Abnormal mast cells in mice deficient in a heparin-synthesizing enzyme

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Heparin is a sulphated polysaccharide, synthesized exclusively by connective-tissue-type mast cells[1] and stored in the secretory granules in complex with histamine and various mast-cell proteases[2]. Although heparin has long been used as an anti-thrombotic drug, endogenous heparin is not present in the blood, so it cannot have a physiological role in regulating blood coagulation. The biosynthesis of heparin involves a series of enzymatic reactions, including sulphation at various positions[1,3]. The initial modification step, catalysed by the enzyme glucosaminyl *N*-deacetylase/*N*-sulphotransferase-2, NDST-2 (refs 4–7), is essential for the subsequent reactions. Here we report that mice carrying a targeted disruption of the gene encoding NDST-2 are unable to synthesize sulphated heparin. These NDST-2-deficient mice are viable and fertile but have fewer connective-tissue-type

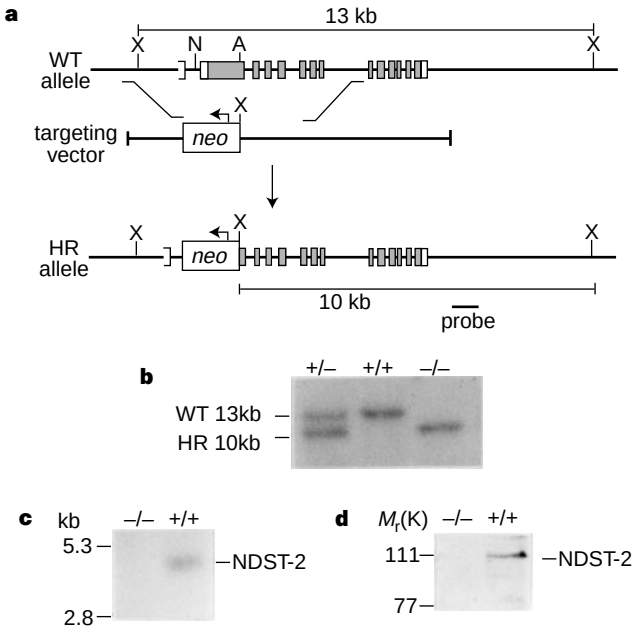

Figure 1 Targeted disruption of the *NDST-2* locus and establishment of a mutant mouse strain. **a**, The *NDST-2* gene (WT, wild type; top), the targeting vector (middle) and the disrupted *NDST-2* locus (HR, homologous recombination; bottom). A 1,329-bp deletion, including 307 bp of 5′-untranslated and 780 bp of coding sequence, was replaced with a *pgk-neo* cassette. The expected size of an *Xba*I digestion product of the gene, hybridizing with the indicated probe, is shown for the wild-type locus (top) and for the mutated allele (bottom). Restriction enzymes: A, *Apa*I; N, *Nhe*I; X, *Xba*I. **b**, Genomic DNA isolated from F_2 litter mates of the intercross of *NDST-2*$^{+/-}$ heterozygous mice was digested with *Xba*I, blotted, and hybridized with the probe in **a**. Genotypes of the progeny are indicated at the top of each lane. **c**, Northern-blot analysis of mRNA from testes of *NDST-2*$^{-/-}$ (left lane) and *NDST-2*$^{+/+}$ mice (right lane). The probe corresponds to the fragment between the *Nhe*I/*Apa*I sites in **a**. **d**, Western-blot analysis of peritoneal cell extracts from *NDST-2*$^{-/-}$ and *NDST-2*$^{+/+}$ mice. Binding of the peptide antibody recognizing NDST-2 (ref. 6, peptide 2) was detected by using electrochemi-luminescence (ECL) (Amersham). The 110K NDST-2 protein is seen in extracts from wild-type mice but not from *NDST-2*-null mice.

