IL-1 Receptor Antagonist Anakinra Enhances Tumour Growth Inhibition in Mice Receiving Peptide Vaccination and β-(1-3),(1-6)-D-Glucan

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Abstract. *Background: Immunotherapy of cancer by vaccination is hampered by tumour-mediated immune suppression, to which pro-inflammatory cytokines such as interleukin-1 (IL-1) and IL-6 contribute. In mouse models, IL-1-receptor antagonist (IL-1 Ra) diminished inflammation and tumour growth when administered during or shortly after tumour inoculation. Materials and Methods: The capacity of IL-1 Ra anakinra to reduce IL-1-induced production of IL-6 in order to improve the efficacy of a subsequent booster vaccination with survivin-derived peptides and soluble β-glucan as adjuvant was tested in colon-26 adenocarcinoma-bearing Balb/c-mice. Results: Bolus administration of anakinra into non-immunized mice with macroscopic tumour significantly lowered serum levels of IL-6 without inhibiting tumour growth. When administered to pre-immunized mice bearing a palpable tumour, IL-1 Ra enhanced growth inhibition of a subsequent booster vaccination, although serum-IL-6 was not reduced and the number of IFN-γ-producing splenic $CD8^+$ T-cells was not increased. Conclusion: Anakinra contributes to growth-inhibition of small tumours, presumably by blocking IL-1 as tumour growth-promoting factor rather than by facilitating an enhanced CTL response.*

Pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α play a key role in chronic inflammation-driven tumourigenesis, and once a tumour is established, in angiogenesis and metastasis (1-6). Furthermore, these cytokines are involved in tumour-induced cachexia and have been shown to contribute, depending on the size of the tumour, to local or systemic suppression of protective innate and adaptive immune responses (7-11). The usage of anti-inflammatory drugs to inhibit or prevent acute and chronic inflammation has a broad rationale in the treatment of autoimmune and rheumatic diseases. Some of these compounds, which include glucocorticoids, non steroidal anti-inflammatory drugs (NSAIDs) and cytokine inhibitors, have also shown beneficial effects against cancer-induced chronic inflammation and cachexia (7, 8, 12, 13). However, little is known about the potential of these drugs to facilitate the re-establishment or therapeutic induction of innate and adaptive immune responses in tumour-bearing hosts. Recently, in one of the first of such studies, the treatment of fibrosarcoma-bearing mice with the synthetic corticoid dexamethasone (DX) was shown to abolish systemic inflammation and to restore responsiveness of the adaptive immune system to immunization with tumour lysate-loaded dendritic cells (DCs) (14).

In the hierarchy of pro-inflammatory cytokines, IL-1 acts as inducer of IL-6 and TNF-α, making it an attractive target for the inhibition of acute and chronic inflammation (6, 8, 15-17). The function of IL-1β is controlled by the naturally occurring IL-1 receptor antagonist (IL-1 Ra) which binds competitively to IL-1 receptors without mediating any biological effect (18). In colorectal carcinomas, the ratio of IL-1 Ra and IL-1β has been found to be significantly diminished and IL-1β has been shown to promote angiogenesis and metastasis in human cancer cell lines and experimental mouse models (1, 4, 6, 19, 20). In mice with established murine colon-26 adenocarcinoma, which produces trace amounts of IL-1β and expresses the type I IL-1 receptor (IL-1 RI), exogenous IL-1 Ra diminished IL-1-induced production of IL-6 *in vivo* and reduced cachexia without inhibiting tumour growth (8, 15, 21). Higher doses of IL-1 Ra have been shown to decrease tumour growth, angiogenesis and metastasis in murine IL-1 producing xenografts when continuously administered from the day of tumour inoculation (20). Anakinra is a recombinant IL-1 Ra clinically approved for the treatment of rheumatic diseases in combination with disease modifying anti-rheumatic drugs (DMARD) and shows

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beneficial effects in a growing number of systemic inflammation-associated diseases (22-24). The effect of IL-1 Ra on antigen-specific immune responses *in vivo* is controversial: initial experiments showed that neither CTL nor $CD4^+$ T helper cell responses were inhibited by IL-1 Ra, whereas recent studies described diminished or impaired activation and expansion of antigen-specific $CD4^+$ and $CD8^+$ T-cells through systemic inhibition of IL-1 receptors (25-28).

