Microenvironment and Immunology

Systemic Cancer Therapy with a Small Molecule Agonist of Toll-like Receptor 7 Can Be Improved by Circumventing TLR Tolerance

Carole Bourquin, Christian Hotz, Daniel Noerenberg, Andreas Voelkl, Simon Heidegger, Laurin C. Roetzer, Bettina Storch, Nadja Sandholzer, Cornelia Wurzenberger, David Anz, and Stefan Endres

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 interleukin (IL)-12p70 and IL-6 as well as by a block in IFN-α production. We show 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 IL-1 receptor–associated kinase 1. On the basis of these findings, we have designed a novel strategy for the treatment of tumors by 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.

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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 TLRs 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 (3M Pharmaceuticals) 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 dendritic cells (6) as well as IFN-α release by plasmacytoid DCs (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 antitumor response was low despite 3 weekly applications of 852A and measurable cytokine responses (9). A recent trial relying on 2 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 lipopolysaccharide (LPS) tolerance (11) whereby repeated activation of TLR4 leads to refractoriness toward further stimulation. TLR tolerance was later shown to affect not only TLR4 but also other TLRs, 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 DCs, which are central to cancer immunotherapy. Here we investigated TLR7 tolerance both in vivo and in vitro and examined the consequences on DCs, in particular with respect to the induction of the anticancer cytokines interleukin (IL)-12p70 and IFN-α. On the basis of 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.

Materials and Methods

Mice, cell lines, and reagents

Female Balb/c or C57BL/6 mice were purchased from Harlan-Winkelmann, 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). The murine colon carcinoma cell line CT26 was from Cell Lines Service 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 American Association for Cancer Research guidelines. Resiquimod (R848) and Malp-2 were purchased from Alexis Biochemicals. LPS was purchased from Sigma-Aldrich. The PTO-modified CpG oligodeoxyribonucleotide 1826 (CpG, 5′-TCATGACGTTCCTGACGTT-3′) was obtained from the Coley Pharmaceutical Group. The cyclooxygenase (COX)-1/2 inhibitor indomethacin was purchased from Sigma-Aldrich and the COX-2 inhibitor NS398 was from Tocris. Recombinant IFN-α was from Miltenyi Biotec.

Generation of DCs

To prepare myeloid DCs, mouse bone marrow cells were cultured in complete RPMI 1640 (10% fetal calf serum, 2 mmol/L L-glutamine, 100 μg/mL streptomycin, and 1 IU/mL penicillin) supplemented with 20 ng/mL granulocyte macrophage colony-stimulating factor and 20 ng/mL IL-4 (Tebu Bio). On day 6 to 7, cells were harvested. Plasmacytoid DCs were generated from bone marrow cells cultured in complete RPMI 1640 supplemented with 1 mmol/L sodium pyruvate, 1% nonessential amino acids (MEM-NEAA), 3.75 × 10⁻⁵% 2-mercaptoethanol, and 20 ng/mL recombinant Flt3-L (Tebu Bio). On day 7 to 9, cells were harvested and B220⁺ cells were isolated by magnetic microbeads (Miltenyi Biotec).

Tumor experiments

For tumor induction, groups of 4 to 5 Balb/c mice were injected s.c. on day 0 with 2.5 × 10⁵ CT26 tumor cells. Treatment was initiated on day 7 to 9. For the generation of the DC vaccine, myeloid DCs were prepared as described above and pulsed with γ-irradiated (100 Gy) CT26 tumor cells at a ratio of 5:1 for 24 hours. Subsequently, myeloid DCs were activated in the presence of 6 μg/mL CpG for an additional 24 hours. A total of 2 × 10⁶ activated myeloid DCs per mouse were applied contralaterally followed by treatment with 200 μg 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). 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, horseradish peroxidase (HRP)-coupled donkey-anti-rabbit was from Biomedica. 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 mmol/L Tris-HCl, pH 8.0, 1% NP-40, 150 mmol/L NaCl, 10% glycerol, 2 mmol/L EDTA, pH 8, 1 mmol/L sodium orthovanadate, and 1 mmol/L protease inhibitor cocktail (Roche)]. Lysates underwent SDS-PAGE and were transferred to polyvinylidene difluoride membranes (GE Healthcare). Membranes were blocked with 5% dry milk in PBST (PBS, 0.1% Tween-20). Primary antibodies were diluted in PBST supplemented with 4% bovine serum albumin fraction V (Sigma-Aldrich); secondary antibodies were diluted in blocking solution. Immunoreactive proteins were detected by using a chemoluminescence detection system (ECL, GE Healthcare). Antibodies used were anti–IRAK-1 (IL-1 receptor-associated kinase; clone F-4) from Santa Cruz Biotechnologies, anti–β-actin, and anti-mouse IgG HRP as secondary antibody from Promega.

