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Review

Cancer relapse under chemotherapy: Why TLR2/4 receptor agonists can help

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Abstract

Liver or lung metastases usually relapse under chemotherapy. Such life-threatening condition urgently needs new, systemic anticancer compounds, with original and efficient mechanisms of action. In B16 melanoma mice treated with cyclophosphamide, D'Agostini et al. [D'Agostini, C., Pica, F., Febbraro, G., Grelli, S., Chiavaroli, C., Garaci, E., 2005. Antitumour effect of OM-174 and Cyclophosphamide on murine B16 melanoma in different experimental conditions. Int. Immunopharmacol. 5, 1205–1212.] recently found that OM-174, a chemically defined Toll-like receptor(TLR)2/4 agonist, reduces tumor progression and prolongs survival. Here we review 149 articles concerning molecular mechanisms of TLR2/4 agonists, alone or in combination with chemotherapy. It appears that TLR2/4 agonists induce a well controlled tumor necrosis factor-alpha (TNF-α) secretion, at plasma levels known to permeabilize neoangiogenic tumor vessels to the passage of cytotoxic drugs. Moreover, TLR2/4 agonists induce inducible nitric oxide synthase (iNOS) expression, and nitric oxide is able to induce apoptosis of chemotherapy-resistant tumor cell clones. Finally, TLR2/4-stimulation activates dendritic cell traffic and its associated tumor-specific, cytotoxic T-cell responses. Therefore, parenteral TLR2/4 agonists seem promising molecules to prolong survival in cancer patients who relapse under chemotherapy.

Keywords: Toll-like receptor; TLR agonist; TNF; OM-174; Chemotherapy

Contents

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1. Introduction

Chemotherapy and radiotherapy can delay cancer progression, but liver or lung metastases usually relapse. Toll-like receptor 2/4 agonists (TLR2/4 agonists) are promising molecules against chemotherapy- or radiotherapy-relapsing cancer metastases because:

- In cancer patients, the TLR2/4 agonist OM-174 induces transitory increases in tumor necrosis factor-alpha (TNF-α), a potent inflammatory mediator which selectively enhances the permeability of tumoral neoangiogenic vessels to cytotoxic drugs
- TLR2/4 signaling promotes inducible nitric oxide synthase (iNOS)-dependent apoptosis of chemotherapy-resistant tumor cell clones
- TLR2/4 and TNF-α signaling activate dendritic cell and macrophage traffic and its associated tumor-specific, cytotoxic T-cell responses, further enhancing the anticancer efficacy of cytotoxic drugs.

Here we present a survey of anticancer compounds stimulating TLR2 and/or TLR4 responses.

2. TLR2 and TLR4 signaling

The TLR family comprises at least eleven members, which recognize pathogen-associated microbial structures (for recent review see Takeda and Akira, 2004; Re and Strominger, 2004a; Moynagh, 2005). Prominent among the microbial or parasitic substances stimulating TLR receptors are:

- Muramyl peptides, bacterial lipopeptides, peptidoglycans and lipoteichoic acids (LTA), which stimulate TLR2 responses
- Lipopolysaccharide (LPS), a selective TLR4 agonist (some LPS however may activate TLR2, such as LPS from *Porphyromonas gingivalis*, see Zhou at al., 2005)
- Unmethylated CpG dinucleotides (CGP-DNA), which stimulate TLR9 responses.

TLR2 or TLR4 agonists stimulate the MyD88 signaling pathway in antigen presenting cells such as macrophages and dendritic cells, which induces various genes via the activation of the major transcription factors NF-kappaB and AP-1. This leads to the rapid expression of iNOS and a wide variety of proinflammatory cytokines, chemokines, and their receptors, including TNF-α, interleukin(IL)-1α, IL-1β, IL-1ra, IL-6, IL-8,

IL-10, IL-12p40, IL-23, macrophage inflammatory protein (MIP)-1α, MIP-1β (for review, see Re and Strominger, 2004a). These factors initiate the inflammatory response, increase vascular permeability, direct dendritic cell and macrophage migration from the periphery to the central lymphoid organs, and regulate various aspects of adaptive immunity development.

Striking differences were observed for the kinetics and the pattern of cytokines induced by TLR2 and TLR4 agonists. Thus, the transcription of several cytokine genes remain elevated for extended periods in cells stimulated by TLR2, but rapidly fade in cells stimulated by TLR4 (Re and Strominger, 2004b). Moreover, IL-12, IP-10, interferon(IFN)-β, and IL-15 expression was exclusively induced by the TLR4 agonist LPS and not by TLR2 agonists. In contrast, TLR2 agonists preferentially induced IL-8, IL-10, and IL-23p19.

TLR-signaling results in macrophage activation, and the removal of the infectious organisms (for review, see Klimp et al., 2002; Adam et al., 2003). The immune system is also able to recognize and lyse tumor cells, including those that are resistant to cytostatic drugs, and also destroy tumor-associated vessels, but such defensive antitumoral processes require activation signals. Toll-like receptors (TLRs) and their associated cytokines trigger all major activating phases of such immune response.

3. Microbes and microbial products: a long history of anticancer efficacy accompanied by high toxicity

Microbes or microbial products possess potent anticancer action, but are generally extremely toxic to handle (Wiemannn and Starnes, 1994). Their anticancer action was known since the beginning of the XVIIIth century, when Deidier (1725) reported that infection in cancer patients could be concomitant with the remission of malignant diseases. In 1898, Coley developed a cancer treatment with a mixture of bacterial toxins (Coley, 1898). In primarily inoperable sarcoma, Coley accomplished a cure rate of better than 10% (Wiemannn and Starnes, 1994).

3.1. Lipopolysaccharide

In 1943, Shear et al. found that the antitumor effect of Coley's toxin was due to LPS (lipopolysaccharide; Shear and Turner, 1943), a major component of the outer membrane of Gram negative bacteria (for review see Dobrovolskaia and Vogel, 2002). Unfortunately, because of the LPS toxicity, cancer patients can only be treated with a few ng LPS per kg injected i.v. or μg per kg injected s.c., these doses being too low to obtain a beneficial antitumor effect.

Because of their systemic toxicity, therapeutical use of microbes or microbial products was limited to local application.

3.2. BCG for bladder cancer

Bacillus Calmette-Guérin (BCG) was originally developed as a tuberculosis vaccine, but was later discovered to be effective against superficial bladder tumors. Thus, in 1976 Morales et al. introduced intravesical BCG to treat superficial bladder cancer (Morales et al., 1976). Clinical trials showed complete response rates of 75, 68 and 59% for prophylaxis of recurrence, therapy of carcinoma in situ and therapy of residual disease, respectively (Martinez-Piñeiro, 1992; for review see Böhle, 2000; Van der Meijden and Sylvester, 2003).

BCG is marketed as ImmuCyst® (Sanofi-Pasteur), BCG-Cancer®, TheraCys®, Tice BCG®. ImmuCyst® (Connaught) is the Connaught substrain. BCG-Cancer® (IAF) is the Montreal substrain.

3.2.1. BCG, a TLR2/4 agonist

The cell wall of mycobacteria contains specific molecules (Azuma et al., 1974), which can be recognized by local macrophages and immature dendritic cells. One important component of the cell wall mycobacteria is a peptidoglycan, which is covalently linked to arabinogalactan and mycolic acids. In a series of elegant experiments, the group of Seya et al. clearly showed that this mycobacterial cell wall component stimulates TLR2 and TLR4 responses in immature dendritic cells (Tsuji et al., 2000; Uehori et al., 2003).

3.2.2. TLR2/4-induced dendritic cells' anticancer mechanisms

In patients with superficial bladder cancer, repeated instillations of BCG produce a substantial inflammatory response in the bladder, with secretion of TNF-α and other inflammatory cytokines and pronounced infiltration of immunocompetent cells. The exact immunological mechanism of tumor cell destruction is not known, but dendritic cells seem to play a prominent role (Tsuji et al., 2000; Uehori et al., 2003). Thus, the consensus is that BCG mycobacteria serve as an immune potentiator of lymphocytes, namely an adjuvant, via the maturation of dendritic cells (Tsuji et al., 2000; Uehori et al., 2003). It appears that the effects in bladder cancer are local only. There is no protection against the development of tumors in areas where there is no BCG contact (e.g., the distal ureters and prostatic urethra).