This study tested the capacity of anakinra to improve the efficacy of anti-tumour vaccination against the intradermally (*i.d.*) implanted colon-26 tumour, using two peptides derived from the tumour-antigen survivin and a soluble β-(1-3), (1-6)-D-glucan as adjuvant. β-Glucans are branched glucose polymers derived from the cell wall of a variety of plants and microorganisms and exert pleiotropic activation of the innate immune system in mice and humans (29). Recent studies revealed an adjuvant function of soluble and particulate β-glucans administered by different routes during peptide or protein vaccination (30-32). Survivin is expressed in almost all malignant tissues including murine and human colon cancer (33) and immunization with peptides from this tumour-antigen effectively inhibited tumour growth in different mouse tumour models (32, 34, 35).

Materials and Methods

Mice and cell line. Female, 8-week-old Balb/c-mice (Charles River, Sulzfeld, Germany) were maintained in the pathogen-free animal facility of Epo GmbH (Berlin-Buch, Germany) following institutional guidelines and with approval from the responsible authorities. The adherently growing Balb/c-derived colon-26 carcinoma cell line was kindly provided by Epo GmbH and was maintained in DMEM medium supplemented with 10% FBS and 50 μg/ml gentamicin (all from Biochrom, Berlin, Germany).

Reagents and vaccine. The peptides survivin66-74 (GWEPDDNPI) and survivin85-93 (AFLTVKKQM) were obtained from Biosyntan GmbH (Berlin-Buch, Germany). Composition of the vaccine has been described elsewhere (32). In brief, 50 μg of both peptides were dissolved in 50 μl phosphate-buffered saline (PBS) and emulsified with an equal volume of incomplete Freund's adjuvant (IFA; Sigma-Aldrich, Taufkirchen, Germany). The recombinant human IL-1Ra anakinra (Amgen GmbH, the Netherlands) was adjusted to 1 mg/50 μl PBS. Viscous, aqueous preparation of underivatized β-(1-3),(1-6)-D-glucan (20 mg/ml), derived from the inner cell wall of *Saccharomyces cerevisiae*, was obtained from Biotec Pharmacon (Tromso, Norway) and was dissolved in PBS. Endotoxin level was <0.05 EU/ml.

Anti-inflammatory pre-treatment and tumour-peptide vaccination. To evaluate the effect of anakinra on the growth of an established tumour, mice were subcutaneously (*s.c.*) inoculated with 1×10^6 colon-26 cells and once injected *s.c.* with 1 mg of the drug (n=5) or an equal volume of PBS (n=10) when the tumour volume was between 300-600 mm^3. Tumour-free control mice (n=5) received simultaneously an *s.c.* injection of PBS. The body weight was measured every two or three days between day 7 and day 16. Tumour volume was determined by measuring the width and length of the tumour twice weekly using an electronic calliper. Mice were sacrificed when the tumour volume reached 1.5 cm^3 or the animals showed moribund signs such as ruffled fur and decline of activity. To determine the effect of anakinra on anti-tumour peptide vaccination all mice were first pre-immunized by *s.c.* injection of the peptides into the flank on days 14 and 7 prior to *i.d.* inoculation of 1×10^6 colon-26 tumour cells on day 0. Subsequently, mice received *s.c.* a single dose of anakinra (1 mg/mouse) when the tumour volume was between 100-250 mm^3, followed two days later by a booster immunization with peptides. Alternatively, mice were intragastrically co-administered β-glucan (400 μg/mouse) once daily over three days starting with the booster vaccination, or they received both anakinra and β-glucan addition to the booster vaccination as described above (all groups n=8). Pre-immunized tumour-bearing control mice (n=8) received only *s.c.* PBS once in parallel to the administration of anakinra to the treatment groups. Mice were sacrificed when the tumour size reached 2.0 cm^3 or signs of moribundancy were visible. The animal experiments were performed according to the German Animal protection law and with approval from the responsible authorities.

Quantification of pro-inflammatory cytokines. The concentrations of IL-1α, IL-1β and IL-6 in the peripheral blood were quantified using 'sandwich' cytokine-specific enzyme-linked immunosorbent assay (ELISA) detection kits (eBioscience, Frankfurt, Germany). In brief, 50 μl peripheral blood were taken from all mice by retro-orbital puncture at the indicated time points, and serum was kept frozen (–20˚C) until analysis. Serum was pooled from the mice of each group and time point before the concentration of the cytokines was determined in duplicates following the manufacturer`s instructions.

