Effect of artesunate on immune cells in *ret*-transgenic mouse melanoma model

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The antimalarial artesunate also exerts profound cytotoxicity toward tumor cells. Earlier investigations controversially discussed a possible immunosuppressive function of artemsinin and its derivatives. This poses the question, whether immunosuppressive activity counteracts the anticancer activity *in vivo*. To clarify this issue, we used a transgenic mouse spontaneous melanoma model, in which *ret* transgene is expressed in melanocytes under the control of metallothionein-I promoter. *ret*-transgenic mice were previously reported to accumulate melanoma-specific effector memory T cells and natural killer (NK) cells in the primary tumors and metastatic lymph nodes. In the present investigation, we monitored effects of artesunate on the CD4$^+$ and CD8$^+$ T cells as well as Treg and NK cells from *ret*-transgenic tumor-bearing mice and nontransgenic littermates *in vivo*. In addition, we investigated cytostatic and cytotoxic activity of artesunate on *ret*-tumor cells established from the mouse primary tumor. Artesunate inhibited growth of *ret*-tumor cells and induces their apoptosis in a concentration-dependent manner (0.1–200 μmol/l). Furthermore, we did not find considerable effects of artesunate on the immune function as measured by major cell populations of the immune system; that is, CD4$^+$ and CD8$^+$ T cells as well as Treg and NK cells both from *ret*-transgenic mice and nontransgenic C57BL/6 littermates treated for 2 weeks with a daily dose of 1 mg artesunate. These results indicate that the cytostatic and apoptotic effects of artesunate are not diminished by concomitant immunosuppression. *Anti-Cancer Drugs* 20:910–917 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Artemisinin and its derivatives have initially emerged as valuable drugs for the treatment of otherwise drug-resistant malaria infections. Their pharmacological profile seems, however, to be much broader, as they are also active against various other protozoans, fungi, trematodes, bacteria, viruses, yeast, as well as against cancer cells [1].

By systematic analyses of medicinal plants used in traditional Chinese medicine, we earlier found that the active principle of *Artemisia annua* L., artemisinin, and its semisynthetic derivative, artesunate, not only exert antimalarial activity, but also profound cytotoxicity against tumor cells [2,3]. The inhibitory activity of artemisinin and its derivatives toward cancer cells is in the nanomolar to micromolar range [3,4]. Candidate genes that may contribute to the sensitivity and resistance of tumor cells to artemisinins were identified by pharmacogenomic and molecular pharmacological approaches [4,5]. Target validation was performed using cell lines transfected with candidate genes or corresponding knockout cells. These genes are from classes with different biological function; for example, regulation of proliferation (*BUB3*, cyclins, *CDC25A*), angiogenesis (vascular endothelial growth factor and its receptor, matrix metalloproteinase-9, angiostatin, thrombospondin-1), or apoptosis (*BCL-2*, *BAX*) [5,6]. Artesunate triggers apoptosis both through p53-dependent and p53-independent pathways [6]. Antioxidant stress genes (thioredoxin, catalase, γ-glutamyl-cysteine synthetase, glutathione *S*-transferases) as well as epidermal growth factor receptor and its downstream kinases confer resistance to artesunate [7–10]. Artesunate also induces DNA breakage as shown by single-cell gel electrophoresis and expression of γ-H2AX, an indicator of DNA double-strand breaks. DNA damage is repaired by different pathways; that is, base excision repair, homologous recombination, and nonhomologous end-joining [11]. Furthermore, artesunate inhibits the Wnt/β-catenin pathway [12,13]. Cell lines overexpressing genes that confer resistance to established antitumor drugs (*MDR1*, *MRP1*, *BCRP*, dihydrofolate reductase, ribonucleotide reductase) were not cross-resistant to artesunate, indicating that artesunate is not involved in multidrug resistance [3,6,14]. The anticancer activity of artesunate has been shown in human xenograft tumors in mice [15]. First encouraging therapeutic effects have also been achieved in patients with uveal melanoma [16] and non-small-cell lung cancer [17].

Artemisinins may act as anti-inflammatory agents and inhibit immune functions. Thus, artemisinin and its derivatives were shown to suppress humoral immune

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responses [18]. In addition, artesunate, artemether, and dihydroartemisinin suppressed T-cell activation and proliferation [19,20]. Immunosuppressive effects have also been reported for other semisynthetic artemisinin derivatives [21–25].

