Interleukin 10 gene therapy for Crohn's disease
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Chapter 6

In vivo evaluation of T lymphocyte homing in experimental colitis: imaging of $^{111}\text{In}$ labelled T lymphocytes

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submitted
Chapter 6

Abstract

Background & Aims: Blockade of lymphocyte recruitment to the intestinal mucosa is considered a useful therapy for IBD and anti-α4 antibodies have clinical benefit in patients with active Crohn's disease. The aim of this study was to evaluate a non-invasive 3-dimensional scintigraphic technique to assess lymphocyte homing to the colon in TNBS-induced experimental colitis.

Methods: TNBS sensitized and non-sensitized murine total lymphocytes or CD4+ lymphocytes were radioactively labelled with 111Indium-oxinate (111In). Cells were injected into control mice or mice with TNBS colitis. Specific abdominal radioactive uptake was determined by single photon emission computed tomography (SPECT) using a dedicated pinhole system 48 hours after cell transfer. The severity of inflammation was determined by histological scoring of the colon.

Results: The radioactive colon uptake was most evident in mice with TNBS colitis that received sensitized lymphocytes. The sensitized 111In labelled lymphocytes exacerbated colitis compared with non-sensitized lymphocytes. The colon uptake correlated well with both colon weight and histological score. The use of 111In labelled CD4+ lymphocytes resulted in a similar homing pattern. Administration of an anti-α4 antibody decreased radioactivity colon uptake of the 111In labelled cells compared with the control antibody in mice with TNBS colitis.

Conclusions: Animal pinhole SPECT can be applied for temporal and spatial analysis of the lymphocyte homing process in experimental colitis and allows for optimal evaluation of therapeutic efficacy of new drugs that interfere with lymphocyte migration. Moreover, colon radioactivity uptake can be used as a parameter of disease activity in experimental colitis.
Introduction

Under normal conditions, naïve T lymphocytes migrate randomly from blood to secondary lymphoid tissues for immune surveillance. When naïve T lymphocytes encounter an antigen in the draining lymph nodes of the gut, they differentiate into memory/effectector cells. If such an encounter takes place in the mesenterial lymph nodes, migration is subsequently directed back to the intestinal mucosa. This process is controlled by the expression of different sets of adhesion molecules and chemokines. The integrin α(4)β(7) is the principal gut-homing receptor and functions at several steps in the adhesion cascade by interacting with the mucosal addressin MAdCAM-1 present on endothelial cells. Naïve lymphocytes express low levels of αβ7 but upon activation a significant amount of functionally active αβ7 appears on the surface. During intestinal inflammation MAdCAM-1 is upregulated resulting in an increased influx of lymphocytes. In addition, the chemokine TEC and its receptor CCR9 are involved in the migration of αβ7 memory T lymphocytes to the small intestine. Surprisingly, only few cells in the colon express CCR9, which indicates that effector and memory T lymphocyte homing to the gut may be divisible into particular streams.

In inflammatory bowel disease, increased numbers of activated CD4+ T helper 1 (Th1) lymphocytes, that locally produce pro-inflammatory cytokines, such as interferon γ (IFN-γ) and tumour necrosis factor α (TNF-α), mediate mucosal inflammation and tissue damage. Blockade of lymphocyte recruitment to the intestinal mucosa is considered a useful therapy for inflammatory bowel disease. Indeed, administration of antibodies that bind either the α4 integrin alone or in combination with β7 has resulted in therapeutic benefit in experimental colitis and in patients with active Crohn's disease. In addition, in other models of T lymphocyte mediated disease, such as asthma and rheumatoid arthritis, specific chemokine or chemokine receptor antagonists have been reported to block cell recruitment and these targets are expected to have therapeutic effects in inflammatory bowel disease as well. Although these results are promising, the number of in vivo studies is limited. More insight into the complex migratory pathways of lymphocytes to the intestine and the role chemokines and their receptors...
play herein, is necessary. Several techniques have been used for imaging of lymphocyte migration, such as intravital fluorescence microscopy,\textsuperscript{18,19} bioluminescence assays with luciferase expressing cells\textsuperscript{20} and magnetic resonance imaging of T lymphocytes loaded with superparamagnetic nanoparticles.\textsuperscript{21-24} However, restrictions of these methods include invasiveness, modification of donor cells for detection, or analysis of a limited number of anatomic sites or time points. Previous studies have shown that \textit{in vivo} 2-dimensional planar imaging of radioactive labelled lymphocytes is a technique that allows for \textit{in vivo} detection of lymphocytes on multiple time points in the entire body.\textsuperscript{25-27}

