Targeted Liposomal Delivery of TLR9 Ligands Activates Spontaneous Antitumor Immunity in an Autochthonous Cancer Model

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Accessibility of tumors for highly effective local treatment represents a major challenge for anticancer therapy. Immunostimulatory oligodeoxynucleotides (ODN) with CpG motifs are ligands of TLR9, which prime spontaneous antitumor immunity, but are less effective when applied systemically. We therefore developed a liposome-based agent for selective delivery of CpG-ODN into the tumor environment. A peptide that specifically targets angiogenic endothelial cells in a transgenic tumor model for islet cell carcinogenesis was engrafted into CpG-ODN containing liposomes. Intravenous injection of these liposomes resulted in specific accumulation around tumor vessels, increased uptake by tumor-resident macrophages, and retention over time. In contrast, nontargeted liposomes did not localize to the tumor vasculature. Consequently, only vascular targeting of CpG-ODN liposomes provoked a marked inflammatory response at vessel walls with enhanced CD8+ and CD4+ T cell infiltration and, importantly, activation of spontaneous, tumor-specific cytotoxicity. In a therapeutic setting, 40% of tumor-bearing, transgenic mice survived beyond week 45 after systemic administration of vascular-directed CpG-ODN liposomes. In contrast, control mice survived up to 30 wk. Therapeutic efficacy was further improved by increasing the frequency of tumor-specific effector cells through adoptive transfers. NK cells and CD8+ T cells were major effectors which induced tumor cell death and acted in conjunction with anti-vascular effects. Thus, tumor homing with CpG-ODN-loaded liposomes is as potent as direct injection of free CpG-ODN and has the potential to overcome some major limitations of conventional CpG-ODN monotherapy. The Journal of Immunology, 2009, 183: 1091–1098.

Oligonucleotides containing unmethylated CpG dinucleotides (CpG oligodeoxynucleotides (CpG-ODN)) resembling bacterial DNA have long been recognized as immune-stimulating agents. Cells are able to detect unmethylated CpGs via TLR9, an endosomal receptor expressed on a limited number of immune cells (1). In mice, monocytes are directly activated by CpG motifs to secrete the Th1-like cytokine IL-12 and type IFNs, whereas NK cells respond with increased lytic activity and IFN-γ secretion. In addition, TLR9 stimulation drives dendritic cell and B cell maturation and thus enhances their Ag presentation capability. CpG-ODN are prime candidates for anticancer vaccination because they are, as single agents, able to modulate innate and adaptive immunity.

Naturally occurring antitumor immunity is often weak and ineffective at rejecting solid tumors. In preclinical mouse models, adjuvant CpG-ODN therapy primes antitumor cytotoxicity and IFN-γ secretion by lymphocytes which is highly effective in preventing tumor formation in a prophylactic setting (2). CpG-ODN is also used as a potent single agent (3–7). In these models, CpG-ODN is directly injected into s.c. transplantation tumors and is sufficient to reject small tumors. Surprisingly, local CpG-ODN monotherapy is potent enough to activate spontaneous anticancer immunity mediated by innate and adaptive immune cells, in particular NK and CD8+ T cells (3, 5). However, highest efficacy can only be achieved with direct intratumoral injection into s.c. tumors, whereas injection into the tumor-free flank is less effective (5, 6). Similarly, in a mouse model of mammary adenocarcinoma, direct intramammary CpG-ODN application markedly impairs tumor growth (8), whereas systemic application only prevents tumor formation but has no impact on established tumors (9). Moreover, it has been demonstrated that systemic application of CpG-ODN in mice suppresses T cell activity through IDO induction in spleen (10). This raises the question of how effective systemic CpG-ODN monotherapy would be in dealing with tumors which are not accessible for intratumoral injections. This is especially relevant since several TLR9 agonists are now being assessed in clinical trials ranging from vaccine adjuvants and combination therapies to local or systemic monotherapies (11).
In the RIP1-Tag5 transgenic mouse model of pancreatic islet tumorigenesis, developing insulinomas are deeply embedded in the exocrine pancreas and thus out of reach for intratumoral injections. We found that systemic application of CpG-ODN as a single agent had no impact on established tumors and hence survival of transgenic mice (12). Nevertheless, we observed that systemically applied CpG-ODN had indirect effects on the tumor environment through local uptake by macrophages. We now show that therapeutic success can be enhanced if CpG-ODN was enriched and retained in the tumor microenvironment. We used a vascular targeting peptide (RGR peptide) which specifically homes to tumor blood vessels and lipid vesicle retention in the tumor environment. We further demonstrate a greatly improved therapeutic outcome of systemically applied, tumor-targeted CpG-ODN liposomes through stimulation of spontaneous antitumor immunity and antivascular effects.