TLR2/4 signaling produces TNF-α, which is required for inducing dendritic cell maturation and migration (for review see Banchereau and Steinman, 1998; Re and Strominger, 2004a,b; for review on dendritic cells in cancer therapy see Paczesny et al., 2003; Adema et al., 2005). Thus, TLR or TNF-α receptor-signaling in immature dendritic cells triggers dendritic cell maturation (Sallusto et al., 1998). Migration of mature dendritic cells to the draining lymph nodes is regulated at the level of entry into lymphatic vessels by inflammatory cytokines through up-regulation of CCL21 (Martin-Fontecha et al., 2003). In particular, dendritic cell migration could be increased up to 10-fold by preinjection of inflammatory cytokines such as TNF-α (Martin-Fontecha et al., 2003).

Mature dendritic cells that reach the lymph node induce a rapid and sustained congestion of lymphocyte traffic and their number determines the magnitude of T-cell proliferation and effector response. In this respect, Th2 immunity and antibody responses may not be desirable in cancer where Th1 and cytotoxic T-cell responses are necessary (Paczesny et al., 2003). TLR-dependent cytokines play a pivotal role in the establishment and maintenance of the Th1/Th2 balance, with IL-12 and

IFN-γ committing cells to Th1 lineage differentiation (Re and Strominger, 2004a,b). An IFN-γ and IL-12 rich local environment attracts T cells to the tumor or its draining lymph nodes, where the rich cytokine milieu promotes the development of a $CD4^+$ Th1 antitumor response that eventually gives rise to a cytotoxic $CD8^+$ antitumor cell response. Conversely, in the absence of IL-12 and IFN-γ, IL-4 promotes Th2 development.

The above elements suggest that TLR2 and TLR4 agonists may differ in their ability to influence Th cell differentiation (Re and Strominger, 2004a,b). TLR2 preferentially induced IL-10, a cytokine that inhibits the synthesis of several proinflammatory cytokines, and that belongs to the Th2 response in the mouse. Conversely, the factors specifically induced by TLR4 (IP-10, IL-12, IL-15, and IFN-γ) are all associated with a Th1 lineage commitment. IL-12 is able to stimulate IFN-γ production in T cells, and is the key cytokine that directs the development of human and murine Th1 cells. In the absence of IL-12, T-cell development proceeds by default toward the Th2 lineage. But the picture is somewhat complicated by the observation of Komai-Koma et al. (2004) that TLR2 is expressed on activated T cells as a co-stimulatory receptor for antigen-specific T-cell development and participates in the maintenance of T-cell memory. Moreover, Becker et al. (2003) found that Leishmania lipophosphoglycan activates NK cells through TLR2. Leishmania lipophosphoglycan upregulated both mRNA and the membrane expression of TLR-2 in NK cells. Finally, TLR agonists activate B cells, eosinophil, neutrophils, and several types of epithelia, but their significance in cancer therapy is uncertain. It is interesting to mention that human mast cells express TLR2 and TLR4, and TLR-signaling induced significant release of TNF-α, IL-5, IL-10 and IL-13 (Varadaradjalou et al., 2003). Mast cells accumulate in the stroma surrounding certain tumors, especially mammary adenocarcinoma (for recent review of mast cells in cancer see Theoharides and Conti, 2004).

4. TNF-α as neoadjuvant of local chemotherapy

Generally speaking, solid tumors cannot grow beyond a few mm in diameter, nor metastasise, without neovascularisation (Weidner et al., 1991). This results in heterogeneous tumor perfusion and vascular permeability, and in increased interstitial pressure, all factors which restrict the penetration of chemotherapeutic agents from the circulation into tumor metastases (Jain, 1994, 1999).

TNF-α is a main actor of the inflammatory reaction against microbes, which increases local capillary permeability and stimulates the passage of leukocytes across the vessel, by the activation of leukocyte and endothelial cell adhesive molecules. In 1993, Folli et al. (1993) showed that intravenous TNF-α selectively enhanced [^{125}I]-labeled monoclonal antibody (mAb) uptake in human colon carcinoma xenografts by increasing vascular permeability (Folli et al., 1993). Interestingly after i.v. injection of TNF-α, the mAb concentration in the blood and other normal tissues, such as liver, kidneys, lungs and heart was decreased, resulting in significantly higher ratios of tumor to normal tissue (Folli et al., 1993).

TNF-α is particularly active to permeabilize and destroy neoangiogenic vessels, producing hemorrhagic necrosis of tumors. Recently, some authors showed that TNF-R1 expressed on the surface of tumor endothelial cells is likely to be the most important target of TNF-α antitumor activity (Stoelcker et al., 2000). Ruegg et al. (1998) found that exposure of human endothelial cells to TNF-α and IFN-γ results in a reduced activation of integrin αVβ3, an adhesion receptor that plays a key role in tumor angiogenesis, leading to decreased endothelial cell adhesion and survival. The blockade of this cell surface molecule is known to induce apoptosis of angiogenic blood vessels (Brooks et al., 1994). Finally, Mocellin et al. (2005) hypothesized that higher levels of endothelial nitric oxide synthase (NOS) in tumor vessels rather than in normal surrounding tissues might condition sensitivity to TNF-α, as NOS inhibition significantly reduces endothelial cell sensitivity to TNF-α *in vitro*.

Between 1985 and 1988, recombinant TNF-α (rTNF-α) was made available to medical oncology (for review see ten Hagen et al., 2001; Lejeune, 2002; Leist and Jaattela, 2002). Unfortunately, systemic application in advanced cancer patients showed a very low maximal tolerated dose and tumor response was seen rarely, with unacceptable side effects such as hypotension and organ failure (Lejeune et al., 1998).

In 1998, Lejeune et al. had the idea of infusing high-dose TNF-α to improve tumor penetration of melphalan in patients with locally advanced melanomas and sarcomas of the limbs. Since then, important information was accumulated concerning TNF-α as neoadjuvant of chemotherapy, which is summarized hereafter.

4.1. TNF-α and tumor penetration of chemotherapeutic agents

Lejeune et al. (1998; see also Lejeune, 2002) tried regional application of TNF-α, in order to improve tumor penetration of melphalan in patients with locally advanced melanomas and sarcomas of the limbs. After 90 min of isolated limb perfusion, the limb vessels were intensively rinsed in order to eliminate TNF-α and melphalan. The first single-center pilot trial studied melanoma and sarcoma, giving impressive results: 90% of patients showed complete responses in melanoma and 75% showed complete responses in sarcoma (Lejeune et al., 1998). However this very high response rate was obtained at the cost of a rather heavy toxicity rate, although no TNFα-related death was reported (Lejeune et al., 1998).

Angiographic and histologic studies revealed that TNF-α effects in isolated limb perfusion were due to selective destruction of the tumor-associated vessels and that vessels in normal tissues were spared (Lejeune et al., 1998; Lejeune, 2002). Indeed, Lejeune and others have shown that TNF-α exerts two distinct effects that are selective for angiogenic vessels, namely an early increase of tumor vessels permeability, which can be induced with a low dose of TNF-α, and a later increase in vascular apoptosis, which appears to require higher doses. It was likely suggested that the increase in tumor vessels permeability facilitated accessibility of melphalan in cases of limb melanoma metastases or inextirpable soft-tissue

sarcomas (Lejeune et al., 1998; Lejeune, 2002). Lejeune et al. (1998) suggested that dual targeting is involved, TNF-α increased permeability of angiogenic endothelium, while melphalan induced apoptosis of tumor cells.

4.2. Tasonermin/melphalan combination for limb sarcoma

Four clinical trials in European oncological centres with 260 patients suffering from irresectable limb soft-tissue sarcoma who were destined for amputation or mutilating surgery, after all other treatment options had failed, showed that 80% of the patients experienced a durable limb salvage with isolated limb perfusion with TNF-α and melphalan (see for instance Grunhagen et al., 2006). The rapid tumor response within 2–30 days was characterised by a hemorrhagic necrosis and a complete angiographic disappearance of tumor-associated vessels. Tumor shrinkage greater than 50% was observed in 70% of the patients.

