Treatment with IL-2 and IL-12 Inhibits Tumour Cell Division in SL2 Lymphoma

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Abstract. We examined which mechanism plays a dominant role in the rejection of solid SL2 lymphoma treated with locally applied IL-2 and/or IL-12. This treatment resulted in about 80% cures. There was a moderate influx of leukocytes in the tissue surrounding tumours; yet these cells failed to invade the solid tumours. Potentially cytotoxic cells were not observed in close proximity to areas of tumour cell death, indicating that cell-mediated cytotoxicity is not an important mechanism of tumour rejection in this model. Similarly, inhibition of blood vessel growth and/or blood vessel injury could be ruled out as mechanisms, since tumour rejection was not accompanied by decreased angiogenesis or blood vessel injury. We did observe that many tumour cells die via apoptosis or necrosis and that tumour cell division in cytokine-treated mice is inhibited. In conclusion, IL-2/IL-12-mediated tumour rejection in solid SL2 lymphoma is mainly due to a shifted balance between tumour cell death and tumour growth caused by inhibition of proliferation, rather than to direct cell cytotoxicity or destruction of blood vessels.

The anti-tumour potential of IL-2 and IL-12 is well established (1-5). The mechanisms involved in their anti-tumour action are complex, and different authors define the relative role of various cytokine properties in the overall efficacy of therapy in different ways. Most authors stress the importance of T cells in tumour rejection during IL-2/IL-12 treatment, as IL-2 and IL-12 are both potent T/NK cell stimulators. IL-2 regulates proliferation and differentiation of antigen-activated lymphocytes (6), it augments the cytolytic activity of NK cells and it induces lymphokine-activated killer (LAK) cells. IL-12 is involved in the specific T cell-mediated immune responses (7). Cytotoxic T lymphocytes (CTL) seem to be especially important for IL-12 action as their depletion diminishes its anti-tumour effect (8). On the other hand, T cells are not absolutely necessary for IL-12 anti-tumour action since SCID mice also respond to the treatment (9). NK cell involvement was studied by Kodoma et al. who showed that NK cells are sufficient to mediate IL-12 action (10). Another report suggested that NKT cells are responsible for the anti-tumour effect (11). IL-12 may also serve as an indirect angiogenesis inhibitor (12-14) working via induction of IP-10 and MIG (produced mainly by NK cells), via down-regulation of VEGF (15) and via induction of hypoxia (16). IL-12 may also induce adhesion molecule (ICAM-1, VCAM-1, E-selectin) expression (17).

We examined processes induced by local IL-2 and/or IL-12 treatment and their relative roles in the rejection of solid tumours, using as a model subcutaneous SL2 lymphoma. We studied the distribution of leukocytes, blood vessels, apoptotic, necrotic and proliferating cells within tumours of cytokine- or placebo-treated mice.

Materials and Methods

Mice. Inbred DBA/2 JIco, female mice at the age of 6-8 weeks were obtained from Iffa Credo, France. The Utrecht University Animal Ethics Committee approved all experiments.

Reagents. Recombinant human IL-2 (rhIL-2; specific activity 18x106 IU/ mg) was a gift from Chiron (Amsterdam, The Netherlands). rhIL-2 was reconstituted to 1mg/ml with water. Dilutions were made using phosphate-buffered saline (PBS) with 0.1% bovine serum albumin, fraction V (BSA; Sigma Chemical Co., MO, USA). Recombinant murine IL-12 (rmIL-12; specific activity 2.7x106 IU/mg), a gift from the Genetics Institute (Massachusetts, USA), was diluted in PBS with 0.1% BSA.
Tumour. The DBA/2-derived SL2 lymphoma (SL2) grows subcutaneously (s.c.) as a solid nodule and intraperitoneally (i.p.) in both ascitic and solid form. SL2 was maintained by weekly i.p. transplantation.

Treatment protocol. SL2 cells (2x10^5 per mouse) in RPMI 1640 were injected s.c. in the right flank of naïve syngenic mice at day 0. IL-2 (200 kU) and/or IL-12 (0.25 µg) in 0.2 ml PBS with 0.1% BSA were injected peritumorally daily during a 5-day course, starting on day 10 after tumour cell inoculation. Control mice were treated with diluent. The length (L), breadth (B) and height (H) of the solid tumours were measured. The volume (V) was calculated as

\[ V = \frac{1}{6} \pi L B H. \]

In survival experiments, mice were followed till day 60 and were considered to be cured if they had no outward evidence of tumour at this time. Mice experiencing difficulty with moving or eating, or with tumours with a largest diameter of more than 15 mm, were euthanised.