Materials and Methods

**Mice**

RIP1-Tag5 transgenic mice (provided by D. Hanahan, University of California San Francisco, CA) were used on a C3HeB/Fe background unless stated otherwise (15). For adoptive transfer experiments, mice transgenic for a TCR that recognizes Tag presented by the MHC class I molecule H-2Kk (referred to as TagTCR8 (16); provided by T. Geiger, St. Jude Children’s Research Hospital, Memphis, TN and R. Flavell, Yale University, New Haven, CT) or the MHC class II molecule I-A (TagTCR1 (17); provided by I. Förster, Heinrich-Heine University, Düsseldorf, Germany) bred on a C3HeB/Fe background were used. All mice were kept under specific pathogen-free conditions at the University of Western Australia and all experimental protocols were approved by the Animal Ethics Committee of the University of Western Australia.

**Reagents**

The phospholipids 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) as well as 1,2-dioleoyl-3-trimethylammonium propane (DOTAP) and cholesterol were obtained from Sigma-Aldrich. The chelator lipid 3(nitrilotriacetic acid)ditetradecylamine (NTA3-DTDA) was produced in the Research School of Chemistry (Australian National University) as described previously (18, 19). 1,2-Disteroyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-mPEG2000) and 1,2-dioleoyl-3-dimethylammonium propane (DODAP) were obtained from Avanti Polar Lipids. The tracer lipid Oregon Green-488 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (OG-488-DHPE) was from Invitrogen. NiSO4 was used in all additions of Ni2+ to lipid preparations. The two peptides for engraftment onto liposomes were produced as above, the liposomes were first neutralized by adding sodium phosphate buffer and 10× PBS to give 1× PBS salts in the final suspension. Ni-NTA-DTDA (1 mol% total lipid) was then added and the suspension was mixed and incubated for 30 min at 37°C to allow incorporation of the NTA-DTDA. The NTA-DTDA-containing liposomes were then mixed with either L2 (control) or RGR (targeting peptide) by incubating with the indicated peptide for 30 min at room temperature with occasional mixing. The amount of RGR-targeting peptide used was optimized to give the greatest increase in cell fluorescence in binding assays with NIH-3T3 cells (data not shown). As judged from their ability to target fluorochrome-conjugated CpG-ODN to cells, engrafted liposomes were stable for at least 4 wk when stored at 4°C. For use in experiments liposomes were used within 4 wk of their preparation.

**ELISA**

Primary murine splenocytes (5 × 10^6 cells) were incubated with various dilutions of CpG-ODN and liposome-engrafted CpG-ODN in RPMI 1640 culture medium for 18 h in a 37°C tissue culture incubator. Supernatants were harvested and IL-12 expression was analyzed in a sandwich ELISA (R&D Systems). Strepavidin-HRP (Vector Laboratories) and substrate for peroxide-conjugated secondary Ab (FAST OPD tablets; Sigma-Aldrich) were used for detection in an ELISA plate reader (Victor 1420 Multilabel Counter; PerkinElmer).

**Treatment of RIP1-Tag5 mice**

For histology, 27-wk-old transgenic RIP1-Tag5 mice were treated over 2 wk with biweekly i.v. injections of 100 μl of liposomes, peptide-liposome-CpG-ODN vesicles, or CpG-ODN in PBS (5 μg total). Mice were sacrificed on day 14 and tumors were harvested. For survival analyses, 22- to 23-wk-old RIP1-Tag5 mice were treated over a period of 10 wk with biweekly i.v. injections of 50 μl of liposome/CpG-ODN mixtures as described above and survival monitored up to week 45. In addition, adoptive transfers with combined 2.5×10^6 1 in vitro-activated TagTCR lymph node cells and 2.5×10^6 TagTCR8-derived splenocytes were performed every 2 wk in some treatment groups. In vitro proliferation and adoptive transfers are described elsewhere (14). Briefly, TagTCR8 splenocytes or TagTCR1 lymph node cells were cultured in vitro for 3 days with 10 U of rIL-2/ml and 25 mM Tag peptide 362–568 (SEFLIEKRI for TagTCR8 cells) or 25 mM L2 peptide (20-20) (T. Herrington and J. G. Altin, manuscript submitted for publication).