In the present study, we have evaluated a 3-dimensional non-invasive scintigraphic method (single photon emission computed tomography; \textit{SPECT}) for assessment of radioactively labelled \textsuperscript{111}Indium (In)-oxinate lymphocyte homing to the gut in 2,4,6-trinitrobenzenesulfonic acid (TNBS) induced experimental colitis in mice. TNBS colitis is characterized by a T helper-1 cell mediated transmural colitis and resembles human Crohn's disease.\textsuperscript{28} We performed \textit{SPECT} analysis 48 hours (h) after injection of radioactively labelled cells, since we showed in pilot experiments that colon radioactivity uptake increased over time and peaked at 48 h post injection.\textsuperscript{27} Further, we applied this technique to study blockade of intestinal lymphocyte influx in TNBS colitis with an anti-$\alpha$4 integrin antibody. Our results provide evidence that \textsuperscript{111}In labelled lymphocyte \textit{SPECT} is a suitable tool to assess lymphocyte homing and disease activity \textit{in vivo} in experimental colitis.

\section*{Materials and Methods}

\subsection*{Mice}

BALB/c female mice were purchased from Charles River (Charles River, Someren, the Netherlands) and maintained under standard conditions at our animal care facility. The animal welfare committee approved all experiments. Mice were used at 8 to 10 weeks of age.
Scintigraphy for $^{111}$In labelled T cell migration in vivo

Table 1  Visual uptake and mean colon uptake ratios of SPECT

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Donor</th>
<th>Acceptor</th>
<th>number of mice</th>
<th>Spleen cell type</th>
<th>Ab uptake</th>
<th>Visual uptake</th>
<th>Uptake ratio</th>
</tr>
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<tbody>
<tr>
<td>Exp 1</td>
<td>NaCl</td>
<td>NaCl</td>
<td>5</td>
<td>total</td>
<td>-</td>
<td>+</td>
<td>0.29 ± 0.025</td>
</tr>
<tr>
<td>Exp 1</td>
<td>TNBS</td>
<td>NaCl</td>
<td>5</td>
<td>total</td>
<td>+/−</td>
<td>+</td>
<td>0.22 ± 0.035</td>
</tr>
<tr>
<td>Exp 1</td>
<td>NaCl</td>
<td>TNBS</td>
<td>5</td>
<td>total</td>
<td>+/−</td>
<td>+</td>
<td>0.22 ± 0.036</td>
</tr>
<tr>
<td>Exp 1</td>
<td>TNBS</td>
<td>TNBS</td>
<td>6</td>
<td>total</td>
<td>+</td>
<td>++</td>
<td>0.51 ± 0.033</td>
</tr>
<tr>
<td>Exp 2</td>
<td>NaCl</td>
<td>NaCl</td>
<td>2</td>
<td>CD4$^+$</td>
<td>-</td>
<td>-</td>
<td>0.11</td>
</tr>
<tr>
<td>Exp 2</td>
<td>TNBS</td>
<td>NaCl</td>
<td>2</td>
<td>CD4$^+$</td>
<td>+/−</td>
<td>-</td>
<td>0.25</td>
</tr>
<tr>
<td>Exp 2</td>
<td>NaCl</td>
<td>TNBS</td>
<td>3</td>
<td>CD4$^+$</td>
<td>+/−</td>
<td>+</td>
<td>0.23</td>
</tr>
<tr>
<td>Exp 2</td>
<td>TNBS</td>
<td>TNBS</td>
<td>2</td>
<td>CD4$^+$</td>
<td>+</td>
<td>+</td>
<td>1.18</td>
</tr>
<tr>
<td>Exp 3</td>
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<td>TNBS</td>
<td>5</td>
<td>CD4$^+$ control</td>
<td>+</td>
<td>++</td>
<td>0.72 ± 0.14</td>
</tr>
<tr>
<td>Exp 3</td>
<td>TNBS</td>
<td>TNBS</td>
<td>5</td>
<td>CD4$^+$ anti-α4</td>
<td>+</td>
<td>+</td>
<td>0.33 ± 0.025</td>
</tr>
</tbody>
</table>

