Final thesis

Analysis of tumour infiltrating leukocytes in colon cancer carcinoma in a syngeneic rat model

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Thesis performed at Lunds experimental oncology research department
2010-04-05

LITH-IFM-A-Ex--10/2230--SE
Titel: Analysis of tumour infiltrating leukocytes in colon cancer carcinoma in a syngeneic rat model

Abstract:
Tumour immunity is a balance between immune mediators that promote tumor progression versus mediators that promote tumor rejection. Infiltrating lymphocytes in human colorectal cancer tissues are independent prognostic factors for a better survival and a high number of cytotoxic CD8+ T-cells have been associated with a better prognosis in terms of a longer and disease free survival for the patient. In our syngeneic rat model we induce colon carcinoma subperitoneally by injecting a colon cancer cell line BN7005, a cell line expressing the epitope (Lewis Y) for the BR96 antibody. Tumours are dissected out and treated with different fixatives and then either frozen, snap-frozen or embedded in paraffin followed by sectioning. Immunohistochemistry using monoclonal antibodies against the tumour infiltrating leukocytes was performed on the tissue. The results were seen as an infiltration of different leukocytes in the tumours.

Nyckelord:
Tumour infiltrating leukocytes, Immunohistochemistry, Colon cancer, monoclonal antibodies, BR96 antibody
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Analysis of Tumour infiltrating leukocytes in colon cancer carcinoma in a syngeneic rat model

1. Summary
In our syngeneic rat model we induce colon carcinoma subperitoneally by injecting a colon cancer cell line BN7005, a cell line expressing the epitope (Lewis Y) for the BR96 antibody. When exposing the rats to radioimmunotherapy with beta-emitters ($^{90}$Y, $^{177}$Lu) it has resulted in a complete remission of local disease. A fraction of locally cured rats have developed distant metastases later on.

Tumour immunity is a balance between immune mediators that promote tumor progression versus mediators that promote tumor rejection. Infiltrating lymphocytes in human colorectal cancer tissues are independent prognostic factors for a better survival and a high number of cytotoxic CD8+ T-cells have been associated with a better prognosis in terms of a longer and disease free survival for the patient. A Low-dose total body irradiation has been shown to have immunostimulatory but not immunosuppressive effects in rats. 0.2 Gy decreased the incidence of metastases and increased the number of CD8+ T-cells in the tumor.

1.1 Aim
To establish methods to detect different types of infiltrating leukocytes (TILs) in rat colon carcinoma, optimizing the method for different leukocyte markers and finding the best preparation method regarding both tissue and marker preservation.

1.2 Method
- Immunohistochemistry with primary and secondary antibodies
- Primary antibodies: mouse anti-rat CD8, CD3, CD4, CD68, CD163, Granulocytes, MHCII, CD45RA and CD45
- Secondary antibody: rabbit anti-mouse conjugated to Horse Radish Peroxidase
- Visualization: Slide scanner.

1.3 Results
There is a presence infiltrating T-lymphocytes, macrophages and a few granulocytes.
1.4 Future objectives

- Determine what types of CD4 T-helper cells are present in the tissue and if they are active or not because an infiltration of active CD4 positive cells type II gives a tumour progressive response whilst an infiltration of type I gives a tumour rejection response.
- Determine if there is a presence of T regs in the tissue, which if present would also further a tumour progression.
- Perform a low total body irradiation to try to activate and increase the number of CD8 positive T-cells which are the cells most associated with a good prognosis in colon cancer,
- Examine the changes of TILs during and after radioimmunotherapy, does the number increase or decrease?

2. Introduction

2.1 On Colon cancer

In 2004 the world health organisation, WHO, listed the top ten causes of death by broad income in high income countries. Colon cancer was in 7th place as a disease that killed 0.27 million people that year which made up 3.3% of all deaths that year.\(^1\)

Although most cases of colon carcinoma are curable with an operation where the tumour is removed from the colon, in 20-50% of the cases metastases occur post operation with a peak after two years. The peak is believed to be due to a possible presence of micro metastases before the surgery.\(^15\)

2.2 Magic bullets and Immune surveillance

In the 1900s Paul Ehrlich suggested that the immune system can be used therapeutically to diminish tumours by conjugating antibodies with toxins and targeting them against the cancer cells. A method he called “Magic bullets”. In 1950, Thomas and Burnet extended Ehrlich’s “Magic bullet” theory in their “Immune surveillance hypothesis” saying that the immune system diminishes malignant cells thus hindering them from developing into tumours and shielding the body from emerging cancers. Further studies showed that the immune system destroys a fraction of the tumour cells and hinders their development; a concept called immunoediting.
Immunoediting is the process where a tumour causing a weak immune response are allowed to survive but the tumours causing a stronger immune response (are strongly immunogenic) are selectively destroyed.