**Histology**

For localization of peptide-tagged liposomal vesicles, 100 μl of Oregon Green-488-labeled liposomes were injected into tumor-bearing mice and mice were sacrificed at 2, 4, 6, and 8 h time points. Tumors embedded in Tissue-Tek OCT compound (Sakura Finetek) and 7-μm sections were analyzed using fluorescent microscopy. For fluorescence detection of blood vessels and tumor-resident macrophages, the following Abs were used:

- CD31 (12/1, Acris Antibodies)
- CD86 (286, Serotec, Oxford, UK)
- CD8 (2.1, BD Pharmingen, San Diego, CA)
- CD11c (N418, BD Biosciences, San Diego, CA)
- CD3 (G.7.2.5, BD Pharmingen, San Diego, CA)
- CD11b (clone M1/70, BD Pharmingen, San Diego, CA)
- F4/80 (clone 3.1, BD Pharmingen, San Diego, CA)
used: anti-CD31 (Mec 13.13, 10 μg/ml; BD PharMingen), anti-ICAM-1 (Y8/17.4, 10 μg/ml; American Type Culture Collections), anti-CD68 (FA/11, 10 μg/ml; Abcam), followed by cyanin 3-conjugated IgG (F(ab′)2, goat anti-rat (3 μg/ml; Jackson ImmunoResearch Laboratories). Immuno-histochemistry was performed with the following Abs at 10 μg/ml (BD PharMingen): anti-CD4 (NK1.1), anti-CD8 (Ly-2), anti-CD11b (M1/70), and anti-CD45 (30-F11). Primary Abs were detected with anti-rat or anti-donkey biotinylated secondary Abs (Vector Laboratories) followed by an ABC elite kit (Vector Laboratories). TUNEL assay was performed on frozen sections using an in situ cell death detection kit-fluorescein (Roche). Quantification was performed on tumor sections using a ×20 objective lens. Five independent areas per tumor were selected, digitally photographed, and positive signals counted using automated software (Image-Pro Plus 5.0; Media Cybernetics).

**In vivo killing assay**

The in vivo CTL assay was performed as described (12). For this assay, the F1 generation of RIP1-Tag5/C3H mice and C57BL/6 mice (referred to as RIP1-Tag5/F1) were used. Briefly, 1 × 107 splenocytes/ml were loaded with H2-Kb-restricted Tag peptide IV (404–411, VVYDFLKL) or left without peptide. Targets were labeled with CSFE (Molecular Probes and Invitrogen) in a final concentration of 0.75 μM (high, with peptide IV) or 0.075 μM (low, without peptide IV). One × 107 cells of each population were injected i.v into recipient mice. CTL activity was assessed 18 h after the adoptive transfer using FACS analysis.

**Flow cytometry**

Spleen cell suspensions were prepared from mice injected with Oregon Green-488-labeled liposomes after 2, 24, and 48 h. One × 106 spleen cells were analyzed on a FACScan (BD Biosciences).

**Statistical analysis**

Cumulative survival was calculated by the Kaplan-Meier method and analyzed by the log-rank test. Student’s t test was used for all other statistical evaluation. Statistical values are presented as mean ± SEM. A p value of 0.05 was considered statistically significant.

**Results**

**RGR-engrafted CpG-ODN liposomes specifically home to the tumor vasculature**

RIP1-Tag5-transgenic mice express the oncogene SV40 large T Ag (Tag) under the control of the rat insulin gene promoter (RIP). Oncogene expression leads to progressive growth of insulinomas and death by hypoglycemia at ~30 wk of age (15). Tumor development follows a well-characterized pathway from hyperplastic and angiogenic islets to solid tumors with a distinct angiogenic vasculature (21). In this study, we have engrafted RGR peptides which specifically bind to RIP1-Tag5 tumor blood vessels via polyhistidine residues onto metal chelator lipid NTA3-DTDA-containing liposomes (18). As control, liposomes engrafted with an unrelated peptide (L2) without binding preference to tumor endothelium were used. All liposomes contained 10% DSPE-mPEG2000 to protect them from opsonization and to prolong circulation time. For in vivo homing studies, lipid vesicles also contained the tracer lipid Oregon Green-488-DHPE (referred to as 488-liposomes). A volume of 100 μl of RGR or L2–488-liposomes was injected i.v. into 27-wk-old tumor-bearing RIP1-Tag5 mice and allowed to circulate for 2 h. Tumors were analyzed for the presence of green fluorescence, indicative of liposomes, and compared with staining for vascular (CD31) or macrophage markers (CD68; Fig. 1). For RGR-tagged 488-liposomes, the majority of fluorescent signals colocalized with vascular structures. This pattern was not observed in normal pancreatic tissue or in tumors of L2–488-liposome-treated mice (Fig. 1A). Furthermore, green cellular structures were visible in all tumors, independent of the engrafted peptide, but not in exocrine pancreas. These cells colocalized with tumor-resident CD68+ macrophages (Fig. 1B). Interestingly, green macrophages in RGR–488-liposome-treated mice outnumbered those found in L2 controls as confirmed by quantification of green CD68+ cells in tumors and pancreatic tissue (Fig. 1C). These results show that RGR-tagged liposomes indeed preferentially bind to tumor vasculature. In addition, liposomes are also taken up by macrophages independent of their homing peptide. However, enrichment of liposomes through vascular binding correlates with a higher number of green macrophages in RGR-liposome-treated tumors. The presence of vessel-associated green signals was monitored over time and still detected 48 h after RGR–488-liposome injection, but not in L2–488-liposome-treated mice (supplemental Fig. 1A). To assess nonspecific liposome uptake in the periphery, spleen cells from RGR- and L2–488-liposome-treated mice were analyzed by FACS over time. Green fluorescent splenocytes were detected 2 h and 24 h after liposome injection. However, after 48 h green fluorescence disappeared, indicating clearance from the circulation (supplemental Fig. 1B). These findings show that peptide-tagged liposomes are specifically enriched and retained in tumors through