Quantification of IFN-γ–secreting $CD8^+$ T-cells. IFN-γ-secreting $CD8^+$ T-cells were quantified in splenocyte suspensions from two mice of each group one week after the booster immunization by flow cytometry using cytokine-specific secretion assays (Miltenyi Biotec GmbH, Germany). In brief, the splenic tissue was pressed gently through a 70 μm cell strainer (BD Falcon, Heidelberg, Germany) using a syringe plunger to remove connective tissue and debris. Subsequently, splenocytes were washed twice with RPMI-1640 medium before 1×10^6 cells were seeded in duplicate wells of a 24-well plate and re-stimulated with 5 μg of each peptide over 4 hours. Thereafter, the cells were stained with a FITC-labelled anti-CD8α antibody (clone 53-6.7; eBioscience, Frankfurt, Germany) and for secreted IFN-γ, following the manufacturer's instructions. A total of 1×10^5 cells were measured using a FACS-Calibur and data were analyzed with the CellQuest software (BD) based upon gating on $CD8^+$ T-cells.

Statistics. Data are presented as mean±the standard error of the mean (SEM). Statistical significance of the data was calculated by two-sided, paired Student's *t*-test. Significance levels of $p<0.05$, and $p<0.005$ were chosen.

Results

Anakinra diminishes IL-6 serum levels in tumour-bearing mice. The concentration of IL-1α, IL-1β and IL-6 in the peripheral blood during colon-26 tumour growth and the capacity of a single treatment with anakinra to reduce the

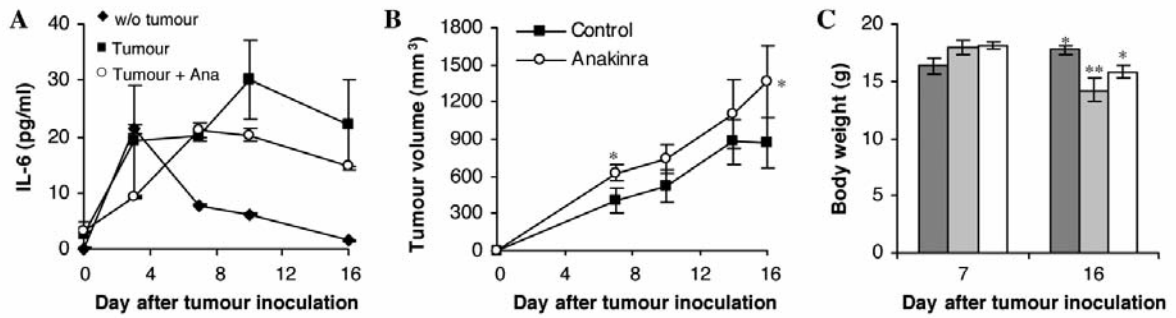

Figure 1. *Effects of anakinra on serum level of IL-6, tumour growth and body weight of colon-26 carcinoma-bearing mice. A-C: Mice remained tumour free (n=5), or were inoculated with 1×10^6 tumour cells on day zero. Tumour-bearing mice received s.c. anakinra (1 mg/mouse; n=5) or PBS (n=10) once when the tumour volume was between 300 and 600 mm^3 (day 8). A: Quantification of IL-6 in the peripheral blood serum pooled from all mice of each group at the indicated time points by ELISA. Mean±SEM values of duplicates are shown. B: Tumour growth curve of untreated versus drug-treated tumour-bearing mice. *Significant (p<0.05) difference between groups by two-sided Student's t-test. C: Determination of body weight in healthy (dark) and drug-treated (grey) or untreated (white) tumour-bearing mice. Significant (*p<0.05, **p<0.005) difference between day 7 and day 16 within the groups by two-sided Student's t-test.*

serum level of these cytokines when the tumour volume was in the range of 300 to 600 mm^3 were determined. IL-1α and IL-1β were not detectable in the serum of tumour-free and tumour-bearing mice by standard ELISA (data not shown). Tumour-free mice showed a transient elevation of serum IL-6 after injection of PBS, which quickly declined, whereas the serum concentration of IL-6 in untreated tumour-bearing mice increased until day ten after tumour inoculation before it markedly decreased (Figure 1A). A single administration of 1 mg anakinra on day 8 after tumour injection inhibited further augmentation of serum IL-6 within 2 days followed by a 33% decline of this cytokine until day 16.