2.3 Immune response

Our immune response can be divided into an adaptive and innate response. The adaptive immune response involves a production of specific antibodies and/or cytotoxic T-cells against antigens. The innate immune response is a non-specific reaction to a foreign object and is caused by activation of defence mechanisms (non-specific).

When the body detects aberrant cells it responds with an immune reaction activating immune cells to destroy or diminish the foreign object. In colon cancer this defence mechanism can be displayed by observing an infiltration of immune cells, more precisely leukocytes (TILs), into the tumour. However the infiltration is not purely harmful for the tumour it can also be beneficial for the tumours progression and thus help it to grow. The difference between the responses causing a tumour progression versus the response causing a tumour rejection is due to what type of cells that have infiltrated the tissue.

The balance of tumour immunity is therefore dependent on the number and activity of cells that promote tumour rejection responses versus the number and activity of cells that mediate a tumour progression response. An overload of CD8 positive T-cells, CD4 positive T-helper cells type I (collaborates with macrophages of type I), natural killer cells, natural killer T-cells, macrophages type I and immune killer dendritic cells will shift the immunity towards a immune response that will favour a rejection of the tumour by killing the tumour cells or preventing them to differentiate and form cell clusters. On the other hand an infiltration of T regulatory cells, CD4-Thelper cells type II, Natural killer cells type II, Myloeid derived suppressor cells, M2 macrophages and B-cells will benefit the tumour and it will continue to progress. (See figure 1)

When the body experiences inflammation it can affect the immunity balance, the inflammation causes immune mediators to form and the mediators can withhold the inflammation or in fact induce it. The effect of the mediators will be that more of the second type macrophages, myeloid derived suppressor cells and CD4 T-regulatory cells will be activated and their number will increase leading to a shift in the immunity balance which will inhibit the systems destructive effect on the cancer cells. In other words the immune surveillance will be inhibited. If a tumour is already present, the
inflammation state gets withheld by inflammation-factors secreted by B-cells. The process becomes a vicious circle where a chronic inflammation causes a blockage of the immune surveillance by the activation and multiplication of the TILs mediating a tumour progression response. This in turn causes carcinogenesis where normal cells are transformed into cancer cells. See figure 2.


Fig 1 and 2: The tumour immunity is maintained by the balance between cells that promote tumour rejection responses and the cells that promote tumour progression responses. Chronic inflammation is a factor causing the immunity to shift towards tumour progression.

2.4 The importance of TILs
Colorectal cancer in human is divided into two groups; microsatellite-stable and microsatellite instable tumours. Microsatellite DNA is repetitive DNA where a dinucleotide DNA sequence repeats itself, how many repeats there are is highly individual. A mutation in the DNA mismatch repair mechanism causes the length of the repeats to vary, resulting in microsatellite instability. The stability can be indicated by the number of infiltrating leukocytes, a lower number of TILs indicate a microsatellite-stable tumour. Microsatellite-stable tumours represent 85% of all colon-cancers.

For human colon-cancer an infiltration of leucocytes is considered a prognostic factor, especially the infiltrate of CD8 positive cytotoxic cells which has been associated with a longer and disease-free survival.
Another significant infiltration is the infiltration of FOXP3+ T regulatory cells in cancerous tissue, it has been correlated to a better survival but an infiltration of FOXP3+ T-regs in normal tissue has been correlated to a bad prognosis. In an article by Pages et al it is stated that an infiltration of CD45RO positive memory T-cells is an important prognostic factor for colon cancer and the tumours microenvironment with the immune response against the cancer will affect the way the tumour progresses.

Another prognostic factor for colon cancer is infiltrating NKT-cells and which is correlated to fewer metastases in the lymph nodes.

There seems to be a decrease in infiltrating cells in higher tumour stages (the higher the tumour stage the more widespread cancer, see table 1) which is hypothesized to be due to the immuno-suppressive effect in more advanced cancers, the infiltration of intraepithelial lymphocytes or macrophages (in the periphery of the tumours) have been noted to decrease as the tumour spreads.