In 1999, the European Medicine Evaluation Agency (EMEA) issued a Marketing authorisation for a human recombinant TNF-αa (Tasonermin, Beromun®, Boehringer Ingelheim International GmbH, Germany; see also Grunhagen et al., 2006) as an adjunct to surgery for subsequent removal of the tumor so as to prevent or delay amputation, or in the palliative situation, for irresectable soft-tissue sarcoma of the limbs, used in combination with melphalan (interestingly Coley's toxin was very efficient in this type of cancer, see Wiemannn and Starnes, 1994). The approval was also based on an elaborate matched control study demonstrating that a TNF-α-based isolated limb perfusion did not worsen the survival of the patients.

5. Chemically pure TLR agonists

5.1. TLR2 agonists

In the 70s, chemically pure bacterial compounds were presumed to be promising candidates to replace viable bacillus Calmette-Guerin in cancer immunotherapy in humans and animals. Unfortunately, muramyl dipeptide required combination with other compounds to be therapeutically effective against the guinea pig line 10 hepatoma (Ribi et al., 1979; McLaughlin et al., 1980). Muramyl dipeptides were reviewed by Masihi (2000). Muramyl dipeptides induced little TNF-α or interleukin-1β, but strong Il-8 secretion. Muramyl dipeptides also substantially upregulated secretion of TNF-α induced by LPS (Beutler et al., 2001). Muramyl dipeptide-Lys(L18) has been found effective to restore decreased neutrophils and platelets in cancer patients, and has been licensed in Japan under the trademark Romurtide® (Namba et al., 1997).

Macrophage activating lipopeptide(MALP)-2 is a synthetic lipopeptide with two long chain fatty acid ester residues which signals through TLR2 (associated with TLR6), activates nuclear transcription factor NF-κB, and induces the synthesis of a number of cytokines and chemokines, depending on its target cell, and also induces maturation of dendritic cells (Schneider et al., 2004). Moreover, MALP-2 induces *in vitro* tumoricidal activity of macrophages. It is also highly active *in vivo* as it induces leucocyte infiltration after intraperitoneal administration in mice or after intratracheal administration in rats. In a pancreatic cancer mouse model, MALP-2 reduced formation of metastases in the lung. MALP-2 was shown to exhibit adjuvant properties when applied intranasally. Studies in BALB/c mice bearing methyl cholanthrene induced fibrosarcoma showed strong haemorrhagic necrosis with complete healing 10 days after MALP-2 application. Schneider et al. (2004) induced a tumor suppressive effect by treatment with MALP-2 in a model of orthotopic pancreatic cancer in mice.

5.2. TLR4 agonists

LPS is among the most potent TLR agonists. LPS-mediated anticancer actions and toxicity are due, at least in part, to the secretion of tumor necrosis factor (TNF-α) by macrophages (for recent review, see Mocellin et al., 2005). Intensive research was therefore dedicated to obtain detoxified LPS-derivatives, retaining TLR4-agonist properties (TNF-α production), for parenteral use. Thus, Takayama et al. (1981) isolated a nontoxic lipid A fraction, inducing tumor and metastases regression in animal models: ONO-4007 (sodium 2-deoxy-2-[3*S*-(9-phenylnonanoyloxy)tetradecanoyl] amino-3-*O*-(9-phenylnonanoyl)-D-glucopyranose 4-sulphate), developed by Ono Pharmaceutical Co (Osaka, Japan).

5.2.1. ONO-4007

Animal studies showed that ONO-4007 (see structure in Fig. 1) had remarkable and selective efficacy against TNF-α-sensitive tumors. In the mouse MM46 mammary tumor model, intravenous ONO-4007 activated tumor-infiltrating macrophages to secrete TNF-α (Yang et al., 1994). ONO-4007 brought about complete cures in about 60% of rats bearing TNF-α-sensitive hepatocellular carcinoma KDH-8 cells, whereas no complete cure was observed in rats bearing TNF-α-resistant tumors, including those bearing cKDH-8/11, which is almost identical to KDH-8 (Kuramitsu et al., 1997;

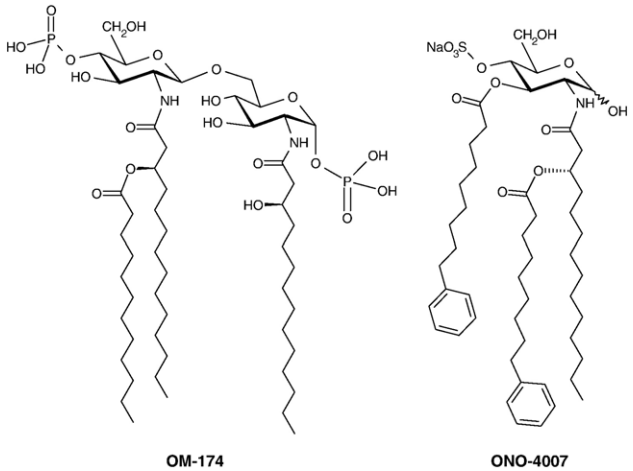

Fig. 1. Chemical structures of OM-174 and ONO-4007.

Matsumoto et al., 1998a; Matsushita et al., 1998, 2003). The concomitant administration of the rabbit anti-TNF-α antibody completely abrogated the therapeutic effects of ONO-4007 in TNF-α-sensitive KDH-8 (Matsushita et al., 1998).

Inagawa et al. (1998) found that a combination therapy of ONO-4007 with cyclophosphamide induced complete regression in 50–100% of BALB/c mice inoculated with Meth A fibrosarcoma, while cyclophosphamide or ONO-4007 alone did not cause complete regression. In a recent study, Takiguchi et al. (2004) found that cyclophosphamide significantly enhanced IL-12 (p40) mRNA compared to that by ONO-4007 alone. Yoshida (2000) established two cell clones from a rat myelomonocytic leukemia c-WRT-7/P2 clone which undergoes differentiation and/or apoptosis by ONO-4007. p53 was found to be one key factor in leading these cells to differentiation or apoptosis, with bcl-2 acting to suppress apoptosis.

In contrast to its effects in mice and rats, ONO-4007 has little or no ability to induce TNF-α production when injected into cynomolgus monkeys, dogs or rabbits (Matsumoto et al., 1998b). A phase I study with intravenous ONO-4007 showed a sharp increase in serum TNF-α levels near the maximum tolerated dose (MTD = 125 mg; De Bono et al., 2000). Moreover, 6 h after administration of the MTD, circulating TNF-α levels were still 20–30% of those peaking at 1 h. ONO-4007 development was discontinued thereafter.

5.2.2. Partial TLR4 agonists

DT-5461, SDZ MLR 953 and GLA-60 are LPS-antagonists (Sato et al., 1995b; Holst et al., 1996; Kiani et al., 1997; Chaby, 1999), which retain partial TLR4 agonist activity. Although their development as anticancer agents was discontinued, their pharmacological profile deserves to be mentioned.

DT-5461 is a synthetic low-toxic lipid A analog developed by Daiichi Pharmaceutical Co (Tokyo, Japan). In animal models, antitumor effects of DT-5461 significantly correlated with intratumoral levels of TNF-α induced by the compound (Kumazawa et al., 1997). Sato et al. (1992) reported that DT-5461 inhibits lung and liver metastasis of tumor in mice, against two highly metastatic tumor cell lines, L5178Y-ML25 T-lymphoma and B16-BL6 melanoma cells in mice. Four intermittent i.v. administrations of DT-5461 at intervals of 4 days resulted in a significant inhibition of liver metastasis caused by i.v. injection of L5178Y-ML25 cells and lung metastasis of B16-BL6 cells in the experimental metastasis models. Intraperitoneal intravenous and intranasal administration of DT-5461 were effective in preventing lung metastasis of the melanoma cells.