**FIGURE 1.** Homing and localization of peptide-tagged liposomes into RIP1-Tag5 tumors. A, RGR or L2-488-liposomes were injected i.v. into 27-wk-old tumor-bearing RIP1-Tag5 mice (n = 6) and allowed to circulate for 2 h. Cryosections of tumor (RGR tumor: RGR-tagged liposomes, specific for tumor vasculature; L2 tumor: L2-tagged liposomes, nonspecific control) or corresponding exocrine pancreatic tissue (RGR pancreas: exocrine pancreas from RGR-tagged green fluorescent liposome-injected mice) were analyzed for green fluorescent liposomes and stained with the vascular marker CD31 (red). Original magnification, ×20; bar, 50 μm. B, Treatment as described in A, colocalization of Oregon Green-488 liposomes (green) with CD68+ macrophages (red) in tumors and exocrine pancreas. Original magnification, ×10; bar, 100 μm. C, Quantification of CD68+ macrophages with internal green signals (yellow in merged histology), indicative of liposomal uptake. (L2 pancreas: exocrine pancreas from L2-tagged green fluorescent liposome-injected mice).

4 The online version of this article contains supplemental material.
vascular targeting, but some are also taken up by the reticuloendothelial system via phagocytosis.

Intratumoral CpG-ODN elicits vessel wall inflammation in RIP1-Tag5 tumors

To exploit the tumor-homing properties of RGR-tagged liposomes, liposomes were loaded with immunostimulatory CpG-ODN (referred to as RGR-liposomes plus CpG). To quantify the amount of CpG-ODN packaged into liposomes, the capacity of RGR-liposomes plus CpG to stimulate IL-12 secretion by splenocytes was compared with unpackaged CpG-ODN of known concentration (supplemental Fig. 2). We determined that a 100-µl mixture of RGR-liposomes plus CpG contains ~5 µg of CpG-ODN. To begin, 27-wk-old, tumor-bearing RIP1-Tag5 mice were treated twice weekly with injections of 100 µl of liposomes alone, RGR-liposomes plus CpG, L2-liposomes plus CpG, CpG-ODN alone, or left untreated for 2 wk. Subsequently, tumors were analyzed for signs of inflammation. RGR-liposomes plus CpG treatment groups showed a significant increase in CD45+ leukocytes in tumors compared with untreated groups or mice treated with RGR-engrafted liposomes or liposomes (Fig. 2A). This finding correlated well with enhanced influx of CD11b+ monocytes/macrophages, which was predominantly observed after RGR-liposomes plus CpG treatment (Fig. 2B). L2-liposomes plus CpG treatment groups and CpG-ODN alone also showed elevated levels of CD45+ and CD11b+ cells in tumors, with the L2-liposomes plus CpG regimen reaching statistical significance over untreated groups. Elevated infiltration is most likely correlated with the nonspecific uptake of liposomes by tumor-resident macrophages as demonstrated in Fig. 1B. Strikingly, only in the RGR-liposomes plus CpG treatment group were CD45+ cells observed to specifically accumulate around vessel walls (Fig. 2C). This was in clear contrast to all other treatment groups (supplemental Fig. 3) and prompted us to investigate tumors for expression of vascular activation markers. Indeed, ICAM-1 expression was specifically up-regulated in the stroma of RGR-liposomes plus CpG-treated tumors, but not in control groups (Fig. 2D). These results demonstrate that peptide-mediated, vascular targeting of CpG-ODN activates innate immune cells which cluster around tumor vessels and elicit an inflammatory response at the vessel wall.