In contrast, artemisinin and derivatives have been shown to enhance immune responses. Artemisinin can increase phagocytosis of peritoneal macrophages and interferon production, enhance the delayed-type hypersensitivity response and acid phosphatase activity in macrophages [26–28]. Furthermore, artesunate stimulated sheep erythrocyte-induced antibody formation [29] and accelerated the immune reconstitution in mice after syngeneic bone marrow transplantation [30].

As possible suppressive effects on T cells by artemisinin-like compounds may be favorable to develop treatment strategies for autoimmune and chronic inflammatory diseases, for example, lupus erythematosus [31–33], autoimmune encephalomyelitis [34], rheumatoid arthritis [35], acute pancreatitis [36], and contact dermatitis [37], immunosuppression may counteract the cytotoxic activity of artemisinins toward tumors.

To clarify the effect of artemisinins on immune functions in the context of cancer therapy, we used a transgenic mouse spontaneous melanoma model, in which *ret* transgene is expressed in melanocytes under the control of metallothionein-I promoter [38]. In contrast to transplantation models, this mouse model closely resembles human melanoma with respect to etiology, tumor genetics, histopathology, and clinical development. After a short latency (20–70 days), around 25% of all transgenic mice develop on the face, back, or on the tail skin melanomas metastasizing to lymph nodes (LNs), liver, lungs, and brain [39]. *ret*-transgenic mice were previously reported to accumulate melanoma-specific effector memory T cells [39] and natural killer (NK) cells [40] in the primary tumors and metastatic LNs. Here, we monitored effects of artesunate on the T cells, and NK cells from *ret*-transgenic tumor-bearing mice and non-transgenic littermates *in vivo*. In addition, we investigated cytostatic and cytotoxic activity of artesunate on the *ret*-tumor cells established from the mouse primary tumor.

Materials and methods

Mice

Mice (C57BL/6 background) that express human *ret* transgene in melanocytes under the control of mouse metallothionein-I promoter–enhancer were kept under specific pathogen-free conditions in the animal facility of German Cancer Research Center (Heidelberg, Germany). Experiments were carried out in accordance with government and institute guidelines and regulations. The survival and general performance of mice was monitored daily. Spontaneous tumor development was assessed macroscopically. C57BL/6 wild-type and *ret*-transgenic mice were injected intraperitoneally with 1 mg/200 µl artesunate (Saokim Ltd., Hanoi, Vietnam) daily for 2 weeks.

Antibodies

The following directly conjugated rat anti-mouse monoclonal antibodies (mAbs) were used for the FACS staining: CD3-PerCP-Cy5.5, CD4-FITC, CD4-PE, CD8-APC-Cy7, CD25-APC, CD45.2-PerCP-Cy5.5, NK1.1-FITC and isotype-matched control mAbs (all from BD Biosciences, San Diego, California, USA) as well as Foxp3-PE (eBiosciences, San Diego, California, USA).

Cell culture

Cells established from primary skin melanomas in *ret*-transgenic mice (*ret*-melanoma cells) were cultured in RPMI 1640 medium supplemented with 2 mmol/l L-glutamine (PAA Laboratories, Pasching, Germany), 10% FCS (PAN Biotech, Aidenbach, Germany) and 0.1% penicillin/streptomycin (PAA). *ret*-melanoma cells (3×10^5/ml) were treated for 5 days with artesunate at different concentrations (0.1–200 µmol/l). The number of live cells at different time points after the treatment was evaluated using trypan blue exclusion. *ret*-melanoma cell morphology was evaluated by the confocal microscope (Leica DM IRB; Leica Microsystems, Wetzlar, Germany).

Apoptosis assay

Apoptosis was assessed by measuring the binding FITC-conjugated annexin-V protein to the phospholipid phosphatidylserine, which is present on the external membrane surface of apoptotic cells; and 1×10^6 cells/ml were stained with the annexin-V–FITC apoptosis detection Kit I and with 1 mg/ml propidium iodide following the manufacturer's instruction (BD Bioscience).

