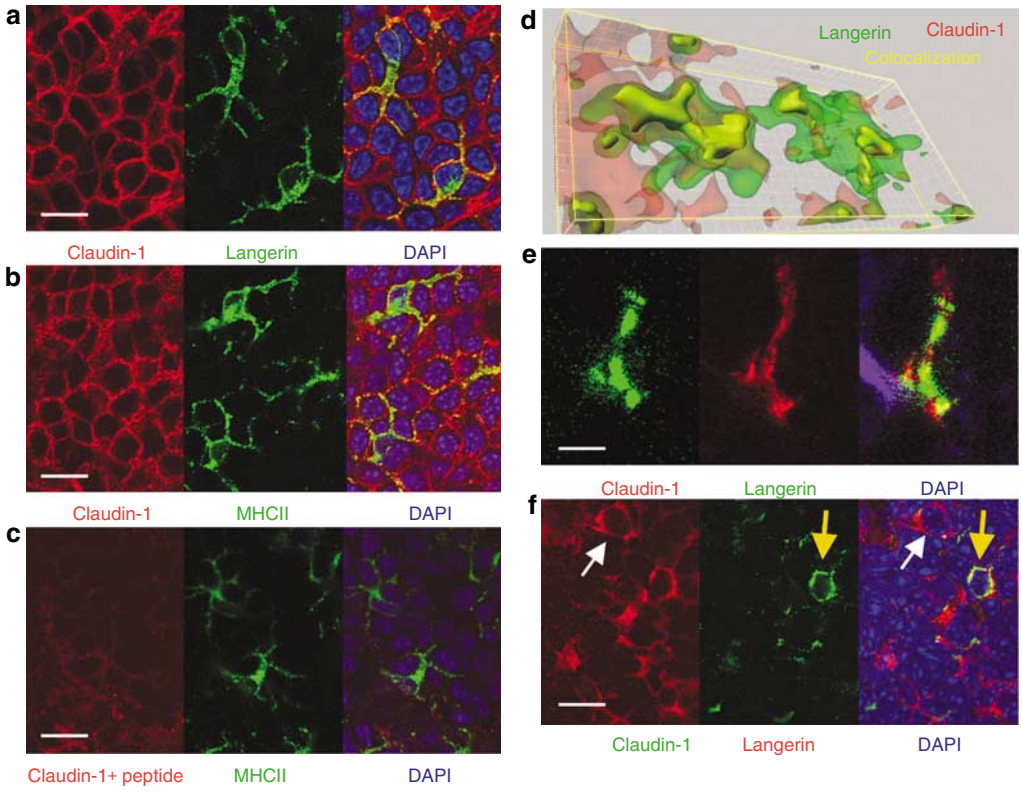

Figure 1. Epidermal and lymph node LC express claudin-1 *in situ*. (**a**–**c**) Epidermal sheets, (**e**) skin, or (**f**) lymph node sections were stained with mAb to CD207 (929F3) or MHC class II (green, red in panel e) and a pAb to claudin-1 (red, green in panel e) pre-absorbed or not with the relevant peptide (+ peptide) corresponding to intracellular epitopes of claudin-1. The sections and sheets were counterstained or not with DAPI. DAPI, DAPI counterstaining plus green and red fluorescence overlay. (**d**) Three-dimensional reconstruction of epidermal sheet stained as in panel a. (**e**) CD207 + claudin-1 + cell (yellow arrow); CD207−claudin-1 + cell (white arrow). Bar = 8.2 μm in panels a and c, 8.4 μm in panel b, and 6.6 μm in panel e and 9.1 μm in panel f.

labeled by epicutaneous application of tetramethylrhodamine-isothiocyanate (TRITC) and in labeled cells that have migrated to draining lymph nodes. Further, we employed a bone marrow culture system that permits the induction of CD207 + LC-like cells and LC that have migrated from skin in organ culture.

RESULTS

Claudin-1 in epidermal LC

Epidermal sheets from steady-state epidermis and skin sections were stained with polyclonal (p) antibody (Ab) to intracellular C-terminal determinants of claudin-1 and mAbs to histocompatibility complex (MHC) class II or CD207 (langerin, clone 929F3) and analyzed by confocal laser microscopy. The claudin-1 Ab labeled interkeratinocyte boundaries as expected. In addition, claudin-1 staining was also present at sites of dendrites and at perinuclear location of LC (Figure 1a, b, and e), suggesting that LC express claudin-1 epitopes. Isotype and normal rabbit serum controls were negative. Secondary antibodies (anti-rabbit and anti-rat) did not cross-react across species barriers (rat and rabbit). When a peptide corresponding to the relevant intracellular C-terminal claudin-1 epitopes was incubated with the anti-claudin pAb before immunostaining, signals were reduced to background (Figure 1c). These results confirmed the specificity of the immunohistologic labeling. The staining pattern of keratinocytes and LC with the claudin-1 pAb was also demonstrated in three-dimensional reconstructions of epidermis (Figure 1d).

To confirm these results, trypsinized epidermal cell suspensions were labeled with CD207 (clone 205C1) and major MHC class II m Ab combinations. Claudin-1 was detected with a mAb, in addition to the pAb. Cells were analyzed by flow cytometry. The vast majority of CD207 + and MHC class II + cells were claudin-1 + (Figure 2a). These results were reproducible with another mAb to CD207 (clone 929F3, data not shown). Claudin-1 staining in both CD207 + and CD207− cells was abolished by pre-incubation of the mAb (Figure 2b) and the pAb (data not shown) with the C-terminal peptide but not with an irrelevant peptide, indicating that it included epitopes detected by the pAb and the mAb. A subpopulation of CD207− and MHC class II cells (keratinocytes) labeled with the anti-claudin-1 Abs as expected (Figure 2a and b). CD11c + and/or CD11b + CD207− or MHC class II + CD207− cells prepared freshly from the dermis were negative for claudin-1 (Figure 3). When dispase was used for dermoepidermal separation, the number of CD207 + cells recovered from steady-state dermis was too low to make a reliable statement regarding claudin-1 expression. When we used trypsin for dermoepidermal