![Fig 3: The correlation between tumour stage and the number of TILs.](image)

<table>
<thead>
<tr>
<th>Tumour stage</th>
<th>TNM staging system</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Invasion of the submucosa</td>
</tr>
<tr>
<td>II</td>
<td>Invasion of muscularis propria</td>
</tr>
<tr>
<td>III</td>
<td>Inv. trough the muscularis propria</td>
</tr>
<tr>
<td>IV</td>
<td>Direct inv. of other organs and/or perforation</td>
</tr>
</tbody>
</table>

Table 1: TNM staging system of cancer

2.5 The syngeneic immunocompetent rat model

In our research group, we use a syngeneic rat model to find ways to develop and improve radio immunotherapy with radio-labelled antibodies. A colon carcinoma cell line called BN7005, expressing the epitope (Lewis Y) for the BR96 antibody, is injected into a rat which causes tumours to form underneath the peritoneum.
When antibodies labelled with 177-Lutetium or 90-Yttrium is directed against tumours in the rat the result has been a complete remission of tumours.

In this project we have inoculated a colon carcinoma cell-line into the immunocompetent animal model which causes the animals to react with an immune response against the cancer cells.

2.6 Questions raised

• Do we have a presence of TILs in our tumour tissue?
• Where are the TILs located in the tissue?
• What kinds of TILs are present?
• How can we detect the TILs?
• How can we optimize the method used for detecting TILs?
3. Material and Methods:

3.1 Tissue preparation and sectioning

Tumours and spleens were dissected from Brown Norway rats, (weight 100-250 g, Harland, Netherlands) and the tumours were treated in four different ways, see list below and the spleen was treated by fixation in PFA and embedding in paraffin.

- fixation in paraformaldehyde and freezing
- fixation in paraformaldehyde and embedding in paraffin
- zinc-fixated followed by embedding in paraffin
- snap-freezing directly after dissection.

All of the tumours and spleen tissue were cut in half before fixation. I received the tissues after fixation and embedding.

3.1.1 Paraffin embedded tissue: tumours and spleen

The paraffin-blocks were chilled with the tissue side down on a tempering plate at 1°C and sliced using a microtome into 4-5µm thick sections. The sections were then placed on a Superfrost Plus slide and a drop of distilled water (dH2O) was added to be able to position the section into place. Using a heating platter the sections were straightened out, the water poured off and the samples dried in RT for an hour followed by two hours in 58°C and then storage at 4°C.

3.1.2 Snap-frozen tumours

Sectioning of tissues that had been snap-frozen was performed in one of the experiments in order to identify the best preparation method for the tissue to be able to optimizing the staining. The tumour was dissected out from the rat, immediately cut in half, covered in Tissue-Tek® (Tissue-Tek® O.C.T.™ compound, Sakura Finetek, The Netherlands) then placed on dry-ice and isopentane. The tissue was then stored in -80°C until the sectioning. Using the Cryostat (Leica CM 3050, Leica Microsystems A/S) the tissue was sectioned into 10 µm thick sections and captured on a Superfrost Plus slide. The slides were left in RT for an hour and stored at -16°C.
3.1.3 Frozen tumours
The tissue was fixed in PFA, covered in Tissue-Tek® and stored in -80 °C until the sectioning and then sectioned in the same way as the snap-frozen tissue.

3.1.4 Zink Fixated
The tissue was fixed in IHC Zink fixative (BD Pharmingen) and embedded in paraffin and treated in the same way as the PFA- fixated tissue concerning sectioning and storage.

3.2 Immunohistochemical staining of paraffin embedded tissue

3.2.1 Removal of paraffin
The samples were dried for 15 minutes at 42°C and placed in Xylene for 2x5 min to remove the paraffin. Then the tissues were treated by the following hydrations steps: incubation in absolute ethanol for 2x5 min to remove any remaining Xylene, absolute ethanol for 2x5 min, 95% ethanol for 2x5 min, 70% ethanol for 2x5 minutes and finally distilled water for 3 min.

3.2.2 Epitope/Antigen retrieval
Epitope retrieval dissolves the cross-linkage achieved from the fixation of the samples and thus unmasking epitopes to which the antibodies can attach. 0.25 L of citrate buffer was prepared by dissolving 0.525g of citric acid in 0.2375 L of TBS-Tween buffer (pH 7.34) and the pH was adjusted to 6.0 by adding HCl. The volume was adjusted to 0.25 L by adding more TBS-T buffer. (For 2L TBS-Tween buffer 6.06g of Tris and 8.77g of NaCl was dissolved in 1900mL of distilled water. 0.5mL of Tween 20 was added, the pH adjusted to 7.4 with HCl and then more distilled water was added to make up a volume of 2L.)
The slides were placed in a container filled with citrate buffer and the container was placed in the “2100 Retriever” a pressure cooker that heats the buffer with the slides in it to 120° C and holds the temperature for 5 minutes then cools off, the whole process took about one hour. After the slides were cooled they were rinsed in distilled water for 5 min.
3.2.3 Inactivation of endogenous peroxidases