Preparation of single-cell suspensions

Fresh spleen, LN, and tumor samples were immediately transferred into PBS and stored on ice. After removal of necrotic tissue and fat, tumor biopsies were cut into small pieces and filtered through the cell strainer. Whole spleens and LN were dissociated and filtered. Spleen samples were depleted of erythrocytes by ammonium chloride lysis and washed twice.

Flow cytometry

Single-cell suspensions were treated with Fc-block and stained with mAbs for 20 min at 4°C. Acquisition was performed by four-color or five-color flow cytometry using a FACSCantoII with FACSDiva software (both BD Biosciences) with dead cell exclusion based on scatter profile or propidium iodide inclusion. FlowJo software (Tree Star Inc., San Carlos, California, USA) was used to analyze at least 100 000 events. Data were expressed as dot plots.

Results

Growth inhibition

As a first step, we measured inhibition of cell growth by artesunate in *ret*-melanoma cells *in vitro*. Inhibition of cell growth was found in a concentration range of 10–200 µmol/l artesunate (Fig. 1a). As untreated control cells grew to the confluence state in an adherent manner (Fig. 1b), 10 µmol/l artesunate suppressed cell growth, but cells were still adherent (Fig. 1c). However, upon treatment with 100 µmol/l artesunate, cells tended to round up and detach, indicating cell death (Fig. 1d).

Induction of apoptosis

In the next experiment, we analyzed apoptosis by annexin-V–FITC staining and flow cytometry. As shown in Fig. 2a, the proportion of apoptotic cells increased with the elevating artesunate concentration and incubation time. Representative flow cytometry histograms of untreated and artesunate-treated *ret*-tumor cells are shown in Fig. 2b and c.

Effect of artesunate on T-cell and NK-cell subsets in wild-type mice *in vivo*

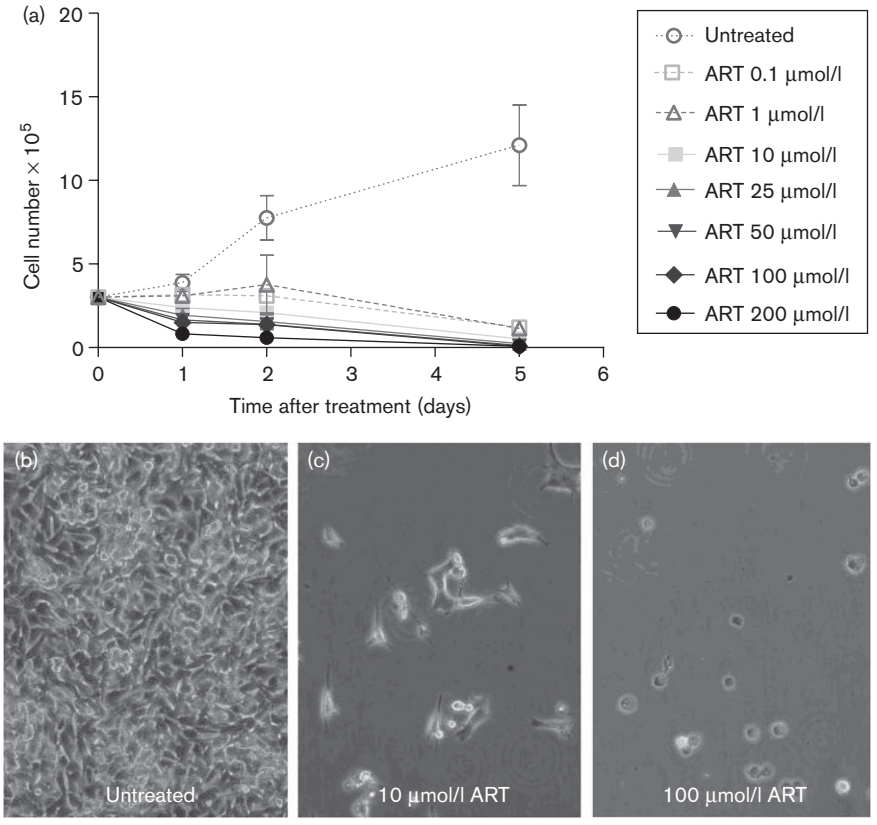
Six mice were treated with artesunate and three mice with solvent as control. T-cell subpopulations were characterized by flow cytometry using specific antibodies toward CD3, CD4, CD25, and regulatory T-cell (Treg) marker Foxp3. Figure 3a shows the gating strategy for spleen cells. Living single cells (left-sided histogram) were gated and presented according to their CD3 and CD4 staining (middle histogram). $CD4^+$ cells were then gaited for their expression of CD25 and Foxp3 to determine the Treg cells (right-sided histogram).