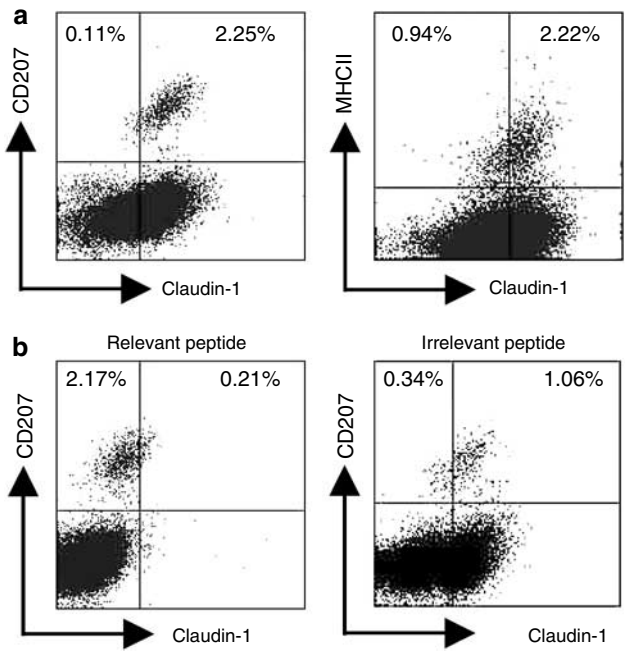

Figure 2. Flow cytometric demonstration of claudin-1 in epidermal LC. Fixed and permeabilized epidermal cell suspensions were stained with mAb to CD207 (205C1) or MHC class II and claudin-1. Results are representative for at least three experiments. Relevant peptide, preabsorption with relevant peptide. Irrelevant peptide, preabsorption with irrelevant peptide.

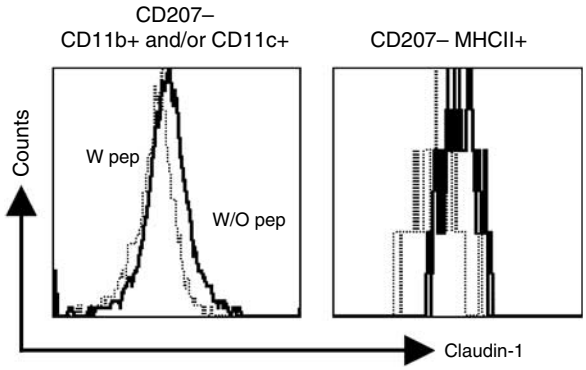

Figure 3. Claudin-1 is not expressed in dermal DC. Freshly prepared dermal cells from steady-state skin were stained with mAb to CD207, CD11b, CD11c, and claudin-1 or to CD207, MHC class II, and claudin-1. Results are representative for at least three experiments. Relevant peptide, preabsorption with relevant peptide.

separation, many CD207 + claudin-1 + cells were observed but may represent contamination from epidermal (follicular) LC as described (Tse and Cooper, 1990, data not shown).

Claudin-1 in LC of skin-draining lymph nodes

Sections of skin-draining lymph nodes were labeled for CD207(929F3) and claudin-1 with the pAb. Lymph node LCs were identified as large cells with dendrites among smaller cells (lymphocytes) as described. CD207 + cells labeled for claudin-1 in variable intensity (Figure 1f). We also detected dendritically shaped claudin-1 + cells, which were clearly CD207− (Figure 1f, white arrow), suggesting that DC other than LC in the lymph node express claudin-1.

Claudin-1 expression in skin-draining lymph nodes was further studied by flow cytometry. CD207 + (205C1 or 929F3) lymph node cells were specifically labeled with the mAb to claudin-1 (Figure 4a and b). Douillard *et al.* (2005) described recently CD8 + CD207 + cells in lymph nodes that may correspond to lymph node-resident LC derived from the blood, in addition to the CD8− skin-derived LC. To see whether both subsets of CD207 + lymph node LC express claudin-1, CD4 and B220-depleted lymph node cells were additionally labeled for CD8. Both CD8 + and CD8− CD207 LC in Balb/C mice were claudin-1 + (Figure 4c and d). These results were confirmed in low-density lymph node cells from C57BL/6 mice (data not shown).

To further characterize the CD207− cells in the lymph node that were claudin-1 +, we stained low-density lymph node cells with CD207(205C1), CD8, CD11b, and CD11c or CD207, and CD205 mAb. CD11c + but not CD11c cells stained for claudin-1 (Figure 4e). We found most CD207 + cells in the CD11b intermediate CD11c intermediate fraction (data not shown) as described by Iezzi *et al.* (2006). All CD11b + and/or CD11c + CD207− cell fractions specifically stained for claudin-1 (Figure 4f). CD205 + CD207− and the CD8 + CD11c + cells were also claudin-1 + (Figure 4g).

We next wanted to confirm claudin-1 protein in LC. To this end, CD207 + lymph node cells from transgenic mice expressing enhanced green fluorescent protein (EGFP) under the control of the CD207 promoter (Kissenpfennig *et al.*, 2005) were sorted and subjected to Western blotting. The blots showed positive and specific signals at the 23 kDa region with the pAb to claudin-1 in EGFP + -sorted lymph node cells (Figure 5a), confirming the presence of claudin-1 protein in lymph node LC.