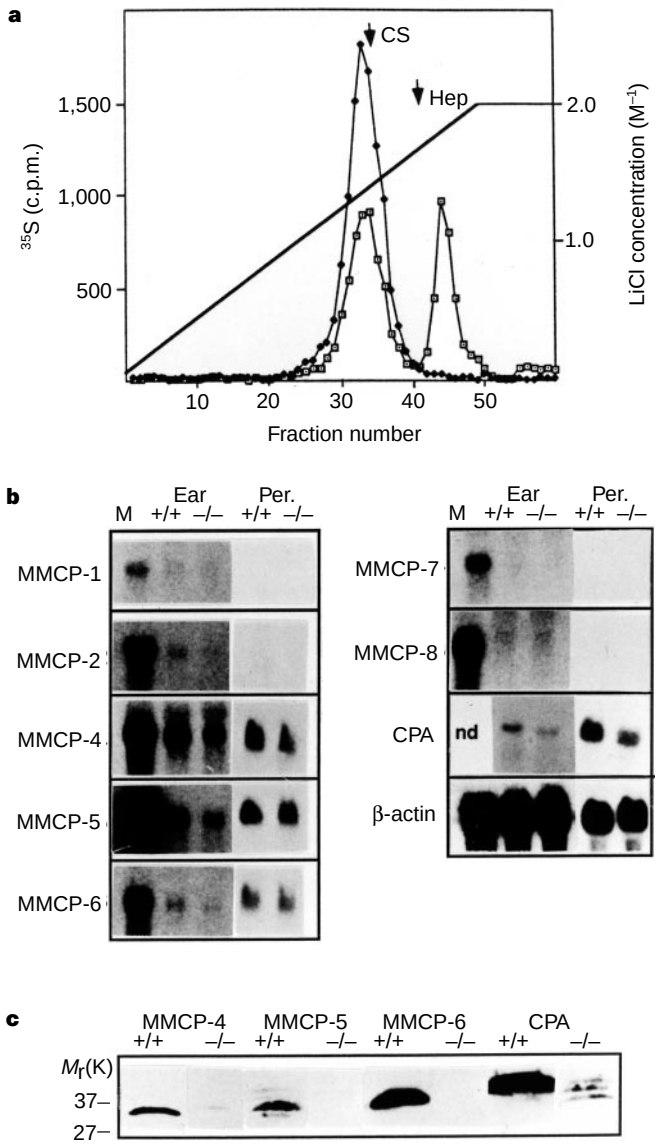

Figure 2 Absence of normally sulphated heparin and mast-cell proteases in peritoneal cells from *NDST-2*$^{-/-}$ mice. **a**, [^{35}S]macromolecules from *NDST-2*$^{+/+}$ (open symbols) and *NDST-2*$^{-/-}$ mice (filled symbols), respectively, were mixed with unlabelled glycosaminoglycan standards (chondroitin sulphate and heparin) and applied to a 1-ml DEAE–Sephacel column that was eluted with a linear gradient of LiCl in 0.05 M acetate buffer, pH 4.0. Elution positions of chondroitin sulphate (CS) and heparin (Hep) are indicated. **b**, Northern blot under high-stringency conditions[25] of total RNA extracted from peritoneal cells (Per.) and ear tissue from *NDST-2*$^{+/+}$ and *NDST-2*$^{-/-}$ mice and from mouse mastocytoma tissue (M). RNA was analysed for transcripts encoding MMCP-1, -2, -4, -5, -6, -7 and -8, and carboxypeptidase A (CPA). Probes were specific for the various mast-cell proteases[25]. β-Actin was used as a loading control. **c**, Western-blot analysis of mast-cell proteases using specific antisera against MMCP-2, -4, -5, -6 and -7 and carboxypeptidase A. Peritoneal-cell extracts from *NDST-2*$^{+/+}$ and *NDST-2*$^{-/-}$ mice were subjected to SDS–PAGE and western blotted. Filters were developed by using the ECL system after incubation with anti-rat or anti-rabbit immunoglobulin conjugated with horseradish peroxidase.

mast cells; these cells have an altered morphology and contain severely reduced amounts of histamine and mast-cell proteases. Our results indicate that one site of physiological action for heparin could be inside connective-tissue-type mast cells, where its absence results in severe defects in the secretory granules.

