C-Reactive Protein Reduces the Relative Number of Tumor-Associated M2 Macrophages and Intratumoral Angiogenesis in Mice

CRP の抗腫瘍効果 -腫瘍関連マクロファージと腫瘍内血管新生に関して-

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Title

C-Reactive Protein Reduces the Relative Number of Tumor-Associated M2 Macrophages and Intratumoral Angiogenesis in Mice

Running title:

C-Reactive Protein Reduces Decreases the M2 Macrophage Accumulation

Authors

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Abstract

Tumor-associated macrophages play a key role in cancer metastasis. On the other hand, C-reactive protein (CRP), a widely used biomarker of inflammation, has been shown to have inhibitory effects on tumor proliferation and metastasis. Here we used an implanted tumor mouse model to assess the effect of CRP on tumor-associated macrophage numbers and on their phenotype, as well as on intratumoral angiogenesis. NR-S1M murine oral squamous cell carcinoma cells were implanted subcutaneously in the backs of anesthetized C3H/HeN mice. Some of the mice were also subcutaneously administered 1 μg of recombinant mouse CRP in 100 μL of phosphate-buffered saline (PBS) (CRP group, n = 10) near the neck every 2 days for 30 days (15 injections in all). Control mice received PBS without CRP. The mice were then sacrificed and the excised tumors were analyzed. Tumor weight and size did not differ between the two groups, but immunohistochemical analysis showed the $F4/80^+$ macrophage (total macrophages) count to be significantly larger in the CRP group ($P = 0.0028$), while the relative number of $CD206^+$ anti-inflammatory M2 macrophages was significantly reduced ($P = 0.0091$). In addition, expression of colony stimulating factor 1 mRNA, which is associated with the M2 macrophage phenotype, was significantly lower in the CRP group. Intratumoral angiogenesis, indicated by the presence of $CD31^+$ vessels within the tumor, was reduced in the CRP group ($P = 0.0028$). These findings suggest that CRP has therapeutic potential against cancer through decreasing the accumulation of M2 macrophages and angiogenesis within tumors.

Key words

Angiogenesis, Colony stimulating factor 1, C-reactive protein, M2 phenotype, Tumor-associated macrophages

Background

Evidence now suggests that in addition to the tumor cells themselves, the tumor stroma (tumor vessels, fibroblasts, inflammatory cells, and so on) plays an important role in tumorigenesis, tumor proliferation, angiogenesis and metastasis. Particularly important are the macrophages that infiltrate tumors. Referred to as tumor-associated macrophages (TAMs), they produce a variety of cytokines affecting tumor growth **(**Qian & Pollard 2010**)**. Macrophages have been classified into several types, and it is well known that the inflammatory (M1) and anti-inflammatory (M2) types are extremes on a spectrum of phenotypes (Mantovani et al. 2002). M1 macrophages, which produce interleukin-12 (IL-12) and tumor necrosis factor (TNF), are effector cells that kill microorganisms and tumor cells and produce large amounts of proinflammatory cytokines. By contrast, M2 macrophages produce IL-1 and IL-10, suppress inflammatory responses, show little cytotoxicity and, if anything, promote tumor angiogenesis (Seth et al. 2009; Qian & Pollard 2010)**.** Particularly important is that M2 TAMs play a key role in tumor metastasis (Mantovani et al. 2002).

C-reactive protein (CRP) is a plasma protein that causes precipitin to react with the group-specific C carbohydrate of Pneumococcus, and is widely used as the biomarker of acute or chronic inflammation, tissue destruction and infectious disease. In addition, several studies have shown that CRP also exerts inhibitory effects on tumor proliferation (Kresl et al. 1999), angiogenesis (Franco-Molina et al. 2010) and distant metastasis (Deodhar et al. 1982). Recently, we found that lymph node involvement in esophageal and lung cancers may have a genetic component – i.e., the CRP 1846C>T genetic polymorphism appears to be an independent factor associated with lymph node metastasis (Motoyama et al. 2009; Minamiya et al. 2010; Motoyama et al. 2013). The CRP 1846 T/T genotype is associated with lower serum CRP levels than the

1846 T/C or C/C genotypes. Moreover, esophageal cancer patients carrying the 1846T/T genotype are more than 3 times more likely to have lymph node involvement than patients carrying the 1846 T/C or C/C genotype. In this study, we tested whether CRP affects TAM numbers or the M2 phenotype fraction within tumors and/or affects angiogenesis.