Statistics

Statistical analysis was done with GraphPad Prism Software, version 5.0b (GraphPad Software). Error bars indicate SEM. Tumor experiments were statistically analyzed by 2-way ANOVA followed by Bonferroni posttest. Cytokine levels were analyzed with 1-way ANOVA followed by Dunnett’s posttest or 2-tailed Student t test as appropriate.

Results

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

Because the in vivo cytokine response to TLR7 stimulation with R848 is potent but short-lived (ref. 16; Supplementary Fig. 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 6 to 120 hours, followed by quantification of serum cytokines 2 hours after the second injection (Fig. 1A). We found that a single injection of R848 induced a state of hyperresponsiveness for up to 24 hours. A second application of R848 6 hours after the first injection resulted in a 4-fold increase in serum levels of IFN-α, a key cytokine for
the induction of antitumor responses (Fig. 1B). An increase in IL-6 induction was seen upon R848 stimulation at a 24-hour interval. In contrast, after 48 hours responsiveness toward the second stimulation was markedly decreased. IFN-α production was entirely blocked when mice were restimulated after 48 hours. Sensitivity toward R848 slowly recovered within 5 days. Hyporesponsiveness was also seen for the induction of IL-12p70 and IL-6 by R848 and lasted for up to 5 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 hours after injection. Because 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 DCs

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 hours later with R848, and cytokine secretion was measured upon secondary stimulation (Fig. 2A). Indeed, pretreatment of bone marrow cells with as little as 0.01 µg/mL R848 entirely blocked the induction of IFN-α by R848 (Fig. 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. Because the main producers of IFN-α upon TLR7 stimulation are plasmacytoid DCs (17), we investigated the effect of R848 pretreatment on plasmacytoid DCs. We observed a complete block in IFN-α secretion in this cell type after prestimulation with 0.1 µg/mL R848 (Fig. 2B). Furthermore, R848 pretreatment inhibited IL-12p70 and IL-6 production in plasmacytoid DCs. We also examined the effect of sequential R848 application in myeloid DCs. As seen with plasmacytoid DCs, 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 DCs 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 DCs

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

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 DCs, LPS (Fig. 4A). To assess whether increased IL-10 levels were responsible for TLR7 tolerance, DCs from IL-10−/− deficient mice were stimulated twice with R848 (Fig. 4B). As observed previously in wild-type DCs, decreased secretion of IL-12p70 and IL-6 was again seen in R848-pretreated cells, showing 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 DCs**

To obtain further insights into the molecular factors mediating TLR7 tachyphylaxis, we determined the levels of the

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Figure 3. R848 induces cross-tolerance in plasmacytoid and myeloid DCs. A, plasmacytoid DCs or B, myeloid DCs (2 × 10⁵ per well) were incubated as in Fig. 2B in medium containing 0.1 μg/mL R848 or medium alone. Six hours later three-fourths of the medium was exchanged by fresh medium without stimulus. Eighteen hours later, three-fourths of 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). Results are representative of 2 to 3 independent experiments. bdl, below detection limit; pDC, plasmacytoid DC; mDC, myeloid DC.
adapter protein IRAK-1 in plasmacytoid DCs following R848 activation. In plasmacytoid DCs, IRAK-1 is essential for the induction of IFN-α through TLR7 (7). IRAK-1 expression levels were sharply decreased as early as 5 minutes after exposure to R848 and remained low for at least 24 hours. A second stimulation with R848 after 24 hours did not increase IRAK-1 expression (Fig. 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 2 hours after R848 injection and was not detectable 48 hours after injection (Fig. 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 (Fig. 1) may result from a priming effect of type I IFN (22). Indeed, pDCs pretreated with recombinant IFN-α show enhanced secondary IFN-α secretion following R848 stimulation (Fig. 5B). Similarly, mice pretreated with recombinant IFN-α show increased IFN-α responses to R848 4 hours after pretreatment. This effect decreases at 24 hours and is lost 48 hours after pretreatment with IFN-α. No serum IFN-α was observed 4 hours after pretreatment with recombinant IFN-α alone (data not shown), showing 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 antitumor therapy