Sato et al. (1995a) found inhibition of tumor-induced angiogenesis by DT-5461 on the neovascularization induced by B16-BL6 melanoma in syngeneic mice. A systemic single administration of DT-5461 caused a marked decrease in the number of vessels oriented toward the tumor mass and in the tumor size during the early phase of vasculogenesis (on day 4 after tumor inoculation), with little or no inhibition in the following phases. Multiple i.v. administrations of DT-5461 at intervals of 4 days (an effective schedule for inhibiting tumor metastasis) significantly reduced the number of capillary vessels and tumor growth over a period of 14 days after the tumor implantation. Multiple systemic administrations of DT-5461 on days 1, 5 and 9 after tumor inoculation caused a high production of TNF-α in tumor sites although this treatment modality induced a low production in serum of tumor-bearing mice. The anti-angiogenic effect of DT-5461 was completely abrogated by anti-mTNF-alpha antiserum, whereas the inhibition of tumor growth by DT-5461 was only slightly diminished. Multiple i.v. administrations of DT-5461 after s.c. implantation of B16-BL6 cells significantly inhibited the growth of primary tumors measured at the time of tumor excision on day 21, and the lung metastasis of melanoma cells as compared with the untreated control in the spontaneous metastasis model. These results suggested that the suppressive effect upon tumor-associated angiogenesis by DT-5461 contributes in part to the inhibition of tumor metastasis.

Perera et al. (1993) evaluated the monosaccharide SDZ MRL 953 in murine macrophages. The compound was able to induce LPS-inducible genes (TNF-α and IL-1β). The nontoxic SDZ MRL 953 was approximately 1000-fold less potent than synthetic lipid A at inducing TNF-α secretion, and perhaps this contributes to the lack of toxicity exhibited by this compound. A phase I study by Kiani et al. (1997) showed that SDZ MRL 953 does not increase serum levels of TNF-α. Indeed, the compound markedly reduced the release of TNF-α induced by pretreatment with endotoxin (Kiani et al., 1997). MRL 953 has been shown to be protective against endotoxic shock and bacterial infection in preclinical *in vivo* models, and is developed by Sandoz Research Institute (Vienna) as immunostimulant in cancer patients (Kiani et al., 1997).

Nakatsuka et al. (1991) found inhibition in mice of experimental metastasis of B16 melanoma by the synthetic lipid A-subunit analogue GLA-60. Probably natural killer cells, but not macrophages, participate as effector cells in depressing metastasis of the melanoma cells into the lung.

6. Systemic TNF-α as neoadjuvant of chemotherapy

6.1. Polyethylene-glycol(PEG)-TNF-α to improve accessibility of cytotoxics to tumors

In these recent years, several attempts were done to reduce TNF-α-toxicity by achieving a targeted delivery or even a better target-specific action of TNF-α, (reviewed in Wajant et al., 2005). Chemically modified TNF-α preparations were designed to maximize therapeutic efficiency and spare normal tissue from detrimental effects. Much effort has been paid to develop less toxic TNF-α muteins for a safe and systemic administration.

PEGylation may increase the therapeutic window of TNF-α mutants by reducing systemic side effects rather than through direct improvement of antitumoral actions (Wajant et al., 2005). PEG-TNF-α induced a selective enhancement of tumor vascular permeability (Yoshioka et al., 2004). The permeability was increased at 1 h, after an i.v. injection of PEG-TNF-α and returned to the basal level at 2 h. Conversely, PEG-TNF-α didn't affect the permeability of normal tissue and inflammation

site. PEG-TNF-α is now under research to increase accessibility of cytotoxics to tumors (Yoshioka et al., 2004).

6.2. Systemic TNF-α induction by TLR2/4 agonists

Beutler et al. (2001) found a profound synergy between LPS and muramyl dipeptide in the release of TNF-α from RAW cells. These authors suggested that, due to the massive release of cytokines which can result in lethal endotoxin shock, costimulation of more than one TLR receptor would provide a more reliable signal (Beutler et al., 2001). This view was confirmed by animal studies showing that oral administration of the TLR2 receptor agonists, muramyl dipeptide and romurtide, have a priming effect on TLR4-induced serum TNF-α (Okutomi et al., 1990; Ueda and Yamazaki, 2001). Moreover, the anticancer efficacy of local BCG is due to its TLR2/4 agonists properties (see before). Finally, IL-10, which is preferentially produced by TLR2 agonists, have an important role to limit LPS- and TNF-α-toxicity; this important aspect will be developed below.

6.2.1. The importance of IL-10

TLR2 stimulation led to release of IL-10, a pleiotropic cytokine that modulates the inflammatory response to septic shock and LPS administration (for recent review see Mocellin et al., 2004). Generally speaking, IL-10 seems to play an important role in mitigating the inflammatory phenomena accompanying the immune response to pathogens (Mocellin et al., 2004). In the absence of IL-10, mice acutely infected with *T. gondii* develop a lethal $CD4^+$ T-cell mediated response distinguished by excessive levels of IL-12 and IFN-γ, large areas of necrosis and severe cellular infiltrate into multiple organs (Wilson et al., 2005). In fact, the potent antimicrobial mechanisms operated by the immune system can also cause significant collateral damage to the host (Mocellin et al., 2004). Destruction of even small areas of critical tissues (e.g. cardiac muscle, central/peripheral nervous system) can be more harmful than the infection itself. A successful immune response must strike a balance between protection from pathogen invasion and pathology of the tissues, and IL-10 seems to play a pivotal role in establishing this equilibrium (Mocellin et al., 2004).

IL-10 protects against LPS-induced toxicity. Thus, Nicoletti et al. (1997) found that LPS-induced lethality in neonatal mice is counteracted by IL-10 and exacerbated by anti-IL-10. Inoue (2000) found that when LPS was given to nude mice, the mortality rate was 100% at 48 h of the observation period. However, mortality was reduced to 30% when IL-10 was added concomitantly (Inoue, 2000).

The mechanism leading to the TNF-α-mediated ischemic insult of tumor tissue is still under investigation (for review see Mocellin et al., 2005). In the 1980s, it was hypothesized that TNF-α-induced coagulation in the tumor vasculature might be one potential mechanism of tumor necrosis. Indeed, systemic inflammation is associated with the activation of the coagulation and fibrinolytic system, and injection of LPS activates the coagulation and fibrinolytic pathways (Suffredini et al., 1989). Monocytic tissue factor (TF), initiating the extrinsic blood coagulation pathway, is often upregulated under septic or inflammatory conditions, and is also known to be induced by TNF-α *in vivo* (Mocellin et al., 2005).

IL-10 has been found to inhibit LPS-induced tissue factor expression by monocytes *in vitro* (Ramani et al., 1993) and Pajkrt et al. (1997) found that IL-10 inhibits the activation of coagulation and fibrinolysis during human endotoxemia.

IL-10 limits TNF-α toxicity by several mechanisms. First, IL-10 inhibits TNF-α production (Mocellin et al., 2004). Second, IL-10 blocks induction of IL-12p35, and IFN-γ (Re and Strominger, 2004a,b). This is an important regulatory mechanism because TLR4-stimulation induces IL-12, which stimulates IFN-γ production in T cells (Ikeda et al., 2002), which in turn induces more TNF-α secretion and amplify its responses. Finally, IL-10 inhibits TNF-α responses such as neutrophil activation (Mocellin et al., 2004).

6.2.2. OM-174-induced TNF-α and IL-10 secretion in cancer patients

OM-174 is a lipid A derivative (see structure in Fig. 1), which was selected for development because it was *in vitro* similarly potent as LPS in inducing an NO response by murine macrophages, and was very efficient *in vivo* in a syngenic model of colon cancer in the rat (see section 5 and the references therein). Moreover its endotoxin toxicity was more than 10^5 times lower than that of LPS (Brandenburg et al., 2000). *In vitro* studies in bone marrow derived macrophages from TLR-2 and/or TLR-4 knock out mice showed that OM-174 stimulates both, TLR4 and TLR2 receptor responses, as measured by TNF-α production (Fig. 2; for effects of OM-174 on hTLR2 see Samsom et al., 2002).

A phase Ia study in 33 patients with solid cancer, resistant to conventional treatments, showed that OM-174 has an acceptable safety profile after intravenous injection of up to 1 mg (Pink and Kieny, 2004). The same study showed that OM-174 produced a biological response, as measured by TNF-α and IL-10 secretion. Other cytokines increasing in plasma after i.v. OM-174 included IL-6, IL-8, and to a lesser extent IFN-γ and IL-1β.

