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Systemic cancer therapy with a small molecule agonist of Toll-like receptor 7 can be improved by circumventing TLR tolerance

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Abstract

Topical application of small molecule Toll-like receptor 7 (TLR7) agonists is highly effective for the treatment of skin tumors, whereas their systemic application has been largely unsuccessful for cancer therapy. One reason may be that repeated systemic application of TLR ligands can induce a state of immune unresponsiveness, termed TLR tolerance. We show here that a single injection of the TLR7 agonist R848 in mice induces a short period of increased response to TLR stimulation followed by a state of hyporesponsiveness lasting several days. This state is characterized by inhibited secretion of the key cytokines IL-12p70 and IL-6 as well as by a block in IFN- α production. We demonstrate for the first time that at the cellular level, TLR7 tolerance occurs in both plasmacytoid and myeloid dendritic cells, two cell populations that play a critical role in the initiation and amplification of antitumor immune responses. We further show that TLR7 tolerance in plasmacytoid dendritic cells is accompanied by downregulation of the adaptor protein interleukin-1 receptor-associated kinase 1 (IRAK-1). Based on these findings, we have designed a novel strategy for the treatment of tumors using cycles of repeated R848 injections separated by treatment-free intervals. We show in CT26 tumor-bearing mice that this protocol circumvents TLR7 tolerance and improves the efficacy of cancer immunotherapy.

Introduction

Invading pathogens are recognized by the innate immune system through pattern-recognition receptors such as the Toll-like receptor (TLR) family. Ligation of their cognate ligand by TLR leads to the maturation of immune cells and to the induction of cytokine and chemokine production (1). Dendritic cells (DC) express many TLR and therefore play a critical role in the initiation of innate and adaptive immunity to pathogens (2). Synthetic TLR agonists can be used pharmacologically to generate strong immune responses and have been investigated for the immunotherapy of cancer. In particular, the topical administration of small molecule activators of TLR7 and TLR8 are effective for the treatment of skin tumors. Topical administration of imiquimod (Aldara 5% cream), a synthetic TLR7/8 ligand of the imidazoquinoline family, is effective as treatment for basal cell carcinoma (3), lentigo maligna (4) and actinic keratosis (5). TLR7/8 ligation leads to potent inflammatory cytokine secretion by macrophages and myeloid DC (6) as well as interferon- α (IFN- α) release by plasmacytoid DC (6, 7).

In patients, the systemic application of TLR7/8 agonists leads to enhanced production of IFN- α and to activation of natural killer (NK) cells (8), suggesting a potential therapeutic benefit in cancer immunotherapy. However, studies investigating the systemic application of TLR7/8 agonists for cancer therapy are rare. In one study exploring the potential of a systemic TLR7/8 stimulus (852A) in metastatic melanoma, the objective anti-tumor response was low despite three weekly applications of 852A and measurable cytokine responses (9). A recent trial relying on two weekly applications of 852A also met with limited success (10). We reasoned that the phenomenon of TLR tolerance might restrict the effectiveness of repeated TLR7/8 stimulation for therapy.

TLR tolerance is a phenomenon initially characterized as "endotoxin tolerance" or "LPS tolerance" (11) whereby repeated activation of TLR4 leads to refractoriness towards further stimulation. TLR tolerance was later shown to affect not only TLR4 but also other TLR such as TLR2 and TLR5 (12, 13). Indeed, TLR7 tolerance has recently been shown to limit the development of autoimmunity (14), but whether it plays a role in the outcome of cancer immunotherapy with TLR7 agonists is not known. Furthermore, although TLR7 tolerance has been described *in vitro* in macrophages (13, 15), it is not known whether this effect occurs in DC, which are central to cancer immunotherapy. Here we investigated TLR7 tolerance both *in vivo* and *in vitro* and examined the consequences on DC, in particular with respect to the induction of the anticancer cytokines IL-12p70 and IFN- α . Based on our findings, we designed a treatment protocol to circumvent TLR7 tolerance. This regimen was effective for

the treatment of established experimental tumors in a murine model of colon carcinoma and proved superior to protocols used previously in clinical studies.

Material and Methods

Mice, cell lines and reagents

Female Balb/c or C57BL/6 mice were purchased from Harlan-Winkelmann (Borchen, Germany). J. Heesemann (Max-von-Pettenkofer Institute, Munich, Germany) generously provided IL-10 deficient mice (C57BL/6 background). All animal studies were approved by the local regulatory agency (Regierung von Oberbayern, Munich, Germany). The murine colon carcinoma cell line CT26 was from Cell Lines Service (Heidelberg, Germany) and was tested for species and cross-contamination prior to shipping by real time PCR. Cells were passaged for less than 6 months according to AACR guidelines. Resiquimod (R848) and Malp-2 were purchased from Alexis Biochemicals (Heidelberg, Germany). LPS was purchased from Sigma-Aldrich (Steinheim, Germany). The PTO-modified CpG oligodeoxyribonucleotide 1826 (CpG, 5'-ccatgacgttctgacgtt-3') was obtained from the Coley Pharmaceutical Group (Langenfeld, Germany). **The Cox 1/2 inhibitor indomethacin was purchased from Sigma-Aldrich (Steinheim, Germany) and the Cox-2 inhibitor NS 398 was from Tocris (Bristol, UK). Recombinant IFN- α was from Miltenyi Biotec (Bergisch-Gladbach, Germany).**

Generation of DC

To prepare myeloid DC, mouse bone marrow cells were cultured in complete RPMI 1640 (10 % FCS, 2 mM L-glutamine, 100 μ g/ml streptomycin and 1 IU/ml penicillin) supplemented with 20 ng/ml GM-CSF and 20 ng/ml IL-4 (Tebu Bio, Offenbach, Germany). On day 6 to 7, cells were harvested. Plasmacytoid DC were generated from bone marrow cells cultured in complete RPMI 1640 supplemented with 1 mM sodium pyruvate, 1% non-essential amino acids (MEM-NEAA), 3,75x10⁻⁴ % 2-mercaptoethanol and 20 ng/ml recombinant Flt3-L (Tebu Bio, Offenbach, Germany). On day 7 to 9, cells were harvested and B220+ cells were isolated by magnetic microbeads (Miltenyi Biotec, Bergisch-Gladbach, Germany).