Histological stainings. \textit{HE} staining: On days 7, 10, 12 and 14, s.c. tumours were fixed in buffered 4% formalin, embedded in paraffin and the sections (5 µm) were stained. Three serial-cut sections from each distinct part of the tumour from each individual mouse were analysed.

\textit{Plastic sections:} Tumours were fixed and embedded as for electron microscopy. One-µm sections were stained with toluidine blue. Three sections from three distinct parts of the tumour from each individual mouse were analysed.

\textbf{Electron microscopy.} The tumours were fixed in 4% paraformaldehyde and 2% glutaraldehyde in PBS (Sigma), trimmed into 3 to 4-mm³ pieces, post-fixed in 1% osmiumtetroxide in 0.1 M sodium-cacodylate (pH 7.4) for 1 h, washed with distilled osmium, embedded in Durcupan ACM resin (Fluka, Bachs, Switzerland) and sectioned to approximately 50-60 nm on a Reichert Ultracuts (Leica Aktiengesellschaft, Vienna, Austria). The sections were stained for 2 min with Reynolds’ lead citrate, examined and photographed with a Philips CM10 electron microscope (Philips, Eindhoven, The Netherlands) at 80 kV.

\textbf{Immunological stainings.} Detection of cell proliferation: Two hours after sacrifice, mice were injected i.p. with 2.5 mg of the S-phase marker bromodeoxyuridine (BrDU; Sigma). The tumours were fixed in methacarn (60% methanol, 30% chloroform, 10% acetic acid) and embedded in paraffin. Subsequent incubations were performed on sections: 1% periodic acid (30 min at 55°C), 5% BSA (30 min); anti-BrDU antibody (60 min, 1:80; R&D, The Netherlands); peroxidase-labelled secondary antibody (1:80, Dako Corp., CA, USA).

\textit{TUNEL method:} Formalin-fixed, paraffin-embedded tissue sections were used. Subsequent incubations were performed: protease K (1:500, 20 µg/ml, 15 min); 3% hydrogen peroxide in PBS (10 min); terminal transferase and biotin-16-dUTP (overnight, 4°C; Boehringer Mannheim, Germany); and peroxidase-conjugated streptavidin (30 min at 37°C in a 1:500 dilution). Staining with \textit{anti-active caspase 3} antibody: Formalin-fixed, paraffin-embedded tissue sections were used. Subsequent incubations were performed: 3% hydrogen peroxide in PBS (20 min); 10% normal goat serum in PBS (20 min); rabbit anti-active caspase 3 antibody (overnight, 4°C, 1:50 dilution; R&D); biotinylated goat anti-rabbit antibody (60 min, 1:100 dilution); and finally peroxidase-conjugated streptavidin (60 min, 1:500 dilution).

\textbf{Detection of blood vessels:} Tumours were snap-frozen in liquid nitrogen, sectioned and fixed in acetone (10 min). Subsequent incubations were performed: 3% hydrogen peroxide in PBS (20 min); 5% normal goat or rabbit serum in PBS (20 min); monoclonal rat anti-CD31 antibody or polyclonal goat anti-collagen IV (45 min, 1:100 dilution; R&D); peroxidase-conjugated goat anti-rat or rabbit anti-goat IgG (60 min, 1:100 dilution).

\textbf{Detection of T cells and macrophages:} Tumours were snap-frozen in liquid nitrogen, sectioned and fixed in acetone (10 min). Subsequent incubations were performed: 3% hydrogen peroxide in PBS (20 min), 5% normal goat serum in PBS (20 min); monoclonal rat anti-CD1, CD2, CD3, TCR, CD11b or F4/80 antibody (overnight, 1:100 dilution; Biosource); peroxidase-conjugated goat anti-rat IgG (60 min, 1:100 dilution).

In all immunological stainings positive cells were visualized with DAB and counterstained with haematoxylin. Three serial-cut sections from three distinct parts of the tumour from each individual mouse were analysed.

