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Expression of Integrin-α_E by Mucosal Mast Cells in the Intestinal Epithelium and Its Absence in Nematode-Infected Mice Lacking the Transforming Growth Factor-β1-Activating Integrin α_vβ_6

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Peak intestinal mucosal mast cell (MMC) recruitment coincides with expulsion of Trichinella spiralis, at a time when the majority of the MMCs are located within the epithelium in BALB/c mice. Although expression of integrin-α_Eβ_6 by MMCs has not been formally demonstrated, it has been proposed as a potential mechanism to account for the predominantly intraepithelial location of MMCs during nematode infection. Co-expression of integrin-α_Eβ_6 and the MMC chymase mouse mast cell protease-1, by mouse bone marrow-derived mast cells, is strictly regulated by transforming growth factor (TGF)-β_1. However, TGF-β_1 is secreted as part of a latent complex in vivo and subsequent extracellular modification is required to render it biologically active. We now show, for the first time, that intraepithelial MMCs express integrin-α_Eβ_6 in Trichinella-infected BALB/c and S129 mice. In S129 mice that lack the gene for the integrin-β_6 subunit and, as consequence, do not express the epithelial integrin-α_Eβ_6, integrin-α_E expression is virtually abolished and recruitment of MMCs into the intestinal epithelium is dramatically reduced despite significant overall augmentation of the MMC population. Because a major function of integrin-α_Eβ_6 is to activate latent TGF-β_1, these findings strongly support a role for TGF-β_1 in both the recruitment and differentiation of murine MMCs during nematode infection. (Am J Pathol 2004, 165:95–106)

Mast cell recruitment is a common feature of helminth infections and contributes to the immunological expulsion of some, but not all, gastrointestinal nematode parasites.1–3 Mast cells are, in addition, thought to play a significant role in allergic responses in the airways and gastrointestinal tract4–7 and in the immune response to bacterial pathogens.8 Although the role of mast cells in the immune expulsion of gastrointestinal nematodes remains incompletely resolved, experimental studies in rats and mice suggest that intestinal mucosal mast cell (MMC)-derived β-chymases increase intestinal epithelial permeability to macromolecules.7,8 MMCs may, therefore, contribute to the expulsion of gastrointestinal nematodes by facilitating the pathotopic translocation of effector proteins, including immunoglobulins, into the gut lumen.9 The predominantly intraepithelial location of mouse MMCs at the time of nematode expulsion is consistent with this hypothesis.9

Previous studies, by our group, have shown that expression of the MMC-specific β-chymase mouse mast cell protease-1 (mMCP-1) is strictly regulated by the multifunctional cytokine, transforming growth factor (TGF)-β_1.10 Indeed, mouse bone marrow-derived mast cells (mBMMCs) cultured in TGF-β_1, together with interleukin (IL)-3, IL-9, and stem cell factor are highly homologous to MMCs.10–13 In agreement with studies on T lymphocytes,14,15 mBMMC expression of the α_E chain of integrin-α_Eβ_7 is also TGF-β_1-dependent.13,16 The presence of integrin-α_Eβ_7 has been demonstrated on the surface of intraepithelial T lymphocytes in vivo,15,17 but its expression by MMCs has not been previously demonstrated, although antibody-blocking studies provide indirect evidence that α_E is required for recruitment and survival of MMCs within the epithelium.15 Given that the ligand for integrin-α_Eβ_7, E-cadherin,19 is expressed exclusively by epithelial cells,20 TGF-β_1-mediated expression of integrin-α_Eβ_7 by mMCP-1-positive mBMMCs15 is consistent with the predominantly intraepithelial location of mMCP-1-positive MMCs during the immune rejection of gastrointestinal nematodes.21 It therefore seems highly likely that, like intestinal epithelial T lymphocytes, intraepithelial MMCs express integrin-α_Eβ_7, through a TGF-β_1-dependent mechanism and that the interaction between integrin-α_Eβ_7 and E-cadherin contributes to their recruitment into the intestinal epithelium during nematode infection.

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TGF-β1 is ubiquitously expressed in most tissue microenvironments but is secreted as a latent complex with latency-associated protein, derived from the N-terminal sequence of the TGF-β1 proprotein. We hypothesize that the interaction of the TGF-β1-latency-associated protein complex with integrin-α6β1 and the subsequent presentation of active TGF-β1 on the surface of epithelial cells is a mechanism of activating latent TGF-β1 that is likely to be important for MMC recruitment and differentiation during gastrointestinal nematode infection. In support of this hypothesis, we have previously shown that integrin-αβ6-null mice are severely compromised in their ability to mount a MMC response during infection with Nippostrongylus brasiliensis. However, the rat-adapted strain of N. brasiliensis used in these studies stimulates relatively poor MMC recruitment in the mouse (PA Knight, unpublished observation). In the current study, β6<sup>-/-</sup> and β6<sup>+</sup> mice were infected with the epithelium-dwelling gastrointestinal nematode Trichinella spiralis, to generate a maximal MMC response. The specific aims of the current study were: 1) to formally demonstrate that MMCs express integrin-αβ6 in vivo; 2) to analyze the effects of the integrin-β6-null mutation on the TGF-β1-dependent expression of the integrin-α6 subunit; and 3) to determine whether MMC recruitment is attenuated by the integrin-β6-null mutation.

**Materials and Methods**

Unless stated otherwise, reagents were purchased from Fisher Scientific UK, Loughborough, UK.