Tumor experiments

For tumor induction, groups of 4 to 5 Balb/c mice were injected subcutaneously on day 0 with 2.5x10⁵ CT26 tumor cells. Treatment was initiated on day 7 to 9. For the generation of the DC vaccine, myeloid DC were prepared as described above and pulsed with γ -irradiated (100 Gy) CT26 tumor cells at a ratio of 5:1 for 24 h. Subsequently, myeloid DC were activated in the presence of 6 μ g/ml CpG for additional 24 h. 2x10⁵ activated myeloid DC per mouse were applied contralaterally followed by treatment with 200 μ g CpG (half

contralaterally and half peritumorally). R848 was applied s.c. peritumorally at the time points indicated. Tumor size was expressed as the product of the perpendicular diameters of individual tumors.

ELISA

Levels of IL-12p70, IL-12p40, IL-6 and IL-10 in murine serum or cell culture supernatants were quantified by ELISA according to the manufacturer's protocol (OPTEIA®, BD Biosciences, Heidelberg, Germany). IFN- α was detected by a self-made ELISA set: capture antibody anti-IFN- α (RMMA-1) and detection antibody anti-IFN- α (rabbit-anti-mouse polyclonal) were from PBL Biomedical Laboratories (New Brunswick, USA), HRP-coupled donkey-anti-rabbit was from Biomeda (Foster City, USA). Detection of the quantitative enzymatic reaction was carried out with the OPTEIA® system.

Western blot

For immunoblotting, cells were harvested and lysed in NP-40 lysis buffer (20 mM Tris HCl, pH 8.0, 1% NP-40, 150 mM NaCl, 10 % glycerol, 2 mM EDTA, pH 8, 1 mM sodium orthovanadate and 1 mM protease inhibitor cocktail, Roche, Mannheim, Germany). Lysates underwent SDS-PAGE and were transferred to PVDF membranes (GE Healthcare, Buckinghamshire, UK). Membranes were blocked with 5% dry milk in PBST (PBS, 0.1% Tween-20). Primary antibodies were diluted in PBST supplemented with 4% BSA fraction V (Sigma-Aldrich); secondary antibodies were diluted in blocking solution. Immunoreactive proteins were detected using a chemoluminescence detection system (ECL; GE Healthcare). Antibodies used were anti-IRAK-1 (clone F-4) from Santa Cruz Biotechnologies (Santa Cruz, USA), anti- β -actin, and anti-mouse IgG HRP as secondary antibody from Promega (Madison, USA).

Statistics

Statistical analysis was performed using GraphPad Prism Software, version 5.0b (GraphPad Software, San Diego, USA). Error bars indicate SEM. Tumor experiments were statistically analyzed by two-way ANOVA followed by Bonferroni post test. Cytokine levels were analyzed with one-way ANOVA followed by Dunetts post test or two-tailed Students T-test as appropriate.

Results

A single injection of the TLR7 agonist R848 leads to long-lasting immune unresponsiveness

As the *in vivo* cytokine response to TLR7 stimulation with R848 is potent but short-lived ((16) and figure S1), we hypothesized that repeated applications would induce more sustained responses that might result in more effective antitumor immunity. Mice were injected twice with R848 at intervals from six to 120 h, followed by quantification of serum cytokines two hours after the second injection (figure 1A). We found that a single injection of R848 induced a state of hyperresponsiveness for up to 24 h. A second application of R848 six hours after the first injection resulted in a four-fold increase in serum levels of IFN- α , a key cytokine for the induction of antitumor responses (figure 1B). An increase in IL-6 induction was seen upon R848 stimulation at a 24 h interval. In contrast, after 48 h responsiveness towards the second stimulation was markedly decreased. IFN- α production was entirely blocked when mice were restimulated after 48 h. Sensitivity towards R848 slowly recovered within five days. Hyporesponsiveness was also seen for the induction of IL-12p70 and IL-6 by R848 and lasted for up to five days. Interestingly, the induction of IL-12p40, a subunit of bioactive IL-12, was not affected by this tachyphylaxis. Thus, a single application of R848 leads to long-lasting immune unresponsiveness that is initiated 24 h after injection. As TLR7 tolerance may represent a severe limitation for therapeutic use, we further investigated this phenomenon at the cellular and molecular level.

The TLR7 agonist R848 induces tolerance in both plasmacytoid and myeloid DC

To determine the cell type responsible for the observed TLR7 tolerance, we treated freshly isolated bone marrow cells with different concentrations of R848. Cells were restimulated 24 h later with R848 and cytokine secretion was measured upon secondary stimulation (figure 2A). Indeed, pretreatment of bone marrow cells with as little as 0.01 μ g/ml R848 entirely blocked the induction of IFN- α by R848 (figure 2A). A similar suppression was seen for the secretion of IL-12p70 and IL-6. IL-12p40 secretion was inhibited only at higher concentrations of R848 prestimulation. As the main producers of IFN- α upon TLR7 stimulation are plasmacytoid DC (17), we investigated the effect of R848 pretreatment on plasmacytoid DC. We observed a complete block in IFN- α secretion in this cell type after prestimulation with 0.1 μ g/ml R848 (figure 2B). Furthermore, R848 pretreatment inhibited IL-12p70 and IL-6 production in plasmacytoid DC. We also examined the effect of sequential R848 application in myeloid DC. As seen with plasmacytoid DC, the production of IL-12p70 and IL-6 was decreased in this population after pretreatment with R848. Thus, we show for

the first time that TLR7 tolerance occurs in DC of both the plasmacytoid and myeloid subtypes and results in abolished secretion of IFN- α and IL-12p70.

R848 induces cross-tolerance in plasmacytoid and myeloid DC

The TLR4 agonist LPS induces not only homo-tolerance, defined as tolerance towards a second stimulation via the same receptor, but also cross-tolerance towards subsequent stimulation via other TLRs (18, 19). To determine whether TLR7 stimulation induces cross-tolerance in DC, bone marrow-derived plasmacytoid and myeloid DC were cultured for 24 h in the presence of R848, washed and incubated for another 24 h in fresh medium with the TLR ligands R848, CpG (TLR9), Malp2 (TLR2/6) or LPS (TLR4). Cytokine levels of prestimulated cells were compared to non-prestimulated controls. We show that exposure to R848 tolerizes plasmacytoid DC towards TLR9 stimulation with a complete block of IFN- α and IL-12p70 secretion (figure 3A). Myeloid DC are tolerized towards TLR2/6, TLR4 and TLR9 with a complete block of IL-12p70 and partial block of IL-6 and IL-12p40 (figure 3B). Thus, R848 induces cross-tolerance to several other TLR in plasmacytoid and myeloid DC.