All the samples were rinsed in TBS-T buffer for 2x5 min. This ensures that the right pH is kept and removes the surface tension so that when the samples are treated with reagents, the reagents will spread out onto the tissue section. The buffer was poured off and a fine tissue paper was used to dry the glass around the tissue section. To all but one sample, 1-2 drops of peroxidase blocking solution (Dako REAL™ Peroxidase blocking solution, code S2023), was added (see fig 6) which inactivates any endogenous peroxidases present in the tissue and thus avoiding background staining, the solution was left on for 15 minutes. The samples were then rinsed again in TBS-Tween buffer for 2x5 min to remove the blocking solution. The buffer was poured off and fresh buffer was added a couple of times to make sure that all of the peroxidase blocking solution had been washed away.

3.2.4 Staining and Mounting

The primary antibodies were diluted in antibody diluent (Dako REAL™ Antibody diluent, code S2022) and titrated to find the optimal dilution for each antibody, the tested dilutions ranged from 1:50 to 1:200. Fifty µL of the primary antibodies were then added to the tissue sections and incubated at room temperature in a humidity chamber over night.

The next day the slides were washed in TBS-Tween buffer for 2x5 min and the secondary antibodies diluted. Polyclonal Rabbit Anti-Mouse Immunoglobulin (Polyclonal Rabbit Anti-Mouse Immunoglobulins/HRP, Code-No P 0260, DakoCytomation) conjugated to horse radish peroxidase (HRP) was diluted to 1:200.

The secondary antibody was added to the slides and incubated in a humidity-chamber at room temperature for 60 min. The antibody was washed off with TBST for 2x5 min.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone</th>
<th>Detected cells</th>
</tr>
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<tbody>
<tr>
<td>Anti CD45</td>
<td>OX-1¹</td>
<td>All leukocytes</td>
</tr>
<tr>
<td>Anti-CD4</td>
<td>OX-38¹</td>
<td>T helper cells</td>
</tr>
<tr>
<td>Anti-CD4</td>
<td>W3/25²</td>
<td>T helper cells</td>
</tr>
<tr>
<td>Anti-CD8a</td>
<td>OX-8¹</td>
<td>T cytotoxic cells</td>
</tr>
<tr>
<td>Anti-CD3</td>
<td>G4.18¹</td>
<td>All T cells</td>
</tr>
<tr>
<td>Anti CD45RA</td>
<td>OX-33¹</td>
<td>B-cells</td>
</tr>
</tbody>
</table>
Anti-CD68 (ED1) / Anti-mononuclear phagocyte

Anti rat- CD163 (ED2)

Anti rat granulocytes

Anti-rat MHCII / Anti-RT1B

IC7¹  Monocyte/macrophage type I

ED2²  Monocyte/macrophage type II

HIS48¹  Granulocytes

OX-6¹  Antigen presenting cells such as B-lymphocytes, dendritic cells and some macrophages

1; BD Biosciences Pharmigen, 2; AbD serotec

Table 2: Monoclonal antibodies used.

Add peroxidase blocking solution to inactivate endogenous peroxidases

Add the primary antibody (mouse anti-rat) to the tissue

Add the secondary antibody (polyclonal rabbit-anti-mouse) conjugated to horse radish peroxidase

Add chromogen (DAB) to HRP, mix and apply to the tissue

Fig 4: The immunohistochemistry procedure.
To stain the tissue section, 20µL of Diaminobenzidine (Dako REAL™ DAB+ Chromogen) containing substrate solution was added to 1mL HRP substrate buffer (Dako REAL™ HRP Substrate buffer) and added to all of the samples (see fig 6). DAB works as a substrate for HRP and its brownish polymeric oxidation product is visualised through precipitation. The cells marked will have a brown circle from the DAB-precipitate around them where the protein is expressed. The slides were left to incubate for 4 min at RT followed by rinsing in TBS-Tween buffer several times.

To stain the tissue sections further Mayer’s Haematoxylin was used, haematoxylin stains features containing nucleic acids such as the cell nucleus and the ribosomes leaving the cell dyed blue. Incubation of the tissues sections in Haematoxylin for one minute was followed by rinsing of the slides in flowing tap water for 10 min.