Claudin-1 in bone marrow cultures

As reported by Valladeau *et al.* (2002), we found CD207 (205C1) + cells in a subpopulation of murine bone marrow cell cultures supplemented with GM-CSF, IL-4 and transforming growth factor (TGF)-β with or without FMS-like tyrosine kinase 3 ligand (Flt3-ligand). Western blotting with the pAb to claudin-1 confirmed the presence of this protein in these cultures (Figure 5b). Cultures without TGF-β produced a much weaker signal for claudin-1 in three independent experiments, indicating that this cytokine-induced accumulation of claudin-1 protein. A subpopulation of CD207 + and CD207− cells specifically labeled for claudin-1 by flow cytometry (Figure 6). Similar results were obtained with another mAb to CD207 (929F3, data not shown). RT-PCR of cultured cells revealed positive signals for claudin-1 but not other epithelial claudins (claudin-3, -4, -6, -8, and -12, Turksen and Troy, 2004; Figure 5c). Addition of TGF-β leads to a stronger claudin-1 band, indicating that this cytokine also induced accumulation of claudin-1 at the mRNA level. There was no effect of Flt3-ligand on claudin-1 expression. Epidermal cells and low-density lymph node cells were

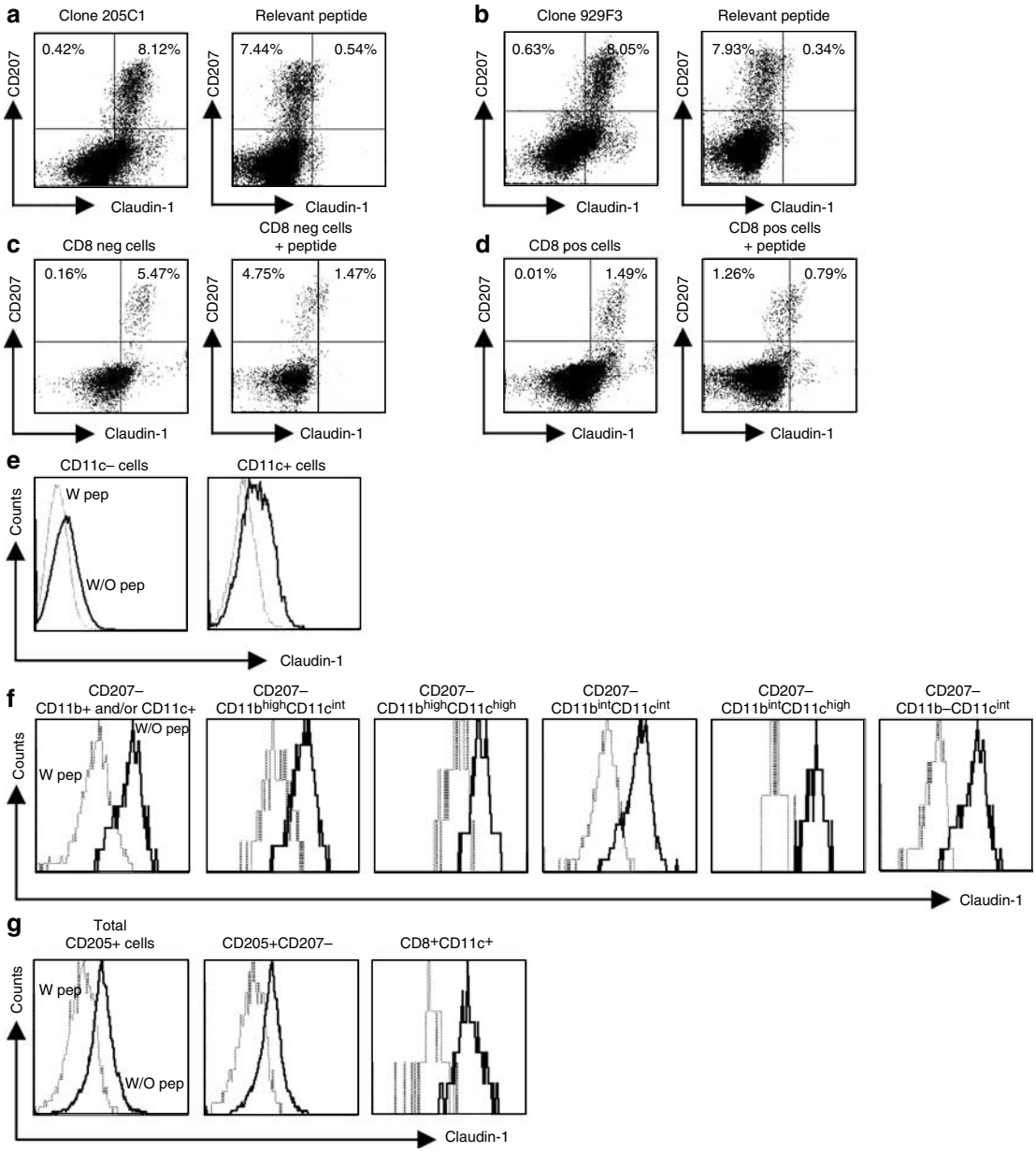

Figure 4. Claudin-1 is expressed in skin-draining lymph node LC. (**a** and **b**, and **e**–**g**) Fixed and permeabilized low-density, skin-draining lymph node cells or (**c** and **d**) CD4 and B220-depleted lymph node cells were stained with mAb to CD207 (205C1) and claudin1 and/or CD8, CD11b, CD11c, CD205. (**c** and **d**) Balb/C strain mice were used. Results are representative for at least three experiments. + Peptide, preabsorption with relevant peptide; W pep, preabsorption with relevant peptide; W/O pep, without peptide preabsorption.

positive for claudin-1 but negative for claudin-3 mRNA (Figure 5d).

Claudin-1 in migrating cutaneous DC

LCs migrate out of skin in organ culture. We used this culture system to characterize claudin-1 expression. After 2 (Figure 7a) or 3 (data not shown) days of culture, CD207+ (205C1) cells stained with claudin-1. The vast majority of CD11c+CD207− cells, referred to as migrating dermal DC cells (Stoitzner *et al.*, 2003), did not label for claudin-1 (Figure 7a). A minor claudin-1+CD11c−CD207− subset was observed under these conditions (Figure 7b).