Figure 3b shows total $CD4^+$ T cells (left side) and the Treg subpopulation (right side) in the spleen. The portions of these cell populations were not significantly different between untreated and artesunate-treated mice (Table 1).

The fractions of $CD4^+$ T cells in LNs also did not differ between untreated and treated mice (Fig. 3c, left side). In contrast, the amount of regulatory T cells in LNs significantly decreased in artesunate-treated mice compared with untreated animals ($P = 0.0347$; Fig. 3c, right side).

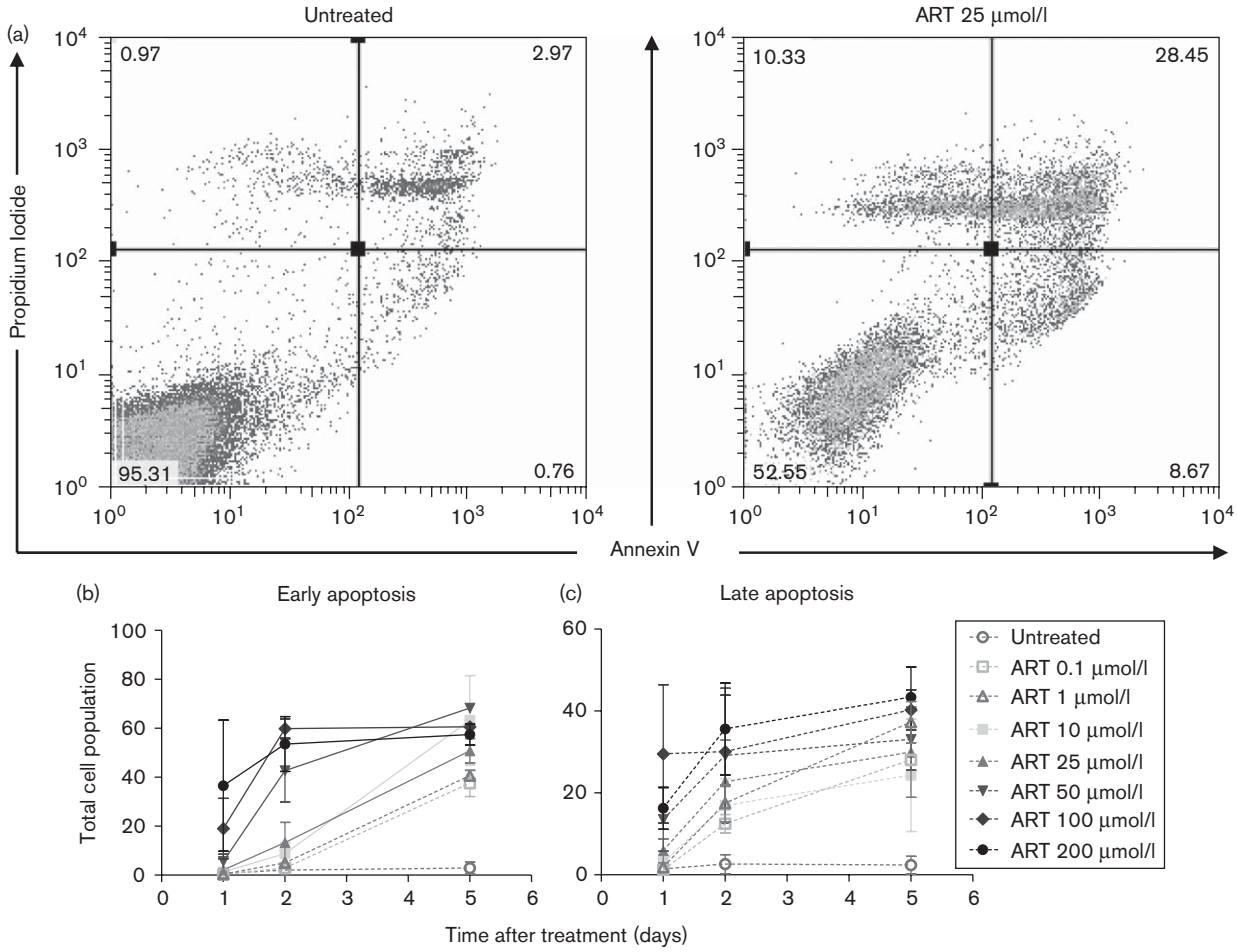
In the next set of experiments, we investigated $CD8^+$ T-cell and NK cell populations. For this reason, eight wild-type mice were daily treated with 1 mg artesunate,