**Antibodies**

Purified and fluorescein isothiocyanate (FITC)-conjugated monoclonal rat IgG<sub>1</sub> anti-mouse IgE (clone 23G3; SouthernBiotech, Birmingham, AL) were purchased from Cambridge Biotechnology, Cambridge, UK. Purified monoclonal rat IgG<sub>1</sub> anti-human integrin-α6 (clone GoH3), control rat IgG<sub>1</sub> (clone R3-34), and FITC-conjugated control rat IgG<sub>1</sub> (clone R3-34) were purchased from BD Biosciences, Cowley, UK. Purified rat IgG<sub>2a</sub> anti-integrin-α6 (clone M290) and anti-integrin-β2 hybridoma supernatant (clone M293) were provided by Dr. Peter Kilshaw (The Babraham Institute, Babraham, Cambridge, UK).14 Purified and fluorescein isothiocyanate (FITC)-conjugated rabbit anti-rabbit IgG (clone M293) and rabbit polyclonal anti-equine tryptase immunoglobulin (IgG) were purified using affinity column (Hi-Trap NHS-activated, 1 ml; Amersham Biosciences, Little Chalfont, UK) to which 1 mg of purified protein had been coupled according to the manufacturer’s instructions. Alkaline phosphatase-conjugated mouse anti-rabbit IgG (clone RG-96) and purified control rabbit IgG were purchased from Sigma-Aldrich Company, Poole, UK. Proliferating cell nuclear antigen (PCNA)-specific and negative control DAKO EPOS conjugates were purchased from DakoCytomation Ltd., Ely, UK. Fluorophore conjugated monovalent goat anti-rat IgG and anti-rabbit IgG Fab fragments (Jackson ImmunoResearch Laboratories, West Grove, PA) were purchased from Stratech Scientific, Soham, UK.

**Western Blot Analysis of Mouse Bone Marrow-Derived Mast Cells with Rabbit Anti-Equine Tryptase Immunoglobulin**

Bone marrow cells, prepared from 10- to 12-week-old male BALB/c mice, were cultured for 12 days ex vivo in the presence of recombinant human TGF-β1 (1 ng/ml; Sigma-Aldrich), recombinant mouse IL-3 (1 ng/ml; R&D Systems, Abingdon, UK), recombinant mouse IL-9 (5 ng/ml; R&D Systems), and recombinant mouse stem cell factor (50 ng/ml; PeproTech EC Ltd., London, UK). The resultant mouse mBMMC cultures were extracted with 20 mmol/L of Tris-HCl, pH 7.5, containing 1 mol/L NaCl. Sodium dodecyl sulfate gels (12%) (Mini-Protein II; Bio-Rad Laboratories, Hemel Hempstead, UK) were run containing molecular weight markers (Bio-Rad Laboratories) and mBMMC extract (equivalent of 5 x 10<sup>4</sup> cells/lane). Gels were either stained with Coomassie Brilliant Blue (Bio-Rad Laboratories), or transferred to polyvinylidene difluoride membrane (Immobilon-P; Millipore, Watford, UK) and probed with 1 μg/ml of affinity-purified rabbit anti-equine tryptase IgG followed by alkaline phosphatase-conjugated mouse anti-rabbit IgG (1/20,000 dilution). Blots were developed using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT; Sigma-Aldrich).

**Parasite Infections**

All experiments involving laboratory animals were performed in accordance with the United Kingdom’s Animals (Scientific Procedures) Act 1986. Integrin-β6-null (S129 strain background; S129 β6<sup>-/-</sup>) mice were originally obtained from Dr. Kairbaan Hodivala-Dilke (Cell Adhesion and Disease Laboratory, GKT School of Medicine, St. Thomas’ Hospital, London, UK) and backcrossed with S129 β6<sup>+/+</sup> controls (B&B Universal, Hull, UK). Breeding colonies of S129 β6<sup>-/-</sup>, S129 β6<sup>+/+</sup>, and BALB/c mice were maintained at the Easter Bush Veterinary Centre animal facility. Maintenance, infection, and recovery of Trichinella spiralis larvae were performed using standard methods. Mice, 8- to 15-week-old, age- and sex-matched BALB/c (B&B Universal, Hull, UK), S129 β6<sup>+/+</sup> (B&B), and S129 β6<sup>-/-</sup>, were infected by gavage with 250 muscle larvae in 0.2 ml of phosphate-buffered saline (PBS) (0.1% agar, freshly isolated from muscle cysts from 30- to 30-day-old infected BALB/c mice. Groups of mice (n = 4) were killed on days 7 and 13 (S129) or 14 (BALB/c) after infection. Adult worms were isolated from the small intestine of S129 mice using a modified Baerman’s technique and samples of jejunum were prepared as described below. Samples were also prepared from age-matched uninfected controls.

**Isolated Jejunal Epithelial Whole Mounts**

Intact sheets of jejunal epithelium that included both villi and crypts were isolated by vascular perfusion with ethylenediaminetetraacetic acid (EDTA) from 8- to 15-week-old female BALB/c mice 14 days after infection with T. spira-
controls. For immunocytochemistry, 10 days after infection with /H11032.

7.4 PBS containing 0.5% Tween 80. Sections were then washed thor-
oughly with PBS before proceeding with immunocyto-
chemistry.

Jejunal Samples for Histochemistry, Immunocytochemistry, and RNA Analysis

Age- and sex-matched S129 β6−/− and β6+/+ mice (n = 4) were killed on days 7 and 13 after infection with T. spiralis, along with uninfected controls. Freshly isolated samples of jejunum were collected into RNA-Later (Ambion, Huntingdon, UK) for RNA isolation (0.5 cm) and into Carnoy’s fixative for histochemistry (3 cm).

Samples of jejunum (3.0 cm) from mice killed on day 13 were embed-
ed in OCT compound (BDH Laboratory Supplies, Dorset, UK) and snap-frozen in dry ice-chilled isopentane. Adult worms were isolated from the reminder of the small intestine.