Prestimulation with R848 leads to increased IL-10 secretion upon secondary stimulation

The induction of the anti-inflammatory cytokine IL-10 has been shown to decrease immune responses upon treatment with TLR7 and TLR9 agonists (20, 21). We therefore examined whether IL-10 production played a role in the tolerance observed following repeated stimulation with R848. Interestingly, in sharp contrast to the results obtained for proinflammatory cytokines, pretreatment with R848 led to increased levels of IL-10 when cells were restimulated with the TLR agonists R848, CpG and, in the case of myeloid DC, LPS (figure 4A). To assess whether increased IL-10 levels were responsible for TLR7 tolerance, DC from IL-10-deficient mice were stimulated twice with R848 (figure 4B). As observed previously in wild-type DC, decreased secretion of IL-12p70 and IL-6 was again seen in R848-prestimulated cells, demonstrating that IL-10 is not the main factor responsible for TLR7 tolerance on the cellular level.

Prestimulation with R848 leads to degradation of IRAK-1 in plasmacytoid DC

To obtain further insights into the molecular factors mediating TLR7 tachyphylaxis, we determined the levels of the adapter protein IRAK-1 in plasmacytoid DC following R848 activation. In plasmacytoid DC, IRAK-1 is essential for the induction of IFN- α through TLR7

(7). IRAK-1 expression levels were sharply decreased as early as 5 min after exposure to R848 and remained low for at least 24 h. A second stimulation with R848 after 24 h did not increase IRAK-1 expression (figure 5A). To investigate IRAK-1 levels in treated mice, IRAK-1 expression was analyzed in splenocytes from mice injected once with R848. IRAK-1 was decreased in treated animals 2 h after R848 injection and was not detectable 48 h after injection (figure 5A). The rapid and persistent decrease in IRAK-1 following R848 treatment may thus represent an essential mechanism for TLR7 tolerance. The increased production of IFN- α seen when mice were restimulated with R848 at an early time point after the first injection (figure 1) may result from a priming effect of type I interferon (22). Indeed, pDC prestimulated with recombinant IFN- α show enhanced secondary IFN- α secretion following R848 stimulation (figure 5B). Similarly, mice pretreated with recombinant IFN- α show increased IFN- α responses to R848 4 h after prestimulation. This effect decreases at 24 h and is lost 48 h after prestimulation with IFN α . No serum IFN- α was observed 4 h after prestimulation with recombinant IFN α alone (data not shown), demonstrating that the measured serum cytokine levels are induced *de novo* by R848 stimulation.

A fractionated R848 treatment regimen circumvents TLR7 tolerance and translates into efficient anti-tumor therapy

To prevent a rapid decline of cytokine levels (figure S1), we designed a treatment protocol to take advantage of the early hyperresponsiveness by repetitively injecting R848 at very short intervals. The repeated treatment consisted of four s.c. injections of R848 at 0, 4, 6 and 24 h and resulted in enhanced levels of IFN- α and IL-6 and longer-lived cytokine responses (figure 6A). Indeed, with the repeated regimen IL-12p70 was still detectable 8 h and 26 h after the first injection, whereas it was barely detectable in mice 8 h after a single application. Thus, four injections of R848 within 24 h lead to enhanced and sustained cytokine titers before the appearance of tolerance. In contrast to this protocol, four injections given at 24-hour intervals lead to a substantial suppression of cytokine secretion and to a state of hyporesponsiveness towards repeated stimulation (data not shown). In a recent clinical trial in patients with metastatic melanoma, the synthetic TLR7 agonist 852A was administered systemically three times a week with limited success (9). According to our data, such a scheduling may result in tachyphylaxis. To circumvent tolerance, we applied a **fractionated** protocol in cycles at 5-day intervals, so that the refractory state would be resolved (see figure 1). To test the efficacy of this regimen, mice with established s.c. CT26 tumors were treated from day 7 with (i) one administration of 30 μ g R848 every three days, (ii) four injections of 10 μ g R848 per cycle according to the repeated protocol every five days, or (iii) were left untreated. Thus, the cumulative dose of R848 was lower in the fractionated regimen than in

the single injection regimen. The fractionated treatment protocol potently reduced the growth of established tumors (figure 6B). In contrast, single injections given every third (figure 6B) or every fifth day (not shown) had a limited success in preventing tumor growth. Indeed, the fractionated protocol was as efficient as a published DC vaccine combined with the TLR9 agonist CpG (23, 24) (figure S2). Thus, cycles of repeated R848 applications separated by 5-day intervals may improve the therapeutic efficacy of TLR7 agonists by circumventing tolerance.

Discussion

Despite the strong immune activation induced by TLR7 ligands and their extensive success for the immunotherapy of skin tumors when applied topically, their systemic use for the treatment of cancer has not met expectations. One drawback for the use of TLR ligands is the immune suppression due to the phenomenon of TLR tolerance, best characterized for TLR4 stimulation by repeated administration of low-dose LPS. Recently, *in vivo* tolerance was also described for TLR7: repeated applications of a synthetic TLR7 agonist, at a low dose that did not result in measurable cytokine responses, blocked the induction of the proinflammatory cytokines TNF- α and IL-6 and limited the course of inflammation in autoimmune encephalomyelitis (14). Whether this tachyphylaxis phenomenon can also be observed after *in vivo* application of an immunoactive dose of a TLR7 agonist and whether it affects cytokines essential for the anticancer efficacy of TLR7 agonists such as IL-12 and IFN- α is however to date not known. We observed that a single injection of the TLR7 agonist R848, after a short phase of hyperresponsiveness, completely blocked the induction of IFN- α by a subsequent stimulation and strongly reduced production of the bioactive IL-12p70 for up to five days.