Fig. 1



Inhibition of *ret*-melanoma cell growth by artesunate (ART) *in vitro*. (a) Cell numbers were counted at different time points of the ART treatment *in vitro* using trypan blue exclusion. (b–d) Confocal microscopy of untreated (b) and treated melanoma cells (c and d). Original magnification, × 200.

Fig. 2



Artesunate (ART) induces apoptosis in *ret*-melanoma cells. Cells were incubated with ART at different concentrations. Apoptosis was evaluated by annexin-V and propidium iodide (PI) staining followed by flow cytometry. (a) Representative dot plots of untreated and ART-treated cells at day 2 after the onset of experiments. (b and c) Data for early apoptotic (annexin-V^+PI^-) cells (b) and late apoptotic (annexin-V^+PI^-) cells (c). Results (means ± SEM) of three independent experiments are expressed as a percentage of apoptotic cells of total cell population.

whereas four wild-type control mice were left untreated. Using specific antibodies against CD3, CD8, and NK1.1, the cells were processed by a similar gating strategy as described in Fig. 2a. Again, no significant differences in numbers of $CD8^+$ or NK cells in the spleen were measured (Fig. 4a; Table 1). In LNs, both the cell populations were also found in a similar amount (Fig. 4b).

DNA genotyping in *ret*-transgenic mice

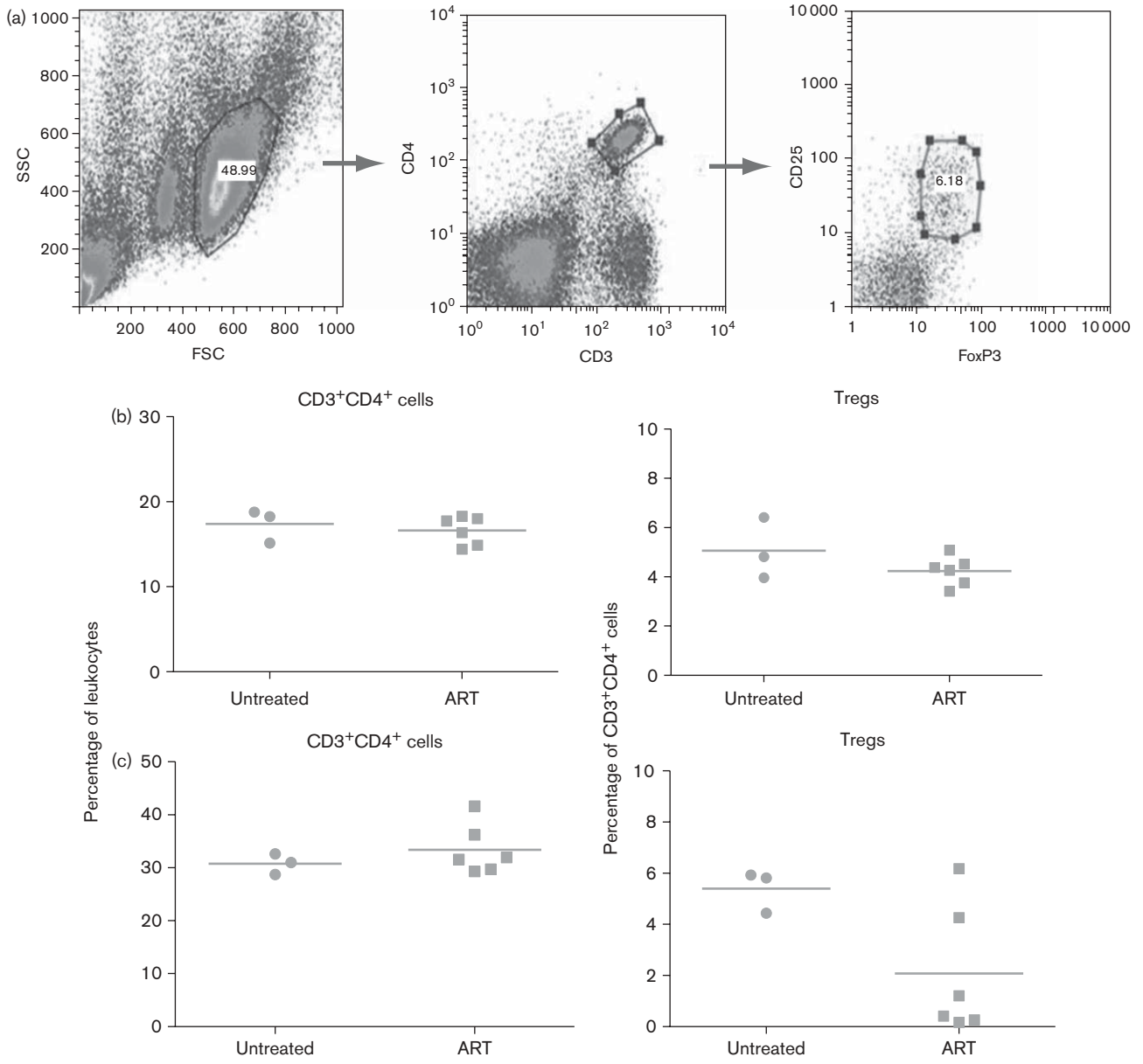
ret-transgenic C57BL/6 mice were crossed with wild-type C57BL/6 mice, which resulted in heterozygous inheritance of the *ret* transgene. To separate *ret*-positive and *ret*-negative offspring, we performed PCR-based DNA genotyping (data not shown).