Snap-frozen samples were also pre-
pared from age-matched female BALB/c mice (n = 4) 14 days after infection with T. spiralis, and from uninfected controls. For immunocytochemistry, 10-μm cryostat sec-
tions from frozen jejunal samples were mounted on Snow Coats X-tra-charged slides (Surgipath Europe, Peterborough, UK) and snap-frozen in dry ice-chilled isopen-
tane. Fixation and 3,3’-diaminobenzidine substrate. Sections were washed and rehydrated in three changes of PBS before proceeding with immunocyto-
chemistry.

Jejunal Samples for Histochemistry, Immunocytochemistry, and RNA Analysis

Age- and sex-matched S129 β6−/− and β6+/+ mice (n = 4) were killed on days 7 and 13 after infection with T. spiralis, along with uninfected controls. Freshly isolated samples of jejunum were collected into RNA-Later (Ambion, Huntingdon, UK) for RNA isolation (0.5 cm) and into Carnoy’s fixative for histochemistry (3 cm). Samples of jejunum (3.0 cm) from mice killed on day 13 were embedded in OCT compound (BDH Laboratory Supplies, Dorset, UK) and snap-frozen in dry ice-chilled isopentane. Adult worms were isolated from the reminder of the small intestine.

Snap-frozen samples were also prepared from age-matched female BALB/c mice (n = 4) 14 days after infection with T. spiralis, and from uninfected controls. For immunocytochemistry, 10-μm cryostat sections from frozen jejunal samples were mounted on Snow Coat X-tra-charged slides, air-dried for 10 minutes, and stored at −20°C. For histochemical mast cell evaluation, Carnoy’s-fixed, paraffin-embedded 4-μm microtome sec-
tions of jejunum were stained overnight in 0.5% toluidine blue in 0.5 mol/L HCl, pH 0.5, and counterstained with 1% eosin solution. Toluidine blue-stained sections prepared from S129 β6−/− and β6+/+ mice on day 13 were also washed thoroughly with PBS and probed with anti-PCNA or control EPOS antibodies as directed by the manufacturer (DakoCytomation Ltd.). PCNA-specific labeling was visualized with 3,3’-diaminobenzidine substrate (Vector Laboratories Ltd., Peterborough, UK).

Fixation and 3,3’-Diaminobenzidine Pretreatment of Frozen Sections

Frozen sections were thawed for 10 minutes at 21°C, fixed in absolute methanol for 10 minutes at −20°C, and air-dried for a further 10 minutes under forced air. Sections were washed and rehydrated in pH 7.4 PBS containing 0.5% Tween 80 (Sigma-Aldrich). Eosinophil autofluorescence was quenched using a modified version of the procedure described by Kingston and Pearson.

Noneosinophil-derived endogenous peroxidase activity was quenched for 30 minutes with 1% H2O2 in pH 7.4 PBS containing 0.5% Tween 80. Sections were then washed with PBS and incubated for 3 minutes with 3,3’-diaminobenzidine substrate. Sections were washed thor-
oughly with PBS before proceeding with immunocyto-
chemistry.

Immunocytochemistry: Standard Conditions and Reagents

Unless stated otherwise, immunocytochemical proce-
dures were performed at 21°C under humidified condi-
tions in a Sequenza immunostaining center (Thermo Shandon, Runcorn, UK). Antibodies and blocking sera were diluted in staining buffer (pH 7.4 PBS containing 0.5 mol/L NaCl and 0.5% Tween 80). After immunocytochem-
ical labeling, samples were washed in PBS and mounted with no. 1.5 (0.17-mm-thick) glass coverslips (BDH Labor-
atory Supplies) using Mowiol (pH 8.5) mounting media (EMD Biosciences, San Diego, CA).

IgE and mMCP-1 Dual-Immunofluorescent Labeling in Isolated Jejunal Epithelium

Non-specific immunoglobulin interactions were blocked for 1 hour with staining buffer containing 10% heat-inac-
tivated normal mouse serum (NMS). Samples were then incubated for 30 minutes with rat IgG1, anti-mMCP-1 or control rat IgG1, diluted to 5 μg/ml in staining buffer and 10% NMS. After washing with PBS, slides were incubated for 30 minutes with Rhodamine Red-X (RRX)-conjugated goat anti-rat IgG Fab fragments, diluted to 4 μg/ml in staining buffer and 10% NMS. Samples were washed in PBS, blocked for 1 hour in staining buffer containing 10% heat-inactivated normal rat serum (NRRS), and then incubated for 30 minutes with FITC-conjugated rat IgG1, anti-
mouse IgE or FITC-conjugated control rat IgG1, diluted to 5 μg/ml in staining buffer and 10% NRRS. Samples were then washed with PBS and counterstained for 10 minutes with DRAQ5 (Biostatus Limited, Shepshed, Leicest-
shire, UK), diluted to 5 μmol/L in PBS.

Triple-Immunofluorescent Labeling of Frozen Sections

Non-specific immunoglobulin interactions were blocked for 1 hour with staining buffer and 10% NMS. Sections were then incubated for 1 hour with staining buffer and 10% NMS containing rat anti-mouse integrin-αE (rat IgG2a, 2 μg/ml), integrin-β6 IgG2a (1/50 dilution of hybridoma super-
natant), control rat IgG2a (2 μg/ml), rabbit anti-equine tryptase Ig (2 μg/ml), or control rabbit IgG (2 μg/ml). Samples were washed with PBS and incubated for 30 minutes with staining buffer and 10% NMS containing Cyanine-5 (Cy5)-conjugated goat anti-rat or goat anti-rabbit Fab fragments (2 μg/ml) as appropriate.

