Early response measurements of NGR-TNF efficacy using MR and immunohistochemical methods

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Introduction: Targeted delivery to the tumor of picogram doses of TNF-α can be achieved by coupling TNF-α with CNGRC (NGR-TNF), a peptide that targets tumor neovasculature. NGR-TNF can improve response to chemotherapy (1) by altering tumor vasculature and tumor microenvironment. To assess NGR-TNF efficacy, the direct effect of NGR-TNF on these tumor characteristics should be evaluated, rather than measuring its effect on tumor growth. Moreover, to avoid exposure to a treatment which may cause side-effects it would be desirable to evaluate NGR-TNF efficacy at an early stage of the treatment. The aim of this study was to asses NGR-TNF efficacy at an early stage in lymphoma bearing mice, using dedicated MR methods and immunohistochemistry.

Materials and methods: RMA lymphoma cells were injected subcutaneously in the flank of female C57BL/6 mice. At a diameter of 5 mm NGR-TNF was injected intraperitoneally in a dose of 100 pg/mouse in a group of 6 mice. As a control six mice were injected with normal saline. Tumor growth was measured daily using callipers. Since the effect of NGR-TNF on tumor growth has been observed already on the first day after treatment (1), MR measurements took place on the first treatment day on a 7T/200 mm horizontal-bore SMIS system. Inversion recovery snapshot fast low angle shot (IR-FLASH) was performed to measure T_1 relaxation time (TR/TE = 5/2.7 ms, inversion times = (52 + n*320) ms, where n = 0,1,…16, image matrix size 64 x 64, FOV 3 x 3, SLT 1.6 mm). Then Sinerem, an ultra small particles of iron oxide (USPIO) blood pool contrast agent, was injected 2 hours after treatment, since a maximum effect of NGR-TNF combined with doxorubicin has been observed when doxorubicin was administered 2 hours after NGR-TNF (unpublished data). Pixel-by-pixel T1 maps were generated from a three-parameter fit of the image intensities. For each tumor two T_1 maps before USPIO and three after USPIO were obtained. From each map, the mean T_1 of the whole tumor was calculated and normalized to the value of the first time point. Also, for each tumor a threshold value (TV) was calculated from the formula TV = $< T_1 >$ -1.5 SD, where $< T_1 >$ and SD are the mean T_1 and the standard deviation values at time point #1. At all time points, the percentage of pixels that were below this threshold was determined. The statistical significance ($p < 0.05$) of differences between the percentage of pixels below TV in the control and treated tumors was determined by means of a two-tailed student t-test. (2;3) In two separate groups of treated and control mice CCI-103F and pimonidazole were used as markers of hypoxia. CCI-103F was injected before treatment and pimonidazole, combined with the perfusion marker Hoechst33342 and the proliferation marker BrdUrd, were administered two hours after treatment. Tumors were removed, stored in liquid nitrogen and then cut for immunohistochemical staining and image analysis (4). The hypoxic fraction (HF), perfused fraction (PF) and BrdUrd labelling index (LI) of each tumor section was computed.