Two NDSTs, NDST-1 and NDST-2, have been described[5–9], and the cloning of a third isoform was recently reported[10]. Connective-tissue-type mast cells express little or no NDST-1, but contain large amounts of the transcript encoding NDST-2 (ref. 7). To establish a mouse strain with defective synthesis of heparin, we introduced a mutation into the *NDST-2* gene. Using gene targeting in embryonic stem cells, the sequence encoding the cytoplasmic and *trans*-Golgi membrane domain, as well as part of the luminal, catalytically active domains of the enzyme, was deleted by inserting a neomycin-resistance cassette (*neo*; Fig. 1a). Homologous recombination in the *NDST-2* locus was found in two clones, which, after hybridization with a *neo* probe, showed no additional sites of integration (results not shown). Germline transmission was achieved with both clones. Heterozygous mice did not display any obvious abnormalities compared to their littermates.

We analysed genotypes of 167 pups (Fig. 1b) from intercrosses between heterozygous mice, which showed the expected mendelian hereditance (25.1% wild type, 52.1% heterozygous, 22.8% homozygous), indicating that NDST-2 is not crucial for embryonic development. Northern-blot analysis of testis messenger RNA (Fig. 1c) and polymerase chain reaction with reverse transcription (RT-PCR) of testis and liver RNAs (results not shown) showed that the replaced part of the gene is not expressed in homozygous mice. Furthermore, the NDST-2 protein is present in extracts of peritoneal cells of *NDST-2*$^{+/+}$ mice, as shown by western blotting (Fig. 1d), whereas no NDST-2 could be detected in cells isolated from *NDST-2*$^{-/-}$ mice, indicating that this was a true null mutation. Female and male *NDST-2*$^{-/-}$ mice were fertile and showed no obvious pathological phenotype at 20 months. In addition, histological examination of liver, kidneys, testis, lung, heart, skeletal muscle and spleen revealed no apparent defects.

To test whether *NDST-2*$^{-/-}$ cells could synthesize heparin, peritoneal cells that are a mixture of macrophages, lymphocytes and mast cells were labelled with ^{35}S-sulphate, followed by purification and characterization of ^{35}S-labelled macromolecules. Equal amounts of [^{35}S]chondroitin sulphate, which is synthesized by both lymphocytes and macrophages, were recovered from *NDST-2*$^{+/+}$ and *NDST-2*$^{-/-}$ cells, whereas the amount of [^{35}S]heparin and/or heparan [^{35}S]sulphate was greatly reduced in *NDST-2*$^{-/-}$ cells (Table 1). Results were similar in four experiments. To distinguish between heparin and heparan sulphate, [^{35}S]polysaccharide chains were released from their core proteins by alkali and analysed by DEAE ion-exchange chromatography, when heparin elutes later in the salt gradient owing to its higher charge density. None of the mutant cell [^{35}S]macromolecules eluted at the position of the heparin standard (Fig. 2a), indicating that ^{35}S-labelled heparin was absent from *NDST-2*$^{-/-}$ cells. Preliminary results using ^{3}H-glucosamine-labelled peritoneal cells indicate that the unsulphated heparin precursor is formed in *NDST-2*$^{-/-}$ mast cells but in about half the normal amount, which corresponds roughly to the observed reduction in the number of mast cells (Table 1). The lack of ^{35}S-sulphated heparin in NDST-2-deficient cells indicates that this enzyme is crucial for heparin biosynthesis. The role of NDST-2 in the biosynthesis of heparan sulphate is less obvious: structural analysis of liver heparan sulphate (J.L., I.E., E.F. and L.K., unpublished results) reveals no differences in the *N*-sulphation patterns of polysaccharides isolated from control and *NDST-2*$^{-/-}$ mice—58% of the glucosamine residues in both preparations carry *N*-sulphated groups (data not shown), so NDST-1 and -3 might compensate for the lack of NDST-2 in the biosynthesis of heparan sulphate. Proteoglycans carrying heparan sulphate have been implicated in several physiological processes, including organogenesis, during embryonic development[11–17]. Our results, demonstrating a lack of influence of NDST-2 on embryonic development and in adult life, indicate that the other NDST isoforms may be more important in the biosynthesis of heparan sulphate, an idea supported by studies of mice with a targeted mutation in *NDST-1* (M.R., J.L., I.E., L.K. and E.F., unpublished results).

