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Abstract

Cancer chemotherapy, particularly one usingregimens employing high-dose cytotoxic drugs such as cyclophosphamide (CTX), has been considered to be immune suppressive[Ed1]. On the contraryHowever, we observed that a single administration of high-dose CTX administration abolished the tumors arising from subcutaneous injection of a mouse hepatoma cell line, and subsequently induced specific tumor immunity. The aAbsence of T cells or Ddepletion of T cells, specifically $CD4^+$ T cells, abrogated the CTX-mediated tumor regression[Ed2]. CTX treatment induced the rapid recruitment of $CD4^+$ T cells into the tumors, and these recruited cells started toinitiated expression of LAMP1/CD107a, a cytotoxic granule molecule, LAMP1/CD107a, and granzyme B without in the absence of antigen presentation at draining lymph nodes and proliferation in the tumor tissues. Moreover, CTX enhanced the expression of a CC chemokine, CCL3, in tumor tissues, and CTX-mediated tumor regression was attenuated in mice deficient in CCR5, the receptor for this chemokine. Consistently, less lessreduced CTX-induced accumulation of intratumoral LAMP1/CD107a-expressing $CD4^+$ T cells was less observed in mice receiving splenocytes derived from CCR5-deficient mouse-derived splenocytesmice than in those receiving splenocytes derived from WT mouse-derived splenocytesmice. Thus, CTX induces the expression of CCL3 to, which induces the intratumoral migration of $CD4^+$ T cells with expressing cytotoxic molecules to induce , leading to tumor eradication and subsequent specific tumor immunity.

Key words: CC chemokine, $CD4^+$ T cell, cytolytic T cell, LAMP1/CD107a.

Abbreviations: allophycocyanin, APC; carboxy fluorescein diacetate, succinimidyl ester, CFSE; cytolytic T lymphocytes (CTLs); cyclophosphamide, CTX; draining lymph node, dLN; flow cytometry, FCM; fluoresceinfluorescence isothiocyanate, FITC; glyceraldehyde-3-phosphate dehydrogenase, GAPDH; hepatocellular carcinoma, HCC;

hypoxanthine phosphoribosyltransferase, HPRT; mean fluorescence intensity, MFI; regulatory T cells, Tregs.

1. Introduction

Cyclophosphamide (CTX) is oxidized to 4-hydroxycyclophosphamide, [1], which enters cells and spontaneously decomposes to phosphoramide -mustard. At a physiological pH of 7.4, this component generates causes covalent linkage of DNA alkyl groups of the DNA [2]. The resultant inter-strand crosslink creates denaturation-resistant DNA fractions, thereby inhibiting DNA replication and leading to subsequent apoptosis [3]. CTX exerts itsThe cytotoxic action of CTX is exerteds against highly -proliferativeng cells, particularly lymphocytes , as well asand cancer cells, thereby depletingcausingand causes the depletion of lymphocytes in from the peripheral blood and tissue [4]. Consequently, CTX, particularly at its high -doses (100 to 200 mg/kg), has been employed as one of the most potent immune osuppressive drugs to combat life-threatening autoimmune diseases and to prevent graft-versus-host disease after allogenic bone marrow transplantation [5].

Regulatory T cells (Tregs), characterized by the expression of CD4, CD25, and Foxp3, accumulate in tumor tissues of both tumor-bearing humanspatients as well as and in those of mice and can contribute to immune tolerance to cancer cells[Ed3] [6]. A Llow -doses of CTX can deplete Tregs in the blood and lymphoid organs of tumor-bearing mice and decrease cause a decrease in the number of Tregs that infiltrating infiltrate tumor tissues [7, 8]. The This decrease in infiltrating Tregs can [Ed4]improve the ability of cytotoxic T cell- and/or NK cell-mediated antitumor immunity, not only in tumor-bearing mice but also in advanced cancer patients [9]. Moreover, low-dose CTX can induce the polarization from of Th2 to Th1 and can eventually exert anti-metastatic effects [10]. Recent observations have suggested that a single high -dose of CTX can induce specific tumor immunity [11]. Hence, we examined the effects of a single high -dose of CTX on the tumors arising from subcutaneous injection of a mouse hepatoma cell line.;, and provedWwe demonstrate that it high-dose CTX can eradicate the tumors in a T cell-dependent (, depending on T

cells, particularly $CD4^+$ T onescell) manner., in contrast totOur results contrast with the widely -held view that a high-dose CTX can be immune osuppressive.

2. Materials and Methods

2.1 Mice

Specific pathogen-free male five5- to seven7-week old BALB/c mice (WT mice) and BALB/c-nu mice (nude mice) were purchased from Charles River Japan [Ed5]. CCR5-deficient ($CCR5^{-/-}$) mice were backcrossed with BALB/c mice for more than eight8 generations [12]. CD45.1 BALB/c congenic mice were obtained from the Jackson Laboratories (Bar Harbor, ME). All mice were kept maintained under the specific pathogen-free conditions. All animal experiments were approved and performed according to the Guideline for the Care and Use of Laboratory Animals of Kanazawa University.

2.2 Tumor cell line

A murine hepatocellular carcinoma (HCC) cell line, BNL 1ME A.7R.1 (BNL), was purchased from the American Type Culture Collection and kept maintained at low passage throughout the study. The cells were cultured as previously described [13].

2.3. Tumor injection

The left flanks of eight8-week old male WT, nude, or $CCR5^{-/-}$ mice were subcutaneously (s.c.) inoculated subcutaneously (s.c.) with 5×10^5 BNL cells in 100 μL of PBS. Tumor sizes were evaluated three times a week using calipers, and tumor volumes were calculated by using the following formula: Tumor volume (mm^3) = (the longest diameter) × (the shortest diameter) × (depth) /2. When the tumor volume became reached 40 to 80 mm^3, the mice were intraperitoneally (i.p.) injected with 150 mg/kg CTX (Figure 1 A). In some experiments, mice were i.p. injected with anti-CD4 (GK1.5, 100 μg/bodyanimal) or anti-CD8 antibody (53.6.7, 200 μg/bodyanimal), twice on [Ed6] Day 1 and 14 (Figure 1D). In another series of experiments, at 30 days after the first BNL inoculation of BNL cells into, the mice, the animals whose tumors had

completely disappeared regressed after the treatment, were inoculated again with 5 × 10^5 BNL cells and 2.5 × 10^5 colon 26 (Col26, a murine colon carcinoma cell line) cells into the right and left flank, respectively. As a control, WT mice were inoculated with 200 μl of PBS into the left flank instead of the first BNL inoculation. The day of the second BNL inoculation was determined designated as day 0.

2.4. Adoptive transfer of splenocytes

Single cell populations were obtained from the spleens of CD45.1 congenic WT mice, and were then the cells were labeled with 2 μM of carboxyfluorescein diacetate, succinimidyl ester (CFSE, Life Technologies Inc.). Ten million CFSE-labeled cells in 200 μl of PBS were intravenously transferred to CD45.2 WT mice intravenously via the tail vein one day after CTX administration (Figure 3A). Similarly, 1 x 10^7 CFSE-labeled splenocytes from $CCR5^{-/-}$ mice were intravenously injected intravenously into the tail vein one day after CTX administration (Figure 5A).

2.5. Flow cytometry (FCM)

Single cell suspensions were prepared from tumors, draining lymph nodes (dLNs), and spleens as previously described [13] and were stained with various combinations of phycoerythrin (PE)-labeled anti-LAMP1/CD107a, allophycocyanin (APC)-labeled anti-CD4, or fluoresceinfluorescence isothiocyanate (FITC)-labeled anti-CD8 antibody (BD Biosciences). FITC-rat IgG, PE-rat IgG, or APC-rat IgG (BD Biosciences Japan) were used as negative controls. The eExpression of each molecule was determined as previously described [14].