Claudin-1 under inflammatory conditions

We asked whether claudin-1 may be modulated in CD207+ cells under inflammatory conditions. Murine ear skin was painted with DNFB. After 6 hours, the animals were killed and epidermis was separated from dermis by trypsin or dispase, and epidermal and dermal cells were prepared. Epidermal LC identified with CD207 (205C1; Figure 8) or MHC class II (data not shown) were claudin-1+. Claudin-1 signals in CD207+ cells under these inflammatory conditions did not differ from steady state.

LCs migrate to skin-draining lymph nodes at increased numbers under inflammatory conditions (Stoitzner *et al.*,

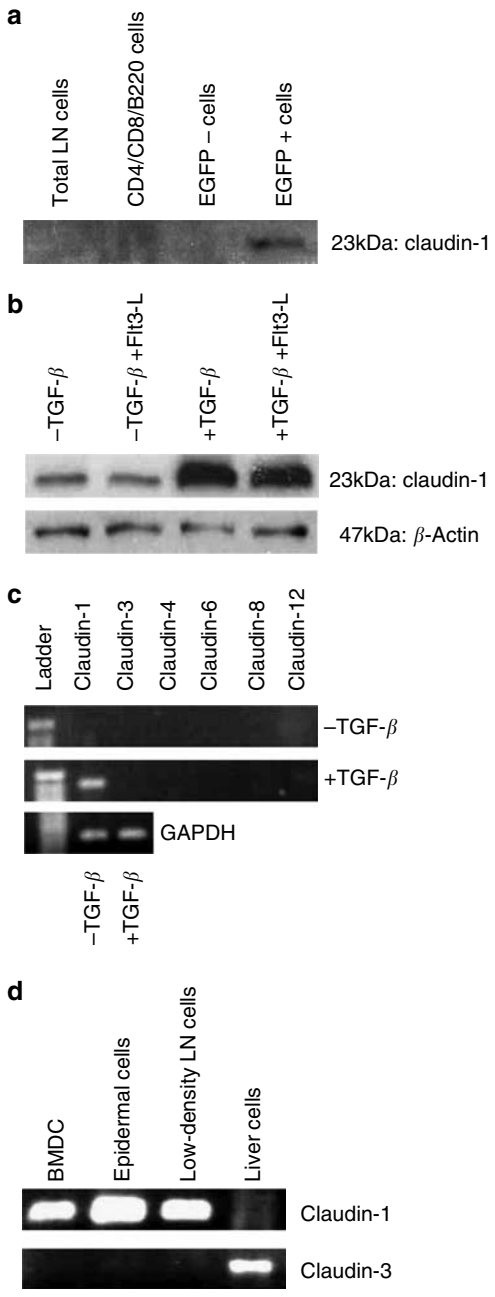

Figure 5. Claudin-1 protein and mRNA in LC. Sorted EGFP+ (2×10^4) and EGFP cells (2×10^5) from CD4, CD8, and B220-depleted, total (total LN cells) skin-draining lymph node cells and enriched cells (CD4/CD8/B220 cells, 2×10^5) from (**a**) langerin/EGFP transgenic mice (**b**) and bone marrow cells cultured in GM-CSF, IL-4, with or without Flt3-ligand, (**b** and **c**) with or without TGF-β, (**d**) epidermal cells, or (**d**) low-density lymph node cells were subjected to (**a** and **b**) Western blotting or (**c** and **d**) RT-PCR. (**a** and **b**) Claudin-1 signals in Western blots from the 23 kDa region, β-actin signals the 47 kDa region. RT-PCR signals for claudin-1 after 35 cycles, RT-PCR signals for GAPDH after 25 cycles.

2003) and can be tracked with epicutaneously applied fluorochrome. To investigate claudin-1 expression in LC after migration from skin, we used the transgenic mice expressing EGFP under the control of the CD207 promoter. Abdominal

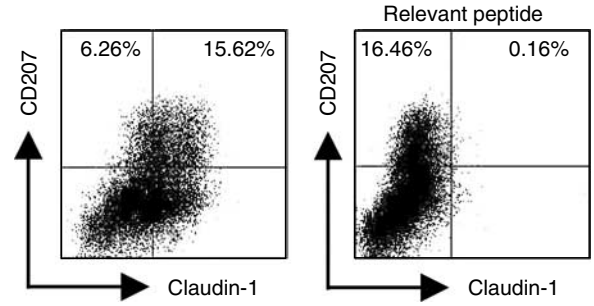

Figure 6. Claudin-1 in LC-derived from bone marrow. Bone marrow-derived LCs were stained for claudin-1, CD207, and CD11c. Results are representative for at least three experiments. Relevant peptide, preabsorption with relevant peptide.

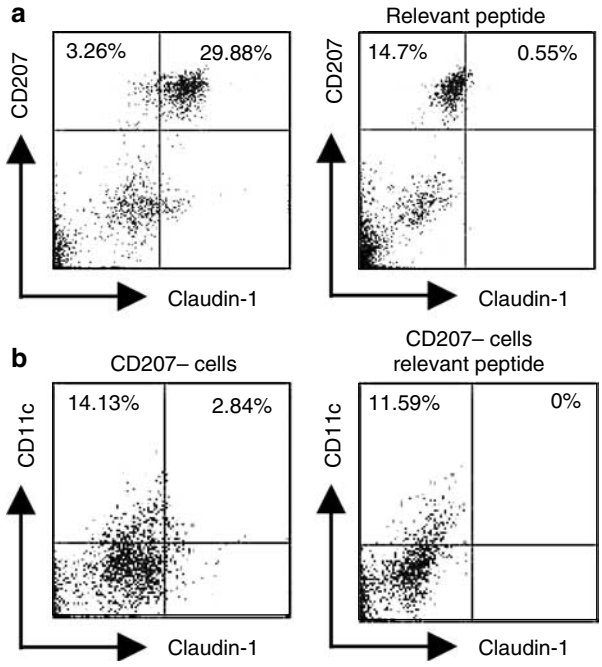

Figure 7. Claudin-1 in DC migrating from skin organ cultures. DC migrating from skin organ cultures after 2 days were stained with mAb to CD207, claudin-1, and CD11c. Results are representative for at least three experiments. Relevant peptide, preabsorption with relevant peptide.