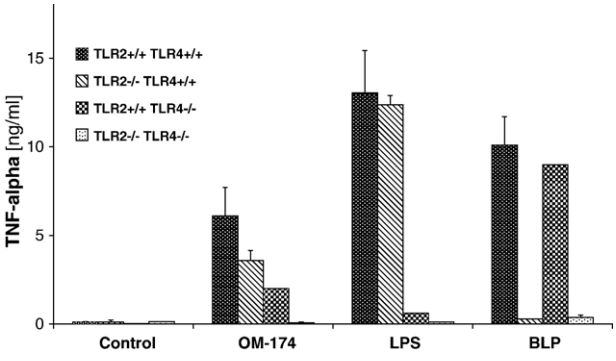

Fig. 2. Stimulation of TLR2 and TLR4 responses by OM-174 in bone marrow derived macrophages from TLR-2 and/or TLR-4 knock out mice. Values are given as mean±SD. Concentrations of tested compounds were: OM-174 10 μg/ml, LPS 100 ng/ml and bacterial lipopeptide 0.5 μg/ml.

Fig. 3 (top) shows plasma TNF-α and IL-10 levels after the intravenous administration of OM-174 1 mg/m^2. Plasma TNF-α peaked 1 h after OM-174 administration, with plasma IL-10 levels increasing thereafter (see Fig. 3, top). This latter IL-10 increase may explain the good tolerance of i.v. OM-174. Thus, plasma TNF-α levels rapidly decayed after 1 h, reaching low levels (<5% of the peak) in about 3 h (Fig. 3, top). This contrasts with plasma TNF-α levels induced by ONO-4007, which remained at about 20% of the peak, 6 h after ONO-4007 administration (De Bono et al., 2000). A rapid inhibition of TNF-α release by IL-10 (Fig. 3, top) may explain such differences. Thus, OM-174 induces a TNF-α window of about 1 h, which reduces TNF-α toxicity and which should be sufficient to facilitate tumor penetration of intravenous cytotoxics.

Fig. 3 (bottom) shows peak plasma TNF-α levels as a function of the intravenous OM-174 dose. It can be seen that peak plasma TNF-α levels increased smoothly with the increase in dose. Again, this contrasts with ONO-4007, whose peak plasma TNF-α levels increased threefold after a slight increase in dose (75 to 125 mg, cf. De Bono et al., 2000). On the other hand, Nooteboom et al. (2002) found that TNF-α increases endothelial permeability of cultured human umbilical venular endothelial cells (HUVEC) at concentration equal or higher than 50 pg/ml, and Folli et al. (1993) showed that intravenous or intratumoral TNF-α selectively and potently enhanced tumoral vascular permeability. Therefore, one should expect that OM-174-induced TNF-α levels will open, at the level of the tumor, a "Vascular Permeability Window" of 1–2 h, which should be utilized to increase cytotoxic tumor penetration in combined chemotherapy/immunotherapy clinical settings.

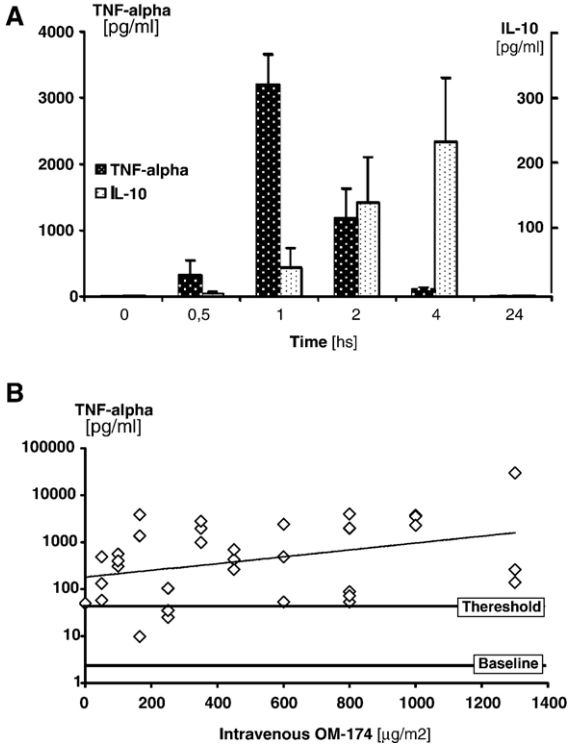

Fig. 3. Top: Plasma TNF-α and IL-10 levels after intravenous administration of OM-174 1 mg/m^2. Values are given as mean ± SEM. Bottom: Peak plasma TNF-α levels as a function of the intravenous OM-174 dose in cancer patients. Baseline indicates mean baseline values. Threshold indicates the minimum TNF-α level to increase endothelial permeability of cultured human umbilical venular endothelial cells (HUVEC; Nooteboom et al., 2002). $P<0.05$ (ANOVA linear regression analysis).

7. iNOS-dependent apoptosis of chemotherapy-resistant tumor cells

In addition to TNF-α-release, the TLR2/4 agonist OM-174 may induce iNOS tumoral expression, as described by the group of Jeannin et al. in a rat colon cancer model (Lagadec et al., 1999; Onier et al., 1999a,b). On the other hand, the efficacy of conventional chemotherapy in solid tumors is limited because tumors frequently have mutations in the p53 gene (for recent reviews see Malats et al., 2005; Munro et al., 2005). p53 is a key regulator in cell apoptosis, and cancer cells deficient in p53 expression frequently fail to respond to chemotherapy. Also, chemotherapy only kills rapidly dividing cells. Members of the tumor necrosis factor family, however, induce apoptosis regardless of the p53 phenotype (Lee et al., 1999).

7.1. iNOS-dependent tumor cell apoptosis

7.1.1. Macrophage iNOS expression

NO derived from TLR-stimulated macrophages participates in local tumoricidal activity against many types of tumors (for review, see Umansky and Schirrmacher, 2001; Lechner et al., 2005). Large amounts of NO produced for relatively long periods of time (days to weeks) by inducible NO synthase in macrophages after challenge with lipopolysaccharide, are cytotoxic for various pathogens and tumor cells (Umansky and Schirrmacher, 2001). This cytotoxic effect against tumor cells was found to be associated with apoptosis (programmed cell death). The mechanism of NO-mediated apoptosis involves accumulation of the tumor suppressor protein p53, damage of different mitochondrial functions, alterations in the expression of members of the Bcl-2 family, activation of the caspase cascade, and finally DNA fragmentation.

The above mechanism also participates in the host defense against metastasis. Thus, the Kupffer cell, a liver-specific macrophage, is a major NO producer and activated Kupffer cells play an important role in preventing the development of malignant tumors (Chen et al., 2002; see below). It is interesting to mention that NO derived from activated NK cells also participates in local tumoricidal activity against many types of tumors (Umansky and Schirrmacher, 2001; Lechner et al., 2005).

7.1.2. Perivascular iNOS expression

Pro-inflammatory cytokines stimulate specific receptors in target cells, leading to the activation of the NF-κB intracellular signal transduction pathway, which in turns provokes iNOS gene expression (for review see Umansky and Schirrmacher,

2001; Lechner et al., 2005). In 1991, Li et al. found that nitric oxide, produced by cytokines (TNF-α and INF-γ)-activated vascular endothelial cells, provoked the lytic destruction of M-5076 reticulum cell sarcoma (Li et al., 1991). Since then, increasing amount of evidence has been accumulated indicating that perivascular NO production influences circulating tumor cell survival and cancer metastasis (for review see Xie and Huang, 2003). Indeed, metastasis is largely an unsuccessful process, because most circulating tumor cells demise shortly after reaching distant organs (Xie and Huang, 2003).

Kong et al. (1996) proposed that NO produced by host cells (or exogenously applied) might block or attenuate the adhesion of tumor cells to the venular side of the microcirculation, where NO inhibits tumor cell adhesion. Wang et al. (2000) found that the injection of B16F1 melanoma cells into the mesenteric vein can induce the rapid local release of nitric oxide (NO) in the liver, causing apoptosis of the melanoma cells in the liver sinusoids and inhibiting the subsequent formation of hepatic metastases. Mortensen et al. (2004) showed that a high expression of endothelial cell nitric oxide synthase in peri-tumoral microvessels predicts increased disease-free survival in patients with colorectal cancer.