Targeted CpG-ODN liposomes activate antitumor CTLs

In RIP1-Tag5 mice, Tag is presented in the draining lymph node of the pancreas and CTL activity against the dominant H-2-Kb-restricted, naturally presented Tag peptide IV (22) can be induced in vivo at all stages during tumor progression (12, 15). Collectively, this argues that tumors in RIP1-Tag5 mice do not induce systemic tolerance. Nevertheless, untreated RIP1-Tag5 mice progressively develop cancer and these tumors harbor few CD8+ or CD4+ T cells (15). We therefore asked whether the increased numbers of CD45+ cells in tumors and concomitant vessel activation in response to RGR-liposomes plus CpG could trigger specific antitumor immunity. Various treatment groups were histologically analyzed to assess the degree of T cell infiltration. Tumors from RGR-liposomes plus CpG groups showed a significant increase in infiltrating CD8+ and CD4+ T cells, whereas treatment with liposomes, L2-liposomes plus CpG, or CpG-ODN alone had no impact on the number of infiltrating T cells compared with untreated RIP1-Tag5 tumors (Fig. 3, A–C). The majority of CD8+ T cells were scattered throughout the tumor, but some T cells accumulated around vascular structures (Fig. 3A). To assess the function of these infiltrating T cells, we analyzed their capacity to lyse Tag peptide IV-loaded splenocytes. As shown previously, naive RIP1-Tag5 mice bearing a considerable tumor burden at 27 wk

**FIGURE 2.** Vascular targeting of CpG-ODN liposomes elicits vessel wall inflammation. A, 27-wk-old, tumor-bearing RIP1-Tag5 mice (n = 7–9) were treated for 2 wk with biweekly injections of liposomes alone (lipos), RGR-liposomes, RGR-liposomes + CpG, L2-liposomes + CpG, free CpG-ODN, or left untreated. At 29 wk, tumors were analyzed by immunohistochemistry and CD45+ leukocytes were quantified (three to five independent sections per tumor; original magnification, ×20), *, p = 0.0015 and **, p = 0.0045 compared with untreated tumors. B, Treatment as in A, histological quantification of CD11b+ monocytes/macrophages in tumor tissues; *, p < 0.0001 and ***, p = 0.003 compared with untreated tumors. C, Representative photographs of CD45+ cells in tumors of RGR-liposomes + CpG treatment groups and comparison to CD31 vascular staining. Upper panel, Original magnification ×10; bar, 100 µm. Insets indicate area of higher magnification shown in lower panels. Original magnification, ×20; bar, 50 µm. Dotted lines delineate “blood lakes,” arrows point to endothelial cell lined, CD31+ blood vessels. D, Representative photographs of tumors from different treatment groups stained with fluorescently labeled (Cy3, red) anti-ICAM-1 Abs. Original magnification, ×10; bar, 100 µm. Please note: in this experiment, liposomes only were compared with RGR-engrafted liposomes to analyze potential effects of the RGR peptide. Since no difference was observed, liposomes were used as controls in all subsequent experiments.
CpG, and free CpG-ODN and stained with anti-CD8 Abs. Representative photographs are shown for liposomal-only and RGR-liposomes + CpG treatment groups. Upper panel, Original magnification, ×4; bar, 250 μm. Insets indicate area of higher magnification shown in lower panels, original magnification 20x, bar length, 50 μm. Arrows point at a vascular structure. 

B. Quantification of infiltrating CD8+ T cells. *p = 0.005 and **p = 0.015 compared with untreated. C. Quantification of infiltrating CD4+ T cells. *p = 0.0005 and **p = 0.002 compared with untreated. D. Untreated 29-wk-old RIP1-Tag5 (C3H × C57BL/6)F1 mice (n = 3–5) or after biweekly treatment were assessed for in vivo CTL activity against the Tag-specific peptide IV. Right panel, Combined in vivo kill data for spleen (spleen), pancreatic lymph nodes (panc LN), and CD8+ and CD4+ T cells. In all three treatment groups, therapeutic efficacy was considerably enhanced when compared with single treatments without immunotherapy (Fig. 4C), demonstrating that effector cells are indeed limiting. The

FIGURE 3. Activation of spontaneous antitumor immunity after vascular targeting of CpG-ODN liposomes (lipos) into RIP1-Tag5 tumors. A. Tumors were harvested from untreated RIP1-Tag5 mice (n = 7–9) or after biweekly treatment with liposomes, RGR-liposomes + CpG, L2-liposomes + CpG, and free CpG-ODN and stained with anti-CD8 Abs. Representative photographs are shown for liposomal-only and RGR-liposome + CpG treatment groups. Upper panel, Original magnification, ×4; bar, 250 μm. Insets indicate area of higher magnification shown in lower panels, original magnification 20x, bar length, 50 μm. Arrows point at a vascular structure. 