The degree of maturation and differentiation of mast cells is

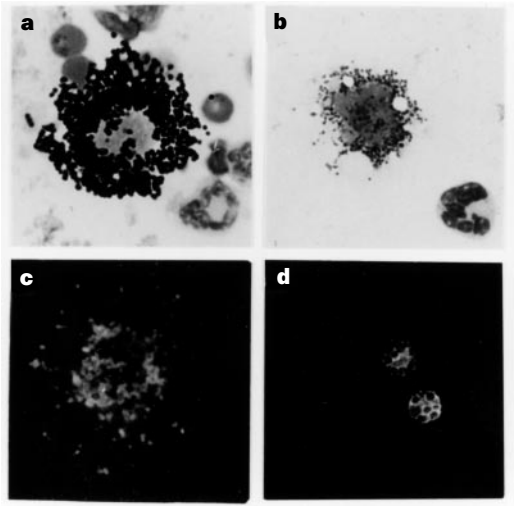

Figure 3 Absence of normal peritoneal mast cells in *NDST-2*$^{-/-}$ mice. **a**–**d**, Cytospins of peritoneal cells from *NDST-2*$^{+/+}$ mice (**a**, **c**) and *NDST-2*$^{-/-}$ mice (**b**, **d**) were stained with May–Grünwald, followed by 5% Giemsa stain in PBS (**a**, **b**), or fixed in ethanol and allowed to bind polyclonal rabbit antibodies against histamine (Euro-Diagnostica), followed by incubation with Cy3-labelled goat anti-rabbit immunoglobulin (Jackson ImmunoResearch) (**c**, **d**). The presence of neutrophils in **a** and **b** is caused by intraperitoneal injection of 2 μg lipopolysaccharide (LPS) 8 h before the peritoneal lavage. Neutrophil numbers were comparable in LPS-injected *NDST-2*$^{+/+;}$ and *NDST-2*$^{-/-}$ mice (15–25% ($n = 7$ for both control and mutant mice).

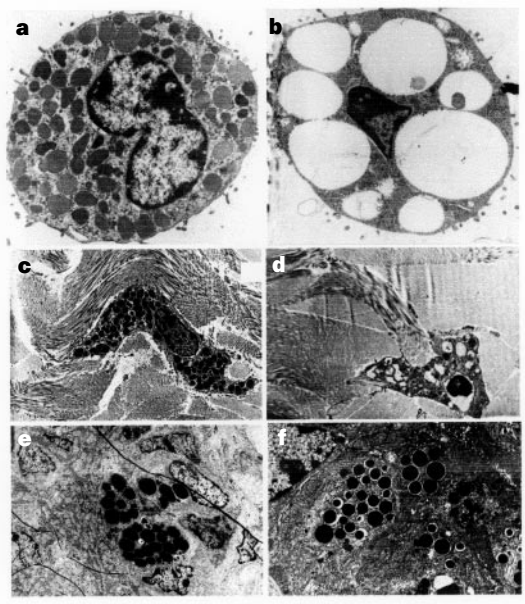

Figure 4 Altered morphology of heparin-containing mast cells. **a**–**f**, Transmission electron micrographs of mast cells from the peritoneum (**a**, **b**), from the skin of the back (**c**, **d**), and from the jejunum of the intestine (**e**, **f**) of *NDST-2*$^{+/+}$ mice (**a**, **c**, **e**) and *NDST-2*$^{-/-}$ mice (**b**, **d**, **f**).

Table 1 Properties of *NDST-2*$^{-/-}$ and *NDST-2*$^{+/+}$ peritoneal cells

Source of cells	^{35}S-chondroitin sulphate (c.p.m. × 10^{-3}/10^6 cells)*	^{35}S-heparan sulphate/heparin (c.p.m. × 10^{-3}/10^6 cells)†	Mast cells (%)‡	Tryptase activity (units)§	Chymase activity (units)§	Histamine content (nmol/10^6 cells)‖
NDST-2$^{+/+}$ mice	4.9	8.3	2.0 ± 0.36 (n = 3)	1.0 ± 0.91 (n = 11)	0.67 ± 0.52 (n = 11)	1.7 ± 0.22 (n = 4)
NDST-2$^{-/-}$ mice	4.9	1.0	0.56 ± 0.19 (n = 3)	0.027 ± 0.031 (n = 11)	0.016 ± 0.023 (n = 11)	0.11 ± 0.044 (n = 4)