Kupffer cells or other types of perivascular cells may contribute to the above antimetastatic process. Recently, Qi et al. (2004) found that NO is produced by hepatic parenchymal cells mainly located in the periportal zones and that the arrest of the B16F1 melanoma cells causes an eNOS-dependent local burst of NO by the sinusoidal lining cells and hepatocytes in the periportal areas.

A low level of perivascular NO production may induce NO resistance, and the NO-resistant tumor cells may usurp NO to undergo progression (Xie and Huang, 2003). Therefore, restoration of iNOS expression and reversal of NO resistance may prevent tumor metastasis (Xie and Huang, 2003).

7.1.3. Tumoral iNOS expression

Pro-inflammatory cytokines can provoke iNOS expression in tumor cells. The most prominent cytokines involved in iNOS stimulation are TNF-α, IL-1β and IFN-γ (for recent review, see Lechner et al., 2005). NO can induce apoptosis of tumor cells, such as pancreatic cancer, breast cancer and colon cancer cells (Lechner et al., 2005). NO-induced apoptotic cell death is characterized by biochemical and morphological changes such as DNA and nuclear fragmentation, cell shrinkage, membrane blebbing and apoptotic bodies formation.

NO-mediated tumor cell apoptosis usually requires synergistic NOS II induction and high NO output with a high concentration in the tumor microenvironment. In most *in vitro* studies, only a combination of multiple cytokines was able to elicit a profound iNOS gene expression, whereas a single stimulus exhibited only a moderate effect in specific cell types (Lechner et al., 2005; Baillat et al., 2005). Sasagawa et al. (2000) showed that IFN-γ or TNF-α alone had no demonstrable cytotoxic effects in Hepa1−6 cells, whereas IFN-γ and TNF-α in combination induced apoptosis drastically. Aggarwal et al. (1985) presented evidence that TNF-α receptors on tumor cells are upregulated by IFN-γ. This suggests that two or more signal transduction pathways are necessary to upregulate iNOS expression fully in cancer cells.

7.2. TLR2/4-induced tumor iNOS expression

Animal studies by the group of Jeannin et al. revealed that OM-174 induces iNOS tumoral expression, and this seems to be an important anticancer mechanism (Lagadec et al., 1999; Onier et al., 1999a,b). Jeannin et al. generated a peritoneal carcinomatosis by injecting syngeneic rats with PROb tumor cells obtained from a chemically-induced rat colon cancer. At the beginning of the treatment, the carcinomatosis is composed of numerous nodules up to 3 or 4 mm in diameter that evolve, in untreated rats, towards progressive and metastatic tumors that finally lead to the death of the host. Using OM-174, Jeannin et al. developed a curative treatment leading to the complete regression of 95% of established tumors while all the untreated rats died. The authors considered this treatment as the most effective strategy in their model in which several immuno- and chemotherapies have been tested without demonstrating significant beneficial effects.

The main events leading tumor regression by OM-174 in the rat colon cancer model can be summarized as follows. In the first hours of its administration, OM-174 stimulates Toll-like receptors in macrophages and monocytes, to release cytokines, particularly IL-1β, IFN-γ and TNF-α. IL-1β and IFN-γ induce iNOS expression in tumor cells, followed by tumor cell apoptosis. In addition, iNOS expression if facilitated by a reduction in the tumor cell product TGF-β1, which downregulates iNOS expression.

7.3. TNF-α-induced apoptosis of chemotherapy-resistant tumor cells

Doxorubicin-induced apoptosis is p53-dependent, and Co et al. (2005) recently found that TNF-α restores doxorubicin-induced cell apoptosis in p53-deficient tumor cells through downregulation of the anti-apoptotic protein p21. These authors suggested that a combined treatment TNF-α/doxorubicin is an effective chemotherapeutic strategy for p53-deficient cancers (Co et al., 2005).

TNF-α induced apoptosis is mediated by nitric oxide (and/or activation of death receptor domains). Nitric oxide donors increase the chemotherapeutic effectiveness of some cytostatics in low, subtherapeutic doses and delay the development of drug resistance to cyclophosphamide (for review see Konovalova et al., 2003). This type of combined therapy results in significant increase in life span and number of survivors among mice bearing leukemias P388 and L-1210. A similar effect was observed for intracerebral leukemia P388 transplantation. In this case the life span of mice treated with cyclophosphamide and NO donor increased by three times in comparison to therapy with cyclophosphamide alone. The coinjection of nitric oxide donor and cytostatics improved the antimetastatic activity of the cytostatics. Indeed, the index of melanoma B16 metastasis inhibition by cyclophosphamide monotherapy was only 50%, and reached 80% by adding a NO donor.

7.3.1. nrTNF-α mutein/doxorubicin combination for lung cancer

Several recombinant human tumor necrosis factor-α (rHuTNF-α) muteins were prepared by protein engineering techniques (Kamijo et al., 1989). Nakamura et al. (1991) obtained a novel recombinant tumor necrosis factor-alpha (nrHuTNF-α) mutant (mutant 471), in which 7 N-terminal amino-acids were deleted and Pro8Ser9Asp10 was replaced by ArgLysArg. In nude mice bearing nasopharyngeal carcinoma, local or intravenous nrHuTNF-α induced tumor hemorrhagic necrosis and the survival time was prolonged (Dong et al., 1998). More importantly, nrHuTNF-α exerted a remarkable synergistic antitumor effect when combined with carboplatin (Dong et al., 1998). Similar results were obtained in nude mice bearing laryngeal carcinoma, for the nrHuTNF-α/cyclophosphamide combination (Dong et al., 2001).

Phase II and phase III trials in lung cancer showed that systemic administration of nrHuTNF-α remarkably enhanced the anticancer action of doxorubicin (Zhou et al., 2003). In total, 44% of the 133 patients exhibited complete or partial remission of lung tumors in response to this combination therapy strategy. nrHuTNF-α has been recently approved by the Chinese SDA (the Chinese FDA).

8. TLR2/4-immunotherapy as neoadjuvant of chemotherapy

8.1. OM-174-induced dendritic cell maturation and migration

In addition to cytokine release, OM-174 possesses important immunological properties, which were extensively investigated by the group of Jeannin et al. in their intraperitoneal rat colon cancer model (Lagadec et al., 1999; Onier et al., 1999a,b; Larmonier et al., 2004). Five days after the beginning of the treatment, mature dendritic cell invade tumor nodules, likely by chemotaxis, followed by infiltrating macrophages. OM-174 induces dendritic cell maturation. Mature dendritic cell could then migrate to the draining lymph nodes where tumor-specific lymphocytes are activated.

The above studies of Jeannin et al., were confirmed and extended by others. Thus, Pajak et al. (2003) found that intravenous or subcutaneous OM-174 induced within hours the migration and the maturation of murine dendritic cells in BALB/c mice. OM-174 induced the migration of dendritic cells from the periphery to the T-cell areas of lymphoid organs, and their maturation into cells expressing high levels of MHC class II and co-stimulatory molecules, with a potency close to that of LPS.

The adjuvant properties of OM-174 were investigated by Meraldi et al. (2003), who demonstrated that OM-174 induces a protective response when administered with the synthetic C-terminal fragment 242–310 from the circumsporozoite protein of *Plasmodium berghei*. Subcutaneous injections of *Pb*CS 242–310 in combination with soluble adjuvant OM-174 induced long lasting peptide-specific antibody titres comparable to those obtained by immunization with incomplete Freund's adjuvant (IFA). The *ex vivo* evaluation of the $CD8^+$ T-cell response by IFN-γ ELISPOT assay revealed that the injection of *Pb*CS 242–310 with OM-174 induced, compared to IFA, a similar increased frequency of peptide-specific lymphocytes in the draining lymph nodes. In the spleen however, OM-174 was eight-fold more efficient than IFA. The $CD8^+$ T cells are specific for the sequence *Pb*CS 245–253, a well-known H-$2K^d$-restricted cytotoxic T-cell epitope, and are cytotoxic as shown in a chromium release assay. Immunization of BALB/c mice with this polypeptide in combination with OM-174 conferred a protection after challenge with live *P. berghei* sporozoites.