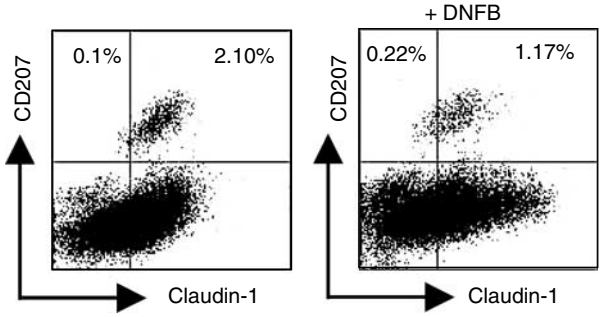

Figure 8. Claudin-1 in epidermal LC after DNFB painting. Epidermal cells were stained with mAb to CD207 and claudin-1 6 hours after DNFB painting. Results are representative for at least three experiments. + DNFB, after DNFB painting.

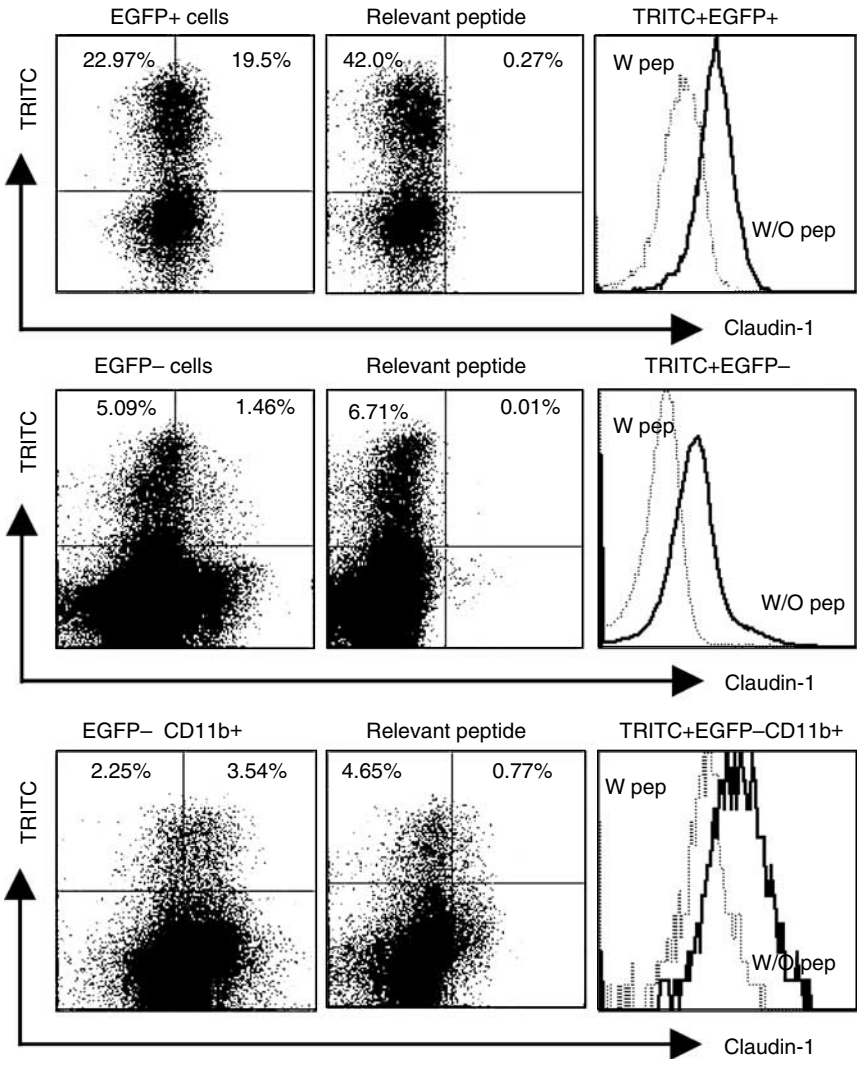

Figure 9. Claudin-1 is expressed in TRITC-traced LC and CD11b+CD207− cells after migration to the lymph node. Low-density lymph node cells from langerin-EGFP transgenic mice were stained with mAb to claudin-1 and CD11b 3 days after TRITC painting. Results are representative of three experiments. Relevant peptide, preabsorption with relevant peptide.

skin of naïve mice was painted with the fluorochrome TRITC and skin-draining lymph nodes were analyzed after 3 days (Kissenpfennig *et al.*, 2005). A subpopulation of TRITC+EGFP+ and TRITC+EGFP− including TRITC+CD11b+EGFP− lymph node cells was positive for claudin-1, demonstrating that skin-derived LC continue to express claudin-1 (Figure 9). These results were confirmed with lymph node cells from nontransgenic mice labeled with the CD207 mAb 205C1 (data not shown). EGFP+ or CD207+TRITC+ and EGFP+ or CD207+TRITC− cells, respectively, exhibited the same staining intensity for claudin-1 (data not shown), suggesting that TRITC uptake and transport to lymph nodes by LC was not associated with significant modulation of this protein. These experiments showed further the presence of TRITC+ non-LC in the lymph node, among them CD11b+ cells of which a subpopulation is claudin-1+.