Methods

Tumor cells

The NR-S1M murine oral squamous cell carcinoma cell line, kindly provided by Dr. Matsumoto, was used. The NR-S1 cell line is a murine oral squamous cell carcinoma cell line that spontaneously arose in the C3H/He mouse strain. NR-S1 has a small probability of metastasizing to lymph nodes (Usui et al. 1976). Derived through in vivo selection in C3H/He mice from the parental NR-S1 cells, NR-S1M cells are highly metastatic cell lines (Matsumoto et al. 2010). The cells were cultured in RPMI-1640 (Sigma-Aldrich, Saint Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO, Grand Island, NY) and antibiotics (100 U/mL penicillin G, 100 μg/mL streptomycin and 250 μg/mL amphotericin B; GIBCO) in a humidified incubator at 37°C under an atmosphere of 5% CO_2/95% air.

Animal model

Female C3H/HeN mice (5 weeks old) were purchased from CLEA (Tokyo, Japan). Then after a 1-week acclimation period, the mice were anesthetized using diethyl ether, and NR-S1M cells ($5x10^6$ cells) were implanted subcutaneously in their backs, near the tail, using a syringe with 27-gauge needle. Simultaneously, 10 of the mice (CRP group) were subcutaneously administered 1

μg of recombinant mouse CRP (r-CRP, R&D Systems, Minneapolis, MN) in 100 μL of phosphate-buffered saline (PBS) into their backs, near the neck. Alternatively, 10 mice (control group) were subcutaneously injected with 100 μL of PBS without r-CRP (control). Thereafter, mice in their respective groups were administered r-CRP or PBS every 2 days for 30 days (15 injections in all). In this model, the serum CRP level reportedly increases gradually, reaching about 12 ng/mL on day 18 **(**Sasaki et al. 2013**)**. The mice were sacrificed 1 day after the last injection, and the tumors were excised. For analysis, each tumor was divided in two at the largest cut surface in the sagittal plane, and one sample was frozen in Tissue-Tek O.T.C. compound (Sakura Finetek Japan, Tokyo, Japan) for preparation of frozen sections, and the other sample was frozen at -80°C for analysis of cytokine mRNA. Animal experiments were performed in accordance with the policies of the animal ethics committee of Akita University Graduate School of Medicine.

Assessing tumor size

Tumors weights were measured when the mice were sacrificed. In addition, digital images of the largest surface of each tumor were captured using a digital scanner. The area of that surface was then determined using Image J 1.64r (National Institute of Health, USA).

Immunohistochemistry

After cutting the largest surface of each tumor into 4-μm-thick frozen sections, immunohistochemistry was performed. Sections were fixed in acetone for 5 min at 4°C and then soaked in PBS for 3 min, after which nonspecific binding sites were blocked by incubating the sections for 30 min in 10% goat serum (Nichirei, Tokyo, Japan). The sections were then

incubated for 60 min in the presence of the primary antibody, followed by incubation for 30 min with peroxidase-conjugated goat anti-rat secondary antibody (Histfine Simple Stain MAX-PO (Rat); Nichirei). The color was then developed by incubation for 3 min with diaminobenzidine (DAB kit; Nichirei).

Macrophage identification

To evaluate the total macrophage population, a rat monoclonal anti-F4/80 antibody (1:200 dilution, MCA497; AbD Serotec, San Diego, CA) was used. M2 macrophages were identified using a rat monoclonal anti-CD206 antibody (1:200 dilution, MCA2235; AbD Serotec). In this analysis, we defined F4/80 positivity as reflecting the total macrophage population, while CD206 positivity reflected only the M2 macrophage population. M1 macrophages belong to the fraction remaining after pulling the $CD206^+$ macrophages from the $F4/80^+$ population.

Evaluation for angiogenesis

Angiogenesis was evaluated immunohistochemically using rabbit polyclonal anti-CD31 antibody (1:200 dilution, ab28364; abcam, Cambridge, UK).

Analysis for the immunochemical staining

For each section, seven digital images were captured at 200x magnification under a light microscope, in random order, avoiding areas of necrosis. After counting the DAB-positive cells or vessels in each image, the average of five images, excluding the minimum and maximum values, was calculated.