IgE and integrin-αE were labeled simultaneously using Fab fragment complexes. Samples were blocked for 1 hour with staining buffer and 10% NRRS. During this time, primary rat antibodies were incubated for 20 minutes at 21°C with fluorophore-conjugated anti-rat IgG Fab frag-
ments in a small volume, 10 μl per 1 μg of primary antibody, of staining buffer in a microcentrifuge tube. Rat anti-mouse IgE and control IgG1 were labeled with FITC-
conjugated Fab fragments at a ratio of 5 μg of Fab fragments per μg of primary rat IgG1. Rat anti-human
integrin-α₆ and control IgG₂a were labeled with RRX-conjugated Fab fragments at a ratio of 5 μg of Fab fragments per μg of primary rat IgG₂a. The resultant Fab fragment complexes were diluted to 4 μg/ml of primary antibody with staining buffer and 10% NRTS and incubated for 10 minutes at 21°C to block any unbound Fab fragment paratopes. Tissue sections were then incubated for 1 hour with the following combinations of Fab fragment complexes (mixed 1:1 to yield a final concentration of 2 μg/ml of each primary/control antibody): IgE-specific Fab FITC and integrin-α₆-specific Fab RRX; IgE-specific Fab FITC and integrin-α₆-specific control Fab RRX; IgE-specific Fab FITC and isotype-matched control Fab RRX; isotype-matched control Fab FITC and integrin-α₆-specific control Fab RRX; and isotype-matched control Fab FITC and isotype-matched control Fab RRX.

Microscopy and Imaging

Bright-field images were acquired with a Sony DXC-390P 3CCD color video camera (Scion Corp., Frederick, MD) mounted on an Axiovert 100 inverted microscope (Carl Zeiss, Welwyn Garden City, UK). The RGB video signal from the camera was digitized using Scion Image (Scion Corp.) mounted in a G4 Macintosh computer (Apple Computer, Cupertino, CA) fitted with a CG-7 frame grabber (Scion Corp.).

Fluorescent images were acquired using an MRC-600 confocal laser-scanning microscope (CLSM; Bio-Rad Laboratories) mounted on an Axiovert 100 inverted microscope equipped with Plan-Apochromat objective lenses (Carl Zeiss). Fluorophores were excited and imaged sequentially using the 488-nm (FITC), 568-nm (RRX), and 647-nm (DRAQ5 and Cy5) lines from a 15 mW Kr/Ar laser (Bio-Rad Laboratories).

Toluidine blue-based mast cell counts were determined from 20 jejunal villus-crypt units (VCU) per mouse. The proportion of mast cells expressing PCNA in samples from β₆⁻/⁻ and β₆⁺/⁺ S129 mice on day 13 after infection was determined by evaluation of 200 toluidine blue-positive mast cells per mouse. Manual cell counting and stereology measurements (Cavalieri method) were performed on images of fluorescently labeled samples using custom software developed for Object-Image. Object-Image is a public domain software package, based on NIH Image, developed by Norbert Vischer (The University of Amsterdam, Amsterdam, The Netherlands) and is available via the Internet at http://simon.bio.uva.nl/object-image.html. Images were prepared for publication using Object-Image and Photoshop (Adobe Systems (UK), Uxbridge, UK).

Detection of Integrin Transcripts by Semiquantitative Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from RNA-Later (Ambion)-fixed jejunal samples using Tri-Reagent (Sigma-Aldrich) and contaminating DNA removed using DNA-free DNase (Ambion). One mg of RNA was reverse-transcribed using 2.5 mmol/L (dT)$_{15}$. A one-twentieth volume was amplified by PCR using gene-specific primers for α₆ (sense), CCCCCTACTCTCTTTAGGACGATCAA; AEF (anti-sense), TATCAGTATCAAAAACGCGATG that give a RT-PCR product of 197 bp, and β₇ (B7D (sense), GCTCTCTGGAATCTACGA, B7F (anti-sense), TCACCTGAAAATCTCAGCG) that give a RT-PCR product of 278 bp, or for the housekeeping gene GAPDH, with equivalent quantities of nonreverse-transcribed RNA as negative controls. Reaction conditions were optimized to ensure the number of thermocycles used coincided with the amplification phase of the PCR. Amplifications were performed for 40 seconds at 94°C, 40 seconds at 55°C, and 120 seconds at 72°C for 34 thermocycles for α₆ and β₇, and for 40 seconds at 94°C, 40 seconds at 60°C, and 120 seconds at 72°C for 32 thermocycles for GAPDH, in a final magnesium concentration of 1.5 mmol/L, pH 8.3. Resultant PCR products were visualized on ethidium bromide-stained 1.6% agarose gels. Images were acquired with a Kodak Digital Science Image Station 440CF (Eastman-Kodak, Rochester, NY) and analyzed using Kodak 1D Image Analysis software. PCR product identities were confirmed by Southern blotting following standard protocols and hybridized using digoxigenin-labeled gene-specific probes. Internal probe sequences for the α₆/β₇ RT-PCR products were as follows; α₆; AEE (sense), AGTGTCTTTAATGAGCAGGGTT; β₇; B7E (sense), ACTGGAAGCAGGACACAACT. Primers and internal complementary probes were designed using RawPrimer (The Virtual Genome Center, http://alces.med.umn.edu/rawprimer.html).