To elucidate the cellular basis for the observed block in IFN- α and IL-12p70, we investigated TLR7 tolerance in cell culture using primary bone marrow cells and bone marrow-derived DC. DC in particular play an essential role for the initiation and development of antitumor immunity (25) and are one of the main producers of the antitumor cytokines IFN- α and IL-12p70. Myeloid DC are susceptible to tolerance via the receptors TLR2 and TLR4 (12, 26) but tolerance to TLR7 in myeloid DC has to our knowledge not been previously described. Here we clearly show tolerance in primary bone marrow cells and in myeloid DC following TLR7 activation, characterized by a block in IL-12p70 and IL-6 production. In sharp contrast to the block in proinflammatory cytokines, we observed increased secretion of the anti-inflammatory cytokine IL-10 in tolerant cells subsequent to TLR restimulation, as seen previously for TLR4 stimulation of myeloid DC (26). However, we show using IL-10-deficient mice that this cytokine is not essential for tolerance in myeloid DC. Interestingly, the p40 subunit of IL-12 was not affected by tolerance *in vivo*. The p40 subunit is synthesized in excess over p35, the other subunit of the IL-12 heterodimer, which is rate-limiting for the production of bioactive IL-12 (27). Thus, the p40 protein may not be as sensitive towards TLR tolerance as the p35 subunit. *In vitro*, p40 was less suppressed and required higher doses of R848 for tolerance induction than the other cytokines in our study. Prostaglandin E2 (PGE2)-conditioned DC have a cytokine profile similar to R848-prestimulated DC: they produce enhanced levels of IL-10, decreased IL-12p70 and unchanged amounts of IL-12p40

(28, 29). We however observed no significant change in R848-induced tolerance in the presence of Cox1/2 inhibitors, suggesting that prostaglandins are not determinant for tolerance induction (figure S3). Indeed, tolerance was not mediated by a soluble factor, as supernatant from R848-treated cells did not transfer tolerance (data not shown). Taken together, these results suggest that tolerance is not mediated by soluble factors such as anti-inflammatory cytokines or prostaglandins, but is cell-intrinsic.

Several molecular mechanisms have been described for TLR4 tolerance, such as a decreased accumulation of NF κ B (13), a reduced activation of the MAP kinase p38 (26) or increased expression and activation of the regulator molecule IRAK-M (14). In addition, decreased signaling through the adapter molecule IRAK-1 has been shown to mediate TLR7 tolerance in murine macrophages (15, 19). We found that IRAK-1 was depleted within minutes of R848 stimulation in plasmacytoid DC and was maintained at low levels for at least 24 hours. Since IRAK-1 is essential for the induction of IFN- α through TLR7 and TLR9 stimulation in plasmacytoid DC (7), this finding is sufficient to explain the block in IFN- α secretion. The downregulation of IRAK-1 was confirmed *in vivo*, as this protein was not detectable in the spleen 48 h after R848 injection. Residual levels of IRAK-1 were however observed in the spleen 2 h after injection. These low levels of IRAK-1 may be sufficient, in the presence of an enhancing factor, to mediate the increased IFN- α response seen at early time points after TLR7 stimulation. Type I interferon, which is increased in serum for up to six hours after TLR7 stimulation, may represent such an amplification factor of IFN- α (22). IRAK-1 is not involved in TLR-dependent proinflammatory cytokine release by plasmacytoid DC (7), suggesting that IRAK-1 degradation is not responsible for the hyporesponsiveness affecting IL-6 and IL-12. Further studies are necessary to determine the mechanisms involved in decreased secretion of these cytokines in this cell type.

In a recent clinical study on TLR tolerance, subjects were injected with the TLR4 agonist LPS (30). *In vitro* restimulation of PBMC from these subjects with TLR ligands showed both homo- and heterotolerance, including a decreased ability to secrete type I interferons after TLR7 stimulation. This suggests that TLR7 tolerance may occur in humans and may affect IFN- α secretion by plasmacytoid DC. Both the cytokines IFN- α and IL-12 are believed to play central roles in the immunological control of cancer (31, 32). The short duration of cytokine secretion following systemic administration of small molecule TLR7/8 agonists together with the long-lasting immune tolerance induced may therefore pose severe limitations to the use of these compounds for the immunotherapy of cancer. Taking advantage of the hyperresponsiveness in the early phase following administration, we designed a protocol relying on cycles of repeated R848 treatment within 24 h interspaced by 5 days to circumvent tachyphylaxis. This intensive regimen was superior to other treatment options, including

R848 application protocols used previously in clinical studies. Indeed, protocols consisting of single injections at two to four-day intervals may result in short-lived cytokine titers followed by induction of tolerance that decreases treatment efficacy.

The difference in therapeutic efficacy observed between topical and systemic treatment with TLR7 agonists may result from a variety of mechanisms. Local effects not directly related to cytokine production by DC may largely contribute to the efficacy of topical TLR7 agonists. Imiquimod-activated DC can directly kill tumor cells via perforin, granzyme B and TRAIL-dependent mechanisms (33), and imiquimod itself may have a direct proapoptotic effect on tumor cells (34, 35). Skin lesions treated topically with imiquimod show infiltrates of cytotoxic T cells and DC (33, 36), suggesting that topical treatment leads to the local production of chemotactic factors for effector cells. Thus, systemic and topical administration may differ substantially in their modes of action. It is probable that topical application of TLR7 agonists does not induce tolerance either systemically or locally. We have observed that R848 at concentrations of 0.5 ng/ml and below does not induce tolerance in DC (figure S4), suggesting that the very low serum concentrations observed after topical application (37, 38) do not induce systemic tolerance. In TLR7-treated skin, persistent upregulation of IFN- α and IFN-inducible genes was demonstrated (39, 40), suggesting that topical treatment with TLR7 agonists does not result in local tolerance either. It is possible that untolerized circulating DC, which infiltrate the treated skin areas *de novo* (39), contribute to cytokine production in the skin (39, 40). Whether the difference in tolerance between topical and systemic treatment plays a role in the observed difference in treatment efficacy is however unclear.

Mechanistically, TLR7 ligands exert their antitumoral effect on several cell types including DC and T cells (41, 42). In addition, Butchar and colleagues demonstrated that TLR7 ligands improve the outcome of treatment with monoclonal antibodies by increasing Fc- γ receptor expression and thus enhancing antibody-mediated cellular cytotoxicity (43). We have demonstrated earlier that application of TLR7-activating RNA oligonucleotides can prevent growth of natural killer (NK) cell-sensitive tumors by activation of NK cells via TLR7-expressing DC (44). Furthermore, small molecule TLR7 agonists and immunostimulatory RNA block the suppressive function of regulatory T cells (45). In conclusion, according to the present study a key feature of therapy with TLR7 agonists will be to provide sustained and long-lasting stimulation rather than short-lived immune activation by immune modifying agents. Given that TLR tolerance is a general phenomenon, our findings have implications for all immunotherapeutic interventions relying on **systemic** application of TLR agonists as immune response modifiers.