* [^{35}S]macromolecules sensitive to chondroitinase ABC digestion.
† [^{35}S]macromolecules sensitive to treatment with HNO_2 at pH 1.5.
‡ Mast cells were detected and counted after combined stainings with May–Grünwald/Giemsa and fluorescent antibodies against histamine (Fig. 3 legend).
§ Enzyme activities were assayed using chromogenic peptide substrates: tryptase, H-D-Ile-Pro-Arg-pNA (S-2288; Chromogenix); chymase, MeO-Suc-Arg-Pro-Tyr-pNA (S-2586, Chromogenix). One unit of tryptase/chymase activity corresponds to the activity that yields an optical absorbance change at 405 nm of 1.0 per hour for 10^6 cells.
‖ Histamine was quantified by enzyme immunoassay (Immunotech). Results are shown with standard deviations.

intimately linked to the repertoire of proteases they express[18,19]: for example, mature connective-tissue mast cells express the chymases known as mouse mast-cell protease-4 (MMCP-4), -5 and -2, the tryptases MMCP-6 and -7, and carboxypeptidase A, whereas mast cells of the mucosal type preferentially express MMCP-1 and -2. Measurement of mast-cell protease activity in peritoneal cell extracts from *NDST-2*$^{-/-}$ mice revealed barely any chymase or tryptase activity (Table 1). Western blot analysis showed that MMCP-4, -5 and -6 and carboxypeptidase A antigens were present in peritoneal cells from *NDST-2*$^{+/+}$ mice (Fig. 2c; MMCP-2 and -7 were not detectable). In contrast, peritoneal cell extracts from *NDST-2*$^{-/-}$ mice were virtually devoid of protease antigens (Fig. 2c). MMCP-2, -4, -5 and -6 and carboxypeptidase were expressed in both *NDST-2*$^{+/+}$ and *NDST-2*$^{-/-}$ animals, as indicated by northern blots of total RNA extracted from ears and peritoneal cells (Fig. 2b). Signals were generally weaker in the *NDST-2*$^{-/-}$ mice, in agreement with the observed reduction in the number of mast cells (Table 1). Thus, the absence of heparin does not affect the levels of mast-cell protease mRNA but it does affect the amount of protease stored in the secretory granules. Presumably, the high negative charge of normally sulphated heparin is required for the assembly of positively charged mast-cell mediators, including the mast-cell proteases and histamine. Further, as connective-tissue-type mast cells from both *NDST-2*$^{+/+}$ and *NDST-2*$^{-/-}$ mice express the same repertoire of proteases, the absence of heparin proteoglycan does not affect the degree of mast-cell maturation.

To find out the effect of a lack of heparin on the mast-cell phenotype, we stained peritoneal cells with toluidine blue (0.1% in PBS buffer) to quantify the number of mast cells present. The cell population from control mice showed the normal frequency of connective-tissue-type mast cells (2%), but we detected no toluidine-blue-staining in homozygous littermates (data not shown). May–Grüwald/Giemsa staining revealed *NDST-2*$^{-/-}$ mast cells with a reduced number of weakly staining, smaller granules and large empty vacuoles (Fig. 3b). The *NDST-2*$^{+/+}$ mast cells, by contrast, contained numerous densely stained granules (Fig. 3a). Histamine-positive cells were detected in the *NDST-2*$^{-/-}$ peritoneal cell population by immunostaining (Fig. 3d): these cells had a raspberry-like appearance, with most of the histamine being present between the large vacuoles, probably in the small granular structures observed after May-Grünwald and Giemsa staining (Fig. 3b).