2.6. Quantitative (q)RT-PCR

qRT-PCR was done performed to quantify the chemokine mRNA of chemokines as previously described [12]. Hypoxanthine phosphoribosyltransferase (HPRT) or

glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified as an internal control. The nucleotide sequences of the used primers used were are described in Table 1.

2.7. Determination of intratumoral chemokine contents

Protein lysates were obtained from frozen tumor tissues, and their the protein concentrations were determined as previously described [12]. CCL3 and CCL4 levels Then,were then measured in the protein lysates were used for the determination ofto measure CCL3 and CCL4 by using specific enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems) according to the manufacturer's instructions.

2.8. DoubleTwo-color immunofluorescence analysis

Resected tumor tissues were processed for doubletwo-color immunofluorescence analysis as previously described [12]. The obtained sections were incubated with optimally -diluted rat anti-granzyme B (eBioscience) or isotype-matched control antibodies, and thenand subsequently incubated with the optimally diluted Alexa Fluor 488 Donkey anti-rRrat IgG antibody (Life Technology Inc.). Finally, the sections were incubated with optimally -diluted Alexa Fluor 594-conjugated anti-CD4 or isotype-matched control antibody (BioLegend). Immunofluorescence was visualized on a Keyence BZ-X7100 (Keyence Japan).

2.8. Statistical analysis

The dData were statistically analyzed statistically using the methods indicated in each figure legend. $p < 0.05$ was considered statistically significant.

3. Results

3.1. $CD4^+$ T cells are indispensable to in CTX-mediated eradication of BNL tumors.

Although an immune response is presumed to be involved in continuous low-dose chemotherapeutics-induced mediated tumor eradication regression [7] [9, 15], the roles of anthe immune response in tumor eradication induced by a single high -dose of a chemotherapeutics it still remains to be investigated on the roles of immune response in tumor eradication induced by a single high-dose of chemotherapeutics. In order Tto address this question, we examined the process of tumor eradication regression caused by a single administration of a high -dose of CTX. We observed that CTX (at doses of 100 mg/kg or lower) [Ed7]failed to eradicate the formed tumors established tumors arising resulting from by subcutaneous injection of BNL cells up to 100 mg/kg (Supplementary Figure S1). On the contraryIn contrast, 150 mg/kg CTX completely eradicated the tumors in most of the WT mice (Figure 1 B). Moreover, WT mice whose tumors had disappeared after CTX treatment rejected re-challenged BNL cells, but not cells from an unrelated murine colorectal cancer cell line, Col26 (Figure 1C). These observations would indicate that a single high -dose of CTX could can induce specific tumor immunity as well as tumor abolitionablation. In support of this notion, in vitro treatment with mafosfamide, (an active compound of derived from CTX), induced immunogenic apoptosis in BNL cells, as evidenced by calreticulin exposure and high mobility group box-1 secretion [16] (Supplementary Figure S2). Furthermore, all nude mice exhibited a transient decrease in tumor size but followed by [Ed8]a subsequent progressive increase in tumor sizes upon treatment with the same dose [Ed9]of CTX (Figure 1B), while treatment with an anti-CD4 antibody but not an anti-CD8 antibody treatment significantly reducedinhibited the CTX-mediated tumor regression (Figure 1E). Thus, CTX-mediated tumor eradication may require T cells, particularly $CD4^+$ T cells.

3.2. $CD4^+$ T cells at the tumor site Expression ofdisplay cytotoxic phenotypes by $CD4^+$ T cells in at the tumor site.

FCM analysis revealed that CTX treatment induced the appearance expression of a cytotoxic granule membrane protein, LAMP1/CD107a, in-expressing CD4-positive or CD8-positive cells in tumor tissues but not in dLNs and or the spleens (Figure 2A). Of interestNotably, is that LAMP1/CD107a expression was consistentlyconstantly higher in intratumoral $CD4^+$ cells than in $CD8^+$ ones cells (Figure 2B). LAMP1/CD107a translocates to the cell membrane in cytotoxic cells upon release of granzyme B or perforin, which mediate the cytotoxic effects of these cells.[Ed10] Cytotoxic cells translocate LAMP1/CD107a to cell membrane, when they release granzyme B or perforin to exhibit their cytotoxicity [17]. Hence, we examined evalulated the expression of these molecules[Ed11]. CTX treatment enhanced caused an increase in granzyme B and perforin mRNA expression (Figure 2C), and granzyme B was detected in intratumoral $CD4^+$ cells three 3 days after CTX administration (Figure 2D). Thus, CTX treatment increased the expression of cytotoxic molecule-expressings in $CD4^+$ T cells[Ed12] within tumors and subsequently eradicatedled to BNL tumor regressions.

3.3. Direct migration of Ccirculating $CD4^+$ T cells directly migrate into the tumor site.

To delineate the mechanisms underlying the increase in $CD4^+$ T cells with expressing cytotoxic molecules within tumors, we conducted performed adoptive transfer experiments (Fgiure Figure 3A). Dilution of CFSE dilution, which indicates proliferation, was observed in draining lymph nodes at only sevenat 7 days after CTX injection (Figure 3B). Non-proliferating CFSE-labeled $CD4^+$ T cells appeared in the tumor but without proliferation, at four 4 days day 4 after CTX treatment, whereas some proliferating $CD4^+$ T cells proliferated at day 7were observed seven 7 days after

CTX treatment (Figure 3B). Thus, at day 4 after CTX administration when the tumors started to regress four on day 44 days after CTX administration, circulating $CD4^+$ T cells were recruited into the tumors (without in the absence of antigen priming at dLN) and the proliferation inbegan proliferating to proliferate at the tumor sites. [Ed13]Furthermore, the expression levels of LAMP1/CD107a was expressed to awere similar extent by in intratumoral $CFSE^{high}$ non-proliferating $CD4^+$ T cells at on days 4 and at day 7, and in $CFSE^{low}$ proliferating $CD4^+$ T cells at on day 7 (Figure 3C), suggesting that $CD4^+$ T cells in tumors can release cytotoxic granules. Given that the levels of LAMP1/CD107a were negligible in circulating $CD4^+$ T cells expressed a negligible level of LAMP1/CD107a (Supplementary Figure S3), LAMP1/CD107a expression was initiatedinduced upon migration of $CD4^+$ T cells started to express LAMP1/CD107a after migrating into the tumor sites.[Ed14]

3.4. Crucial roles of CCR5-expressing cells play crucial roles in CTX-mediated tumor eradication.

To understand the mechanism underlying the early phase of intratumoral $CD4^+$ T cell recruitment in the early phase, we examined the mRNA expression levels of several chemokines in the tumor tissue three 3 days after CTX administration. Among the chemokines that we examined, CCL3 and CCL4 mRNA expression was significantly up-regulated while CCL2 and CCL5 mRNA did not change significantly (Figure 4A to D). Moreover, CTX treatment significantly increased intratumoral CCL3 but not CCL4 contents levels significantly (Figure 4E and F). Although CCR1 and CCR5 are receptors for CCL3, we have previously observed that tumor-infiltrating $CD4^+$ and $CD8^+$ T cells expressed CCR5 but not CCR1 [18]. Thus, we inoculated BNL cells into $CCR5^{-/-}$ and WT mice a[Ed15]nd subsequently treated the animals with CTX (Figure 1A). Tumors in $CCR5^{-/-}$ mice recurred with a significantly higher frequency in $CCR5^{-/-}$ mice than those in WT mice (Figure 5A). These observations would indicate the involvement of

CCR5-expressing cells in CTX-mediated tumor regression. We nextNext, we intravenously injected intravenously CFSE-labeled splenocytes from WT- or $CCR5^{-/-}$ mouse-derived mouse splenocytesmice into tumor-bearing WT mice 1 day after CTX injection and removed ; the tumors were then removed four at 4 days after CTX injection (Figure 5B). $CD4^+$ splenocytes obtained from both WT and $CCR5^{-/-}$ mice did not express LAMP1/CD107a (Supplementary Figure S3). However, the expression of LAMP1/CD107a expression in intratumoral $CD4^+$ T cells expressed LAMP1/CD107awas more abundantlyhigher in mice receiving WT mouse-derived splenocytes, compared with than in those receiving $CCR5^{-/-}$ mouse-derived ones splenocytes (Figure 5C and D). Thus, CTX enhances the expression of CC chemokines to induce intratumoral migration of $CD4^+$ T cells withexpressing cytotoxic molecules to, which induce leadsleading to tumor eradication and subsequent specific tumor immunity.