Anakinra had no inhibitory effect on the growth of established colon-26 tumours in vivo but reduced loss of body weight. Whether the anti-inflammatory drug affects tumour growth and cachexia-associated loss of body weight of tumour-bearing mice was analysed. Differences in tumour volume and body weight between the groups on day 7 were due to a lack of randomizing the mice. Anakinra treatment had no inhibitory effect on tumour growth (Figure 1B). Healthy mice gained on average 9% (1.45 g) of their body weight between day 7 and 16 (p=0.022, Figure 1C), whereas untreated tumour-bearing mice lost on average 21% (3.71 g) of their weight during this period (p<0.001). In contrast, drug-treated tumour-bearing mice showed a weight loss of only 13% (2.30 g) (p=0.01).

Anakinra enhances the tumour-inhibitory effect of peptide vaccination on small colon-26 tumours without improving vaccine-specific CD8$^+$ T-cell response. To evaluate whether the anti-inflammatory drug improves the efficacy of anti-tumour peptide vaccination, pre-immunized mice received a single dose of anakinra when the tumour volume was between 100 and 250 mm^3, followed by a peptide booster immunization with or without adjuvant. A further group received a booster immunization with peptides and β-glucan without anakinra and a pre-immunized control group was injected with PBS instead of the peptide booster vaccine. Pre-treatment with anakinra resulted in a slightly greater tumour-inhibition than the co-treatment with β-glucan to the booster vaccination (Figure 2A). However, combining the peptide vaccine with both anakinra and β-glucan induced a superior growth-inhibitory effect being significant in comparison to PBS-treated control mice (p=0.028). In two mice of each group exhibiting either the smallest or largest tumour it was further determined whether the inhibition of IL-1 receptors enhanced the induction of peptide-specific CD8$^+$ T-cells by the vaccine. In the tested mice, which were treated with anakinra before, or with β-glucan during the booster immunization, or received only PBS, a small tumour size was associated with a high percentage of IFN-γ secreting CD8$^+$ T-cells and normal spleen size (Figure 2B). In contrast, large tumours were associated with a lower frequency of activated tumour-specific CD8$^+$ T-cells and with strong splenomegaly in these treatment groups. However, the tested mice which received both anakinra and β-glucan during vaccination exhibited a small or intermediate tumour size accompanied with a low percentage of IFN-γ-producing CD8$^+$ T-cells and mild enlargement of the spleen.

Co-treatment with β-glucan prevents decrease of IL-6 serum levels by anakinra during peptide vaccination. To determine the effect of anakinra on the levels of the pro-inflammatory cytokines IL-1α, IL-1β and IL-6 during anti-tumour peptide vaccination, blood samples were taken from all mice prior to and 3, 5 and 8 days after administration of the drug. The