Radioactivity uptake in the colon was scored visually, as follows: - no uptake (equal to background), +/− equivocal uptake (higher than background but lower than bone marrow uptake), + positive uptake (equal to bone marrow uptake) or ++ manifest uptake (higher than bone marrow uptake). For the uptake ratio five consecutive transverse slices with the highest colon uptake were selected and added. Regions of interest (ROI) were set for the colon, pelvic bone marrow and abdominal background adjacent to the colon and counts in each ROI were determined to determine the ratio of specific activity and non-specific activity. The colon uptake ratio was calculated normalized to the pelvic bone marrow uptake corrected for background activity (counts colon-counts background/counts bone marrow-counts background). Results are expressed as mean ± SEM.

Experiments

The study was divided in three experimental protocols to assess lymphocyte migration by SPECT (Table 1). We first addressed homing characteristics of sensitized and non-sensitized total splenic lymphocytes in TNBS or control mice (experiment 1). Secondly, we evaluated homing characteristics of sensitized and non-sensitized CD4$^+$ lymphocytes in TNBS or control mice (experiment 2). Finally, we studied the ability of an anti-α4 integrin antibody to interfere with recruitment of sensitized CD4$^+$ lymphocytes in TNBS-induced colitis (experiment 3).

Induction of TNBS colitis

Colitis was induced by rectal administration of 0.5-2 mg TNBS (Sigma Chemical Co, St Louis, MO, USA) dissolved in 40°v/v ethanol (Merck, Darmstadt, Germany), using a vinyl catheter positioned 3 centimeters from the anus, as previously described. Control mice
underwent identical procedures, but were instilled with saline (NaCl 0.9\text{w/w}). The donor mice, used for lymphocyte isolation from the spleen, received 1 dose of saline or TNBS and were sacrificed after 7 days. The recipient mice, used for transfer of radioactively labelled lymphocytes and SPECT analysis, were instilled with saline or TNBS twice, separated by a 7-day interval (to induce a Th1 mediated delayed type hypersensitivity response).

**In labelling and adoptive transfer**

BALB/c lymphocytes from either saline or TNBS mice were used as source for radioactive labelling and transfer. Briefly, lymphocyte suspensions from the spleen were prepared using filter cell strainers (Becton Dickinson, New Jersey, USA) and red cell lysis. The cells were labelled with \textsuperscript{111}In either directly or after CD4\textsuperscript{*} cell enrichment. For CD4\textsuperscript{*} cell enrichment, lymphocytes were labelled with the following rat anti-mouse monoclonal antibodies (mAbs): B220 (clone RA3-6B2), Mac-1 (clone M1/70), and CD8\alpha (clone 53-6.7) (gift from Dr. R. Mebius, Vrije Universiteit Medical Center, Amsterdam, The Netherlands) and the mAb-stained cells were removed in a magnetic field using sheep anti-rat IgG coated magnetic beads (Dynal, Hamburg, Germany). CD4\textsuperscript{*} enrichment of total splenic lymphocytes resulted in \textgreater\textsuperscript{60}\text{w/w} CD4\textsuperscript{*} lymphocytes.

\textsuperscript{111}In-oxinate (1.4\text{\(\mu\)Bq, Mallinckrodt, Petten, the Netherlands) was added for each 1x10\textsuperscript{6} cells, and incubated for 15 minutes at room temperature. After washing, 200 \text{\(\mu\)l of cell suspension (approximately 25x10\textsuperscript{6} cells and 25 MBq per mouse) was injected intravenously (iv) in the tail vein of randomly assigned TNBS or saline recipient mice on day 7.**

**Flow cytometry**

In separate experiments, splenocytes from donor mice were stained with fluorochrome-conjugated CD4, CD45RB, CD25, CD4\text{\(\alpha\)B7, CD62L, CD44 (all Pharmingen) mAbs and rat IgG2a and IgG2b isotype controls and analyzed on a FACS Calibur flow cytometer (Becton Dickinson, CA, USA).
In vivo homing assay using SPECT