DISCUSSION

The *in situ* demonstration of claudin-1 in epidermal LC was complicated by expression of this molecule by neighboring keratinocytes. The fixation method used for epidermal sheets or skin sections may induce artifacts making the precise assignment of immunolabel to LC or keratinocytes difficult, although the confocal images and the three-dimensional reconstructions were very suggestive (Figure 1a–e). Flow cytometry, however, clearly indicated the presence of claudin-1 epitopes in LC (Figure 2a and b). Any nonspecific binding was ruled out by absorption with a peptide corresponding to the relevant intracellular C-terminal claudin-1 epitopes. The relevant peptide inhibited the binding of both the pAb and the mAb, indicating that the peptide contained epitopes detected by both reagents. The pAb but not the mAb are known to cross-react with claudin-3. We found no claudin-3 mRNA by RT-PCR in murine epidermal

cells or low-density lymph node cells (Figure 5d). Together, this strongly suggests that the observed immunolabeling was detecting claudin-1 epitopes.

The expression of claudin-1 by a subpopulation of keratinocytes also rendered difficult the molecular demonstration of claudin-1 in LC. As it was impossible in our hands to physically separate LC and keratinocytes to sufficient purity, we used two different approaches. One was to employ transgenic mice in which EGFP is expressed under the control of the promoter of CD207 (Kissenpfennig *et al.*, 2005) and cell sorting of lymph node cells devoid of epithelial cells. The other approach consisted in the use of bone marrow cultures that generate CD207+ LC-like cells. Both approaches were successful for the demonstration of claudin-1 protein (Figure 5a and b). We used EGFP+ and EGFP− sorted CD4, CD8, and B220-depleted lymph node cells to perform Western blotting. Claudin-1 protein was identified in the EGFP+ but not in the EGFP− cell fraction. Presumably, the frequency of non-LC lymph node DC in the EGFP− cell fraction was too low to detect claudin-1 signals. In bone marrow-derived LC cultures, we identified CD207+claudin-1+ as well as CD207−claudin-1+ cells (Figure 6). Under the conditions used here, bone marrow cultures may generate multiple DC subsets and precursors that may not have acquired CD207. Nonetheless, this culture system permitted us to demonstrate claudin-1 mRNA and protein accumulation in response to TGF-β (Figure 5b and c), the cytokine that is critical for the induction of epidermal LC (Borkowski *et al.*, 1996, 1997; Strobl *et al.*, 1996). The addition of Flt3-ligand did not modulate claudin-1 levels (Figure 5b; data not shown). The RT-PCR experiments were positive for claudin-1 but no other epidermal claudin, indicating restricted expression of claudin family genes in LC. Cell sorting with the CD207 mAb 205C1 coupled to Alexa488 was not possible in our hands without permeabilization of cells when we used epidermal, lymph node, or bone marrow culture cells, although the manufacturer claims that this Ab detects an extracellular CD207 epitope. Our results with another CD207 mAb, (clone 929F3) however, closely correlated with data obtained with 205C1 in epidermal cell suspensions, bone marrow cultures and lymph nodes when analyzed in permeabilized cells (data not shown). In agreement with a previous report (Douillard *et al.*, 2005), the presence of a small subpopulation of 929F3+205C1− cells aside from a larger 929F3+205C1+ population in lymph node cells was confirmed in our hands (data not shown).

Claudin-1 expression in epidermal LC was not grossly modulated 6 hours after DNFB painting (Figure 8). Further, TRITC+ and TRITC−EGFP+ (or TRITC+CD207+ and TRITC−CD207+) cells in the lymph node had the same claudin-1 staining intensity (data not shown). Together with the clear claudin-1 signal in LC that migrated from skin to the culture dish (Figure 7a), this suggests that claudin-1 is not grossly modulated by migration and/or inflammatory stimuli. CD207+CD8− cells in skin-draining lymph nodes are thought to represent epidermal LC that have migrated. We demonstrated claudin-1 in these cells under steady-state and inflammatory conditions. We used the fluorochrome TRITC to track LC in lymph nodes using either transgenic mice expressing EGFP under the control of the langerin promoter (Figure 9) or CD207 mAb to identify LC (data not shown), permitting the formal demonstration that migrating LC express claudin-1 under inflammatory conditions (TRITC skin painting) in the draining lymph node with both approaches.

Although claudin-1 was not found in other cutaneous antigen-presenting cells than LC, we found other DC subsets in the lymph node expressing claudin-1. CD207+CD8+ cells in the lymph node are thought to represent blood-borne nonmigrating, lymph node resident LC (Douillard *et al.*, 2005). These cells clearly expressed claudin-1 epitopes (Figure 4d). Claudin-1 expression in LC may thus not be associated with skin origin. The CD207−CD8+ cell fraction staining for claudin-1 (Figure 4d) may contain CD8+CD11c+ blood-borne, resident lymph node DC with high capacity to cross-present antigen (Shortman and Liu, 2002). Claudin-1 expression was demonstrated in this latter subset (Figure 4g). All cell fractions defined by CD11b and CD11c or CD205 among CD207− DC were claudin-1+ (Figure 4e and f). This indicated that most DCs in skin-draining lymph nodes express claudin-1 under steady-state conditions. The fact that CD11b+CD207− cells were claudin-1+ (Figure 4f) suggested that DC migrating from dermis under noninflammatory conditions increase their claudin-1 protein because dermal DC did not express detectable claudin-1. CD11b is considered as a marker of skin migration in draining lymph node DC (He *et al.*, 2006). This was supported by the observation that all CD205+CD207− lymph node cells were claudin-1+ (Figure 4g). Aside from lymph node-resident CD8+CD11c+ lymph node DC, skin migrating DC, including LC and dermal DC, express CD205 (He *et al.*, 2006). Furthermore, the TRITC tracking experiment directly demonstrated that TRITC+CD11b+CD207− DC in the lymph node expressed claudin-1 (Figure 9). It is, thus, possible that CD207− DC migrating from skin upregulated claudin-1 expression also under inflammatory conditions, once their migration to the lymph node has been initiated or completed. Candidates are dermal DC that are resident in steady-state skin (Stoitzner *et al.*, 2003) or blood-borne monocytes that infiltrate inflammatory foci in skin, which can differentiate into DC-like cells and migrate to the draining lymph node (Randolph *et al.*, 1999; Le Borgne *et al.*, 2006). Our attempts to characterize claudin-1 in dermal antigen-presenting cells 6 hours after DNFB yielded nonreproducible results (data not shown). Thus, it can not be ruled out that claudin-1 was induced under inflammatory conditions in the dermis before migration to lymph nodes.