Effect of artesunate on T-cell and NK-cell subsets in *ret*-transgenic mice *in vivo*

Four mice were treated with artesunate and two mice with the solvent as a control. Figure 5a shows the fraction of $CD4^+$ T cells and Treg in spleens of untreated and treated wild-type *ret*-transgenic mice. In addition, the portions of these cell types in tumor-bearing *ret*-transgenic mice have been determined. The mean values of $CD4^+$ T cells in tumor-bearing *ret*-transgenic mice were slightly higher as compared with $CD4^+$ T cells of wild-type mice. There were, however, no differences in treated or untreated mice of both strains (Fig. 5a, left side; Table 1). Similar observations were made for Treg cells (Fig. 5a, right side; Table 1).

The amount of $CD8^+$ T cells and NK cells in untreated or treated wild-type and *ret*-transgenic mice are shown in Fig. 5b. As $CD8^+$ T cells were slightly enriched in tumor-bearing *ret*-transgenic mice compared with wild-type animals, there were again no differences between treated and untreated groups (Fig. 5b, left side). The amount of NK cells was higher in *ret*-transgenic mice than in nontransgenic littermates (Fig. 5b, right side).

Fig. 3



Effect of artesunate (ART) on $CD4^+$ T cells and Treg in C57BL/6 wild-type mice *in vivo*. Mice were treated intraperitoneally with 2 mg ART daily for 2 weeks. Cells were stained with monoclonal antibodies for CD3, CD4, CD25, and the Treg marker FoxP3 followed by flow cytometry. (a) Dot plots are representative of mouse spleen. (b and c) Data for $CD4^+$ T cells and Treg in spleens (b) and lymph nodes (c) from untreated and ART-treated mice are expressed as the percentage within leukocytes. Individual results of three to six mice are shown. FSC, forward scatter; SSC, side scatter.

Discussion

In the present investigation, we have shown that artesunate inhibited growth of *ret*-tumor cells and induces their apoptosis. This indicates that artesunate may act both in a cytostatic and cytotoxic manner.