Results and Discussion: A significantly smaller mean tumor volume on the first day after treatment with NGR-TNF as a single agent was observed compared with tumor volume the first day after injection of normal saline, as reported previously (1). All tumors showed a small but consistent increase in the T_1 relaxation time at time point #2 (fig.1); suggesting an effect of the anesthesia on the T_1 possibly due to (micro-)edema. Just after USPIO administration (#3, fig.1), treated and control animals had similar decrease in T_1 relaxation time. The decrease just after USPIO is proportional to the tumor blood volume and is expected to be the same for tumors of the same cell line. At the time points #4 and #5 T_1 further decreased in time in both control and treated group. The decrease of T_1 in time is due to an increase in the amount of contrast agent within the imaged pixels, which can be attributed to extravasation of the contrast agent from the vascular space into the interstitial space. The decrease in T_1 was more pronounced in the treated than in control animals ($p > 0.05$). The percentage of pixels below the threshold increased with time in both treated and control groups which was significantly different between the treated and control tumors at time point #4 and #5 (35.4 ± 7.3 and 12.9 ± 2.2, respectively, $p = 0.01$, N=5). This suggests that leakage of the USPIO from the vasculature is greater in NGR-TNF treated tumors than in controls, indicating vessel damage and/or increased vessel permeability.On microscopical observation a considerable overlap between hypoxic areas before and after treatment is noted (fig. 2), suggesting that no gross changes in hypoxia occur after treatment with NGR-TNF. A trend for a significant decrease in hypoxia ($p = 0.076$) and an increase in labeling index (average = 29.4%; $p = 0.081$) was observed after NGR-TNF treatment. The percentage of proliferating cells that are hypoxic seemed to increase after NGR-TNF treatment ($p = 0.078$). A decrease in hypoxic fraction and an increase in proliferation may be explained by an increased delivery of oxygen due to increased tumor blood flow after NGR-TNF (5). Increased proliferation may also be a direct effect of TNF on tumor cells, since *in vitro* experiments have shown enhanced proliferation rates in normal cells after treatment with TNF (6), although it should be noted that this phenomenon is not observed in vitro in RMA lymphoma cells (unpublished results). In conclusion, two hours after NGR-TNF treatment we observed an increase in vessel permeability, a decrease in tumor hypoxia and an increase in proliferation. Although the latter two would increase tumor growth, we observed a decrease in tumor growth one day after NGR-TNF, possibly because the increase in vascular permeability led to haemoconcentration and increased interstitial pressure, on the longer term (i.e. later than two hours) resulting in an reduction of tumor blood flow (5) and thus in a decrease in tumor growth.

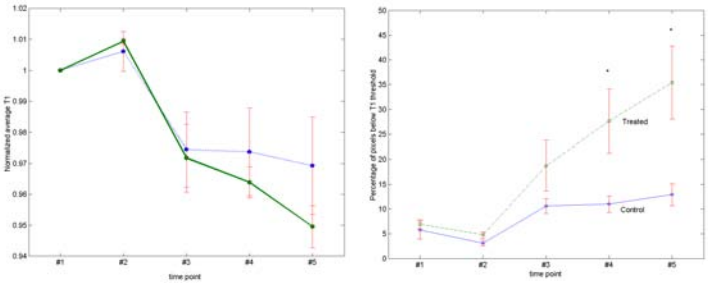

Fig 1. Plot of the normalized average T_1 (left) and the percentage of pixels below the T_1 threshold value (right) at 2 time points (#1 and #2) before USPIO and 3 time points (#3, #4 and #5) after USPIO administration, in treated (green line) and control groups (blue line)

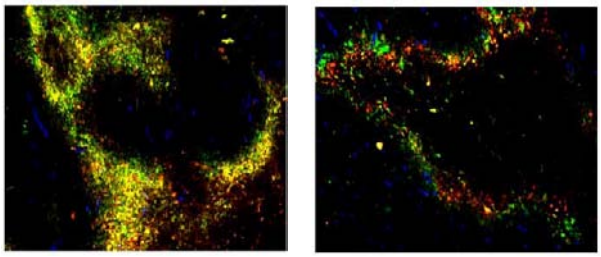

Fig. 2 Photomicrograph of a detail of RMA-T lymphoma of the control group (left) and the group treated with NGR-TNF (right) showing vasculature and hypoxic areas. Vascular structures: blue; hypoxic areas stained by CCI-103F: red; hypoxic areas stained by pimonidazole: green; overlap of CCI-103F and pimonidazole stained areas: yellow.

Literature: 1. Curnis, F., et al. J Clin Invest, *110*: 475-482, 2002; 2. Kauppinen, R. A. NMR Biomed, *15*: 6-17, 2002; 3. Duvvuri, U., et al. Cancer Res, *61*: 7747-7753, 2001. 4. Rijken, P. F., et al. Microvasc Res, *50*: 141-153, 1995. 5. Kallinowski, et al. Br J Cancer, *60*: 555-560, 1989. 6. Sugarman, B. J., et al. Science, *230*: 943-945, 1985.

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