Scintigraphy was performed using a recently described and validated high-resolution pinhole SPECT. Animals were sedated with fentanyl and fluamson (Janssen Pharmaceutica, Beerse, Belgium) and midazolam (Roche, Mijdrecht, the Netherlands). SPECT was performed 48 h after injection of the \(^{111}\)In labelled cells. SPECT reconstruction was performed using a HERMES (Nuclear Diagnostics, Stockholm, Sweden) application program, using filtered back projection adapted to pinhole SPECT. Images were visually interpreted on transversal slices by two experienced nuclear medicine physicians (R.B. and H.V.). Radioactivity uptake in the colon was scored as follows: no uptake (equal to background), equivocal uptake (higher than background but lower than bone marrow uptake), positive uptake (equal to bone marrow uptake) or manifest uptake (higher than bone marrow uptake). To determine the radioactivity uptake semiquantitatively, five consecutive transverse slices with the highest colon uptake were selected and added. Regions of interest (ROI) were set for the colon, pelvic bone marrow and abdominal background adjacent to the colon and counts in each ROI to determine the ratio of specific activity and non-specific activity. The colon uptake ratio was calculated normalized to the pelvic bone marrow uptake corrected for background activity ((counts colon-counts background)/(counts bone marrow-counts background)).

After SPECT the mice were sacrificed and colons were removed through a midline incision. To validate the results of the SPECT, isolated colons were cleaned for standard planar pinhole scintigraphy. In the anti-\(\alpha_4\) versus control antibody experiment, the colon and a blood sample were weighed and counted in a gamma (\(\gamma\))-counter (Packard 5530) after SPECT and planar scintigraphy. Results of the \(\gamma\)-counter were corrected for decay and expressed as counts per minute (cpm)/gram tissue. To correct for non-specific binding, ratios were calculated normalized to radioactivity in blood ((cpm/gram colon-cpm/gram blood)/(cpm/gram blood)).

Blockade of \(\alpha_4\) integrin

Rat mAb specific for \(\alpha_4\) (PS/2, IgG2b) or an irrelevant isotype-matched control antibody (rat IgG2b both mAbs were kindly provided by Biogen, Cambridge, MA, USA) were administered to recipient mice by a single intraperitoneal injection of 200 \(\mu\)g/mouse.
following the second instillation of TNBS. In addition, approximately $25 \times 10^7$ $^{111}$In labelled donor cells were treated for 20 min at 4°C with 20 µg/ml of the appropriate Ab before transfer. Next, the $^{111}$In labelled donor cells were washed, resuspended in saline and equal volumes were injected iv into recipient mice.

**Assessment of inflammation**

The wet weight of the distal 6 cm of the colon was used as an index of disease-related intestinal wall thickening. An experienced pathologist (P.K.) blinded to the experimental protocol performed microscopic evaluation on formalin fixed tissue sections stained with hematoxylin and eosin, as previously described. Two sections of rolled colons were scored using the following parameters 1) percentage of colon involved, 2) fibrosis, 3) edema, 4) erosions and ulcerations, 5) crypt loss, 6) infiltration of mononuclear cells and 7) polymorphonuclear cells. The total score ranges from 0 (normal colon) to a maximum of 20 points (most severe inflammation).

**Statistical analysis**

Differences between groups were analyzed by the Mann-Whitney U test. For comparison of multiple data sets, statistical analysis was performed according to Kruskal-Wallis. Correlation analysis between uptake ratios and histological scores or colon weights was performed by applying the Spearman’s rho correlation test. Results are expressed as the mean ± SEM. A two-tailed p value of less than 0.05 was considered to represent a significant difference.

**Results**

**Phenotype of donor lymphocytes**

First, we analyzed the phenotype of spleen lymphocytes isolated from TNBS sensitized or saline control mice by flow cytometry. TNBS sensitization increased the proportion of CD4+ lymphocytes expressing the memory marker CD45RB+ (69 ± 8°) compared with
Scintigraphy for $^{111}$In labelled T cell migration in vivo

Figure 1 Phenotype of donor CD4$^+$ lymphocytes

Flow cytometry analysis of $\alpha 4\beta 7$ expression on fresh CD4$^+$ lymphocytes isolated from spleens of TNBS or saline control BALB/c mice. The y-axis represents the relative cell number (counts) and the x-axis represents $\alpha 4\beta 7$ expression overlaid onto the isotype control. Gates were set to contain live lymphocytes only. This is a representative image of 13 mice.