A cytostatic effect of artesunate by arresting cells in the G_0/G_1 phase of the cell cycle has been shown by different groups including our own [2,41–43]. In a recent analysis, we found that the CDC25A protein is downregulated in $p53^{+/+}$ and $p53^{-/-}$ HCT116 human colon cancer cells upon treatment with artesunate [6]. This protein is involved in the transition of the cell cycle from G_1 phase to S phase. It is worth speculating that CDC25A might also play a role in *ret*-tumor cells.

The cytotoxic effect of artesunate is associated with the induction of apoptosis. Apoptosis induction by artesunate

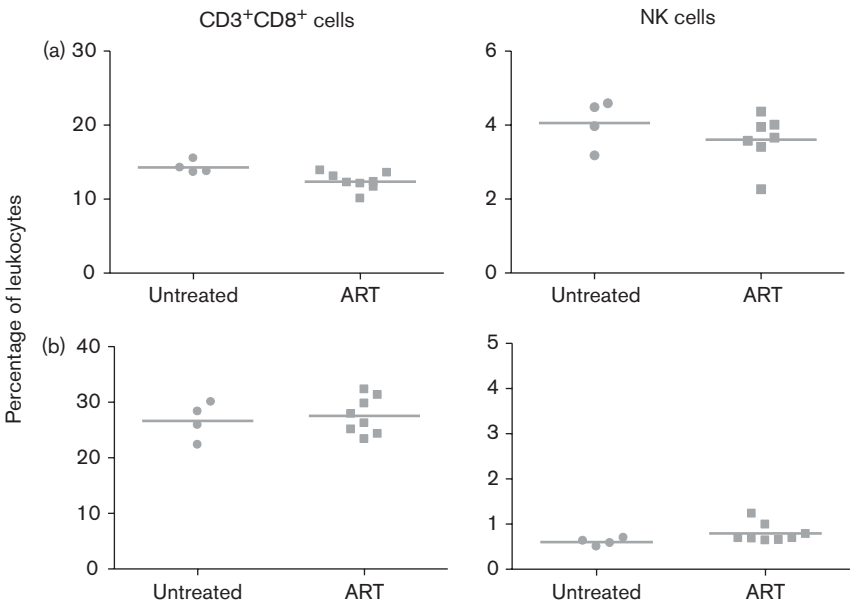
has first been shown by us [2] and was subsequently confirmed by other authors [44–48]. Apoptosis is induced by artesunate in a p53-independent manner [6]. In tumor cells with functional wild-type p53, apoptosis is mediated by the Fas-receptor driven extrinsic pathway of apoptosis [49], whereas in cell lines with mutated p53, the intrinsic, mitochrondrial apoptosis pathway is activated [49–51]. Whether artesunate induces apoptosis by extrinsic or intrinsic apoptosis signaling pathways in *ret*-melanoma cells is unknown. Interestingly, we did not find considerable effects of artesunate on the immune function both from *ret*-transgenic mice and nontransgenic C57BL/6 littermates. Several, but not all earlier investigations found immunosuppressive effects of artemisinin and its derivatives [18–29]. As immunosuppression induced by artemisinin-type drugs is favorable to treat autoimmune diseases, it might be disadvantageous in cancer therapy and might counteract growth inhibition and apoptosis induction. The activation of antitumor immune responses is believed to suppress tumor growth and represents the major paradigm of many immunotherapeutic approaches. Our results indicate that the cytostatic and apoptotic effects of artesunate are not diminished by concomitant immunosuppression. This might have important implications for cancer treatment in a clinical setting. In this study, the mice have been treated with a daily dose of 1 mg. Considering that a mouse has an average weight of 20 g, an artesunate concentration of 1 mg in mice would compare 350 mg in a human weighting 70 kg. This is well above the standard doses to treat malaria (50–100 mg/day). Hence, the lack of immunosupression observed in our study is not because of underdosage. Our point of view is also supported by meta-analyses of clinical trials focusing on side effects during malaria therapy with artemisinins [52]. The drugs are well tolerated without significant adverse effects, and especially without immunosuppressive effects, which might also counteract the efficacy in malaria therapy.