Discussion

A Ssingle high-dose CTX is not widely used for the treatment for of human cancers, including hepatoceullarhepatocellular carcinoma. Rahir et al. have reported that a single high -dose of CTX eradicatesd the tumors arising from subcutaneous injection of mouse mastocytoma cells and subsequently inducesd specific tumor immunity [11]. We have also observed that a single administration of high-dose CTX completely eradicatesd completely tumors in most of immunocompetent mice but not in nude mice, even when CTX is administered after tumor formation. Thus, further investigation of the effects of a single high -dose of CTX in preclinical models is warranted may warrant further investigation on preclinical model. Moreover, depletion of $CD4^+$ T cells and, to a lesser extent, that of $CD8^+$ T cells, attenuated CTX-induced mediated tumor eradication. Furthermore, immunocompetent mice rejected the same cancer cell line, but not a distinct onecell line, after the tumors disappearedtumor regression. Thus, in contrast to the widely -held view that high-dose CTX is acts as an immunosuppressant, it high-dose CTX can induce collaborate with T cells, particularly $CD4^+$ T cells, to cause tumor regression in collaboration with T cells, particularly $CD4^+$ T cells, and can and induce specific tumor immunity.

$CD4^+$ cytolytic T lymphocytes (CTLs) have been proposed to be present in under multiple circumstances several conditions [19], but are frequently viewed as functional variants of the classical $CD4^+$ Th1 subset. However, Mucida and colleagues have proved shown that the helper T cell master regulator ThPOK, can continuously repress the cytotoxic CD8 lineage-associated genes in mature $CD4^+$ helper T cells and that the termination ablation of ThPOK expression in mature $CD4^+$ T cells can reactivate these cytotoxic CD8 lineage-associated genes [20]. These observations indicate the presence of $CD4^+$ CTLs, which are distinct from other $CD4^+$ helper T cell subsets. The authors havey further demonstrated that $CD4^+$ CTLs exhibited enhanced LAMP1/CD107a expression as they were activatedupon activation [20]. Thus,

CTX-mediated infiltrated $CD4^+$ T cells that infiltrate tumors in response to CTX possessed the phenotypes that are characteristic of activated $CD4^+$ CTLs[Ed16].

CTLs contain secretory lysosomes, which are equipped with cytolytic proteins such as granzymes and perforin [21]. After the cell recognizes a target, the secretory lysosomes move along microtubules and polarize at the plasma membrane, where cytolytic proteins were are secreted into the immunological synapse formed between the CTLs and cancer cells [22]. Once Ggranzymes are delivered into the cytosol of cancer cells, and, with the help of perforin, they the granzymes then cleave a variety of intracellular substrates to induce cell death [23]. LAMP1/CD107a appears on the cell surface following the fusion of lysosomes with the plasma membrane, and therefore is therefore used as a functional marker of degranulation of $CD8^+$ CTLs [24] and NK cells [25]. Moreover, LAMP1/CD107a is required for efficient perforin delivery to lytic granules and subsequent cytotoxicity [17]. We observed that infiltrated $CD4^+$ T cells consistently expressed constantly higher levels of LAMP1/CD107a [Ed17]than infiltrated $CD8^+$ T cells. Thus, at least in this model, compared with $CD8^+$ T cells, infiltrated $CD4^+$ T cells can translocate LAMP1/CD107a, a functional marker of cytotoxic granule degranulation, more efficiently than $CD8^+$ T cells translocate LAMP1/CD107a, a functional marker of cytotoxic granule degranulation.

Circulating $CD4^+$ T cells expressed a negligible level of LAMP1/CD107a, while intratumoral $CD4^+$ T cells abundantly expressed LAMP1/CD107a, even immediately after CTX treatment. Thus, it is unlikelythe possibility that a small number of circulating LAMP1/CD107a-positive $CD4^+$ T cells proliferated after entering into the tumor sites is unlikely. Consistently, adoptively -transferred $CD4^+$ T cells did not proliferate within the tumor sites until four 4 days after CTX treatment. Several lines of evidence indicate that $CD4^+$ CTLs can be induced by the combined action of IL-2 and additional inflammatory cytokines, particularly type I interferon [10, 26, 27]. CTX can enhance the expression of IL-2 [28] and type I interferon [29]. Thus, once $CD4^+$ T

cells entered into tumor sites, these cells differentiated into $CD4^+$ CTLs under the influence of these cytokines.

However, high-dose CTX was not effective in all mice and; therefore, improvement of itsimproving the efficacy of high-dose CTX may require additional measures, such as combined treatment with type I interferon [30]. We observed that CTX significantly enhanced the expression of CCL3 and CCL4 in the tumor sites, which is consistent with the a previous report ofn its the effects of CTX on peripheral blood and bone marrow [31]. Moreover, the transfer of splenocytes derived from $CCR5^{-/-}$ mouse-derived splenocytesmice reduced the intratumoral accumulation of $CD4^+$ T cells, particularly those expressing CD107a-expressing ones. These observations would indicate that these chemokines directly attracted directly CCR5-expressing $CD4^+$ T cells into the tumors. Thus, supplementing with the supplementation of CCL3 and/or CCL4 may augment the anti-tumor effects of CTX, as similar to our previous observations in the context of we previously observed on radiofrequency ablation therapy [Ed18][13].

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Gene Name	Forward primer (5'-3')	Reverse primer (5'-3')
Granzyme B	gagccaggagatgtgtgct	gcacgtttggttctttgggtc
Perforin	agttcgtgccaggtgtatgg	gtgggtgattgccacaggta
CCL2	cccactcacctgctgctact	tctggacccattccttcttg
CCL3	gctgacaagctcaccctctgt	ggcagtggtggagaccttca
CCL4	cagcaccaatgggctctga	gccgggaggtgtaagagaaac
CCL5	tccaatcttgcagtcgtgtttg	tctgggttggcacacacttg
GAPDH	catggccttccgtgtttccta	gcggcacgtcagatcca
HPRT	tcctcctcagaccgctttt	cctggttcatcatcgctaatc