Claudin-1 was identified recently in a DC subset in the lung expressing CD207 (Sung *et al.*, 2006). This subset was CD103+ and may correspond to migrating DC. Epidermal LC *in situ* do not express CD103 (Pribila *et al.*, 2004). In our hands, bone marrow-derived CD207+ cells were also negative for CD103 (data not shown) and only a minor subset of CD207+ cells in skin-draining lymph nodes was CD103+ (data not shown). Thus, the relationship between CD207+CD103+claudin-1+ DC in the lung and the claudin-1+ LC described here is currently not clear.

In other reports, expression of claudin-1 in DC was associated with other tight junction proteins (Rescigno *et al*., 2001; Pribila *et al*., 2004). The technical difficulties in localizing precisely tight junction proteins in LC in the epidermis, the lack of specific peptides for inhibition of Ab binding to some of the tight junction proteins, and the cell separation issue precluded a systematic search for these molecules. Rescigno *et al*. (2001) have shown constitutive and inducible expression of several tight junction proteins (ZO-1, occluding, and claudin-1) in a DC line that can send dendrites across an epithelial monolayer, and thus internalize and transport bacteria transepithelially. Pribilia *et al*. (2004) demonstrated ZO-2, an intracellular protein associated with transmembrane proteins of the tight junction, in addition to claudin-1 and -7 in a subset of CD103 + CD207 + DC in the lung. We have preliminary data supporting the expression of ZO-2 in epidermal LC (data not shown).

We screened 12 murine epidermal LC *in situ* by transmission electron microscopy and were unable to identify tight junction-like structures. The characterization of the adhesive functionality of the extracellular domains of claudin-1 is complicated by the fact that it can interact with numerous other members of the claudin family based on its amino-acid sequence similarity. Trypsin may have digested the extracellular domain in freshly prepared epidermal cells. The conserved structure of the extracellular loops of claudins is also probably the reason for the lack of Ab to these domains. Mice with genetic deletion of the claudin-1 gene may be helpful in elucidating its functionality and function in LC and lymph node DC (Furuse *et al*., 2002), although they die in the neonatal period owing to epidermal barrier dysfunction.

In conclusion, we report here the expression of the tight junction protein claudin-1 in LC in the epidermis and after migration to skin-draining lymph nodes under steady-state and inflammatory conditions. All non-LC DC subsets in skin-draining lymph nodes, including non-LC DC that have migrated from skin, expressed claudin-1. Thus, claudin-1 in DC is independent of intraepithelial location and may be involved in migration to lymph nodes, or have functions within the lymph node.

MATERIALS AND METHODS

Mice

C57BL/6, BalbC, or C3H mice were purchased from the animal facility at the Centre Médicale Universitaire (Geneva, Switzerland) or Charles River (l'Arbresle, France) and used at 4–16 weeks of age. For some experiments, Langerin-EGFP knock-in mice were used (kind gift from Dr Adrien Kissenpfennig, Belfast, UK). The experiments were approved by the State Veterinarian's Office (Geneva, Switzerland) and performed according to the Federal Law (Switzerland).

Cell preparations

Bone marrow-derived DC/LC. Bone marrow cells were cultured for 7–8 days in Iscove's medium (Sigma, St Louis, MO) supplemented with 100 U/ml penicillin, 0.1 mg/ml streptomycin, 2 mM L-glutamine solution, 0.05 mM β-mercaptoethanol, and 10% fetal bovine serum (all from Sigma) in presence of 25 ng/ml IL-4 (R&D, Minneapolis, MN or ImmunoTools, Friesoythe, Germany) and 25 ng/ml GM-CSF (R&D or ImmunoTools). Cells were either grown in presence or absence of 8 ng/ml TGF-β (Strathmann Biotec, Hamburg, Germany) and 10 ng/ml Flt3-ligand (R&D or ImmunoTools).

Lymph node DC/LC. Superficial inguinal, axillary, and brachial LN were digested for 30 minutes at 37°C in RPMI 1640 (Gibco/Invitrogen, Paisley, Scotland) containing 10% fetal bovine serum, 1 mg/ml collagenase D (Roche, Penzbeg, Germany) and 40 μm/ml DNase I (Sigma). LN cells were depleted with GK1.5 (CD4), 5367 (CD8), 14.8 (CD220), and sheep anti-rat magnetic beads (Dynal, Osio, Norway) or spun over a 60% Percoll gradient to obtain low-density cells.

Epidermal LC. The dorsal and ventral ear skin was incubated in Hanks-balanced salt solution (Sigma) containing 0.5% of trypsin (Sigma) or DMEM (Sigma) containing 1.2 U/ml dispase (Roche). The epidermis was separated from the dermis and incubated further in Hanks-balanced salt solution containing 0.5% trypsin in the case of dispase separation.

Dermal DC. Dermis freed from epidermis by trypsin or dispase was further incubated at 37°C for 1 hour in RPMI1640 containing 10% fetal bovine serum, 5 mg/ml collagenase D (Roche), 0.37 mg/ml Dnase I (Sigma), and 2.2 mg/ml hyaluronidase (Sigma).

DC migration from mouse ear skin on culture. The dorsal and ventral ear skin was incubated in 24-well plates (Nalge Nunc, Rochester, NY) in DMEM supplemented with 100 U/ml penicillin, 0.1 mg/ml streptomycin, 2 mM L-glutamine, and 0.05 mM β-mercaptoethanol and 10% fetal bovine serum for 2–3 days. The ear leaflets were removed and floating cells recovered.