Results

Surface-Bound IgE as a Phenotypic Marker of Mast Cells in the Parasitized Intestine

Intraepithelial IgE⁺⁺⁺ Cells

mMCP-1 remains highly soluble and diffuses in frozen sections resulting in poor immunocytochemical labeling (data not shown). To address this problem and to confirm previous studies that identified MMCs on the basis of IgE staining, jejunal epithelial whole mounts from T. spiralis-infected (day 14) BALB/c mice (n = 4) were probed with mMCP-1- and IgE-specific antibodies. The distribution of mMCP-1 and IgE staining within the jejunal epithelium confirmed that cell surface-bound IgE is exclusively restricted to mMCP-1⁺⁺⁺ MMCs (Figure 1, A to D; and Table 1). Although this method of sample preparation and fixation preserved the antigenicity of IgE and mMCP-1 (Figure 1; A to D) it was incompatible with integrin immunocytochemistry (data not shown). Subsequent analysis of MMC integrin-α₆β₇ expression was performed on −20°C methanol-fixed, snap-frozen sections of jejunum.
IgE+ve Cells in the Lamina Propria

IgE+ve cells were enumerated in frozen sections of jejunum from *T. spiralis*-infected (day 14) BALB/c mice and uninfected controls. Although the majority of IgE+ve cells within the jejunal mucosa of infected mice were located intraepithelially, substantial numbers of cells exhibiting IgE surface labeling were detected within the lamina propria (Table 2). To confirm that these cells were also mast cells, IgE staining was performed in conjunction with tryptase localization. Anti-equine tryptase resulted in strong intragranular staining (Figure 1F) within >80% of IgE+ve lamina propria cells and >60% of intra-
epithelial IgE\(^\text{+ve}\) cells (Table 2). Western blot analysis of lysates from mBMMCs\(^1\) showed that this antibody recognizes a band of \(\sim 35\) kDa (Figure 2) consistent with the molecular weight (MW) of glycosylated mouse tryptases (mMCP-6 and -7).\(^{36}\) The relative frequencies of tryptase single-positive, IgE single-positive, and tryptase/IgE double-positive cells present in the epithelium and lamina propria were determined using integrin-\(\alpha_6\)-specific labeling to delineate the epithelium.

IgE\(^\text{+ve}\) or tryptase\(^\text{+ve}\) cells were not detected in uninfected controls (Figure 1E). Similarly, few if any, MMCs were detected in control uninfected mice by toluidine blue staining (data not shown). Nonintraepithelial tryptase\(^\text{-ve}\) cells accounted for 1.4 \(\pm\) 0.5% of the total population of IgE\(^\text{+ve}\) cells in the jejunal mucosa of infected mice on day 14 (Table 2). Given that only 0.4 \(\pm\) 0.2% of intraepithelial IgE\(^\text{+ve}\) cells are mMCP-1-negative on day 14 (Table 1), IgE labeling can be used to identify mast cells within the jejunal mucosa of parasitized mice with a \(\geq 95\)% degree of confidence. This is consistent with previous observations in rats and mice in which tryptase/IgE double-positive cells present in the epithelium and lamina propria were determined from a population of 500 cells/sample (\(n = 4\)).

**Table 1.** Surface-Bound IgE as a Phenotypic Marker of Mast Cells in the Parasitized Intestinal Epithelium

<table>
<thead>
<tr>
<th>Labeling</th>
<th>Mean (%)</th>
<th>Standard error of the mean (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgE only</td>
<td>0.4</td>
<td>0.2</td>
</tr>
<tr>
<td>mMCP-1 only</td>
<td>0.9</td>
<td>0.3</td>
</tr>
<tr>
<td>IgE and mMCP-1</td>
<td>98.7</td>
<td>0.4</td>
</tr>
</tbody>
</table>

Exfoliated jejunal epithelium (\(\sim 20^\circ\)C methanol-fixed) from T. spiralis-infected BALB/c mice (day 14) was probed with anti-mMCP-1 and anti-IgE monoclonal antibodies (Figure 1; A to D). The relative frequencies of IgE single-positive (IgE only), mMCP-1 single-positive (mMCP-1 only), and IgE/mMCP-1 double-positive (IgE and mMCP-1) cells present in the epithelium were determined from a population of 500 cells/sample (\(n = 4\)).

**Table 2.** Surface-Bound IgE as a Phenotypic Marker of Mast Cells in the Parasitized Intestine

<table>
<thead>
<tr>
<th>Location</th>
<th>Labeling</th>
<th>Mean (%)</th>
<th>Standard error of the mean (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epithelium</td>
<td>IgE only</td>
<td>30.6</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td>Tryptase only</td>
<td>2.3</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>Dual</td>
<td>49.8</td>
<td>2.7</td>
</tr>
<tr>
<td>Lamina Propria</td>
<td>IgE only</td>
<td>1.4</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>Tryptase only</td>
<td>1.3</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>Dual</td>
<td>14.6</td>
<td>1.9</td>
</tr>
</tbody>
</table>

Frozen sections of jejunum (\(\sim 20^\circ\)C methanol-fixed) from T. spiralis-infected BALB/c mice (day 14) were triple-labeled with rabbit anti-equine tryptase Ig, (cross-reactive with mMCP-6 and/or -7, see Figure 2), anti-IgE, and anti-integrin \(\alpha_6\) antibodies (Figure 1; F to I). Using integrin \(\alpha_6\) staining to delineate the epithelium, the relative frequencies of IgE single-positive (IgE only), tryptase single-positive (tryptase only), and tryptase/IgE double-positive (IgE and tryptase) cells present in the epithelium and lamina propria were determined from five randomly selected 0.31-mm\(^2\) fields of view per sample (\(n = 4\)).

**Figure 2.** Detection of mouse tryptase(s) with rabbit anti-equine tryptase polyclonal antibody in Western blots of mBMMC extracts. Samples and molecular weight standards (MW) were run on 12% sodium dodecyl sulfate gels. Cells were stained with Coomassie Brilliant Blue (MW and lane 1), or transferred to Immobilon-P membrane and probed with affinity-purified rabbit anti-equine tryptase antibody (lane 2). Subsequent incubations with alkaline phosphatase-conjugated mouse anti-rabbit IgG and BCIP/NBT substrate resulted in the detection of a band of \(\sim 35\) kDa, consistent with the expected molecular weight for glycosylated murine tryptase(s), mouse mast cells protease-6, and/or protease-7.