Saline (51 ± 1%) Further, TNBS sensitization decreased expression of CD62L, that is involved in lymphocyte homing to peripheral lymph nodes, on CD4$^+$ lymphocytes (mean fluorescence intensity (MFI): 185 ± 18) compared with saline sensitization (MFI: 377 ± 68). By contrast, expression of CD44, which directs lymphocytes to the high endothelial venules, was higher (MFI: 249 ± 20) in the TNBS sensitized CD4$^+$ population compared with the non-sensitized CD4$^+$ population (MFI: 199 ± 17). As shown in figure 1 CD4$^+$ lymphocytes of saline and TNBS sensitized mice both expressed intermediate levels of the $\alpha 4\beta 7$. 

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Figure 2 Colon radioactivity uptake is increased in mice with TNBS colitis transferred with TNBS sensitized $^{111}$In labelled lymphocytes

SPECT was performed 48 h after transfer of $^{111}$In labelled TNBS sensitized or non-sensitized lymphocytes to TNBS and saline control mice. Colon uptake was calculated normalized to the bone marrow uptake in the pelvis and corrected for background activity. Boxplot of mean radioactivity uptake of the four groups as indicated (donor/acceptor). Overall comparison $p=0.002$, individual comparison between TNBS-TNBS group and the 3 other groups **$p=0.004$.

SPECT assessment of $^{111}$In labelled lymphocyte influx to the colon

Splenic lymphocytes were isolated from both TNBS sensitized and non-sensitized mice and radioactively labelled with $^{111}$In, with an efficiency ranging from 60 to 80%. In saline control mice there was no significant difference in the colon uptake ratio between sensitized and non-sensitized total lymphocytes 48 h after cell transfer (figure 2 and 3). By contrast, mice with TNBS colitis had significantly higher colon uptake ratios after transfer of sensitized total lymphocytes compared with non-sensitized lymphocytes ($p=0.004$, figure 2). Visual uptake assessment on transverse SPECT slices (table 1 and figure 3 on page 138) confirmed the colon uptake ratios. In addition, ex vivo planar scintigraphy of isolated colons showed clear differences between mice with TNBS colitis that received sensitized lymphocytes and the other groups (data not shown). These observations indicate that TNBS sensitization was required for lymphocyte homing to the TNBS exposed colon.
Scintigraphy for $^{111}$In labelled T cell migration in vivo

**Figure 4** TNBS sensitized lymphocytes exacerbate colitis

TNBS or saline control mice were transferred with TNBS sensitized or non-sensitized $^{111}$In labelled lymphocytes as indicated (donor-acceptor). Weight of the last 6 cm of the colon was determined upon sacrifice after SPECT. The TNBS-TNBS mice had significantly lower colon weights than the other mice. Data represent means ± SEM. Overall comparison p=0.0015, individual comparison between TNBS-TNBS group and three other groups $^{**}$p<0.05.

$^{111}$In labelled spleen lymphocytes are functional *in vivo*

The weight of the last 6 cm of the colon was determined upon sacrifice at day 9. The colon weight of mice with TNBS colitis that received sensitized lymphocytes (238 ± 24 mg) was significantly higher than the weights of mice that received non-sensitized lymphocytes (162 ± 19 mg, p=0.028) and saline mice that received sensitized lymphocytes (114 ± 5 mg, p=0.006) or non-sensitized lymphocytes (101 ± 5 mg, p=0.006) (figure 4).