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of age were unable to mount a tumor-specific immune response. Similarly, treatment with liposomes or CpG-ODN alone was insufficient to prime Tag-specific cell lysis (Fig. 3D and Ref. 12). L2-liposomes plus CpG treatment groups showed a consistent, but very low in vivo killing. Strikingly, however, treatment with RGR-liposomes plus CpG elicited a Tag-specific CTL activity (Fig. 3D) correlating with the increased CD8+ and CD4+ immune cells in the tumors (Fig. 3A). This result is remarkable, since systemic CpG-ODN alone activates an innate immune response, in particular NK cells, but fails to elicit tumor-specific CTL activity (12). Therefore, CpG-ODN-containing liposomes when targeted into solid tumors have superior effects by attracting innate immune cells, activating tumor vessels, and, in addition, eliciting tumor-specific adaptive immunity.

Specific tumor targeting of CpG-ODN is therapeutically effective

Having monitored the effects of a 2-wk treatment of RGR-liposomes plus CpG in transgenic mice with a late-stage tumor burden, we next assessed whether these short-term effects would translate into meaningful survival benefits. Untreated RIP1-Tag5 mice succumbed to insulinomas at 30 ± 2 wk. Adoptive transfers of activated CD4+ and CD8+ anti-Tag T cells fail to prolong the survival of these mice since activated effectors are unable to penetrate into solid tumors (12, 15). To test whether liposomes alone or in combination with adoptive transfers of CD4+ and CD8+ anti-Tag T cells had any effect on tumor growth, tumor-bearing 22- to 23-wk-old RIP1-Tag5 mice were treated over 10 wk. Not surprisingly, these treatments were ineffective with no survival benefits (Fig. 4A). Similarly, repeated, systemic injection of CpG-ODN alone had no impact on survival (Fig. 4B), whereas L2-liposomes plus CpG demonstrated a statistically significant survival advantage (mean survival 32 ± 2 wk, p = 0.012 compared with untreated mice) most likely due to limited intratumoral uptake of CpG-ODN packaged in liposomes (Fig. 1). Remarkably, however, in RGR-liposomes plus CpG treatment groups, all mice survived beyond week 35 and 30% were still alive when the experiment was terminated at week 45 (mean survival 39 ± 4 wk).

After RGR-liposomes plus CpG treatment, tumor infiltration by CD4+ and CD8+ T cells was significant but moderate (Fig. 3A). Thus, we postulated that therapeutic success could be improved by increasing effector cell frequency. To test this, RIP1-Tag5 mice were treated with CpG-ODN alone, L2-liposomes plus CpG, and RGR-liposomes plus CpG in combination with adoptive transfers of preactivated anti-Tag CD4+ and CD8+ T cells. In all three treatment groups, therapeutic efficacy was considerably enhanced when compared with single treatments without immunotherapy (Fig. 4C), demonstrating that effector cells are indeed limiting. The
CD8^+ T cells and NK cells are major effectors for long-term survival

Having established considerable therapeutic efficacy of intratumoral RGR-liposomes plus CpG, we attempted to identify the effector cell population responsible for long-term survival. To test potential effectors of the adaptive immune system, CD4^+ and CD8^+ T cells were depleted during RGR-liposomes plus CpG treatment of tumor-bearing RIP1-Tag5 mice. A 10-wk treatment regimen was started at the age of 23 wk and survival was monitored up to week 45. Without depletion or with control IgG Ab injection, 30–40% of RGR-liposomes plus CpG-treated RIP1-Tag5 mice survived beyond week 45 (Figs. 4B and 5). Depletion of CD4^+ T cells had no impact on the survival rate (Fig. 5A), whereas depletion of CD8^+ T cells significantly reduced survival benefits, with death occurring at 38 ± 3 wk (Fig. 5B; p = 0.0078 compared with IgG-treated controls). Although there were no long-term survivors, this result is still significantly better compared with untreated RIP1-Tag5 mice, implying that CD8^+ T cells are not the only important effectors. Since Cpg-ODN are known to be potent activators of the innate immune system, including NK cells (5, 12), we also depleted NK cells in RGR-liposomes plus CpG-treated RIP1-Tag5 mice. NK cell depletion reduced survival to the same extent as CD8^+ T cell depletion, with survival to 37 ± 3 wk, which was still better than that of control RIP1-Tag5 mice (Fig. 5B; p = 0.0047). Since both CD8^+ and NK effector cells impact on survival, we assessed possible synergistic effects in RGR-liposomes plus CpG treatment groups with combined CD8^+ and NK cell depletion. Long-term survival was significantly impaired and mice succumbed to tumors at the age of 34 ± 4 wk (Fig. 5C; p = 0.0006 compared with IgG controls). This result demonstrates that CD8^+ and NK cells are indeed major players for intratumoral CpG-ODN liposomal therapy. However, combined depletion was not sufficient to reduce survival to that of untreated experimental groups (p = 0.024, CD8^+NK cell depletion compared with untreated controls). This implies that tumor-targeted CpG-ODN liposomes exert even more complex effects, possibly within the tumor environment, which impact on tumor growth and overall survival.
Antivascular effects of intratumoral CpG-ODN liposomes