Table 1. Nucleotide sequences of the primers used for qRT-PCR

Legends to Figure Legendss

Figure 1. Eradication of BNL tumors are eradicated by a single high-dose CTX administration. (A) Schematic representation of the experimental procedures. Five hundred thousand BNL cells were inoculated into the left flank of WT or nude mice. CTX was intraperitoneally injected at a dose of 150 mg/kg into the mice when the tumor volume reached 40 to 80 mm^3. Tumor volumes were measured every two to three days after CTX treatment. **(B)** Tumor volumes were determined measured after CTX treatment in BNL tumor-bearing WT (left panel) or nude mice (right panel). The bBlue and red lines indicate the tumor volumes in the animals whose tumors disappeared regressed after CTX treatment and those whose tumors recurred after the treatment, respectively. The parentheses indicate the numbers of mice with a recurrent tumors among the total mouse numbersnumber of mice. *p* values were calculated with using the Fisher's exact test. *, $p< 0.05$. **(C)** WT mice whose BNL tumor disappeared after CTX treatment or naïve WT mice subcutaneously received BNL cells and Col26 cells subcutaneously at into the right and left flank, respectively. Tumor-free time intervals were determined until 21 days after injection[Ed19]. The bBlue and red lines indicate re-challenged and naïve mice, respectively. Each group consisteds of six6 animals. *p* values were calculated with using the log-rank test. *, $p < 0.05$. **(D)** and **(E)** Mmice were treated, as shown in Figure 1A. The mMice received were intraperitoneally injected with anti-CD4 mAb (100 μg /bodyanimal), anti-CD8 mAb (200 μg /bodyanimal), or PBS twice,at one1 day and 14 days after CTX treatment, intraperitoneal injection with either anti-CD4 mAb (100 μg /body), anti-CD8 mAb (200 μg /body), or PBS as shown in D[Ed20]. Tumor volumes were determined measured in mice injected with PBS (left panel), anti-CD8 mAb (middle panel), or anti-CD4 mAb (right panel) for 21 days after CTX injection. The bBlue and red lines indicate the animals whose tumors disappeared regressed after CTX treatment and those whose

tumors recurred after the treatment, respectively **(E)**. The parentheses indicate the numbers of mice with a recurrent tumors among the total mouse numbersnumber of mice. *p* values were calculated with theusing Fisher's exact test. *, $p< 0.05$; N.S., no significant difference.

Figure 2. CTX treatment inducesd the intra-tumoral accumulation of $CD4^+$ T cells expressing LAMP1/CD107a and cytotoxic molecules. (A) and **(B)** WT mice were treated as shown in Figure 1A. Tumors, draining lymph nodes (dLNs), and spleens were harvested to obtain single cell preparations at the indicated time points after CTX injection. LAMP1/CD107a expression was determined evaluated on in $CD4^+$ cells (upper panel) and $CD8^+$ cells (lower panel) by using FCM analysis. Isotype-matched control rat IgG was used as an isotypea control. Representative results from three independent experiments are shown in A. The mMean fluorescent intensity (MFI) of LAMP1/CD107a were determined onin $CD4^+$ or $CD8^+$ cells was determined. Means ± SD were calculated from three independent experiments and are shown in B. *p* values were calculated with using the Student's Student's *t*- test. *, $p< 0.05$. **(C)** Total RNA was extracted from tumor tissues three at 3 days after CTX treatment to quantify granzyme B (Grz B) and perforin mRNA by qRT-PCR analysis. Data represent the mean ± SD from three independent experiments. *p* values were calculated with using the Student's Student's *t*- test. *, $p< 0.05$. **(D)** DoubleTwo-color immunofluorescence image of CD4 (red) and Grz B (green) in the tumor tissues on day 3 after CTX injection. Merged images with or without DAPI is are shown[Ed21]. Representative results from three independent experiments are shown here.

Figure 3. CTX-induced induces direct intratumoral migration of naïve $CD4^+$ T cells with cytotoxic phenotypes (A) CTX were was intraperitoneally administered into CD45.2-expressing WT mice which had received BNL cells at in the left flank,

similarly assimilar to the schematic [Ed22]shown in Figure 1A. Splenocytes were isolated from CD45.1-expressing congenic mice and were labeled with CFSE. Ten million labeled splenocytes were intravenously injected into the tumor-bearing mice one 1 day after CTX injection. Tumors and dLNs were harvested four at 4 and seven7 days after CTX injection. **(B)** Single cell preparations were obtained from tumor tissues or dLNs, and subsequently gated based on CD4 expression. CD45.1 and CFSE expression among the $CD4^+$ cells was determined assessed by FCM analysis four at 4 (left panels) and seven7 days (right panels) after CTX injection. Representative results from three independent experiments are shown here. The proportion of $CD45.1^+$ cells among the total number of $CD4^+$ cells was determined. Means ± SD were calculated from three independent experiments and are shown in each panel. *p* values were calculated with using the Student's Student's *t*- test. **, $p< 0.01$; *, $p< 0.05$; N.S., no significant difference. **(C)** Single cell preparations were obtained from tumor tissues four at 4 and seven7 days after CTX treatment, and subsequently gated with based on CD4 (left panels). LAMP1/CD107a expression was determined evaluated assessed on in either $CFSE^{high}CD4^+CD45.1^+$ or $CFSE^{low}CD4^+CD45.1^+$ cells (right panel). Representative results from three independent experiments are shown here.

Figure 4. Enhanced CCL3 expression is enhanced in the tumor tissues after CTX treatment. WT mice were treated as shown in Figure 1A. **(A)** to **(D)** Total RNA was extracted from tumor tissues three at 3 days after CTX treatment to quantify CCL2 **(A)**, CCL3 **(B),** CCL4 **(C)**, or CCL5 mRNA **(D)** by qRT-PCR analysis. Means ± SD were calculated from three independent experiments and are shown here. *p* values were calculated with using the Student's Student's *t*- test. **, $p< 0.01$; *, $p< 0.05$; N.S., no significant difference. **(E)** and **(F)** Protein lysates were extracted from tumor tissues after CTX treatment to quantify CCL3 **(E)** and CCL4 protein content levels **(F)** by ELISA. Means ± SD were calculated from three independent experiments and are

shown here. *p* values were calculated with using the Student's Student's *t*- test. *, $p<$ 0.05; N.S., no significant difference.

Figure 5. Essential involvement of aA CCR5-mediated signal is essential in CTX-mediated intratumoral migration of naïve cytotoxic CD4$^+$ T cells. (A) WT or CCR5$^{-/-}$ mice were treated as shown in Figure 1A. Each group consisteds of at least five5 animals. Tumor recurrence was defined as the point at which the when tumor volume resumed reachedreturned to the same size as that at initial volume at the time of CTX treatment[Ed23]. Recurrence-free curves are shown here. *p* values were calculated with using the log-rank test. *, $p < 0.05$; N.S., no significant difference. **(B)** to **(D)**. After WT mice were treated as shown in Figure 1A, 10^7 CFSE-labeled splenocytes from CFSE-labeled WT- or CCR5$^{-/-}$ mice-derived 10^7 splenocytes were intravenously injected into the tumor-bearing WT mice one 1 day after CTX injection (B). Tumors were harvested at four4 days after CTX injection to obtain single cell preparations. LAMP1/CD107a expression was determined assessed on in CFSE^{high}CD4$^+$ cells of from mice receiving splenocytes from WT or CCR5$^{-/-}$ mice. Representative results from three independent experiments are shown in C. Means ± SD were calculated from three animals on for the proportion of CFSE^{high}LAMP1/CD107a$^+$ cells among the total number of [Ed24]CD4$^+$ cells and are shown in D. *p* values were calculated with using the Mann-Whitney's *U* test. *, $p< 0.05$.

Legend to Supplementary Figures

Figure S1. Tumor volumes after treatment with different doses of CTX at different doses. CTX was intraperitoneally injected at the indicated doses into wild-typeWT mice when the tumor volume reached 40 to 80 mm^3. Tumor volumes were measured every two to three days after CTX treatment.

Figure S2. MAFO treatment induced immunogenic apoptosis of BNL cells in vitro. (A) and (B) After incubating BNL cells (1 x 10^5 cells/ml) in the medium containing in the presence or the absence of 30 μM mafosfamide (MAFO) for 20 hours, the labeling of BNL cells labeled with expression of propidium iodide (PI) and annexin-V (A) or PI and calreticulin (B) on BNL cells was determined measured by using FCM analysis. Representative images from three3 independent experiments are shown here. **(C)** After treating BNL cells as mentioned described above, the cells were incubated for another an additional 24 hours with fresh medium. The cConcentration of high mobility group box-1 (HMGB-1) in the medium was determined by using a specific ELISA (Sinotest, Tokyo, Japan). *p* values were calculated with using the Student's Student's *t*- test. *, $p<$ 0.05.