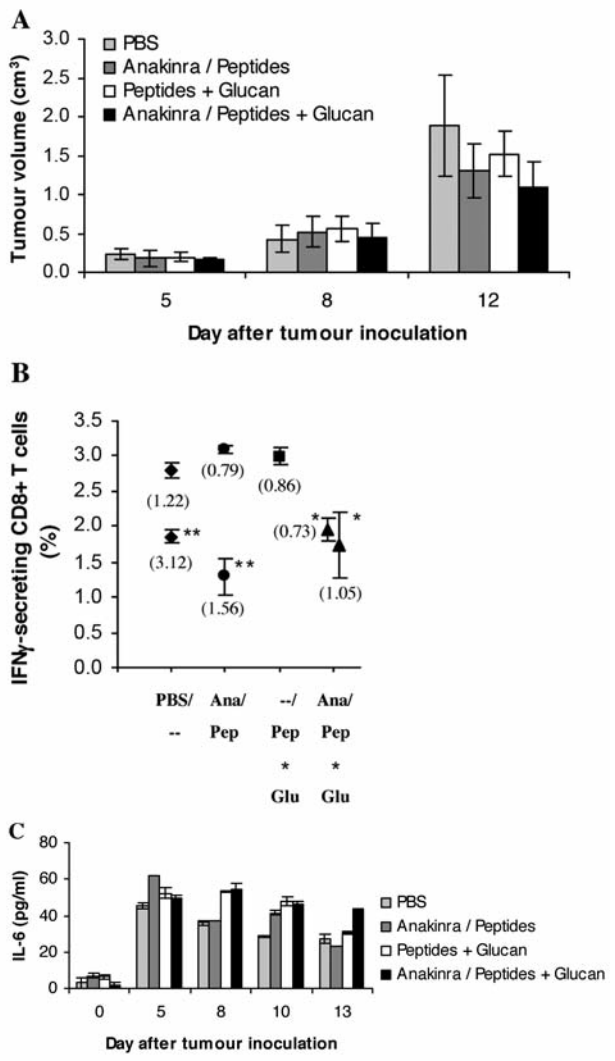

Figure 2. *Effects of anakinra on colon-26 tumour growth, induction of tumour peptide-specific CD8+ T-cells and IL-6 serum levels during peptide vaccination. A-C: Mice were immunized twice on day-7 and day-14 and inoculated s.c. with 1×10⁶ tumour cells on day zero. Mice received once s.c. anakinra (1 mg/mouse) when the tumour volume was between 100 mm³ and 250 mm³ (day 5) followed by a booster immunization with peptides (day 7) and co-administration of β-glucan from day 7 to day 9. Control mice lacked either the pre-treatment with anakinra or co-treatment with the adjuvant, and a third group received only PBS on day 5 and lacked a booster immunization (all groups: n=8). A: Evaluation of tumour growth after anti-inflammatory treatment prior to the booster immunization with peptides plus β-glucan. Data show the mean±SEM of eight mice in each group at the indicated days. *Significant (p<0.05) difference from PBS-treated control group by two-sided Student's t-test. B: Quantification of peptide-specific CD8+ T-cells as a percentage of total CD8+ T-cells in the spleen of tumour-bearing mice one week after the booster immunization by flow cytometry. +/++Mice showing mild or strong splenomegaly, respectively. Data represent the mean±SEM of samples from individual mice measured in duplicates, and the number in brackets indicates the tumour volume. C: Quantification of IL-6 in pooled serum from all mice of each group at the indicated time points by ELISA. The mean±SEM values of duplicates are shown.*

levels of IL-1α and IL-1β were also below detection limits in pre-immunized tumour-bearing mice (data not shown). Similar to non-immunized tumour-bearing mice, the IL-6 serum levels of pre-immunized mice were low prior to tumour inoculation followed by a strong increase after injection of the tumour (Figure 2C). In PBS-injected mice, a 40% decrease of serum IL-6 was observed between day 5 and 13. The pre-treatment with anakinra on day 5 significantly lowered the serum concentration of IL-6 in the absence of β-glucan by 62% between day 5 and 13 ($p<0.001$). In contrast, the anti-inflammatory drug failed to notably diminish systemic IL-6 levels in mice subsequently co-administered with β-glucan (–13%; $p=0.09$). Similarly, mice treated only with peptides and β-glucan nearly maintained concentrations of serum IL-6 until day ten, however, this was followed by a decline of 35% ($p=0.024$).

Discussion

This study aimed to transiently reduce tumour-induced systemic inflammation in pre-immunized colon-26 carcinoma-bearing mice by the blockade of IL-1 receptors early after tumour inoculation and to use this potentially less immune-suppressed 'gap' for a booster immunization. Serum levels of IL-1α and IL-1β incolon-26 tumour-bearing mice were below the detection limits of standard ELISA as shown by others (13, 36). However, trace amounts of IL-1β were reported to induce significant production of IL-6 in these mice which was diminished by administration of IL-1 Ra in a similar range observed in the current study (7, 8, 16, 21, 36). The early increase of serum IL-6 in tumour-free and some of the tumour-bearing mice could be trauma-related due to injection of PBS or tumour cells, respectively (14). The slightly but not significantly reduced loss of body weight and the failure of anakinra to inhibit the growth of established colon-26 tumours have been described elsewhere, also from repeated *s.c.* or intratumoural administration of IL-1 Ra (8). This could be due to the high sensitivity of the tumour to picomolar concentrations of IL-1β and the drug's short half-life of 4-6 hours (15, 37). Nevertheless, weakly delayed tumour growth in non-immunized mice between day 7 and day 10 independent of treatment with anakinra implicates recognition of the tumour by the immune system which could be enhanced by vaccination with tumour-specific peptides in combination with a potent adjuvant (14, 30-32, 38-40). To reduce impairment of the host-immune response by IL-1β-induced inflammation, pre-immunized mice were administered with anakinra at the stage of a small palpable tumour, two days prior to a booster vaccination with peptides and β-glucan. However, the reduction of systemic levels of IL-6 by the blockade of IL-1 receptors appeared to be of minor importance for the tumour-inhibitory effect during vaccination: The mice exhibiting the smallest tumours