Table 1 Effect of ART on lymphocyte subsets in *ret*-transgenic tumor-bearing mice and nontransgenic littermates *in vivo*

	Untreated (%)	ART (%)
Wild-type mice		
Spleen $CD4^+$ T cells	17.4 ± 2.0	16.6 ± 1.7
Spleen Treg	5.1 ± 1.3	4.2 ± 0.6
Spleen $CD8^+$ T cells	14.3 ± 0.9	12.4 ± 1.2
Spleen NK cells	4.5 ± 0.6	3.7 ± 0.6
Lymph node $CD4^+$ T cells	30.8 ± 1.9	33.4 ± 4.7
Lymph node Treg	5.4 ± 0.8	2.1 ± 2.5
Lymph node $CD8^+$ T cells	26.7 ± 3.4	27.6 ± 3.3
Lymph node NK cells	0.6 ± 0.1	0.8 ± 0.2
ret-transgenic mice		
Spleen $CD4^+$ T cells	21.9 ± 7.5	22.2 ± 5.2
Spleen Treg	13.1 ± 5.8	12.7 ± 6.3
Spleen $CD8^+$ T cells	9.5 ± 5.1	11.4 ± 4.2
Spleen NK cells	1.8 ± 0.3	1.2 ± 0.7

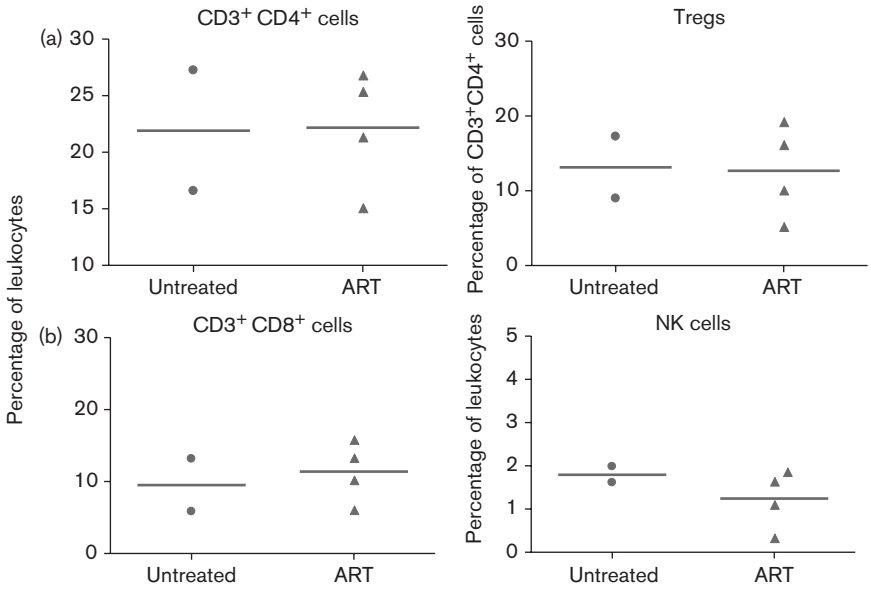
Mice were treated intraperitoneally with 2 mg ART daily for 2 weeks or left untreated. Data represented are means ± SEM.
ART, artesunate; NK, natural killer.

Fig. 4



Effect of artesunate (ART) on $CD8^+$ T cells and NK cells in C57BL/6 wild-type mice *in vivo*. Mice were treated intraperitoneally with 2 mg ART daily for 2 weeks. Cells were stained with monoclonal antibodies for CD3, CD8, the natural killer (NK) marker NK1.1 followed by flow cytometry. (a and b) Data for $CD8^+$ T cells and NK cells in spleens (a) and lymph nodes (b) from untreated and ART-treated mice are expressed as the percentage within leukocytes. Individual results of four to eight mice are shown.

Fig. 5



Effect of artesunate (ART) on $CD4^+$ and $CD8^+$ T cells as well as Treg and natural killer (NK) cells of the spleen from *ret*-transgenic tumor-bearing mice *in vivo*. Mice were treated intraperitoneally with 2 mg ART daily for 2 weeks. Splenocytes were stained with monoclonal antibodies for CD3, CD4, CD8, CD25, FoxP3, and NK1.1 followed by flow cytometry. Individual results of two to four mice are shown.

In conclusion, artesunate inhibited proliferation of *ret*-tumor cells and induced apoptosis, but had no effect on immune function as measured by major cell populations of the immune system, that is, $CD4^+$ and $CD8^+$ T cells as well as Treg, and NK cells.

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