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Figure legends

Figure 1: A single injection of the TLR7 agonist R848 leads to long-lasting immune tolerance *in vivo*

A: Mice (n=4 per group) were stimulated s.c. with 20 µg R848 and restimulated with 20 µg R848 s.c. 6 to 120 h later. **B:** Two hours after the first injection (white bars) or after the second injection (black bars), serum was collected and cytokine levels were determined by ELISA. Asterisks (*, p < 0.05; **, p < 0.01; ***, p < 0.001) indicate significant differences of serum cytokines after restimulation compared to serum from mice after the first injection.

Results are representative of two independent experiments.

Figure 2: The TLR7 agonist R848 induces tolerance in both plasmacytoid and myeloid DC

A: Primary bone marrow cells (4×10^5 per well) were stimulated for 6 h with the indicated concentrations of R848. Cells were washed and rested for 18 h. Restimulation was performed with 1 µg/ml R848 for 6 h. Cytokine levels in the supernatants were determined by ELISA. **B:** Bone marrow-derived plasmacytoid DC or myeloid DC (2×10^5 per well) were incubated in medium containing 0.1 µg/ml R848 or medium alone. Six hours later $\frac{3}{4}$ of the medium was exchanged by fresh medium without stimulus. 18 h later cells were washed and restimulated with 0.1 µg/ml R848 for 24 h. Cytokine levels in supernatants were determined as in A. Asterisks (*, p < 0.05; **, p < 0.01; ***, p < 0.001) indicate significant differences between cells that were prestimulated with R848 (black bars) and cells that were not prestimulated (white bars). “bdl” indicates below detection limit. Results are representative of three independent experiments.

Figure 3: R848 induces cross-tolerance in plasmacytoid and myeloid DC

A: Plasmacytoid DC (pDC) or **B:** myeloid DC (mDC) (2×10^5 per well) were incubated as in figure 2B in medium containing 0.1 µg/ml R848 or medium alone. Six hours later $\frac{3}{4}$ of the medium was exchanged by fresh medium without stimulus. 18 h later cells were washed and restimulated with 0.1 µg/ml R848, 5 µg/ml CpG (TLR9), 50 ng/ml Malp2 (TLR2/6) or 5 µg/ml LPS (TLR4) for 24 h. Cytokine levels in the supernatants were determined by ELISA. Asterisks (*, p < 0.05; **, p < 0.01; ***, p < 0.001) indicate significant differences between cells that were prestimulated with R848 (black bars) and cells that were not prestimulated

(white bars). "bdl" indicates below detection limit. Results are representative of two to three independent experiments.

Figure 4: Prestimulation with R848 leads to increased IL-10 production in DC upon secondary stimulation

A: Plasmacytoid or myeloid DC (2×10^5 per well) were stimulated with R848, then rested and restimulated with R848, CpG or LPS as described in figure 3. **B:** Myeloid DC from IL-10-deficient mice were pretreated with R848, rested and restimulated with R848 as in figure 3. **A** and **B:** IL-10 levels were analyzed in supernatants 24 h after the second stimulus. Asterisks (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$) indicate significant differences between cells that were prestimulated with R848 (black bars) and cells that were not prestimulated (white bars). Results are representative of two independent experiments.

Figure 5: Prestimulation with R848 leads to degradation of IRAK-1 in plasmacytoid DC

A, left panel: Bone marrow-derived plasmacytoid DC were stimulated with R848, washed and restimulated with R848 after 24 h. Cells were lysed at the indicated time points after the first R848 stimulation or after R848 restimulation. **A, right panel:** For *in vivo* analysis, mice were injected once with 20 μ g R848 and sacrificed at the indicated time points after injection. Whole splenocytes of individual mice were lysed for protein analysis. Cell lysates were subjected to SDS-PAGE and immunoblot with anti-IRAK-1 and anti- β -actin (loading control) antibodies. Results are representative of two independent experiments. **B, left panel:** Plasmacytoid DC were prestimulated with 50 U/ml or 1000 U/ml recombinant IFN- α for 4 h. Cells were washed and restimulated with R848 for 18 h. IFN- α levels in the supernatant were determined by ELISA. Data are shown relative to cells without prestimulation and are representative of two independent experiments. **B, right panel:** Mice (n=3 to 5 per group) were treated with one injection of 10^4 U recombinant IFN- α followed by restimulation with 20 μ g R848 4 h to 48 h later. 2 h after R848 injection, serum IFN- α was analysed by ELISA. Results are shown relative to mice without prestimulation and are pooled from two independent experiments. Asterisks (**, $p < 0.01$; ***, $p < 0.001$) indicate significant differences between cells or mice that were prestimulated with IFN- α (black bars) and cells or mice that were not prestimulated (white bars).

Figure 6: A fractionated R848 treatment regimen circumvents TLR7 tolerance and translates into efficient anti-tumor therapy

A: Mice (n=4 to 5 per group) were injected once s.c. at 0 h or 4 times (repeated) at 0, 4, 6, and 24 h with 20 µg R848. Serum cytokine levels were determined by ELISA at the indicated time points after the first injection (2, 6, 8 or 26 h). Results are representative of two independent experiments. **B:** Mice (n=6 per group) were inoculated with 2.5×10^5 CT26 colon carcinoma cells s.c. on day 0. One cohort of mice was treated every 3 days from day 7 (grey arrows) with a single s.c. injection of 30 µg R848. A second group received 4 fractionated s.c. injections of 10 µg R848 per treatment cycle at 0, 4, 6, and 24 h every 5 days from day 7 (black arrows). Control animals were left untreated. Tumor growth was monitored until day 27. Asterisks (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$) indicate significant differences to the single injection group (R848 once). Results are representative of three similar experiments.