Figure S3. $CD4^+$ T cells in the circulation or spleen merely expressed minimal levels of CD107a[Ed25]. (A) and (B) Single cell preparations were obtained from the peripheral blood (A) and spleen (B) of both WT mice and $CCR5^{-/-}$ mice and were subsequently gated based on CD4 expression. CD107a expression among the $CD4^+$ cells was determined by FCM analysis. The sSolid lines and, dashed lines indicate the results of from the WT mice and $CCR5^{-/-}$ mice, respectively. Isotype controls are shown represented by the gray-filled histograms. Representative results from three independent experiments are shown here.

Legend to Supplementary Figures

Figure S1. Tumor volumes after treatment with different doses of CTX. CTX was intraperitoneally injected at the indicated doses into WT mice when the tumor volume reached 40 to 80 mm^3. Tumor volumes were measured every two to three days after CTX treatment.

Figure S2. MAFO treatment induced immunogenic apoptosis of BNL cells in vitro. (A) and (B) After incubating BNL cells (1 x 10^5 cells/ml) in the presence or the absence of 30 μM mafosfamide (MAFO) for 20 hours, BNL cells labeled with propidium iodide (PI) and annexin-V (A) or PI and calreticulin (B) was measured by FCM analysis. Representative images from three independent experiments are shown. **(C)** After treating BNL cells as described above, the cells were incubated for an additional 24 hours with fresh medium. The concentration of high mobility group box-1 (HMGB-1) in the medium was determined using a specific ELISA (Sinotest, Tokyo, Japan). *p* values were calculated using Student's *t*-test. *, $p< 0.05$.

Figure S3. $CD4^+$ T cells in the circulation or spleen expressed minimal levels of CD107a[Ed1]. (A) and (B) Single cell preparations were obtained from the peripheral blood (A) and spleen (B) of both WT and $CCR5^{-/-}$ mice and were subsequently gated based on CD4 expression. CD107a expression among the $CD4^+$ cells was determined by FCM analysis. The solid lines and dashed lines indicate the results from the WT and $CCR5^{-/-}$ mice, respectively. Isotype controls are represented by the gray-filled histograms. Representative results from three independent experiments are shown.

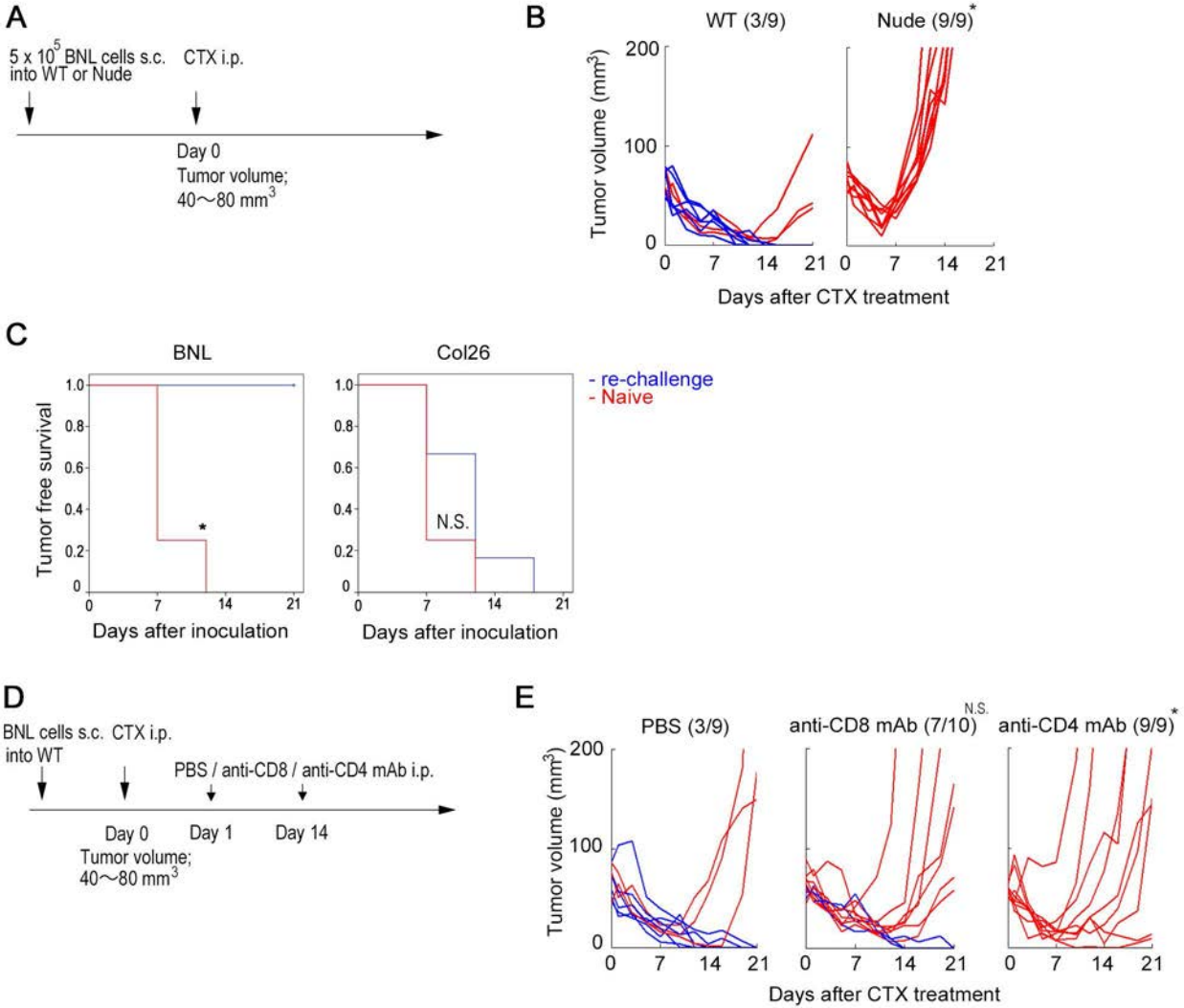
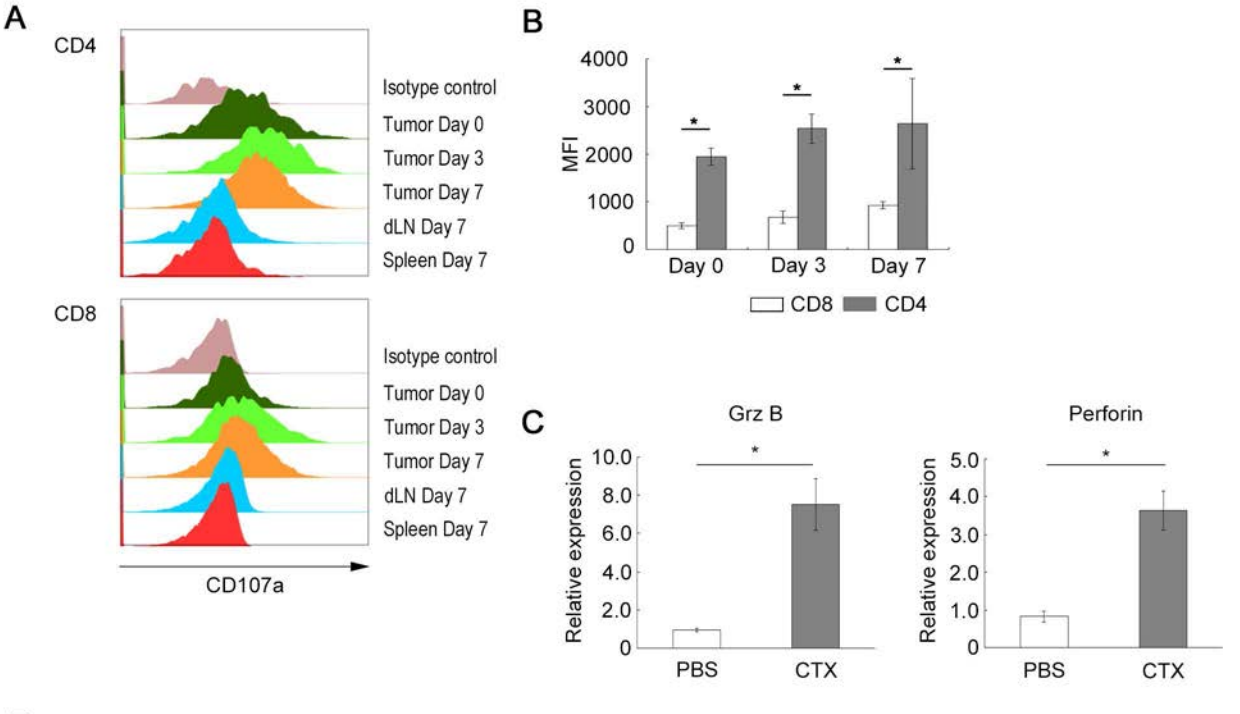