To test whether *NDST-2*$^{-/-}$ mast cells could still initiate an inflammatory response, we injected mice intraperitoneally with IgE and then with antibodies against IgE, and studied the neutrophil influx. We found no obvious difference in the recruitment of neutrophils in the peritoneum of *NDST-2*$^{+/+}$ and *NDST-2*$^{-/-}$ mice at 3 and 7 h after injection (*NDST-2*$^{+/+}$: 38.2 ± 13.7% neutrophils at 3 h (n = 8) and 39.8 ± 8.2% at 7 h (n = 8); *NDST-2*$^{-/-}$: 31.9 ± 14.7% neutrophils at 3 h (n = 8) and 36.1 ± 14.0% at 7 h (n = 8)). *In vitro*, *NDST-2*$^{-/-}$ cells responded specifically to the addition of IgE and anti-IgE by releasing a substantial amount of their stored histamine (27 ± 7.8 pmol per 10^6 *NDST-2*$^{-/-}$ peritoneal cells (n = 4), compared to 56 ± 8.1 pmol per 10^6 NDST-2$^{+/+}$ peritoneal cells (n = 4). As the number of mast cells is reduced in the mutant mice (Table 1), the amount of histamine released per peritoneal mast cell is actually similar or even larger in *NDST-2*$^{-/-}$ as compared to *NDST-2*$^{+/+}$ mice, despite the marked overall decrease in histamine in *NDST-2*$^{-/-}$ mast cells (Table 1).

Examination of the peritoneal cells using transmission electron microscopy revealed striking differences between mast cells from *NDST-2*$^{+/+}$ mice (Fig. 4a) and *NDST-2*$^{-/-}$ mice (Fig. 4b): *NDST-2*$^{+/+}$ mast cells contained numerous electron-dense granules, whereas *NDST-2*$^{-/-}$ mast cells have only a few granules and apparently 'empty' vacuoles. Mast cells from skin tissue sections from *NDST-2*$^{-/-}$ mice had similar abnormalities (Fig. 4d) (skin mast cells from *NDST-2*$^{+/+}$ mice were normal; Fig. 4c). May–Grünwald/Giemsa staining and histamine immunostaining showed that the number of mast cells in skin from the back of *NDST-2*$^{-/-}$ mice was reduced by about half. Skin mast cells contain heparin, whereas intestinal mucosa mast cells contain chondroitin sulphate[20]. As NDST enzymes are not involved in the biosynthesis of this latter polysaccharide, it was important to verify that mucosal mast cells were unaffected by the absence of the *NDST-2* gene. Transmission electron microscopy on sections of the jejunum of the intestine confirmed the presence of mast cells with electron-dense granular structures in both *NDST-2*$^{+/+}$ (Fig. 4e) and *NDST-2*$^{-/-}$ mice (Fig. 4f).

Mast cells effect allergic reactions and participate in other acute and chronic inflammatory conditions[21]. Our NDST-2-deficient mice provide a model for studying the role of heparin in mast-cell function *in vivo* and in the development of mast cells, as well as its effect on their mediator content. □

Methods

Construction of the targeting vector. A 15-kb genomic clone was isolated from a 129/Sv genomic library (Stratagene) and characterized[7]. A 2.8-kb *Eco*RI/*Apa*I fragment was subcloned into a pBSIIKS+ vector (Stratagene). Subsequently, a *pgk-neo* vector was cloned into the *Apa*I and an *Nhe*I site in the opposite direction relative to the transcription of the *NDST-2* gene. This ligation generated a 1.5-kb arm of homology fused to the *neo* cassette. Finally, using *Apa*I, a 6-kb arm of homology was subcloned from the genomic clone into the *Apa*I site of the shortarm *neo* vector.

ES cells and generation of mutant mice. Embryonic stem (ES) cells were cultured as described[22]. The targeting vector, linearized with *Not*I, was electroporated into R1 ES cells[23]. Clones that had survived selection in 350 μg ml^{-1} G418 (Gibco BRL) were Southern blotted using an 800-bp *Sph*I/*Apa*I fragment, located 3′ of the targeting vector, as probe. The two clones that had undergone homologous recombination were injected into C57BL/6 blastocysts. Chimaeric male founder mice were crossed with C57BL/6 females to obtain heterozygous F_1 offspring. Tail biopsies were genotyped as described[22]. By intercrossing heterozygous mice, an *NDST-2*-deficient mouse strain was established and the phenotype was analysed using F_2 littermates. Crossing of the chimaeric male founder mice with 129/SvJ female mice confirmed that the phenotype did not differ in an inbred 129 Sv/SvJ genetic background (results not shown).