To dehydrate the tissue sections, the slides were placed in 70% ethanol for 2x3 min, 95% ethanol for 2x3 min, Abs. ethanol for 2x3 min and xylene for 2x5 min.

After the tissue sections had been dehydrated they were mounted by placing a drop of Pertex on the tissue sections and covering it with a cover-glass. The slides were allowed to dry over night in 4°C and the next day visualized using a slide scanner microscope (Mirax Midi with fluorescence option, digitizer, Zeiss, Software: Mirax scan and Mirax viewer)

The tissue prepared with the snap-frozen technique was sectioned in the cryostat, put on a Super-frost slide, air-dried for 30 min and stored in -80 °C over night. The next day the tissue was fixated to the glass in acetone for 10min and air-dried for 30 min. After the fixation, the protocol was the same as for paraffin-embedded tissue starting from the inactivation of endogenous peroxidases (no antigen-retrieval step was performed before the inactivation-step). The frozen tissue, fixed in PFA were also sectioned in a Cryostat and captured on a Super Frost slide, then treated in the same way as the paraffin-embedded tissue with the addition of an air-drying step for 15 minutes in 42 °C after the antigen retrieval.

The zink-fixated tumour was treated in the same way as the PFA-fixated tumours.

To compare the HRP-conjugated secondary antibody a fluorescent secondary antibody was used. The antibody only consists of the F(ab’)2 part
of the antibody and it is conjugated to a fluorophore (Alexa Fluor 488 F(ab’2) fragment of goat anti-mouse IgG (H+L) Invitrogen) which will emit light when subjected by a specific wavelength.

The method for staining on paraffin-embedded tissue follows the standard protocol stated above with the exceptions that the blocking step of endogenous peroxidases was not performed, instead a blocking step was performed with normal serum from goat, the goat serum was added to the tissue after the rinsing in a phosphate buffer saline called called DPBS 1x buffer (GIBCO, 14040, Lot 407221, ref 14040-091 from Invitrogen) which washes off any unbound primary antibodies. Incubation with the secondary antibody, diluted in DPBS for 60 min and rinsing in DPBS, 2x5 min followed. The tissue was mounted in Vectashield with DAPI and the cover-glass secured with nail-polish. The sections were visualized in a Cell observer microscope with Axio Vision software (Zeiss).

In the material part of this paper a second secondary antibody is mentioned, swine anti-rabbit which was going to further enhance the staining but there was no difference compared to just using one secondary antibody so it was excluded from the process.

4. Results

To test the reactivity of the antibodies directed at leukocytes the first experiment was performed on spleen tissue from the same rat strain. The antibody mouse anti-rat CD3 directed against all T-lymphocytes was titrated, to find the optimal dilution. At 1:200 the immune cells were detected all over the tissue and thus 1:200 was considered an appropriate dilution, see figures 5-7. By testing on a spleen a conclusion can be drawn that the anti-CD3 antibody works on PFA-fixated and paraffin-embedded tissue.
**Fig 5:** Mouse anti-rat CD3 on spleen tissue, dilution 1:50, scale bar represents 2000 µm.

**Fig 6:** Mouse anti-rat CD3, spleen tissue, dilution 1:100, scale bar represents 2000 µm.

**Fig 7:** Mouse anti-rat CD3, spleen tissue, dilution 1:200. Scale bar represents 2000 µm (left) and 50 µm (right)

To identify the possible infiltration of T-cells the same antibody was also used on tumour tissue from the rat model, the tumour was dissected from the same rat as the spleen. See figure 8. The resulting staining of the tumour tissue shows a presence of T-cells that have infiltrated the tumour.
Figure 8 shows the staining with anti-CD3 at a dilution of 1:200 where the cells were easily visualized. The brown circled cells are the infiltrating CD3 positive T-lymphocytes. The infiltrate was most abundant in the tumour margin and around necrotic areas.

The secondary antibody was also titrated; and at 1:200 the background was light enough to enable visualization of the infiltrating cells. When staining the tumour tissue with mouse anti-rat CD8 at a 1:50 dilution the infiltrating cells present were very dark so a further dilution of the primary antibody (CD8) was performed, and at a dilution of 1:200 the cells were nicely stained. See figure 9.
**Fig 9:** CD8+ T-cells stained with Mouse anti-rat CD8a for cytotoxic T-cells. Scale bar represents 1000 µm (left) and 50 µm (right).
Using a fluorescent secondary antibody was an interesting alternative to the HRP-conjugated one; the antibody was used on paraffin-embedded tissue. The figure above shows the resulting images taken in a fluorescence microscope. The cells are nicely stained and one can clearly see the CD8 glycoprotein being expressed on the cell surface. The green colour is the secondary antibody stained with Alexa 488 and the blue is from a colouring of the cell-nucleus by DAPI.