Bourquin, Hotz *et al.*: R848 tolerance

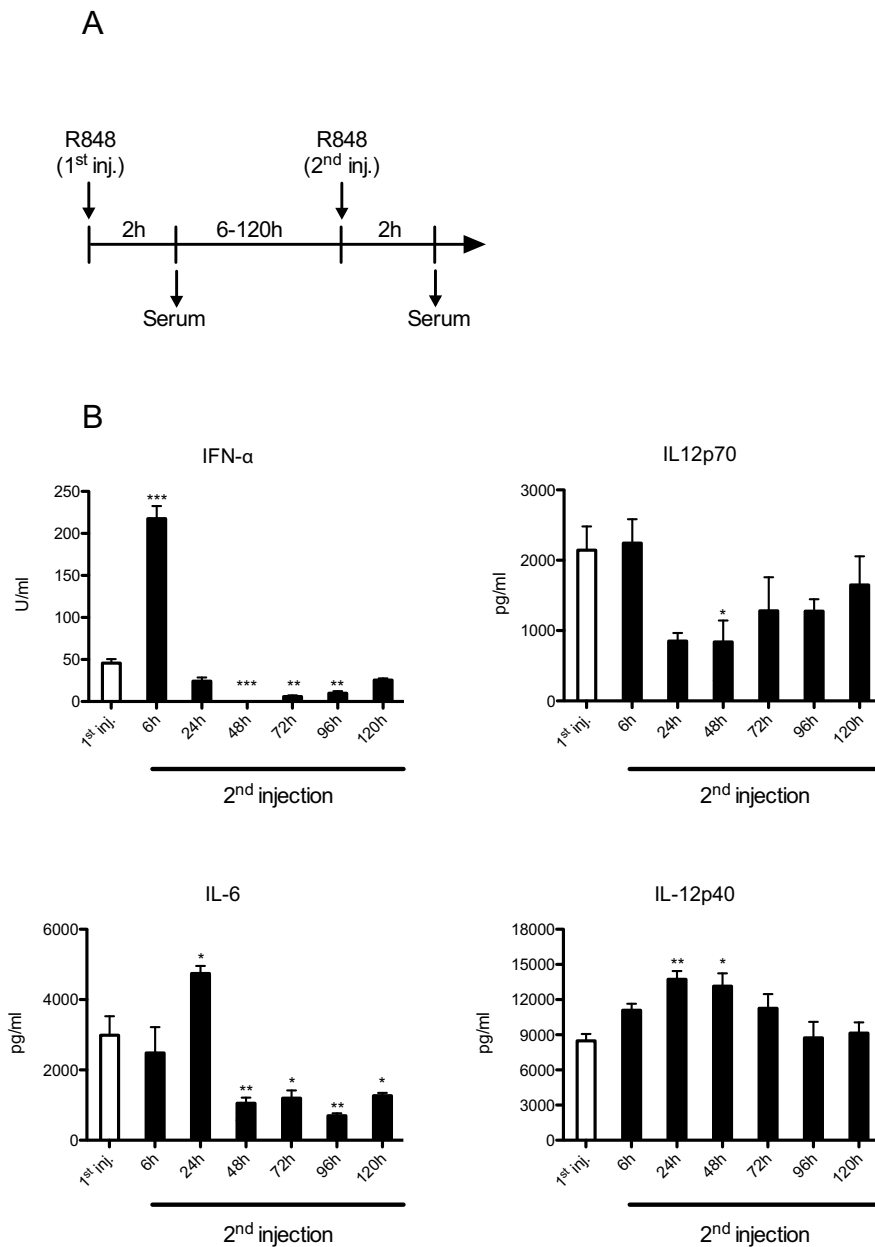


Figure 1

Bourquin, Hotz *et al.*: R848 tolerance

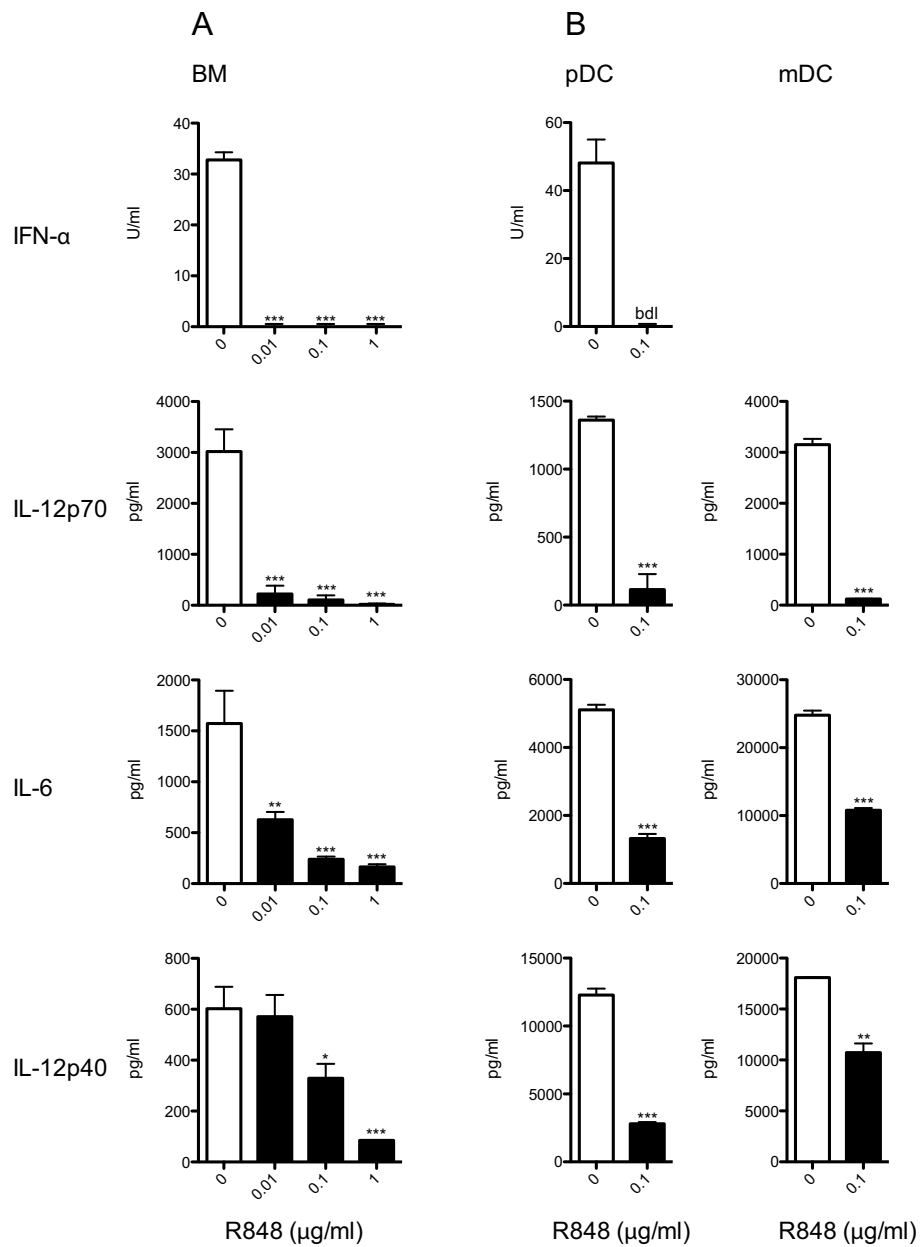


Figure 2