Untreated RIP1-Tag5 tumors display vessels that are heterogeneous in caliber and lack architectural hierarchy. In addition, intratumoral “blood lakes” are described, which are nonendothelium-lined cavities (23, 24). A striking feature of RGR-liposomes plus CpG-treated RIP1-Tag5 tumors was the presence of predominantly large vessels on a background of increased numbers of blood lakes (Fig. 2C). This observation prompted us to quantify CD31-positive vascular structures in different treatment groups. This indeed confirmed a significant reduction in blood vessel density specifically with RGR-liposomes plus CpG treatment (Fig. 6A). We further assessed apoptotic events in 29-wk-old RIP1-Tag5 tumors after a 2-wk treatment period and found a marked increase in TUNEL- cells throughout tumor tissues when mice were repeatedly injected with RGR-liposomes plus CpG (Fig. 6B). In the RGR-liposomes plus CpG group but not control tumors, some of these signals were in close vicinity to CD31-positive vessels (Fig. 6C). This demonstrates that intratumorally enriched CpG-ODN activates vessels but also affects endothelial cell survival. Although the sequence of events is difficult to determine, we show here that innate immune cell and vessel activation strongly correlate with influx of adaptive immune cells, antitumor CTL activity, and increased numbers of apoptotic cells. Vessel death may occur simultaneously or as a consequence of vessel activation. These antivascular effects are specific to tumor-targeted CpG-ODN and contribute to CD8+ and NK cell-mediated tumor immunity, resulting in delayed tumor growth and long-term survival benefits.

Discussion

We and others have previously shown that CpG-ODN monotherapy is sufficient to eradicate s.c. growing tumors when directly injected into the tumor mass. However, systemic application of CpG-ODN is only partially effective for murine transplantation tumors and therapeutically inefficient in an autochthonous tumor model (5, 12). In this study, we show that the therapeutic efficacy of CpG-ODN is substantially enhanced by targeted liposomal delivery into the tumor environment.

Liposomes are particularly useful as carrier of drugs, proteins, or nucleic acids, including CpG-ODN (25–27). More advanced developments combine the drug delivery capacity of liposomes with direct targeting of tumor cells or stroma via mAbs or peptides (28, 29). We have recently described a new methodology which uses metal chelator lipids to engraft polyhistidine-tagged proteins to the surface of liposomes (18, 30). This approach was used here to anchor the tumor vessel-specific RGR peptide with a chain of His residues onto liposomes. Targeting of angiogenic vessels is an attractive approach to specifically deliver reagents into tumors, especially if tumor-specific targeting molecules have not been identified. Several investigators have described in vivo tumor-homing capacities of liposomes tagged with tumor endothelium-specific peptides or Abs such as RGD and NGR peptides and VCAM-1-specific Abs (31–33). Similar to these studies, we also observed that vascular-directed lipid vesicles localize to different intratumoral compartments compared with nonspecific control liposomes (31, 33). Lipid particles coated with cyclic RGD as targeting ligand for αvβ3 integrin, for instance, specifically bind to the tumor vessel wall, whereas control liposomes extravasate into tumor parenchyma (31). VCAM-1-specific targeting of angiogenic vessels results in selective binding to tumor vasculature as opposed to nonspecific accumulation in tumor tissue by controls (33). In contrast to these studies, however, we observed that specific vascular targeting also enhanced liposome uptake by tumor-resident macrophages resulting in an overall increase of phagocytosis in the tumor environment.

We further used tumor vascular targeting to investigate the therapeutic efficacy of an immune stimulatory agent, CpG-ODN, in a novel and so far unexplored context. Free CpG-ODN has been extensively analyzed as adjuvant and direct stimulator of antitumor immunity. More recently, CpG-ODNs were encapsulated in various lipid formulations with superior immunostimulatory activity. CpG-ODN-containing liposomes when administered i.v. or s.c. triggered higher plasma cytokine levels, NK cell activation, and a more vigorous adaptive immune response compared with free CpG-ODN (25–27). This is most likely due to enhanced uptake of liposomes by APCs, including macrophages. Interestingly, liposome-CpG-ODN-Ag complexes are sufficient to cross-prime CD8+ T cells in vivo, independent of CD4+ T cell help (26). As a vaccine adjuvant, immunization with liposomal CpG-ODN in combination with tumor Ag such as a melanoma-specific peptide delayed the growth of established B16 melanomas (26). Although the enhanced antitumor effects of liposomal CpG-ODN is encouraging, its use as vaccine adjuvant requires coadministration of tumor-associated Ags. In contrast, direct intratumoral injections of free CpG-ODN does not require prior identification of tumor Ags, but this administration route is limited to anatomically accessible tumors. Our approach enables high concentration targeting into inaccessible tumor environments.