Next, tissue sections of the colons were examined microscopically to assess whether recruitment of $^{111}$In labelled lymphocytes was associated with histological signs of inflammatory activity (figure 5). Histological scoring indicated that transfer of the sensitized lymphocytes exacerbated disease in TNBS colitis compared with non-sensitized lymphocytes (histological score 12 ± 0.4 and 7 ± 0.8, p=0.006). By contrast, no pathological changes were detected in the colons of saline control mice (figure 5). These data indicate that $^{111}$In labelled lymphocytes were functional *in vivo*, as they aggravated
Figure 3  Increased radioactivity on SPECT of mice with TNBS colitis transferred with TNBS sensitized $^{111}$In labelled lymphocytes

SPECT was performed 48 h after transfer of $^{111}$In labelled TNBS sensitized or non-sensitized lymphocytes to TNBS and saline control mice. Transverse slices from the abdominal region at the pelvic level. The amount of $^{111}$In uptake is colour-coded from low (black) to high (yellow-white). The four images are representative for the four groups (donor-acceptor): A) in the NaCl-NaCl mouse uptake is low, B) in the TNBS-NaCl mouse uptake is equivocal, C) in the NaCl-TNBS mouse uptake is equivocal, and D) in the TNBS-TNBS mouse uptake is manifest.
Figure 5  TNBS sensitized lymphocytes exacerbate colitis

H&E stained colon sections. The extent of mucosal inflammation was graded (see materials and methods) and the mean total histological scores per group (donor-acceptor) are shown. The photos A) NaCl-NaCl mouse and B) TNBS-NaCl mouse, show normal colon architecture with a small number of leukocytes in the mucosa. C) NaCl-TNBS mouse with inflammation (significantly less severe than in D), consisting of edema and influx of inflammatory cells, but ulcerations and fibrosis are absent. D) TNBS-TNBS mouse with severe colitis characterized by edema, an extensive influx of inflammatory cells, ulcerations, crypt loss and fibrosis. Original magnifications: 33 x.

disease activity. To determine whether SPECT could be used to assess disease activity in vivo, we performed a correlation analysis between the colon uptake ratios and the histological scores (figure 6A) or the colon weights (figure 6B). Both the total histological scores and colon weights correlated with the colon uptake ratios in mice with TNBS colitis (for both p<0.001). Hence, the colon uptake ratio is a reliable parameter for the severity of colitis in vivo.
**Figure 6 Colon uptake ratio correlated with parameters of colitis**

Mice with TNBS colitis were transferred with TNBS sensitized or non-sensitized $^{111}$In labelled lymphocytes. Each symbol represents an individual mouse.

A) Significant correlation between histology (x-axis) and colon uptake ratio (y-axis) ($p<0.001$). Correlation coefficient $R^2=0.933$.

B) Significant correlation between colon weight (x-axis) and colon uptake ratio (y-axis) ($p<0.001$). Correlation coefficient $R^2=0.836$.

**Increased migration of CD4$^+$ lymphocytes to the colon**

Since the CD4$^+$ cell population is primarily responsible for transferring TNBS-induced colitis, we analyzed the specificity of $^{111}$In labelled CD4$^+$ spleen cell scintigraphy for migration to both inflamed and healthy colons (experiment 2, table 1). As expected, transfer of sensitized CD4$^+$ lymphocytes to mice with TNBS colitis resulted in a higher colon uptake ratio than transfer of non-sensitized CD4$^+$ lymphocytes. Saline control mice that received sensitized or non-sensitized CD4$^+$ lymphocytes showed low colon uptake ratios similar to those found after transfer of total lymphocytes (table 1). Visual uptake assessment on transverse SPECT slices (table 1) and *ex vivo* planar scanning of isolated colons (data not shown) confirmed the SPECT results. Mice with TNBS colitis that received sensitized CD4$^+$ lymphocytes had a more severe colitis upon histological evaluation compared with mice receiving non-sensitized CD4$^+$ lymphocytes (histological score of 14 and 10 respectively), although no statistical analysis was performed.
Scintigraphy for $^{111}$In labelled T cell migration in vivo

Figure 7 Colon radioactivity uptake is decreased after anti-α4 antibody injection

SPECT was performed of mice with TNBS colitis 48 h after transfer of $^{111}$In labelled TNBS sensitized lymphocytes and injection of a control or anti-α4 antibody.

A) Colon uptake ratio was calculated normalized to the bone marrow uptake in the pelvis. Boxplot of mean radioactivity uptake of the two groups as indicated (*p=0.012).