**The Integrin-\(\alpha_6\) Subunit Is Preferentially Expressed on Intraepithelial IgE\(^\text{+ve}\) Cells**

Frozen sections of jejunum from T. spiralis-infected (day 14) BALB/c mice and uninfected controls (\(n = 4\)) were labeled with integrin-\(\alpha_6\)- and \(\beta_7\)-specific antibodies together with IgE- and integrin-\(\alpha_6\)-specific Fab fragment complexes. Using integrin-\(\alpha_6\) to differentiate the epithelium and lamina propria, the relative frequencies of IgE single-positive cells and IgE/integrin double-positive cells present in each location were determined and stereology was used to calculate IgE\(^\text{+ve}\) cells mm\(^{-2}\) (Figure 3). Although integrin-\(\alpha_6\)- and \(\beta_7\)-positive cells were present, IgE\(^\text{+ve}\) cells were not found in sections from uninfected mice (data not shown). Infection with T. spiralis was associated with a substantial IgE\(^\text{+ve}\) cell recruitment, with the majority (79.1 \(\pm\) 1.5%) of IgE\(^\text{+ve}\) cells located within the epithelium (Figure 3A). Nearly all of the IgE\(^\text{+ve}\) cells present within the jejunal mucosa expressed integrin-\(\beta_7\), regardless of their location (Figure 3B). Integrin-\(\alpha_6\) was expressed by 17.1 \(\pm\) 1.3% of lamina propria IgE\(^\text{+ve}\) cells and was detected on a significantly (\(P < 0.01\); unpaired Student’s \(t\)-test with Welch correction) higher proportion (54.5 \(\pm\) 2.9%) of intraepithelial IgE\(^\text{+ve}\) cells (Figure 3B).
Infection with *T. spiralis* was associated with IgE\(^+\)ve cell recruitment in both S129 \(\beta_\alpha^{+/+}\) and S129 \(\beta_\alpha^{-/-}\) mice (Figure 4E and Figure 5H). However, the frequency and distribution of IgE\(^+\)ve cells differed markedly in the two genotypes (Figure 5A). As observed in BALB/c mice (Figure 3A), IgE\(^+\)ve cell counts per unit area did not differ significantly between the epithelium and lamina propria in S129 \(\beta_\alpha^{+/+}\) mice (Figure 5A), despite the fact that the majority of the IgE\(^+\)ve cell population (74.1 ± 0.6%) was located intraepithelially (Figure 5B). IgE\(^+\)ve cell hyperplasia also occurred in S129 \(\beta_\alpha^{-/-}\) mice. However, only 18.0 ± 2.5% of IgE\(^+\)ve cells were located intraepithelially (Figure 5B), and there was a significant reduction (\(P < 0.01\), unpaired Student’s t-test with Welch correction) in the population density of intraepithelial IgE\(^+\)ve cells compared to that in S129 \(\beta_\alpha^{+/+}\) mice (Figure 5A). By contrast, the population density of IgE\(^+\)ve cells, when compared with infected \(\beta_\gamma^{+/+}\) controls, increased by more than fivefold in the lamina propria (Figure 5A) and by more than 2.5-fold in the jejunal mucosa as a whole (\(P < 0.01\), unpaired Student’s t-test with Welch correction).

The pattern of integrin-\(\alpha_\delta\) expression in S129 \(\beta_\delta^{+/+}\) mice was similar to that observed in BALB/c mice (Figure 3B), with 64.4 ± 8.0% of epithelial and 25.2 ± 7.2% of lamina propria IgE\(^+\)ve cells exhibiting strong integrin-\(\alpha_\delta\) staining (Figure 5C). Integrin-\(\alpha_\delta\)-positive cells were rare in sections from S129 \(\beta_\delta^{-/-}\) mice and were typically IgE\(^-\)ve (Figure 4H). Only 0.2 ± 0.2% of epithelial and 1.2 ± 0.3% of lamina propria IgE\(^+\)ve cells showed evidence of integrin-\(\alpha_\delta\) staining in S129 \(\beta_\delta^{-/-}\) mice (Figure 5C). Virtually all IgE\(^+\)ve cells, regardless of their location, expressed integrin-\(\beta_\delta\) in both S129 \(\beta_\delta^{+/+}\) and S129 \(\beta_\delta^{-/-}\) mice (Figure 5D).

### Deletion of the Integrin-\(\alpha_\delta\) Gene Virtually Abolishes Integrin-\(\alpha_\delta\) Expression by IgE\(^+\)ve Cells and Inhibits Their Recruitment into the Jejunal Epithelium

Frozen sections of jejunum (\(n = 3\)) from *T. spiralis*-infected (day 13) and uninfected control S129 \(\beta_\delta^{+/+}\) and S129 \(\beta_\delta^{-/-}\) mice (\(n = 4\), frozen material from one of the S129 \(\beta_\delta^{-/-}\) mice was damaged during processing) were labeled with integrin-\(\alpha_\delta\)- or \(\beta_\gamma\)-specific antibodies together with IgE- and integrin-\(\alpha_\delta\)-specific Fab fragment complexes (Figure 4). IgE\(^+\)ve cells were not detected in sections from uninfected mice (Figure 4, A to D). Jejunum from uninfected S129 \(\beta_\delta^{+/+}\) mice contained abundant intraepithelial integrin-\(\alpha_\delta\)-\(\gamma_\delta\)-ve cells (Figure 4C) and integrin-\(\beta_\gamma\)-ve cells (Figure 4A). Integrin-\(\beta_\gamma\)-ve cells were also present in large numbers in the jejunal epithelium of S129 \(\beta_\delta^{-/-}\) mice but were predominantly restricted to the lamina propria (Figure 4B). Integrin-\(\alpha_\delta\)-\(\gamma_\delta\)-ve cells were extremely rare in samples from uninfected S129 \(\beta_\delta^{-/-}\) mice and expressed substantially lower levels of integrin-\(\alpha_\delta\) (Figure 4D).