A
5 x 10^5 BNL cells s.c. into WT or Nude
CTX i.p.
Day 0
Tumor volume;
40～80 mm^3
B
WT (3/9)
Nude (9/9)*
Tumor volume (mm^3)
200
100
0
0 7 14 21
Days after CTX treatment
C
BNL
Col26
- re-challenge
- Naive
Tumor free survival
1.0
0.8
0.6
0.4
0.2
0
*
N.S.
Days after inoculation
D
BNL cells s.c. into WT
CTX i.p.
PBS / anti-CD8 / anti-CD4 mAb i.p.
Day 0
Day 1
Day 14
Tumor volume;
40～80 mm^3
E
PBS (3/9)
anti-CD8 mAb (7/10)N.S.
anti-CD4 mAb (9/9)*
Tumor volume (mm^3)
Days after CTX treatment

Figure 1.


A
CD4
Isotype control
Tumor Day 0
Tumor Day 3
Tumor Day 7
dLN Day 7
Spleen Day 7
CD8
Isotype control
Tumor Day 0
Tumor Day 3
Tumor Day 7
dLN Day 7
Spleen Day 7
CD107a
B
MFI
4000
3000
2000
1000
0
Day 0
Day 3
Day 7
CD8
CD4
C
Grz B
Relative expression
10.0
8.0
6.0
4.0
2.0
0
PBS
CTX
Perforin
Relative expression
5.0
4.0
3.0
2.0
1.0
0
PBS
CTX

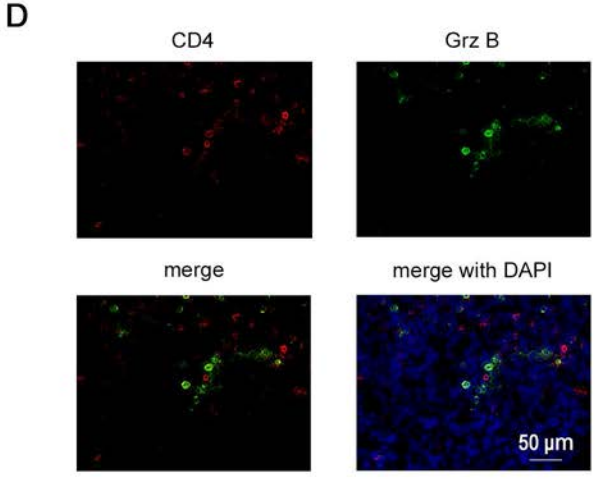

D
CD4
Grz B
merge
merge with DAPI
50 μm

Figure 2.