B) Isolated colons were also counted in a γ-counter. Results of the γ-counter were corrected for decay and expressed as counts per minute (cpm)/gram tissue. To correct for non-specific binding, ratios were calculated normalized to radioactivity in blood ((cpm/gram colon-cpm/gram blood)/cpm/gram blood) (*p=0.05).

due to the limited number of mice per group (n=2). No difference was observed in histological score in the healthy control mice that received sensitized or non-sensitized CD4+ lymphocytes (histological score of 2 and 3 respectively).

Blockade of α4 integrins inhibits migration into the intestine

Mice with TNBS colitis received a single injection with anti-ζ4 antibody or a control antibody, 4 h before transfer of TNBS sensitized $^{111}$In labelled CD4+ lymphocytes (experiment 3). SPECT after 48 h demonstrated that there was a significant decrease in colon uptake ratio after treatment with the anti-ζ4 antibody compared with the control antibody (p=0.012, figure 7A). Visual uptake assessment on the transverse SPECT slices (table 1) and ex vivo scanning of the isolated colons (figure 8) confirmed the difference between the anti-ζ4 antibody treated and control mice. In addition, γ-counting of the
Figure 8 Blockade of CD4⁺ cell migration to the colon after anti-α4 antibody injection

*Ex vivo* planar scintigraphic images of isolated cleaned colon show distinct colon radioactivity uptake in a mouse with TNBS colitis transferred with sensitized ¹¹¹In labelled CD4⁺ lymphocytes (panel A). Colon radioactivity uptake is decreased after treatment with an anti-α4 antibody (panel B). Images are representative of 5 mice per group.

Isolated colon corrected for weight and normalized to blood radioactivity showed a decreased radioactivity in the group treated with the anti-α4 antibody as compared with the group treated with the control antibody (figure 7B, p=0.05). The induced colitis was not severe (maximal histological score 8) and there was no significant difference in colon weights or histological scores between the two groups. Together, these data indicate that the anti-α4 antibody partially inhibited CD4⁺ cell migration to the colon in TNBS colitis. Moreover, this inhibition can be adequately detected by SPECT.

**Discussion**

The data presented here show that animal pinhole SPECT can be applied to study lymphocyte migration *in vivo* in experimental colitis, allowing non-invasive temporal and spatial assessment of lymphocyte migration to the inflamed colon. We visualized homing of TNBS sensitized lymphocytes to the inflamed intestine by SPECT and confirmed our findings with standard planar scintigraphy of isolated colons. TNBS sensitized
Scintigraphy for $^{111}$In labelled T cell migration in vivo

Lymphocytes remained functional after $^{111}$In labelling as they exacerbated signs of colitis. In addition, the colon radioactivity uptake correlated well with the histological scores of intestinal inflammation and can thus be used as a parameter of disease activity in experimental colitis. Finally, blockade of lymphocyte homing by an anti-α4 antibody was detected by SPECT in vivo and could be confirmed by planar scintigraphy of isolated colons.

Several radioactive lymphocyte-labelling techniques have been compared for in vivo biodistribution studies. $^{27}$-$^{30}$-$^{37}$ $^{111}$In-oxinate seems the most appropriate radiolabel for combined spatial and temporal in vivo analysis of T lymphocyte distribution, because of its adequate labelling efficiency, long half-life ($T_{1/2} = 67.2$ h), and gammaphoton energy peaks (171 keV and 245 keV) that allow satisfactory $\gamma$-camera photon detection. $^{38}$ For pinhole SPECT, a minimal amount of radioactivity in the target organ is needed for imaging. With a labelling efficiency of 60-80% in our study, this resulted in a required mean dose of 1.40 MBq/10$^6$ cells, which is considerably higher than the dose used for the above mentioned in vivo indium labelling and toxicity studies. Detrimental effects of isotope labelling, induced by radio-ionising effects, chelate toxicity and chemical toxicity of indium or its decay product cadmium may affect cell viability. $^{39}$-$^{42}$ However, our results clearly confirm in vivo functionality of the transferred $^{111}$In labelled lymphocytes. First, specific colon uptake was significantly higher in the inflamed intestine as compared with the saline controls, indicating active migration to the intestine. After intravenous injection, lymphocytes pass the lungs and then the liver and spleen. Most non-viable lymphocytes are trapped in the liver and it is believed that free radiolabel is not reutilized by lymphocytes but probably transferred to non-circulating cells like liver macrophages. $^{43}$-$^{45}$-$^{46}$ Secondly, we previously showed that colon uptake increased over time and peaked at 48 h post injection in mice with TNBS colitis, $^{29}$ suggesting continuous lymphocyte migration to the colon in time. Finally, $^{111}$In labelled TNBS sensitized lymphocytes exacerbated signs of intestinal inflammation. Together these data indicate that $^{111}$In labelled lymphocytes homed to the inflamed intestine and that $^{111}$In labelled sensitized lymphocytes were actively involved in the pathogenesis of colitis.