One of the major findings we report is that tumor-targeted CpG-ODN-containing liposomes were sufficient to prime a tumor Ag-specific cytotoxic T cell response. This is in stark contrast to i.v. injection of free CpG-ODN in the same mouse model which effectively primes innate immunity but fails to elicit tumor-specific adaptive immunity (12). Interestingly, our data mimic the results of intratumoral injections of free CpG into s.c. growing tumors which were rejected through a concerted action of NK and CD8+ effectors (5). However, through vascular-directed CpG-ODN liposomes, we have now developed an improved strategy where treatment of autochthonous tumors is efficient through the circulation, with important clinical implications for many deeply embedded primary cancers and metastases.

A striking feature of RGR-liposomes plus CpG treatment was the inflammatory response of the vessel wall. This in turn correlated with significantly increased leukocyte entry into tumor tissue compared with controls. Furthermore, adoptively transferred antitumor T cells reach the tumor side under RGR-liposomes plus CpG treatment but not without manipulation of the tumor environment as shown previously (12, 15, 24, 34). Once in the tumor, these effector cells encounter a substantial number of tissue-resident TLR9 receptors most likely amplifies immune activation further. Long-term survival was mainly mediated by CD8+ and NK cells and presumably direct tumor cell lysis. Moreover, enrichment of CpG-ODN liposomes on the vessel wall reduced vascular density over time and thus indirect antivascular effects also impacted on tumor growth. In summary, vascular-directed CpG-ODN liposomes activate innate and adaptive immunity and alter the tumor environment, which results in greatly improved therapeutic efficacy compared with free CpG-ODN injections.

The ability to combine intratumoral targeting with immune modulation using liposome technology has enormous potential for clinical evaluation. We envision that other combinatorial approaches could result in further survival benefits. For instance, peptide-tagged and nontagged liposomes could be loaded with CpG-ODN and tumor Ag for coadministration. In this setting,...
CpG-ODN would act locally and as a vaccine adjuvant. Synergistic action of local CpG-ODN with radiotherapy or depletion of regulatory T cells has also been reported (35, 36).

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Disclosures

J.G.A. and C.R.P. declare a commercial interest in Lipotek Pty Ltd.

References


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Supplementary Figure Legends

FIGURE 1
Liposome uptake by RIP1-Tag5 tumors and splenocytes over time. A, RGR or L2-engrafted Oregon Green-488-liposomes were injected i.v. into 27-week-old tumor-bearing RIP1-Tag5 mice (n=4) and allowed to circulate for 48 h. Tumors were analyzed for green fluorescent signals, original magnification 40x, bar length, 25 μm. B, Spleen cell suspensions from RGR- and L2-488-liposome treated mice (n=3) were analyzed by FACS 2 h, 24 h and 48 h after i.v. injection. Histograms represent representative graphs and indicate % green fluorescent cells.

FIGURE 2
IL-12 stimulation of primary splenocytes. To estimate the amount of liposome-loaded CpG-ODN, the effectiveness of free and liposomal CpG-ODN to stimulate IL12 secretion of primary splenocytes was compared. Splenocytes were incubated with increasing amounts of free CpG-ODN or dilutions of liposomal CpG-ODN for 18 h. IL12 production in culture supernatant was determined by ELISA. Liposomes without CpG-ODN served as controls.

FIGURE 3
Intratumoral localization of CD45+ leukocytes in comparison to CD31+ blood vessels after different treatment regimens. 27-week-old, tumor-bearing RIP1-Tag5 mice (n=7-9)
were treated for 2 weeks with bi-weekly injections of liposomes alone (lipos), RGR-liposomes, RGR-liposomes+CpG, L2-liposomes+CpG, free CpG-ODN or left untreated. At 29 weeks, tumors were analyzed by immunohistochemistry for CD45$^+$ leukocytes (left panels) and CD31-positive blood vessels (right panels). Arrows indicate accumulation of CD45$^+$ cells around blood vessels. This is mainly observed after RGR-liposomes+CpG treatment. In all other treatment groups, CD45$^+$ cells were more evenly distributed throughout tumor tissue. Original magnification 20x, bar length, 50 μm.
Supplementary Fig. 1

A

B
Supplementary Fig. 2

The graph shows the concentration of IL-12p40 (ng/ml) following the administration of different dilutions of CpG-ODN liposomes, CpG liposomes, and CpG-ODN liposomes+CpG liposomes. The x-axis represents the dilution factors, and the y-axis shows the concentration of IL-12p40. The error bars indicate the standard error of the mean.
Supplementary Fig. 3

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