showed the highest serum concentrations of IL-6, although they had received anti-inflammatory pre-treatment. Furthermore, serum IL-6 was diminished by anakinra only in the absence of co-treatment with β-glucan, which implicates an adjuvant-induced production of IL-6, potentially independent from IL-1β. Indeed, soluble 1-3,1-6 β-glucans are well known for their pro-inflammatory effects, including the induction of IL-1β and IL-6 production by DCs or macrophages *in vitro* and *in vivo* following binding to dectin-1 (42, 43). Thus, due to its short half-life, the anti-inflammatory drug could have failed to inhibit production of IL-6 by IL-1β newly induced by the later treatment with the adjuvant (37, 43). The mere fact that serum levels of IL-6 were not diminished is not sufficient to prove that systemic inflammation was completely unaffected by anakinra. Other markers of systemic inflammation such as serum concentration of TNF-α, C-reactive protein or serum A amyloid protein and the number of circulating polymorphonuclear neutrophils could exhibit treatment-related changes (14). The stronger increase of serum IL-6 after tumour inoculation in pre-immunized than observed in non-immunized mice may rely on a sensitization of macrophages and DCs by the previous vaccinations.

The comparable levels of IFN-γ^+ $CD8^+$ T-cells in mice of the control and treatment groups with normal or enlarged spleens, respectively, indicate that both the adjuvant and the anti-inflammatory drug failed to facilitate the expansion of tumour-reactive CTLs by the booster vaccination. This could be due to impaired maturation of peptide-presenting DCs and macrophages and expansion and accumulation of $CD11b^+GR1^+$ immature myeloid derived suppressor cells (MDSCs) in the peripheral blood and spleen of tumour-bearing hosts (9-11, 44-46). Indeed, in most of the tested mice, splenomegaly was associated with high tumour burden and a low frequency of IFNγ-producing $CD8^+$ T-cells and *vice versa*. A single administration of anakinra at the stage of a small tumour could have been ineffective to attenuate the expansion of MDSCs and enlargement of the spleen. Accordingly, an effective reduction of the spleen size and percentage of splenic MDSCs was shown after multiple administrations of IL-1 Ra into mice starting shortly before tumour inoculation (9). Nevertheless, a recent study revealed significant reduction of splenic MDSCs in melanoma-bearing mice when repeatedly administered with IL-1 Ra at tumour volumes >1000 mm^3 (47).

The lack of an activatory effect of anakinra on peptide-specific $CD8^+$ T-cells could also rely on its recently shown detrimental effects on peptide vaccination comprising the inhibition of antigen-specific priming of naïve T-cells and of the expansion of activated $CD8^+$ T-cells (26-28). The inhibitory effect of the drug on the growth of small tumours may instead be mediated by the blockade of IL-1β as pro-angiogenic and pro-metastatic tumour-promoting factor (1, 4, 19, 48). The systemic treatment of T- and NK cell-deficient mice bearing IL-1β-producing xenograft tumours with IL-1 Ra upon tumour inoculation has been shown to be associated with marked down-regulation of intratumoural IL-8, VEGF and neovessel density (19). The orally administered soluble β-1,3-D-glucan may have contributed similarly to tumour inhibition through the recently reported suppression of angiogenesis besides its well-established adjuvant immune-activatory effect on peptide vaccination (30-32, 49). Taken together, the inhibitory effect of systemic IL-1 Ra on the growth of small colon-26 tumours prior to a peptide booster vaccination may be attributed to the inhibition of IL-1β as tumour-promoting factor instead of to improved induction of peptide-specific $CD8^+$ T-cells. When the malignant tissue has exceeded a critical size, inhibition of IL-1β by a single administration of IL-1 Ra may be insufficient to diminish tumour growth.

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