\textbf{RT PCR.} Total RNA was extracted from frozen tissue sections using the StrataPrep Total RNA Microprep Kit (Stratagene Europe, Amsterdam, The Netherlands). Reverse transcription was carried out with the Senscript RT-enzyme kit (Qiagen, Leusden, The Netherlands) with 1 µg oligo dT24-primers and 10U RNase Inhibitor (both Invitrogen, Breda, The Netherlands) in a total volume of 20 µl. PCR of cDNA was performed in 30 µl of Taq reaction buffer (1.5 mM MgCl₂, 20 mM Tris pH 8.4, 50 mM KCl) containing 0.2 mM dNTP, 0.1 unit Taq DNA Polymerase (Amersham Biosciences, Roosendaal, The Netherlands) and 333 nM of each primer. Cycling conditions were: 5 min at 94°C; followed by 25-35 cycles 30 sec at 94°C, 45 sec at 57°C, 45 sec at 72°C, and finally 7 min at 72°C. The sequences of primer pairs were as follows: GAPDH: forward 5'-CCATCACCATCTTCTACGGAGG-3'; reverse 5'-TCCACAAGTCACTGCTTGCACTTCAAGAAATAT-3'; reverse 5'-CAGGTTGGGGGAGGAGG-3'; reverse 5'-TACGACCTCTCAAGAAATAC-3'.

\textbf{Histological findings.} Solid tumours were formed about 5 days after tumour cell injection (Figure 2A). On day 7 the tumours were palpable; consisting of compact nodules surrounded by loose connective tissue. There were no infiltrating cells or sepa, yet necrotic areas were already present (Figure 2B). In day-10 tumours, sepa and some infiltrating cells were present; there was an influx of leukocytes (Figure 2C).

\textbf{Results} Efficacy of the treatment. All mice treated with placebo developed large tumours (Figure 1A). In the groups treated with IL-2 or IL-12, 64% and 50%, respectively, of the animals were cured (Figure 1B,C). The best therapeutic effect was achieved with combined treatment: 86% of cured animals (Figure 1D). This was impressive since, at the beginning of treatment (day 10), the mice already had 50 to 200-mm³ s.c. tumours.

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On day 12, after 3 daily injections of placebo or IL-2 and/or IL-12, some infiltrating cells were present in the
periphery of tumours. Slightly more infiltrating cells were present around tumours from cytokine-treated mice. In all groups, leukocytes failed to infiltrate the solid tumours. Many small blood vessels were present in the connective tissue surrounding the tumours (Figure 2D). They were activated with thickened endothelial cells (Figure 2E). Only a few necrotic cells could be found at the borders of the tumour nodule, whereas many necrotic cells were situated in the dense tumour tissue; in Figure 2F numerous picnotic nuclei are visible. Tumour necrosis was probably not a result of hypoxia or lack of nutrition, since the tumours were well vascularized and we could frequently observe normal blood vessels surrounded by dead tumour cells (Figure 2G).

On day 14 most tumours from placebo-treated mice were larger than those from cytokine-treated mice. The tumours from placebo-treated mice contained extensive necrotic and viable areas. In contrast, the tumours from cytokine-treated mice consisted mainly of necrotic debris and a few, small patches of viable cells.

Cell death. Examination of HE sections from day-12 tumours allowed identification of many apoptotic cells (Figure 3A), either situated in the neighbourhood of large necrotic areas or individually spread in the solid tumour tissue. A few apoptotic cells were observed at the rim. We confirmed these results with TUNEL (detection of the

Figure 1. Anti-tumour effect of local IL-2 and/or IL-12 treatment. Mice were injected s.c. with 2x10⁵ SL2 cells on day 0. On days 10-14 placebo (A), 200 kU IL-2 (B), 0.25 µg IL-12 (C), or 200 kU IL-2 and 0.25 µg IL-12 (D) were administered daily. Tumour volume was measured every other day beginning on day 8. Data shown are pooled from 2 individual experiments (each n=7); each line represents a single tumour.
Figure 2. Histological findings. Mice (n=7) were injected s.c. with 2x10^5 SL2 cells on day 0. An overview picture of a tumour dissected on day 5 (A); centre of a tumour dissected on day 7 (B); connective tissue of tumour dissected on day 12 after daily placebo injections from day 10 until 12 (C). Pictures of tumour dissected on day 12, 8 h after last injection of daily course of 200 kU IL-2 and 0.25 μg IL-12 given from day 10 until 12: connective tissue (D), blood vessel with active endothelium (E), necrotic area (F), blood vessel (G). Sections shown were stained with HE. Symbols used in the picture - a: adipose tissue, c: connective tissue, e: epidermis, m: muscle, n: necrotic area, t: tumour, red arrows: blood vessel, yellow arrows: cells crossing the endothelium, yellow arrowheads: thickened endothelial cell.
Figure 3. Apoptosis of tumour cells. Mice (n=7) were injected s.c. with 2x10^5 SL2 cells on day 0 and treated with 200 kU IL-2 and 0.25 µg IL-12 daily from day 10 until 12. Tumours were dissected 8 h after the last injection. HE staining (A), TUNEL staining (B), and active caspase 3 staining (C). Arrows point to apoptotic cells.