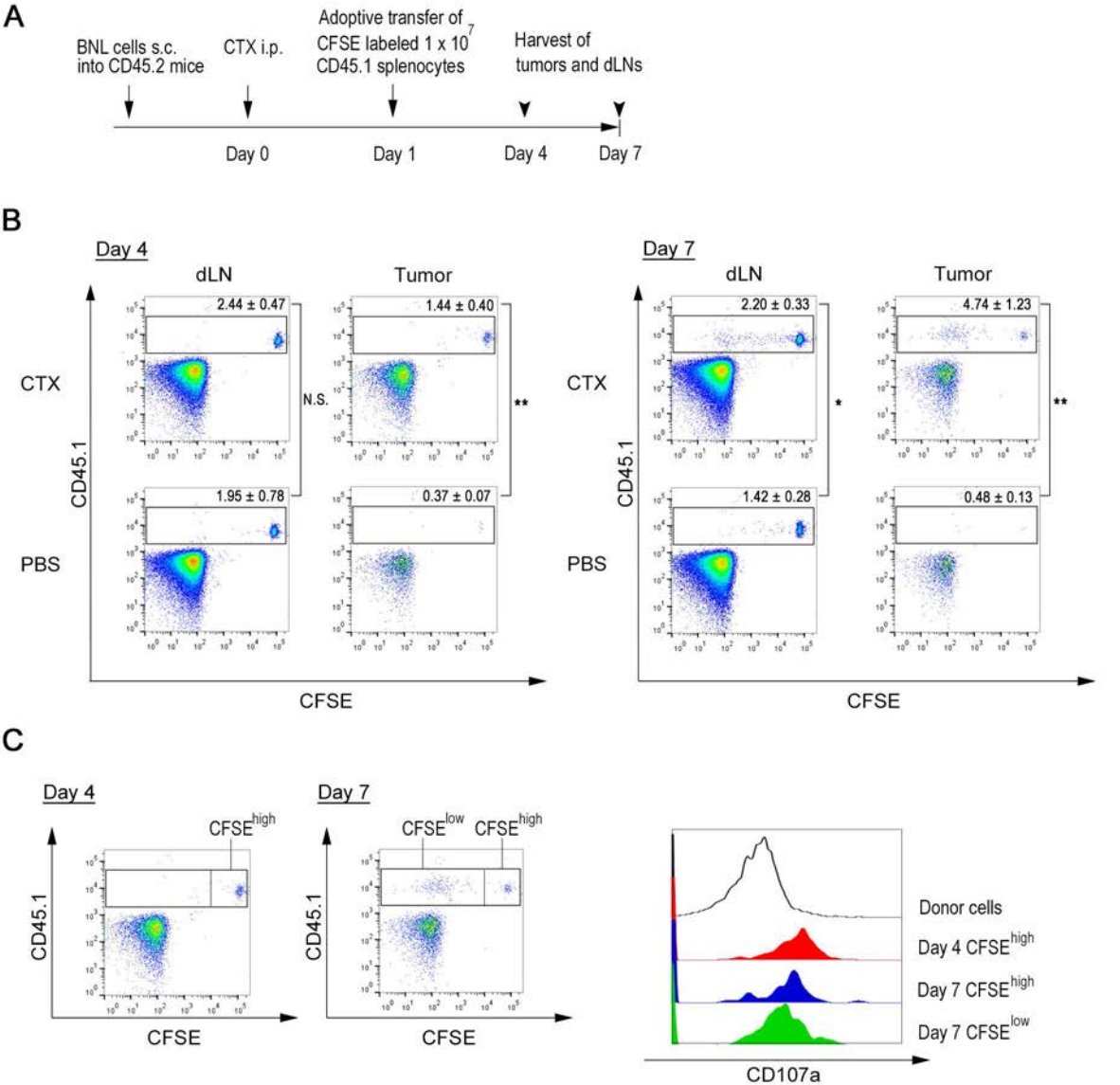

A
BNL cells s.c. into CD45.2 mice
CTX i.p.
Adoptive transfer of CFSE labeled 1 x 10^7 CD45.1 splenocytes
Harvest of tumors and dLNs
Day 0
Day 1
Day 4
Day 7
B
Day 4
dLN
Tumor
CTX
PBS
2.44 ± 0.47
1.44 ± 0.40
1.95 ± 0.78
0.37 ± 0.07
N.S.
**
CD45.1
CFSE
Day 7
dLN
Tumor
CTX
PBS
2.20 ± 0.33
4.74 ± 1.23
1.42 ± 0.28
0.48 ± 0.13
*
**
CD45.1
CFSE
C
Day 4
CFSE^high
CD45.1
CFSE
Day 7
CFSE^low
CFSE^high
CD45.1
CFSE
Donor cells
Day 4 CFSE^high
Day 7 CFSE^high
Day 7 CFSE^low
CD107a

Figure 3.

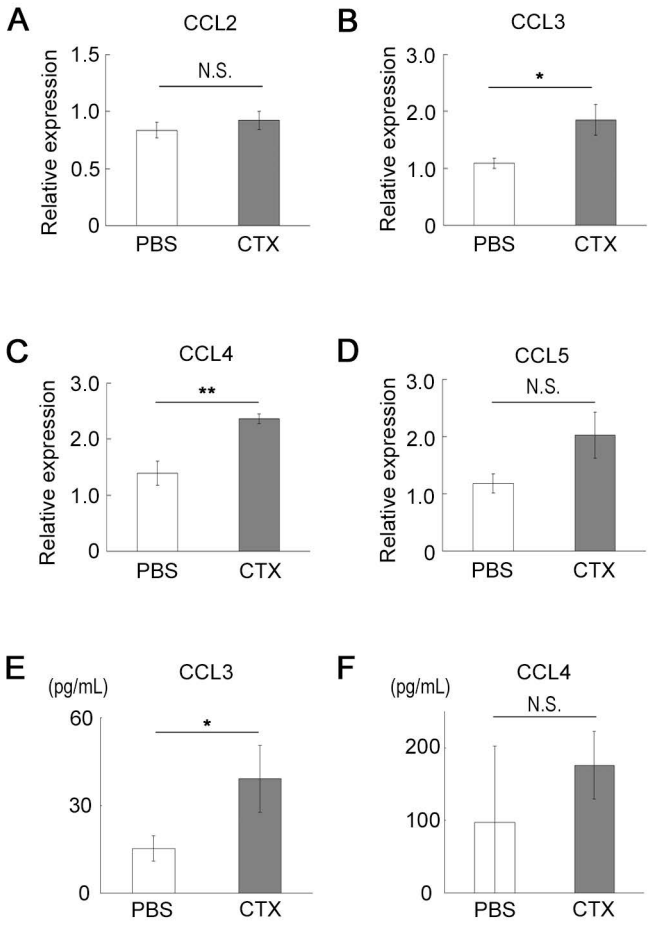

A
CCL2
Relative expression
1.5
1.0
0.5
0
N.S.
PBS
CTX
B
CCL3
Relative expression
3.0
2.0
1.0
0
*
PBS
CTX
C
CCL4
Relative expression
3.0
2.0
1.0
0
**
PBS
CTX
D
CCL5
Relative expression
3.0
2.0
1.0
0
N.S.
PBS
CTX
E
CCL3
(pg/mL)
60
30
0
*
PBS
CTX
F
CCL4
(pg/mL)
200
100
0
N.S.
PBS
CTX

Figure 4.