Finally, it is interesting to mention that OM-174 decreased the production of the suppresive cytokine TGF-β1 in the PROb colon carcinoma syngeneic BDIX rat model (see review by Reisser et al., 2002). TGF-β family members help to restrain the growth of mammalian tissues (for review see Siegel and Massague, 2003). Tumor cells overproduce this cytokine to create a local immunosuppressive environment that exacerbates the invasive and metastatic behaviour of the tumor cells themselves. There is a growing interest in therapeutically targeting TGF-β1-mediated processes in cancer progression, such as breast cancer metastasis to the bone (Siegel and Massague, 2003). In decreasing TGF-β production, OM-174 can also reduce the production of immunosuppressive T regulatory cells (Ghiringhelli et al., 2005).

8.2. Immune mechanisms of OM-174 as chemotherapy adjuvant

D'Agostini et al. (2005) found that OM-174 is able to reduce tumor progression and to prolong the survival of mice in the B16 melanoma experimental model. At the doses employed, OM-174 reduced tumor growth and increased survival time similarly to a single high dose of cyclophosphamide. More striking effects were achieved by means of the combination of the two agents in a protocol consisting of a single intraperitoneal administration of cyclophosphamide (200 mg/kg) followed by five intraperitoneal injections of OM-174 (1 mg/kg). Immunological studies of treated and control mice revealed that the antitumor activity of OM-174, alone or in combination with cyclophosphamide, is mediated by the stimulation of natural killer (NK) and cytotoxic T lymphocyte responses as well as by a significant increase in the absolute number of NK1.1, CD4 and CD8 positive cells.

8.3. Chemotherapy-enhanced immune mechanisms

Dying tumor cells, particularly those killed by chemotherapy, can engage with antitumor immune responses (for review see Lake and Robinson, 2005). Of these, of particular importance for TLR2/4-based therapies are, tumor antigen "surge" by chemotherapy and chemotherapy-facilitation of TLR-induced cytotoxic T-cell responses.

8.3.1. Tumor antigen "surge" by chemotherapy

Several arguments suggest that tumor cell lysis may produce a "surge" of tumor antigens and that cytokines may facilitate the immune response to such antigens. Thus, tumor lysis is thought

to be at the origin of the transient antigen marker surge often seen during the first chemotherapy cycle (Al-Karim et al., 2002). This differs from the emergence of drug resistance, which is characterized by more obvious marker rises with successive cycles of therapy. Sørbye and Dahl (2003) found a clinically relevant CEA (carcinoembryonic antigen marker) surge in four of 27 patients on therapy, in association with the introduction of a more effective chemotherapy regimen for metastatic colorectal cancer (oxaliplatin).

A transient increase in tumor markers after chemotherapy has been seen in *responding* patients with nonseminomatous testicular cancer and breast cancer. Thus, Horwich and Peckham (1986) found a transient rise in human chorionic gonadotropin (the marker "surge") in 9 patients with metastatic nonseminomatous germ cell tumor of the testis (41%) following initiation of chemotherapy with bleomycin, etoposide, of whom one relapsed. None of the other 13 patients (without apparent "surge") have relapsed (follow-up, 24–60 months) (see also Shetty, 1987). Loprinzi et al. (1986) investigated the influence of carcinoembryonic antigen (CEA) in chemotherapy response of women with metastatic breast cancer. During the first 4 months of treatment, serial CEA levels in some responding patients initially rose significantly and then declined.

Weisenthal et al. (1991) investigated the effect of prior cancer chemotherapy on human tumor-specific cytotoxicity *in vitro* in response to immunopotentiating biologic response modifiers. Tumor-specific cytotoxicity was measured in fresh human biopsy specimens by a modification of the differential staining cytotoxicity assay. ImuVert, a cytokine inducer derived from Serratia marcescens, which produces broad-spectrum activation of both macrophages and lymphocytes, was dramatically more effective when it was tested in tumors obtained from patients with previously treated, chemotherapy- responsive adenocarcinomas (breast and ovary) than when it was tested in tumors obtained from either previously untreated patients or previously treated patients with chemotherapy-refractory adenocarcinomas (colon, lung, pancreas, stomach, kidney, gallbladder, uterus, and prostate). Similar findings, relating to prior chemotherapy treatment status, were obtained for TNF-α and IFN-γ, but not for interleukin-2 or IFN-α. Weisenthal et al. (1991) speculated that the response to chemotherapy produces massive release and processing of tumor antigens, and that this response leads to a state in which the human immune system is primed (via in situ vaccination) to respond to exogenous macrophage-activation signals with potent, specific antitumor effects. Another possibility is that the chemotherapy-dependent tumoral mass reduction, decreases immunosuppresive mechanisms.

8.3.2. Facilitation of cytotoxic T-cell responses

Cytotoxic drugs have been reported to sensitize cancer cells to cytotoxic T-cell lysis through Fas-mediated apoptosis (Yang and Haluska, 2004). *In vitro* treatment of melanoma cells with 5-fluorouracil or dacarbazine was reported to sensitize cells to antigen-specific cytotoxic T-cell lysis through perforin/granzyme- and Fas-mediated pathways (Yang and Haluska, 2004). Stimulation of the TNF receptor family member CD40, in cervical carcinoma cell lines expressing a TAP-dependent human papillomavirus 16 E6 Ag epitope resulted in their enhanced killing by specific cytotoxic T cells (Hill et al., 2005). Moreover, chemotherapeutic agents that suppress protein synthesis and reverse the CD40-mediated dissociation of the translational repressor eukaryotic initiation factor 4E-binding protein, such as 5-fluorouracil, etoposide, and quercetin, dramatically increase the susceptibility of cervical carcinoma cells to CD40L-induced apoptosis (Hill et al., 2005).

9. Therapeutic potential of OM-174 as adjuvant of chemotherapy

Based on the evidences presented so far, OM-174 deserves to be investigated to prevent or treat cancer relapse under chemotherapy. Intravenous OM-174 should be given 30 min before cytotoxic administration, for opening a "Tumor Permeability Window" of 1–2 h. Between cytotoxic administrations intervals, i.v. OM-174 can be given repeatedly, to stimulate antitumor immunity. Primary targets are immunogenic solid tumors.

9.1. Immunogenic solid tumors

9.1.1. Metastatic malignant melanoma

Numerous chemotherapeutic agents have shown activity in the treatment of metastatic malignant melanoma, such as dacarbazine, but patients finally relapse (Lugovic et al., 2005). Cytokines in combination with cytotoxic drugs had a profound effect upon widely metastatic disease (for review see Heaton and Grimm, 1993). Indeed, the standard treatment of melanoma consists of the surgical removal of the cancer if possible, the chemotherapy agent dacarbazine and/or biologic therapy with Proleukin® (IL-2) or interferon (INF-α).

Melanoma is a model of immunogenic tumor. Dalerba et al. (2003) stress that in melanoma, primary lesions can sporadically undergo spontaneous regression associated with tumor infiltration by immune effectors (3–7% of cases). Bulk TIL cultures purified from melanoma, demonstrate substantial *in vitro* lytic activity against autologous cancer cells. Finally, classical immunotherapeutic interventions are known to be active against melanoma, such as systemic administration of cytokines (IL-2, INF-α) or adoptive transfer of autologous lymphocyte effectors (LAK, lymphokine-activated killer cells; TIL).

OM-174 has proven efficacy in enhancing cyclophosphamide anticancer action in murine B16 melanoma (D'Agostini et al., 2005). Therefore, the dacarbazine/OM-174 combination deserves to be tested against metastatic malignant melanoma.

Dendritic cell vaccines are in development for advanced melanoma, and OM-174 presents one clear advantage with respect to dendritic cell vaccines, i.e.: the logistics and technical complications of manufacturing autologous tumor cell treatments for individual patients have resulted in a paucity of standardized, multicenter, randomized clinical trials. Moreover, convincing survival advantages by melanoma vaccines have yet to be reported (Elliott and Dalgleish, 2004).

9.1.2. Colorectal cancer

Recent studies clearly showed that colorectal carcinoma is immunogenic, and colorectal carcinoma is now considered as a target for immunotherapy (for review see Dalerba et al. 2003). Monoclonal antibodies such as cetuximab and bevacizumab are promising molecules against colorectal cancer (see however the modest results with Edrecolomab, a monoclonal antibody; Chau and Cunningham, 2002, 2006).