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**Figure 3.** IgE\(^+\)ve cells in the jejunal mucosa of *T. spiralis*-infected mice express integrin-\(\alpha_\delta\)-\(\beta_\gamma\). Frozen sections of jejunum from infected BALB/c mice were triple-labeled with anti-integrin-\(\alpha_\delta\) or anti-integrin-\(\beta_\gamma\) together with anti-IgE and anti-integrin-\(\alpha_\delta\) (see Figure 4). Using integrin-\(\alpha_\delta\) staining to differentiate the epithelium (filled bars) and lamina propria (open bars), IgE\(^+\)ve cells \(\text{mm}^{-2}\) (A) and the relative frequencies of IgE\(^+\)ve cells expressing integrin-\(\alpha_\delta\) or integrin-\(\beta_\gamma\) (B) were determined for each location using five randomly selected 0.31-mm\(^2\) fields of view per sample. The mean percentage of intraepithelial IgE\(^+\)ve cells ± 1 SEM is provided in parentheses (A). Data are expressed as mean values ± 1 SEM (\(n = 4\)). Differences observed between the epithelial and lamina propria compartments were significant at \(P < 0.01\) (**).
mucosa of expressing integrin-β7 was repeated in an independent study in S129 mice. The percentages of toluidine blue-positive cells in the lamina propria (Figure 4, open bars) and MMCs (filled bars) were compared between S129 and S129 mice on day 7 after infection. The relative frequencies of IgE+ve cells expressing integrin-αE (G) or integrin-β7 (D) in the jejunal mucosa of S129 mice were compared 

Note that mast cells are present in greater numbers in β6−/− mice but are predominantly restricted to the lamina propria. Scale bars, 50 μm.

tode infection express the integrin-αE-β7, that these are predominantly intraepithelial mast cells, and that their percentage within the MMC compartment is highly dependent on the integrin-αE-β7 level. A major difference between this and our previous study, showing a general reduction in mast cell recruitment in integrin-β6−/− mice when compared with controls infected with rat-adapted N. brasiliensis, is that with T. spiralis there was substantially enhanced recruitment of mast cells in the β6−/− mice compared with β6+/+ controls. The intestinal nematode T. spiralis induces a particularly potent MMC response when compared with N. brasilienensis and this is strikingly evident from the present study.

Because we were unable to co-localize mMCP-1 and integrin-αE in frozen sections or in whole mounts of exfoliated epithelium, we used surface-bound IgE to identify MMCs, as described by others (Figure 1 and Tables 1 and 2). Our preliminary co-localization of mMCP-1 and IgE in exfoliated epithelium and of IgE and tryptase in lamina propria mast cells is in agreement with previous studies in which Carnoy’s fixation and toluidine blue staining showed that virtually all IgE-bearing cells are mast cells in parasitized rodent intestines. Using surface IgE as a marker, we have established that the αE- and β7-integrin subunits are expressed on the surface of the mucosa of S129 (open bars) and MMCs (filled bars) S129 mice were triple-labeled with anti-integrin-αE or anti-integrin-β7 together with anti-IgE and anti-integrin-αE (see Figure 4). Data are presented as mean values ± 1 SEM (n = 3) for IgE+ve cells mm−2 (A), the percentage of IgE+ve cells located intraepithelially (B), and the relative frequencies of IgE+ve cells expressing integrin-αE (C) or integrin-β7 (D). Differences observed between β6−/− and β6+/+ S129 mice were significant at P < 0.05 (*) and P < 0.01 (**).

**Discussion**

The results described here, show, for the first time, that a significant proportion of MMCs recruited during nema-
Mast Cells derive from hematopoietic progenitor cells of, as yet, an incompletely defined phenotype.\textsuperscript{38,39} Despite the massive mast cell hyperplasia induced by intestinal nematode infection, there is little evidence of mast cell progenitor (Mcp) proliferation in the bone marrow or circulation, with the main site of proliferation and differentiation occurring in the jejunum.\textsuperscript{40–42} Differentiation of Mcps into mature mast cells occurs after the cells leave the blood stream and is regulated by factors in their local tissue microenvironment.\textsuperscript{38} Mcps homing to the small intestine has been shown to be dependent on expression of integrin-\( \alpha_{E}\beta_{7} \).\textsuperscript{43} Intraepithelial Mcps can be readily isolated from normal intestinal epithelium and the numbers increase within 4 days of infection with \textit{T. spiralis}.\textsuperscript{30} The microenvironment that permits the intraepithelial expansion of Mcps includes expression of stem cell factor\textsuperscript{44} and TGF-\( \beta_{1} \);\textsuperscript{45} by epithelial cells and of IL-3 by intraepithelial T cells.\textsuperscript{46} There are also reports that IL-9-expressing cells can be located within allergic airway epithelium,\textsuperscript{47} suggesting that enteric epithelium could, similarly, support the presence of IL-9-secreting lymphocytes. These observations support the hypothesis that the conditions for MMC differentiation and proliferation exist within the jejunal epithelium.