Skin painting

Mice were painted with 20 μl of 0.5% 2,4-DNFB (Sigma) in acetone/olive oil (4:1) on the dorsal and ventral side of both ears and killed after 6 hours. TRITC (Molecular Probes, Eugene, OR) was diluted to 10% in dimethylsulfoxide. This stock was diluted further to 0.5% in a 1:1 acetone/dibutylphtalate mix. Hundred microliters were painted onto the abdomen.

Immunostaining

Flow cytometry. Cells were fixed in Cytofix/Cytoperm (BD Pharmingen, Franklin, NJ) for 20 minutes on ice followed by two wash steps using Perm/Wash buffer (BD Pharmingen). All stainings were performed in Perm/Wash buffer. The following antibodies were used: Anti-langerin (CD207, rat IgG1, clone 929F3, kind gift from Dr Stephan Beissert, Department of Dermatology, Munster, Germany, or clone 205C1 mouse IgM coupled to Alexa488 (Dendritics-AbCys, Paris, France) followed by goat anti-rat FITC (Caltag/Invitrogen, Carlsbad, CA), goat-anti-rat A488 (Molecular Probes Cat no. A11006) or goat anti-rat-biotin (BD Pharmingen), and Streptavidin-APC (BD Pharmingen) or clone 929F3 directly coupled to A488 or 546 or clone 205C1 coupled to biotin (all from Dendritics-AbCys) followed by Streptavidin-PE (Caltag), anti-claudin-1 mAb (mouse IgG1, Zymed, San Francisco, CA) labeled with APC using a Zenon

mouse IgG1 labeling kit (Molecular Probes) and anti-MHC class II-PE (ratIgG2b, BD Pharmingen), CD8α-PE (rat IgG2a, BD Pharmingen), CD11b-PE-Cy7 (rat IgG2b, BD Pharmingen), CD11c (hamster IgG1, BD Pharmingen) coupled to PE or APC or CD205 coupled to A488 (rat IgG2a, Serotec, Kidlington UK). Multiparameter analyses were acquired on a FACScalibur (BD Pharmingen) and analyzed with Cellquest software (BD Pharmingen).

Epidermal sheets. The dorsal and ventral ear skin was incubated for 20 minutes at 37°C in 0.5 M NH_4SCN. The epidermis was fixed in a 1:1 mix of acetone/methanol. Epidermal sheets were incubated anti-claudin-1 (polyclonal rabbit, Zymed) followed by goat anti-rabbit Alexa488 (Molecular Probes Cat no. A11008) in combination with anti-langerin or anti-MHC II mAb and subsequently with secondary antibodies (goat anti-rat FITC, Alexa488 or Alexa568, and goat anti-rabbit Alexa568 or Alexa488). Sheets were mounted with Vectashield containing DAPI and images were acquired on a confocal laser microscope (LSM 510 or LSM 510 Meta, Zeiss, Oberkochen, Germany). Three-dimensional reconstructions were made with Imaris (Bitplane, Zurich, Switzerland) software. To control the specificity of the claudin-1 staining, a C-terminal peptide (Santa Cruz Technology, Santa Cruz, CA) was used for both *in situ* staining and flow cytometry.

Western blots

Cells were lysed with a buffer containing NaCl 0.15 M, Tris 0.05 M, pH 6.8, EDTA 1 mM, protease inhibitors (Roche), and Triton 1%. Protein concentration in the supernatant was determined with a Bradford assay (Bio-Rad, Munich, Germany). Samples were separated by 10% SDS-PAGE gel and transferred to a nitrocellulose membrane (Amersham, Piscataway, NJ). The blocked membrane was incubated with anti-claudin-1 or anti-β-actin (Abcam, Cambridge, UK) and subsequently with goat anti-rabbit horseradish peroxidase (Bio-Rad, Hercules, CA). For the chemiluminescence, ECL detection reagents (Amersham Pharmacia, Piscataway, NJ) were used. The membrane was exposed to a film and developed using a Kodak XP 2000 film developer device.

RT-PCR

RNA was extracted using Trizol (Gibco BRL, Invitrogen, Paisley, UK) and chloroform (Gibco BRL) or an RNeasy Plus Mini kit (Qiagen, Hilden, Germany). The RNA was treated with RNase-free DNase (Promega, Madison, WI) to remove remaining DNA. RT-PCR was performed using random primers (Promega), 10 mM dNTPs (Invitrogen), RNasin Plus (Promega), 5 × first strand buffer, dithiothreitol, and Superscript (Invitrogen). cDNA was then amplified by PCR using Taq Polymerase (Invitrogen) under the following conditions: denaturation 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 1 minute for 35 cycles. The following primers were used: mouse *claudin-1*, 5′-CTGGGTTTCATCCTGGCTTC-3′ and 5′-TTGATGGGGGTCAAGGGGTC-3′; mouse *claudin-3*, 5′-AAAGAATTCTGCTCCTGCCCACCGCGCGAC-3′ and 5′-AAACTC GAGAAGTAGCTGCAGTGGCCACC-3′; mouse *claudin-4*, 5′-TAGGGGGCAAGTGCACCAAC-3′ and 5′-CCCCAGCAAGCAGT TAGTGG-3′; mouse *claudin-6*, 5′-ATCTTGGGGATCGTCCTGAC-3′ and 5′-TTTGAGCATCAGCCACCAAG-3′; mouse *claudin-8*, 5′-GCCTCAGTGGAGAGTGTCTG-3′ and 5′-AGAACAGTGCTCCTC CAGCG-3′; mouse *claudin-12*, 5′-TAACTGGAGGAAACTGCGGC-3′ and 5′-CCCCTGAGCTAGCAATAGTG-3′.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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