In this study we compared migration of TNBS sensitized lymphocytes with non-sensitized lymphocytes. The immunologically active moiety of TNBS links to autologous
colonic proteins, which become immunogenic and are presented via MHC-class II on antigen presenting cells to Th1 lymphocytes. TNBS sensitized CD4+ lymphocytes acquire an effector/memory phenotype and preferentially migrate to the tissues with high exposure to the inciting antigen. We showed that adoptive transfer of total splenic lymphocytes or CD4+ lymphocytes from TNBS sensitized mice, a population that included CD4+ memory lymphocytes (CD45RBhigh, CD62Lhigh and CD44high), resulted in a significantly higher colon uptake ratio in the inflamed colon compared with transfer of lymphocytes from non-sensitized mice. Moreover, the mice with TNBS colitis that received sensitized lymphocytes had a more severe colitis as determined by histological scoring and colon weights. These data are consistent with a study showing that transferred splenic lymphocytes isolated from rats with TNBS colitis migrated to the colon of recipients and exacerbated disease. The colon uptake ratio correlated well with the total histological score and the colon weight of mice with TNBS colitis. Indeed, the number of lymphocytes in the colon is one of the histological parameters of colitis severity. These findings prove that the colon uptake ratio assessed by SPECT can be used as a parameter of disease activity in vivo.

The presence of lymphocytes in the colon was determined visually and semi-quantitative by means of a ratio on transverse slices. The uptake ratio of colon to bone marrow uptake eliminates variables dependent on the exact amount of radioactivity injected intravenously and normalizes subjects within and in-between groups. This is important for comparison, since the exact amount of radioactivity and number of labelled lymphocytes injected intravenously is mostly unknown due to toxic cell loss and possible partial paravascular administration. The homing pattern of lymphocytes was further confirmed by the presence of radioactivity in the isolated colon of recipient mice as determined by planar scanning and γ-counting.

Based on the increasing knowledge of the molecular basis of leukocyte trafficking, new therapeutic strategies are designed that intervene with lymphocyte migration to the inflamed intestine. For optimal evaluation of therapy efficacy and understanding of the mechanism of action of new drugs, serial analysis of lymphocyte migration in animal models is a prerequisite for pre-clinical development. Treatment of mice with TNBS colitis with the anti-α4 antibody served as an example for the possible applications of
Scintigraphy for $^{111}$In labelled T cell migration in vivo

SPECT. We showed that animal pinhole scintigraphy of mice with TNBS colitis successfully detected inhibition of lymphocyte migration to the intestine by a single injection of an anti-α4 antibody. In line with previous studies, homing of CD4$^+$ lymphocytes to the inflamed intestine was partially blocked by a single injection of an anti-α4 antibody. Planar scanning and γ-counting of the isolated colon confirmed the SPECT findings. The set-up of this experiment was not therapeutic and we found no difference in our parameters of inflammation between administration of the anti-α4 or control antibody. For a therapeutic effect, earlier administration during the course of TNBS colitis of the anti-α4 antibody may be necessary. Efficacy of new therapeutic strategies with for example chemokine antagonists can be assessed by the here-described technique.

Collectively, the results of this study indicate that SPECT of $^{111}$In labelled lymphocytes is a straightforward and reliable technique to assess lymphocyte migration to the colon in experimental colitis and the radioactive uptake ratio of the colon can be used as a parameter of disease activity in vivo. Moreover, SPECT will improve the pre-clinical development of drugs that intervene with lymphocyte migration to the inflamed intestine.

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Chapter 6


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