As stated earlier the CD3 antibody stains all T-lymphocytes which consist of CD4 positive cells and CD8 positive cells and thus should the number of cells stained by the anti-CD3 be the sum of the number of cells stained by anti-CD4 and anti-CD8. Below is the figure (11) of the staining with the anti-CD4 marker (clone OX-38), it stains T-helper cells (both Th1 and Th2) expressing the CD4 glycoprotein on their surface. The number of cells stained with anti-CD8 seems to be higher than the number stained with anti-CD3 and very few (or none in some slides) CD4- positive cells were found which was puzzling.
Fig 11: Anti-CD4 (OX-38) staining on PFA-fixated and paraffin embedded tumour tissue. Scale bar represents 2000 µm (left) and 100µm (right).

The anti-CD4 antibody (OX-38) was not recommended to be used on PFA-fixed tissue by the manufacturer which could explain the poor results seen in fig 11 and had also only been tested on frozen tissue. An attempt to successfully stain the cells on frozen tissue was performed; the result can be seen in fig 12.

Fig 12: Mouse anti-rat CD4 (OX-38) on frozen tumour tissue. Scale bar represents 2000µm (left) and 100µm (right).
The result from using anti-CD4 (OX-38) on frozen tissue did unfortunately not improve the result, and therefore the anti-CD4, clone OX-38 was disclaimed. Compared to staining of paraffin-embedded tissue the frozen tissue sections breaks more easily and the background colour was more intense making it hard to detect any infiltrating cells.

Another anti-CD4 (clone W3/25) was ordered from another manufacturer reported to work on paraffin embedded tissue, the result seen in figure 13.

![Fig 13: Mouse Anti- rat CD4 (W3725), overview with scale bar representing 1000 µm (left) and zoomed (right) where scale bar represents 50 µm.](image)

The cells stained brown by the anti-CD4 clone W3/25, figure 13 are very dark and are not stained in the same way as the CD8+ cells. All markers stained by the primary antibodies are glycoproteins expressed on the cell-surface and cells should therefore be circled by brown DAB-product precipitation instead of the overall staining.
Figure 14 displays paraffin-embedded tumour section stained for all leukocytes. The stained tissue has a diffuse brown precipitate in a large area. The staining is a bit grainy and it is difficult to determine a definite infiltration.

Fig 14: Mouse anti-rat CD45, for all lymphocytes, scale bar represents 500 µm (left) and 50 µm (right).
Fig 15: Mouse anti-rat CD45RA, B-cells, scale bar represents 1000 µm (top) and 20 µm (bottom).

The expected infiltration of B-cells was lower than the results seen in figure 15 as B-cells are developed in the bone marrow and are most present in the lymph nodes, spleen and blood.
A few granulocytes were detected albeit hard to determine because of the dark staining of the entire cells.
Fig 17: Mouse Ant-rat RT1B, stains B-cells, dendritic cells and some Macrophages, diluted 1:50, overview. Scale bar represents 1000 µm (top) and 50µm. (bottom)

The resulting staining of B-cells with RT1B were poor see fig 17, if the aim is to stain just B-cells it is preferably achieved using anti-CD45RA.
A few macrophages type II have infiltrated the tissue. These cells sometimes called TAMs largely infiltrate intensively growing tumours and promote tumour progressive response, they are important for the tumours angiogenesis\textsuperscript{4}. Our tumours growth is extensive and thus we should assume that the tissue would have a larger infiltration of these cells than shown above in figure 18.
A diffuse infiltrate of macrophages type I was observed although the cells were darkly stained and hard to interpret. The staining was all-over the cell instead of at the cell-surface. Type I macrophages produces cytokines of type I that gives a tumour rejection response and nitric oxide which works as a toxin for the tumour.

Another method used was snap-frozen tissue below, compared with paraffin-embedded tissue stained with anti-CD8. See figure 20 and 21.
The number of stained CD8-positive cells in the snap-frozen tissue was estimated to be approximately the same as in the paraffin-embedded tissue so the preparation method of the tissue seems to be equally good. However the frozen tissue was more difficult to section and it is easier to achieve thinner sections when using the microtome compared to the cryostat as the sections tend to fall apart easier when frozen albeit this might be due to laboratory malfunction or a lot of necrosis present in the tissue.