Interestingly, a combination of autologous tumor cells and the TLR2/4 agonist BCG gave significant clinical benefit in surgically resected patients with stage II colon cancer (Vermorken et al., 1999). OM-174 induces the migration and maturation of murine dendritic cells *in vivo* (Pajak et al., 2003), a newly recognized, essential factor in the immunotherapeutical approach of colorectal cancer (for review see Dalerba et al., 2003). Finally, OM-174 is active in the PROb model of colon cancer in rats, both alone (Onier et al., 1999a,b; Larmonier et al., 2004) or in combination with cisplatin, 5-FU (manuscript in preparation) or cyclophosphamide (Bruno Chauffert, unpublished data).

9.1.3. Ovarian cancer

Recently, a panel of experts in ovarian cancer stressed the necessity to develop immunotherapeutical treatments to combine with chemotherapy (Balkwill et al., 2003). Dendritic cell based therapies were recommended, but "*adoptive transfer methods are costly and time consuming and utilize extensive resources*" Again, OM-174 presents a clear advantage with respect to the logistics and technical complications of dendritic cell vaccines. Finally, intraperitoneal administration of OM-174 can be a treatment of choice because of several reasons (Balkwill et al., 2003). Ovarian cancer usually involves the peritoneal or serosal surfaces or at least areas proximal to these. Most cytokines and antibodies tend to have long residence time in the peritoneum. The presence of large numbers of mononuclear leukocytes in the peritoneal cavity of these patients offers a challenging target for activation (Balkwill et al., 2003).

9.2. Hypoxic tumors

Hypoxic cells are found in the majority of human solid tumors (approximately one third of the tumor cell population seems permanently or intermittently hypoxic). Hypoxic tumor cells can undergo genetic and adaptive changes that allow them to survive and proliferate (Li and Jackson, 2002). Thus, hypoxic growth can result in a tumor with more aggressive growth characteristics and more malignant phenotype (Harris, 2002).

Tumor hypoxia has been correlated with metastasis and resistance to chemotherapy and radiotherapy (for review see Brown, 1999; Höpfl et al., 2004). The level of hypoxia in primary tumors has been linked both clinically and experimentally to the incidence of metastases.

Recently, De Ridder et al. (2006) showed that OM-174 strongly enhanced the effect of radiation in hypoxic EMT-6 tumor cells. Therefore, tumor hypoxia can be an additional circumstance where OM-174 can help as neodajuvant of chemotherapy and/or radiotherapy.

9.2.1. Head and neck cancer

Head and neck cancer are normally hypoxic, many oral carcinomas respond poorly to chemotherapy approaches and their responses to radiation therapy have been highly variable. The short term clinical response of irradiated cervical lymph node metastases, originating from squamous carcinoma primaries in the head and neck, is better in well oxygenated metastases (Gatenby et al., 1988).

Tirapazamine (SR 259075) is developed by Sanofi-Aventis for hypoxic head and neck cancer (Hicks et al., 2004). Tirapazamine is a novel bioreductive agent with selective cytotoxicity against hypoxic cells. Under hypoxic conditions, tirapazamine becomes a free radical species that causes single- and double-strand DNA breaks, accounting for the drug's two effects: hypoxic cytotoxicity and hypoxic sensitization. Normal tissues are not hypoxic to the same degree as tumor cells, and therefore little or no toxicity is expected from the agent.

Phase III studies are actually undergoing to demonstrate efficacy of tirapazamine associated to cisplatin and radiotherapy in cancers of head and neck. Therefore, hypoxic head and neck cancer treated with tirapazamine can be a target indication to test a parenteral TLR2/TLR4 agonist on cancer relapse.

9.2.2. TLR and hypoxia-induced resistance to chemotherapy

Frederiksen et al. (2003) reported that the NO donor, glyceryl trinitrate, reversed the hypoxia-induced resistance to doxorubicin in human PC-3 and mouse TRAMP-C2 prostatic adenocarcinoma cells. Moreover, Van den Berge et al. (2001) found that chronic hypoxia upregulated the mRNA and protein expression of inducible nitric oxide synthase (iNOS) in EMT-6 tumor cells exposed to interferon IFN-γ and IL-1β. Therefore, chronic hypoxia may potentially be exploited to increase tumor cell radio- or chemo-response through the cytokine-inducible iNOS pathway (Van den Berge et al., 2001).

De Ridder et al. (2006) showed that the efficacy of OM-174 to enhance the effect of radiation in hypoxic EMT-6 tumor cells, was associated with iNOS-induction inside tumor cells.

9.3. OM-174 as palliative of cytotoxic-toxicity

Myelodepression is the most common and the most commonly fatal complication of antineoplastic drug therapy and may represent a serious hindrance to the management of cancer in older individuals (currently 60% of all neoplasms occur in persons aged 65 years and older, and this percentage is expected to increase as the population ages) (Balducci et al., 2001). Indirect arguments suggests that OM-174 can perhaps be used as a palliative of chemotherapy-induced myelodepression. Thus, TLR4-signaling strongly stimulates GM-CSF production (Sheridan and Metcalf, 1972) and GM-CSF may significantly decrease the hematopoietic toxicity of chemotherapeutic agents (Janik et al., 2001; Rinehart et al., 2003). Moreover, intravenous and subcutaneous administration of human recombinant IL-1β prevented or corrected chemotherapy-induced leukopenia in

patients with extensive solid neoplasms and non-Hodgkin's disease (Gershanovich et al., 2001).

OM-174 can perhaps also be useful as a palliative of chemotherapy-induced immunodepression. Indeed, aggressive chemotherapy is poorly tolerated in immunodepressed elderly patients, where deep immunodepression may result in fatal opportunistic infections (Vuckovic-Dekic et al., 1992). Immunodepression associated with cancer or any of its major modalities of treatment (surgery, irradiation, or chemotherapy) has been effectively alleviated with immunoactivators, such as thymopentine (Bernengo et al., 1992), isoprinosine (Imunovir^{R}) (O'Neill and Glasky, 1987) or Thymex L (Vuckovic-Dekic et al., 1992).

10. Conclusions

BCG, a TLR2/4 agonist, is the most successful immunotherapy of solid cancer (superficial bladder cancer), and the only exemple where immunotherapy works better than chemotherapy. Unfortunately, BCG and related microbial products are very toxic to handle for systemic administration, and their use is therefore limited to local application.

Another successful example, although limited to local application, is TNF-α. TLR2/4 agonists induce TNF-α, which selectively destroys neoangiogenic vessels. At low doses, TNF-α permeabilizes tumor vessels to the passage of chemotherapeutic agents. Isolated limb perfusion with TNF-α improves tumor penetration of melphalan in patients with locally advanced melanomas and sarcomas of the limbs.

Liver or lung metastases usually relapse under chemotherapy. Such life-threatening condition urgently needs new, systemic anticancer compounds, with original and efficient mechanisms of action. Chemically defined TLR2/4 agonists are promising molecules for systemic administration in cancer patients who relapse under chemotherapy.

The TLR2/4 agonist, OM-174 is a promising molecule against cancer metastases. Studies in cancer patients showed that intravenous OM-174 induces well tolerated TNF-α secretion, at plasma levels known to permeabilize neoangiogenic tumor vessels to the passage of cytotoxic drugs. A delayed IL-10 secretion suppresses TNF-α production and shortens the time interval of permeabilizing plasma TNF-α levels to 1–2 h. Therefore, intravenous OM-174 should be given 30 min before cytotoxic administration, for opening a "Tumor Permeability Window" of 1–2 h.

In animal cancer models, TLR2/4-stimulation activates dendritic cell traffic and it's associated tumor-specific, cytotoxic T-cell responses. Moreover, it induces iNOS expression, and NO is able to induce apoptosis of chemotherapy-resistant tumor cell clones. Such immune mechanisms of TLR2/4 agonists strongly enhances chemotherapy efficacy in animal cancer models. Therefore, between cytotoxic administrations intervals, TLR2/4 agonists can be given repeatedly, to stimulate antitumor immunity against immunogenic solid tumors.

Taken all the above elements together, parenteral TLR2/4 agonists seem promising to prolong survival in cancer patients who relapse under chemotherapy. Further developmental studies will tell us if TLR4/2 receptor agonists fulfill such therapeutic hope.

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