Bourquin, Hotz *et al.*: R848 tolerance

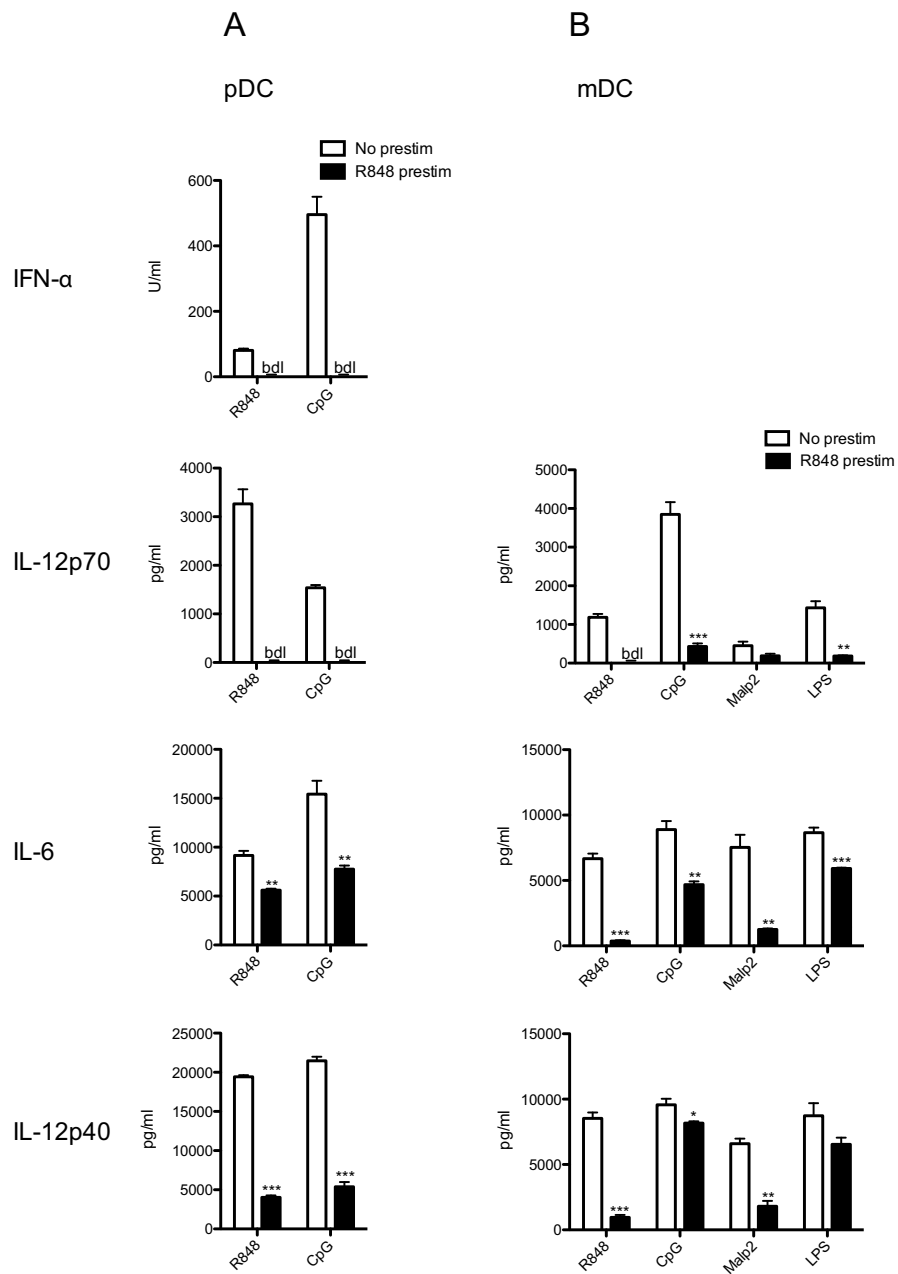


Figure 3

Bourquin, Hotz *et al.*: R848 tolerance

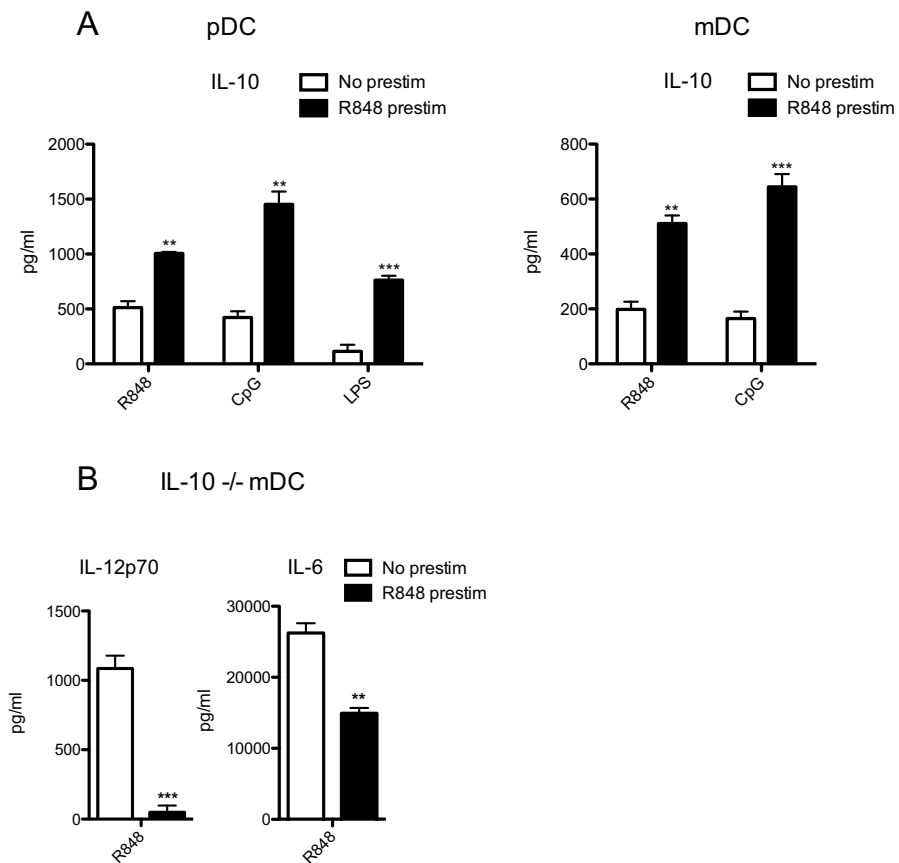


Figure 4