To prevent a rapid decline of cytokine levels (Supplementary Fig. 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 4 s.c. injections of R848 at 0, 4, 6, and 24 hours and resulted in enhanced levels of IFN-α and IL-6 and longer-lived cytokine responses (Fig. 6A). Indeed, with the repeated regimen, IL-12p70 was still detectable 8 hours and 26 hours after the first injection, whereas it was barely detectable in mice 8 hours after a single application. Thus, 4 injections of R848 within 24 hours lead to enhanced and sustained cytokine titers before the appearance of tolerance. In contrast to this protocol, 4 injections given at 24-hour intervals lead to a substantial suppression of cytokine secretion and to a state of hyporesponsiveness toward repeated stimulation (data not shown). In a recent clinical trial in patients with metastatic melanoma, the synthetic TLR7 agonist 852A was administered systemically 3 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 Fig. 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 mg R848 every 3 days, (ii) 4 injections of 10 mg R848 per cycle according to the repeated protocol every 5 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 (Fig. 6B). In contrast, single injections given every third (Fig. 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 (refs. 23, 24; Supplementary Fig. 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 5 days.

To elucidate the cellular basis for the observed block in IFN-α and IL-12p70, we investigated TLR7 tolerance in cell culture by using primary bone marrow cells and bone marrow–derived DCs. DCs 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 DCs are susceptible to tolerance via the receptors TLR2 and TLR4 (12, 26), but tolerance to TLR7 in myeloid DCs has to our knowledge not been previously described. Here we clearly show tolerance in primary bone marrow cells and in myeloid DCs 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 DCs (26). However, we show using IL-10–deficient mice that this cytokine is not essential for tolerance in myeloid DCs. 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 toward 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–conditioned DCs have a cytokine profile similar to R848-prestimulated DCs: They produce enhanced levels of IL-10, decreased IL-12p70, and unchanged amounts of IL-12p40 (28, 29). However, we observed no significant change in R848-induced tolerance in the presence of COX-1/2 inhibitors, suggesting that prostaglandins are not determinant for tolerance induction (Supplementary Fig. S3). Indeed, tolerance was not mediated by a soluble factor, as supernatants 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-kB (13), a reduced activation of the mitogen-activated protein 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 DCs and was maintained at low levels for at least 24 hours. Because IRAK-1 is essential for the induction of IFN-α through TLR7 and TLR9 stimulation in plasmacytoid DCs (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 hours after R848 injection. Residual levels of IRAK-1 were, however, observed in the spleen 2 hours 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 IFN, which is increased in serum for up to 6 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 DCs (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 TLR7 tolerance, subjects were injected with the TLR4 agonist LPS (30). In vitro restimulation of peripheral blood mononuclear cells from these subjects with TLR7 ligands showed both homo- and heterotolerance, including a decreased ability to secrete type I IFNs after TLR7 stimulation. This suggests that TLR7 tolerance may occur in humans and may affect IFN-α secretion by plasmacytoid DCs. Both the cytokines IFN-α and IL-12 are believed to play central roles in the immunologic 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 hours interspaced by 5 days to circumvent tachyphylaxis. This fractionated regimen was superior to other treatment options, including R848 application protocols used previously in clinical studies. Indeed, protocols consisting of single injections at 2- to 4-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 DCs may largely contribute to the efficacy of topical TLR7 agonists. Imiquimod-activated DCs 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 DCs (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 bone marrow cells (Supplementary Fig. 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 shown (39, 40), suggesting that topical treatment with TLR7 agonists does not result in local tolerance either. It is possible that untolerized circulating DCs, 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 DCs and T cells (41, 42). In addition, Butcher and colleagues showed 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 shown earlier that application of TLR7-activating RNA oligonucleotides can prevent growth of NK cell–sensitive tumors by activation of NK cells via TLR7-expressing DCs (44). Furthermore, small molecule TLR7 agonists and immunostimulatory RNA block the suppressive function of regulatory T cells (45). In conclusion, according to this 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.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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