Figure 4. Vascularization of tumours. Mice (n=7) were injected s.c. with 2x10^5 SL2 cells on day 0, and were treated with placebo or 200 kU IL-2 and 0.25 µg IL-12 daily from day 10 until 12, and tumours were dissected 8 h after the last injection. Sections from placebo-treated mice: consecutive sections: CD31 staining (A), collagen IV staining (B). Sections from placebo-treated mice: collagen IV staining (C). Sections from IL-2/IL-12-treated mice: collagen IV staining (D).
characteristic DNA fragmentation) and active caspase 3 (apoptosis-related protein) stainings. Typical examples of cells considered apoptotic are shown in Figure 3B and C. The results from HE, TUNEL and active caspase 3 staining indicated a similar pattern of apoptotic cell distribution. There were no differences in occurrence or distribution of apoptotic cells in tumours from different treatment groups.

Similarly, most necrotic cells in the day-12 tumours were present within the solid tumour. At this time point necrotic areas were of similar size in tumours of all groups. Day 14 tumours from all groups were very necrotic. The placebo-treated tumours, however, were bigger and also had large areas of viable cells. The number of apoptotic cells in these tumours was comparable to the number of apoptotic cells in tumours of day 12. On the other hand, tumours from cytokine-treated mice consisted mainly of necrotic cells with very few viable areas. In these tumours detection of apoptosis was impossible due to the overwhelming false-positive staining of necrotic cells.

Vascularization. Day-12 tumours from different groups were stained for CD31 (Figure 4A) and for collagen IV (Figure 4B). A dense blood vessel network was observed using both markers, although more vessels seemed to be visualized with collagen IV, as shown on consecutive sections (compare Figure 4A and 4B). In all tumours were areas of diverse vessel density: especially a high number of blood vessels (Figure 4B) and "normal" density areas (Figure 4C, D). We were not able to observe differences in the numbers of blood vessels between tumours from placebo (Figure 4C) and cytokine-treated mice; blood vessels in cytokine-treated mice were, however, more diluted (Figure 4D). To confirm the observations regarding the number of blood vessels, we measured levels of CD31 mRNA expression in different tumours.

Expression of CD31 mRNA. CD31 is specifically expressed on endothelial cells. We were not able to detect differences in the levels of expression of CD31 in the different treatment groups on day 12 after tumour cell injection (Figure 5). Although truly quantitative data on mRNA levels can only be obtained by real-time RT PCR techniques, the data acquired by conventional RT PCR did not indicate extensive differences in CD31 mRNA levels between the treatment groups.

Electron microscopical studies of blood vessels. Electron microscopical studies of blood vessels were performed in order to confirm the observation that blood vessels of normal morphology were present in the areas of dead tumour cells. We examined numerous blood vessels in viable and necrotic patches of tumours. Blood vessels of normal morphology were found in tumours from all treatment groups, surrounded both by viable (Figure 6A) and by necrotic (Figure 6B) tumour cells.

Infiltration by leukocytes. We performed stainings for macrophages (F4/80, CD11b) and T cells (CD1, CD2, CD3, and TCR). Some macrophages were present in the loose connective tissue surrounding tumours (Figure 7A, B), but only sporadically within tumour nodules from all groups. There were no differences in number of macrophages in day-12 tumours versus day-14 tumours (data not shown).

We were not able to distinguish T cells using any of the above-mentioned antibodies, since the SL2 tumour (T cell lymphoma) expressed CD1, CD2, CD3 and TCR, making distinction between tumour cells and T cells very difficult. We based lymphocyte detection on morphological examination of thin plastic sections stained with toluidine blue. There were on average twice as many lymphocytes in the connective tissue surrounding cytokine-treated tumours.

![Figure 5. Expression of CD31 mRNA in tumours. Mice were injected s.c. with 2x10^5 SL2 cells on day 0 and treated with placebo (mouse 1 and 2), 200 kU IL-2 (mouse 3 and 4), 0.25 µg IL-12 (mouse 5 and 6), or 200 kU IL-2 and 0.25 µg IL-12 (mouse 7 and 8) daily from day 10 until day 14. Tumours were dissected 8 h after the last injection. Total RNA was extracted from tumour sections and subjected to RT-PCR analysis with GAPDH and CD31. PCR was carried out in duplicate for each mouse.](image-url)
Figure 7D) as around placebo-treated tumours (Figure 7C). Lymphocytes were not, or only sporadically, found within the tumours. There were no differences in the numbers of lymphocytes in day-12 tumours versus day-14 tumours (data not shown).