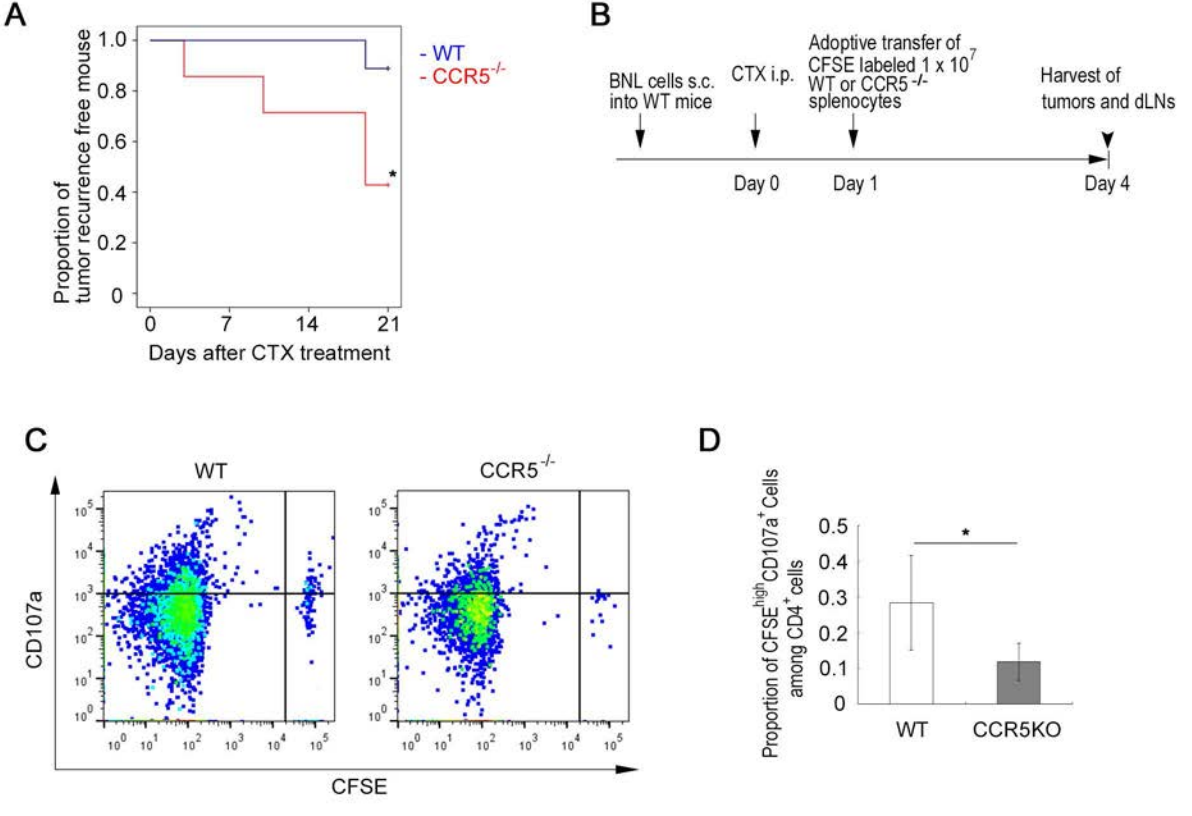
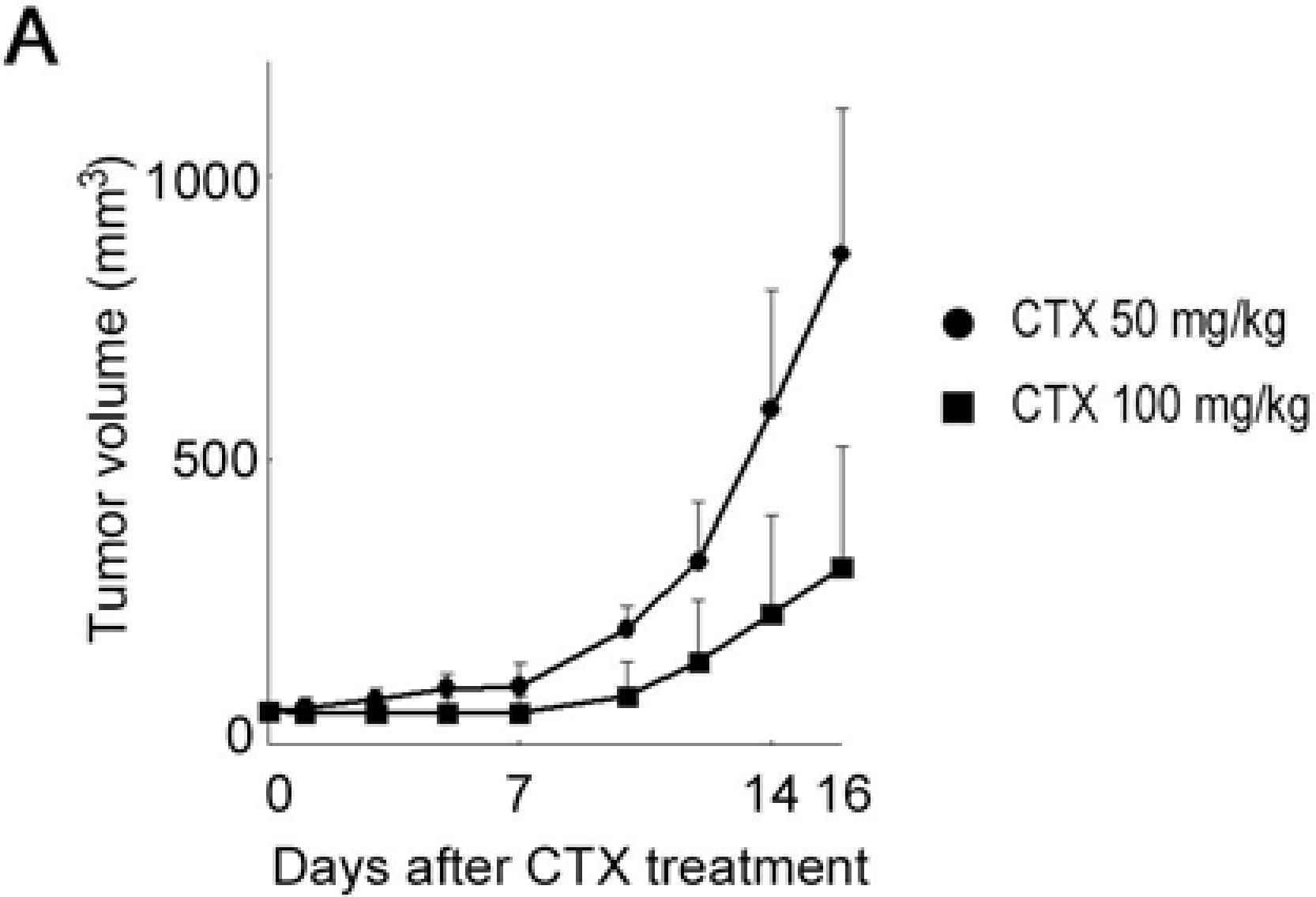
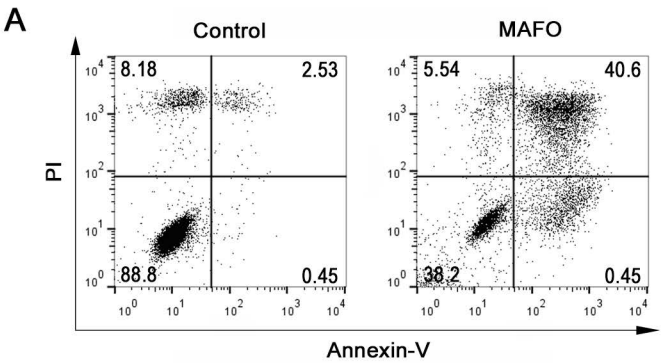
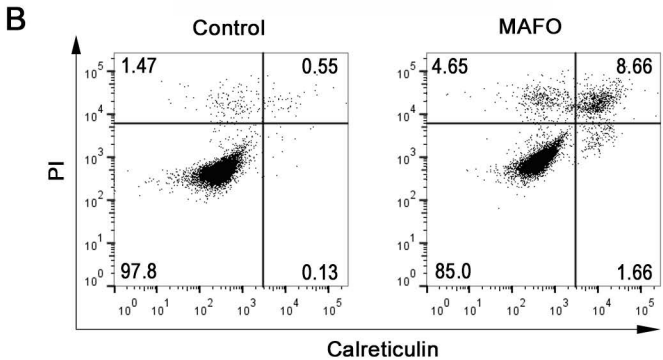

A
Proportion of tumor recurrence free mouse
1.0
0.8
0.6
0.4
0.2
0
0
7
14
21
Days after CTX treatment
- WT
- CCR5-/-
*
B
BNL cells s.c. into WT mice
CTX i.p.
Adoptive transfer of CFSE labeled 1 x 10 7 WT or CCR5 -/- splenocytes
Harvest of tumors and dLNs
Day 0
Day 1
Day 4
C
WT
CCR5 -/-
CD107a
CFSE
D
Proportion of CFSE high CD107a + Cells among CD4 + cells
0.5
0.4
0.3
0.2
0.1
0
*
WT
CCR5KO

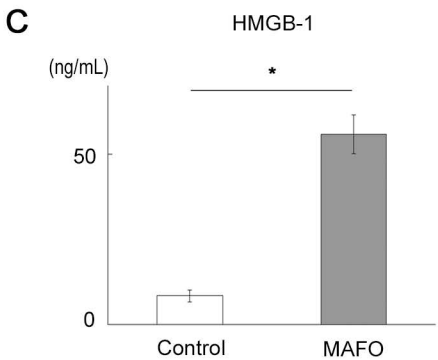
Figure 5.



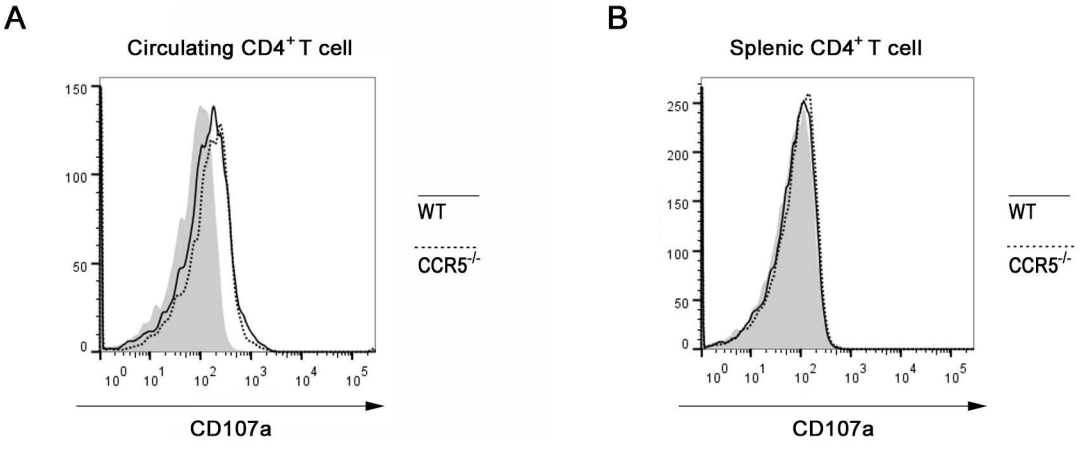
Supplementary Figure 1.







Supplementary Figure 2.



Supplementary Figure 3.