**Fig 21:** Paraffin-embedded tumour, fixed in PFA, stained with mouse anti-rat CD8.

**Fig 22:** Zink fixated tumour tissue, stained with anti-CD45. Scale bar represents 500 μm (top) and 50 μm (bottom).
When treating the tissue with Zink instead of PFA, the result after staining got very dark. Above in figure 22 is one of the few tissues that were fairly bright. A few diffusely stained cells were detected albeit oddly stained since the cells are far too dark, almost black.

The sectioning and the staining of the tissue that had been prepared with the snap-frozen technique was not as satisfactory as the tissues embedded in paraffin and fixed in paraformaldehyde, therefore we performed most of our experiments on paraffin-embedded tissue.

5. Discussion
The aim of this thesis project has been to establish and evaluate methods to detect and characterise tumour infiltrating leukocytes with immunohistochemistry in a colon carcinoma using our experimental syngeneic rat model. The rat was inoculated sub intraperitoneally with a colon cancer cell-line; BN7005. The tumours were dissected out after 13 days and divided in two-halves followed by fixation.

The importance of infiltrating immune cells previously have been established by many researchers and it is evident that TILs are an important prognostic factor for colon cancer.

In 2003 Kobaek-Larsen et al characterized azoxymethan-induced colon tumours by immunohistochemistry in their BDIX rat strain and found that the tumours histopathologically resembles tumours occurring in humans with colon cancer, they concluded that their experimental model could therefore be used as an appropriate model for predicting how colon cancer is affected by the infiltration of TILs in humans.

When looking at the tumour tissue from our model there was a minor infiltrate of macrophages (stained by Mouse anti-rat CD163 for type II and CD68 for type I). The type II (M2) sometimes also called TAMS for tumour associated macrophages are associated with tumour progression and the type I activated by interferon gamma (IFN-γ) can kill the tumour cells with nitric oxide as well as activating the tumour rejecting CD8+ T-cells and are thus associated with tumour rejection.

Macrophages that are activated by IL-4, IL-13 and TGF-β mediate a tumour favourable response and are thus classified as type II, progressive tumours have an abundant infiltration of these cells.
Since there was not a large infiltrate of the type II macrophages/TAMS in the tissue, there is a possibility that the response is not in favour of tumour growth, but tumour rejection thus associated with a good prognosis. However the tumour-growth in our model is extensive therefore it is not possible to draw any conclusions. In figure 19, where macrophages of type I (M1) can be visualized, there is a lot of dark staining but only a few cells are stained in the right way, at the cell surface, most of the dark staining is background staining. So an assumption can be made that the M1-infiltration is fairly low.

The tumours also had a few infiltrating granulocytes albeit a bit hard to determine because of the much too dark cells. A possible explanation could be due to the isotype of the antibody. The isotype of this antibody is IgM compared to the rest of the primary antibodies used that are of IgG isotyp. Since IgM is a larger molecule it is a bit “stickier” than IgG meaning that it is more likely to attach to nonspecific targets and thus causes more background, the secondary antibody used is anti rat –IgG, therefore the reactivity against rat IgM might be low. A granulocyte infiltrate is correlated to an improved prognosis in colorectal cancer\(^1\) and it is an important prognostic factor.\(^2\) Granulocytes, more specifically the eosinophilic cells, are activated in an autocrine fashion by the production of IL-5 coming from the stromal cells inside the tumour. The autocrine signalling keeps the eosinophilic cells alive and they can release toxic granules inhibiting the tumour-growth.\(^13\)

To stain cytotoxic T-cells mouse anti-rat CD8 was used and for T-helper cells of type I and II mouse anti-rat CD4. The type II of the T-helper cells promotes a tumour progression response whilst type I promotes a tumour rejection response. Unfortunately with this marker it is not possible to see if the T-helper cells were of type I or II. Like all markers stained in this project CD8 and CD4 are both glycoproteins expressed on the cell surface of the specific cells and therefore the brown staining from the DAB precipitate was located around the edges of the positive cells.

A reagent which stain all T-lymphocytes was also used, mouse anti-rat CD3 should in theory be the sum of both mouse anti-rat CD8 and mouse anti-rat CD4 stained cells and thus stain as much cells as both of them put together, the marker was used as a positive control as well in some experiments since it stained the tissue sections nicely. However no conclusion could be made about whether or not the staining of CD3 added
up to the staining of CD8 and CD4, the infiltration of CD8 positive T-cells was quite strong. When using anti-CD4 the results were poor, only a few cells were detected. The result was confusing since there were great results with both CD8 and CD3, the staining of CD3 did however resemble the infiltration of CD8 so there is a possibility the CD8 positive cells dominate in the tissue.