Bourquin, Hotz *et al.*: R848 tolerance

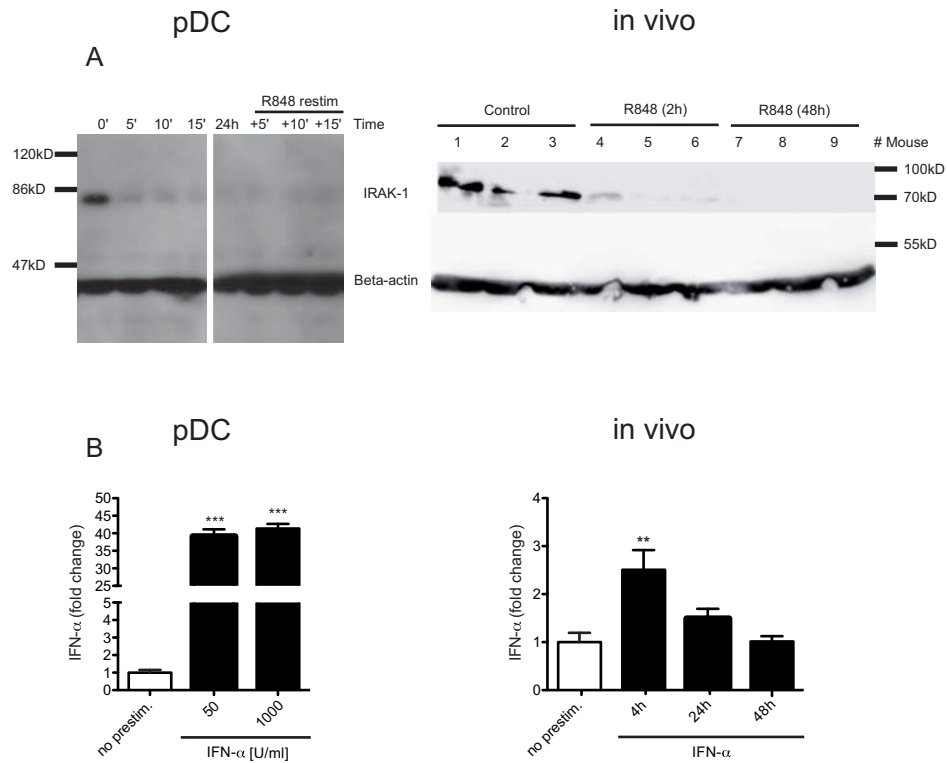


Figure 5

Bourquin, Hotz *et al.*: R848 tolerance

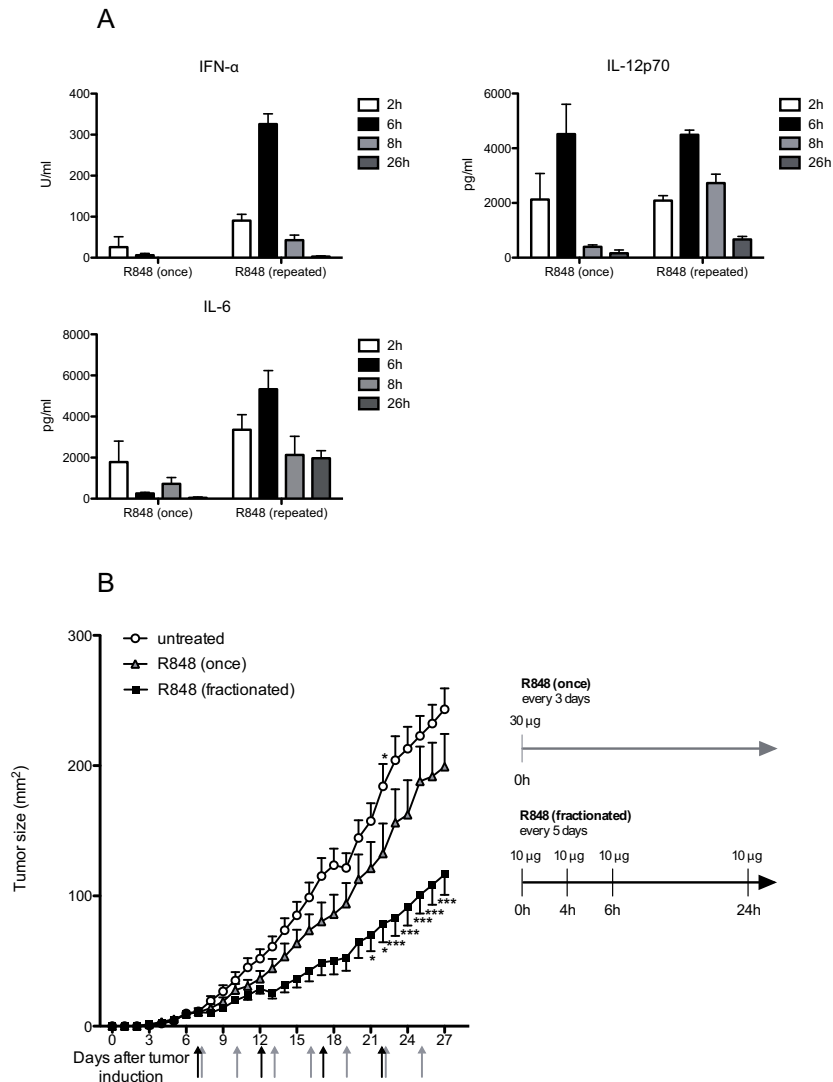


Figure 6