There was striking difference between infection with \textit{T. spiralis}, in which mast cells were very abundant in the lamina propria of \( \beta_{6}^{–/–} \) mice, and the result of a comparable experiment with the rat-adapted strain of \textit{N. brasiliensis}, in which very few MMCs were recruited.\textsuperscript{24} It is, however, recognized that \textit{T. spiralis} promotes a particularly potent MMC response\textsuperscript{48} and the stimulus is presumably strong enough to overcome the apparent inhibitory effect on MMC recruitment of a lack of \( \alpha_{6}\beta_{4} \) integrin.\textsuperscript{22} The reason why there were more MMCs in the \( \beta_{6}^{–/–} \) mice than in \( \beta_{6}^{+/–} \) controls is less obvious. It could be that the slightly larger worm burden on day 13 (Table 3) provides a more potent stimulus. The preliminary analysis on day 13 using PCNA (Table 3) suggests that MMCs were proliferating at the same rate in both groups. An alternative explanation is that the failure of the cells to enter the epithelium and the probable lack of expression of mMCP-1, as described for infection with \textit{N. brasiliensis},\textsuperscript{24} might result in greater retention of MMCs in \( \beta_{6}^{–/–} \) mice. This is analogous to the augmented accumulation of MMCs in mMCP-1/– mice.\textsuperscript{1} It is possible, for example, that some intraepithelial MMCs can migrate directly into the gut lumen as a consequence of the proteolysis of tight junctions by secreted mMCP-1.\textsuperscript{7} Alternatively, the presence of mMCP-1 in the extracellular space may promote cleavage of epithelial stem cell factor and subsequent apoptosis of MMCs\textsuperscript{49} as they are carried up into the villus. Any loss of autoregulatory function because of the absence, or to very low levels, of mMCP-1, may disturb the rate of apoptosis and/or luminal shedding of MMCs. This is further compounded by the fact that the MMCs were unable to migrate into the epithelium.

Because the most likely defect associated with the absence of the \( \beta_{6} \) integrin gene is a lack of activated TGF-\( \beta_{1} \) on the basolateral membranes of enterocytes, it is possible that the reduction in intraepithelial MMCs in \( \beta_{6}^{–/–} \) mice occurs primarily because MCps fail to express \( \alpha_{E} \), do not bind to epithelialy expressed E-cadherin, and, thus have a reduced ability to remain intraepithelial. If the Mcps enter the lamina propria of \( \beta_{6}^{–/–} \) mice and expand and differentiate in the relative absence of TGF-\( \beta_{1} \), then not only would numbers in the lamina propria increase, but the phenotype of the differentiated MMC population would also differ from that described in \( \beta_{6}^{+/–} \) controls. As mentioned above, in our study with \textit{N. brasiliensis}, the few MMCs that were recruited did not express mMCP-1,\textsuperscript{24} and this is consistent with an altered MMC phenotype.

\begin{table}[h]
\centering
\begin{tabular}{|l|c|c|c|}
\hline
 & Day 0 & 7 & 13 \\
\hline
Worm burden & \( \beta_{6}^{+/+} \) & – & 62.3 ± 20.4 & 7.5 ± 5.2 \\
 & \( \beta_{6}^{–/–} \) & – & 63.3 ± 18.4 & 23.8 ± 7.3 \\
Mast Cells/VCU & \( \beta_{6}^{+/+} \) & 0.3 ± 0.1 & 3.2 ± 0.7 & 6.8 ± 0.9 \\
 & \( \beta_{6}^{–/–} \) & 0.2 ± 0.1 & 4.3 ± 1.5 & 19.6 ± 2.1 \\
PCNA stain Mast Cells (%) & \( \beta_{6}^{+/+} \) & ND & ND & 13.4 ± 2.2 \\
 & \( \beta_{6}^{–/–} \) & ND & ND & 13.3 ± 0.7 \\
\hline
\end{tabular}
\caption{Adult Worm Burdens and Jejunal Mast Cell Numbers}
\end{table}

Total adult worm burdens and numbers of Toluidine Blue-positive mast cells per villus crypt were determined for \( \beta_{6}^{–/–} \) and \( \beta_{6}^{+/+} \) S129 mice on days 7 and 13 after infection with \textit{T. spiralis}. Toluidine Blue-positive cells counts were also performed on uninfected \( \beta_{6}^{–/–} \) and \( \beta_{6}^{+/+} \) S129 mice (day 0). The proportion of mast cells in the S phase of cell proliferation (PCNA+++\%\textsuperscript{23}) was determined on day 13 after infection using PCNA immunohistochemistry and Toluidine Blue staining (Figure 4, O and P). Data are presented as mean values ± 1 SEM (n = 4).

* Differences observed between \( \beta_{6}^{–/–} \) S129 mice and \( \beta_{6}^{+/+} \) S129 controls were significant at \( P < 0.01 \) (unpaired Student’s \( t \)-test with Welch correction).
Studies using integrin-deficient mice and, more specifically, antibody neutralization of integrin-αE in T. spiralis-infected mice suggest that integrin-αEβ7 is not required for the retention of MCP and MMCs within the intestinal epithelium but may also be essential for the recruitment of MCPs to the gut mucosa because treated mice lacked MMCs both in epithelium and lamina propria. The present results suggest that the reduced expression of integrin-αE, as a consequence of defective processing of extracellular TGF-β latency, is not only associated with depletion of intraepithelial MMCs, substantial down-regulation of αEβ7 expression, and a paradoxical increase in the frequency of lamina propria MMCs. This report is the first to demonstrate, unequivocally, that MMCs express integrin-αEβ7. Furthermore, the absence of epithelially expressed integrin-αEβ7 is associated with depletion of intraepithelial MMCs, substantial down-regulation of αEβ7 expression, and a paradoxical increase in the frequency of lamina propria MMCs.

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