In humans the infiltration of CD4-positive cells are the most common and that CD8-positive cells are not as common, this contradicts the results gained with our rat model and made us begin to ponder if the CD4-antibody used might be sensitive to the PFA-fixation or to the antigen retrieval step in the protocol, a further study of the data sheet from the manufacturer informed us that the Mouse anti-rat CD4, clone OX-38 from BD Biosciences Pharmigen, was not suitable for use on paraffin-embedded tissue. The marker was therefore also evaluated on frozen tissue but there was no difference in the amount of infiltration and there was a lot more background staining. The solution was to evaluate another reagent for CD4 from AbD Serotec, mouse anti-rat CD4, clone W3/25, this time the results were somewhat better. The observation is still a higher infiltration of CD8-positive cells compared the few infiltrating CD4-positive cells. We must therefore conclude that in our tissues we have a strong infiltration of CD8 positive T-cells as shown by staining and a lesser infiltration of CD4-positive T-cells. Other antibodies are needed to determine if the CD4+ infiltrate is of type I or type II and to determine if they are in their active form since an active type I CD4+ T-cell promotes a tumour rejection reponse and an active type II a tumour progression response. A solution is to use the CD25 antibody which stains the IL2-receptor expressed on the surface of active lymphocytes. In a similar experiment to ours performed by Kobeak-Larsen their CD8-positive infiltrating lymphocytes were found not active when staining for the IL-2 receptor. And so using anti-CD25 together with an antibody specific for CD4-type I positive cells can reveal to us if our few CD4-positive cells are active or not.

The overall infiltrate of TILs was detected by a leukocyte common antigen reagent, mouse anti-rat CD45 and the result was a bit surprising, in some samples all of the cells were diffusely stained and in others there was no staining at all. The technical problems did not depend on the wrong fixative or preparation method as the antibody is supposed to work with both PFA-fixation and paraffin-embedding. We also tried to exclude the
antigen retrieval step as some marker epitopes might be sensitive to the high temperature used but there was no improvement.

B-cells where stained by anti-CD45RA and by anti-RT1B. The tissue stained with anti-CD45RA show a high infiltration of B-cells, when looking at figure 15 most of the cells in the tissue seems to be slightly stained (left) and when looking at the section in a higher magnification the cell surface seems to be darkly stained at a lot of cells, implying that these cells are in fact B-cells. In the tissue stained with anti-RT1B, the results were weak; the reason could be that it has, according to the manufacturer, only been reported to work on trypsin pre-treated formalin-fixated paraffin-embedded sections.

Some of the tissues stained have got very dark backgrounds and diffusely stained cells which can be caused by cross-reactivity for the secondary antibody.

6. Conclusion

In conclusion we have seen a presence of tumour infiltrating leukocytes in our experimental syngeneic rat model, the infiltration is located all over the tumour in some concentration to the margins. The infiltrate consists mainly of T-lymphocytes such as CD8 positive and CD4-positive T-cells where the CD8-positive cells are associated with a better prognosis as well B-cells.

The cells were detected on a PFA-fixated, paraffin embedded tumour tissue, frozen PFA-fixated tissue and snap-frozen tissue with immuno histochemistry with monoclonal mouse anti-rat antibodies. Both a secondary antibody conjugated to horse radish peroxidase, and a fluorescent secondary antibody was used although not on the same sections.

A strategy to optimize the methods has been to titre the primary antibodies to find the optimal dilution for detection of the cells and to titre the secondary antibody so that background staining was avoided as much as possible. Further optimization has been to find the best preparation method for the tissue, frozen, zink-treated, paraffin or snap-frozen. The resulting images display our preference in using the paraffin, PFA-fixated tissue. The reasons are both staining improvement compared to zink-treated and frozen tissue as well as better sectioning results.
To determine if there is a presence of T regs in the tissue and what kind of CD4+ T-cells are present and if they are active or we need to analyze other markers, for example anti-CD25 as well as examining the changes of TILs during and after RIT, does the number increase or decrease?

7. References


8. **Acknowledgements**

I would like to thank my supervisor Rune Nilsson for all his excellent guidance and support and for his patients with all the lab reports I sent, altered and sent again before he had a chance to read the first version sent…

I also would like to thank Sophie Ericsson and Anna Ebbesson for all of their help and tutoring in the lab and in the office and to Jan Tennvall for his guidance and support.

A big thank you to Kristina Lövgren who introduced me to everything in the new lab and who patiently answered all of my questions.

Last but not least I would like to thank my family.