**Tumour cell proliferation.** Proliferating cells were detected by the BrdU incorporation assay. In day-12 tumours of the different treatment groups, there were no striking differences in the presence of dividing cells. In day-14 tumours, a dramatically reduced occurrence of proliferating cells was observed in cytokine-treated tumours (Figure 8B) versus placebo-treated mice (Figure 8A). These findings indicate that shrinkage of cytokine-treated tumours is accompanied by inhibition of tumour cell division, while growing tumours from placebo-treated mice consist of rapidly proliferating cells.

**Discussion**

Because IL-2 may induce vascular leakage syndrome (18) and IL-12 may act anti-angiogenically (12), our first efforts concentrated on analysis of the tumour vasculature. Both cytokine- and placebo-treated tumours were supplied with a dense vascular network and with numerous, small blood vessels in the connective tissue surrounding the tumour. In contrast to other authors (13), we were not able to detect a decreased number of blood vessels in IL-12-treated tumours.

Most blood vessels from viable patches of tumour had normal morphology (Figure 6A), suggesting that the tumour cells were well supplied with nutrients and oxygen. It was remarkable that functional vessels were frequently surrounded by dead and dying tumour cells (Figure 2G, 6B), as if cytotoxic agents were being supplied by the blood vessels.
Figure 7. Tumour infiltration by immune cells. Mice (n=7) were injected s.c. with $2 \times 10^5$ SL2 cells on day 0 and were treated with placebo or 200 kU IL-2 and 0.25 μg IL-12 daily from day 10 until 12. Tumours were dissected 8 h after the last injection. Sections from placebo-treated mice: F4/80 staining, arrows point to macrophages (A), toluidine blue staining on plastic sections, arrows point to lymphocytes (C). Sections from IL-2/IL-12-treated mice: F4/80 staining, arrows point to macrophages (B), toluidine blue staining on plastic sections, arrows point to lymphocytes (D).

Figure 8. Proliferation of tumour cells. Mice (n=7) were injected s.c. with $2 \times 10^5$ SL2 cells on day 0 and were treated with placebo or 200 kU IL-2 and 0.25 μg IL-12 daily from day 10 until 14. Six h after the last cytokine injection, BrdU was injected i.p. and, 2h later, tumours were dissected. Section from placebo- (A) and IL-2/IL-12-treated mouse (B).
We also examined the role of direct cell-cell cytotoxicity in tumour regression. Examination of HE sections, active caspase 3 and TUNEL stainings revealed patches of necrotic cells and individual apoptotic cells spread throughout the tumour, rather than concentrated at the rim. This lack of massive cell death in the rim of treated tumours was unexpected, since this is the location where leukocytes gather, so cell cytotoxicity should be expected. It is especially unusual in light of the general belief that cytotoxic cells are responsible for tumour rejection after cytokine treatment. Panelli et al. have shown that IL-2 injections stimulate the release of chemoattractants, which induces migration of immune cells (19). Indeed, there are studies showing that 7/7 regressing metastatic melanoma and renal carcinoma lesions were permeated with macrophages and CD8+ T cells, while only 3/7 non-responding tumours showed no infiltration (19). Wigginton et al. (20) have shown that i.p. treatment with IL-2 and IL-12 induces CD4 and CD8 influx in s.c. Renca tumours. In fact, the ascitic SL2 tumour also exhibits an influx of cytotoxic T cells during i.p. treatment with IL-2 and IL-12 (submitted for publication), and macrophages are essential for tumour rejection in this model (21). In solid SL2 tumour, on the other hand, we observed a moderate influx of lymphocytes and macrophages into the connective tissue surrounding the tumour and only sporadically observed leukocytes within the tumour. Given the lack of infiltrating cells within the tumour where necrosis was detected and that few necrotic cells were found in close proximity to potentially cytotoxic cells, we conclude that cell-cell cytotoxicity can be excluded as a major mechanism of tumour rejection in this model.

Since absence of vessels, vessel dysfunction and cell-cell cytotoxicity can be excluded as major tumour rejection mechanisms, we investigated whether there is increased apoptosis and/or decreased proliferation of cells in treated tumours. Increased apoptosis in tumours treated with IL-2/IL-12 has been shown previously. The authors suggest that cytokine treatment may induce tumour rejection by inhibiting tumour cell division, thus redressing the balance between proliferation and cell death. There is an interval of about 4 days between the start of IL-2/IL-12 treatment and inhibition of tumour cell proliferation. This suggests an indirect effect. Nonetheless, it remains unclear as to how IL-2 and IL-12 inhibit cell division in this model.

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References