Isolation of peritoneal cells. Peritoneal cells were obtained by peritoneal lavage with 10 ml cold PBS. Cells were centrifuged at 300*g* and washed once in PBS before use.

Glycosaminoglycan analysis. Peritoneal cells from two control and two mutant mice, respectively (4.5–7 × 10^6 cells), were labelled *in vitro* with

0.5 mCi of carrier-free ^{35}S-sulphate by overnight incubation at 37 °C. ^{35}S-labelled macromolecules, obtained after solubilization of cells with buffer containing 1% Triton X-100, were purified by DEAE ion-exchange chromatography essentially as described[24]. Samples were desalted before treatment with chondroitinase ABC (which degrades chondroitin sulphate) or HNO_2 at pH 1.5 (degrades heparan sulphate and heparin), followed by gel chromatography on Sephadex G-50 (ref. 24).

Antisera. Antisera against MMCP-5, -6 and -7 and carboxypeptidase A were raised in rats against fusion proteins derived from the corresponding full-length clones[25]; antisera against MMCP-2 and -4 were raised in rabbits against non-homologous peptide sequences conjugated with keyhole limpet haemocyanin.

IgE/anti-IgE challenge. For studies of neutrophil recruitment, mice were injected with 1 μg of murine IgE (PharMingen), then with 1 μg rat anti-murine IgE (PharMingen) 20 min later. Peritoneal cells were collected 3 and 7 h after IgE injection. Cytospins were stained with May–Grünwald stain then with 5% Giemsa stain in PBS and the percentage of neutrophils in the peritoneal cell population was determined. For *in vitro* studies of histamine release, 6×10^5 peritoneal cells in 300 μl M2 medium (Sigma) were incubated at 37 °C with 0.25 μg IgE for 12 min, followed by a 12-min incubation with 2.5 μg of anti-IgE. After centrifugation, histamine content in the supernatant and in the residual cells was quantified by EIA (Immunotech). The amounts of histamine released without IgE/anti IgE addition were 27 ± 3.7 pmol per 10^6 *NDST-2*$^{+/+}$ cells ($n = 4$) and 5.7 ± 2.0 pmol per 10^6 *NDST-2*$^{-/-}$ cells ($n = 4$).

Transmission electron microscopy. Cells and tissues were fixed in 2% glutaraldehyde, incubated in 1% OsO_4/PBS, dehydrated and embedded in TAAB-B12 resin. Sections were analysed at 60 kV in a Philips CM10 microscope.

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The chemokine receptor CCR4 in vascular recognition by cutaneous but not intestinal memory T cells

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Lymphocytes that are responsible for regional (tissue-specific) immunity home from the blood to the intestines, inflamed skin or other sites through a multistep process involving recognition of vascular endothelial cells and extravasation[1]. Chemoattractant cytokine molecules known as chemokines[2] regulate this lymphocyte traffic, in part by triggering arrest (stopping) of lymphocytes rolling on endothelium[3–5]. Here we show that many systemic memory T cells in blood carry the chemokine receptor CCR4 (ref. 6) and therefore respond to its ligands, the chemokines TARC and MDC. These cells include essentially all skin-homing cells expressing the cutaneous lymphocyte antigen and a subset of other systemic memory lymphocytes; however, intestinal ($\alpha4\beta7^+$) memory and naive T cells respond poorly. Immunohistochemistry reveals anti-TARC reactivity of venules and infiltration of many CCR4$^+$ lymphocytes in chronically inflamed skin, but not in the gastrointestinal lamina propria. Moreover, TARC induces integrin-dependent adhesion of skin (but not intestinal) memory T cells to the cell-adhesion molecule ICAM-1, and causes their rapid arrest under physiological flow. Our results suggest that CCR4 and TARC are important in the recognition of skin vasculature by circulating T cells and in directing lymphocytes that are involved in systemic as opposed to intestinal immunity to their target tissues.

To identify the circulating lymphocyte subsets that are responsive to different chemokines, we analysed the phenotype of peripheral blood lymphocytes (PBL) migrating to chemokines in a standard trans-well chemotaxis assay[7,8]. We used flow cytometry to identify natural killer cells (CD56$^+$, CD16$^+$, CD3$^-$), B cells (CD19$^+$), and

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