Reverse Transcription-quantitative PCR (RT-qPCR)

Total mRNA expression of IL-4, IL-10, TNF, interferon-γ (IFN-γ), and colony stimulating factor 1 (CSF-1) were assessed using RT-qPCR. Total RNA was extracted from the tumor using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. The extracted RNA was then quantified and assessed for purity using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA). Reverse transcription was performed using a Transcriptor First Strand cDNA synthesis kit (Roche Applied Science, Penzberg, Germany). Briefly, after preheating for 10 min at 65°C, RNA aliquots were reverse transcribed by incubation with Transcriptor reverse transcriptase and deoxynucleotide mix for 10 min at 25°C followed by 30 min at 55°C and 5 min at 85°C. Primers and probes for RT-qPCR were designed at the website of the Roche Applied Science, Universal ProbeLibrary Assay Design Center (https://www.roche-applied-science.com/). Real-time PCR was carried out in a 96-well plate (Roche Applied Science) using a LightCycler 480 Real-Time PCR System (Roche Applied Science). After 5 min of initial denaturation at 95°C, the cycling protocol entailed 45 cycles of denaturation at 95°C for 10 s and annealing and elongation at 60°C for 30 s. Each assay was performed with samples, standard curves, positive controls and reagent controls (reagents without cDNA and H_2O only) in duplicate. The means of the duplicates were analyzed as results. The amplification efficiencies of all PCR runs and the CT values of each sample were calculated using LightCycler 480 Software Version 1.5. The ddCT method was employed for comparative mRNA analysis. All samples were normalized to β-actin as an endogenous housekeeping gene. MIQE guidelines were used when assessing and presenting the results. The sequences of the primers were shown in Table 1.

Statistical analysis

Statistical analysis was performed with JMP 10 (SAS Institute, Cary, NC). Numerical values are depicted as the median (minimum value - maximum value). Differences between the control and CRP group were assessed using the Wilcoxon signed-rank test. Differences were considered significant at $P < 0.05$.

Results

Tumor size

The median tumor weight was 0.38 (0.1-1.07) g in the control group and 0.37 (0.1-1.07) g in the CRP group, which was not a significant difference (Fig. 1). The average area of the largest surface of the tumor was 5.5 $x10^6$ ($2.4x10^6$-$10.2x10^6$) pixels in the control group and $5.1x10^6$ ($1.3x10^6$-$9.3x10^6$) pixels in the CRP group. Again there was no significant difference between the CRP and control groups (Fig. 1).

Total macrophages and their fractions within tumors

The total numbers of macrophages ($F4/80^+$ cells) within the tumors were 141.9 (62.6-225.8) cells/field in the control group and 211.3 (152.2-277.2) cells/field in the CRP group (Fig 2AB, 3A). Thus tumors in the CRP group contained significantly ($P = 0.0028$) more macrophages than the controls. On the other hand, the numbers of M2 macrophages ($CD206^+$ cells) did not differ between the two groups (CRP: 70.8 (38.8-84.2) cells/field vs. control: 63.9 (47.2-109) cells/field (Fig 2CD, 3B). We then calculated the size of the M2 macrophage fraction (%) among the total macrophage population as the number of M2 macrophages ($CD206^+$ cells) / total

number of macrophages ($F4/80^+$ cells). Our calculation showed that the M2 macrophage fraction was significantly ($P = 0.0091$) smaller in the CRP group than the control group (30.7 (21.6-43.2) % vs. 44.2 (27.9-174.1) %) (Fig 3C).

The remaining cell fraction, which included the M1 macrophages ($F4/80^+$ cells – $CD206^+$ cells), was found to be 55.8 (25.8-72.1) % in the control group and 69.3 (56.8-78.4) % in the CRP group. Thus the fraction containing the M1 macrophages was significantly ($P = 0.0091$) larger within tumors in the CRP group. Apparently, CRP administration increases the total number of TAMs, while decreasing the M2 phenotype fraction.

Expression of M1- and M2-associated cytokine mRNA in the tumor

Using RT-qPCR we found that expression of INF-γ and TNF mRNA, which is associated with the M1 macrophage phenotype, did not differ between the CRP and control groups (Fig. 4). By contrast, expression of CSF-1 mRNA, which is associated with the M2 macrophage phenotype, was significantly ($P = 0.0376$) weaker in the CRP group than the control group (Fig. 4). Expression of IL-4 and IL-10 mRNA, which is also associated with the M2 macrophage phenotype, did not differ between the two groups.

Angiogenesis in the implanted tumor

The area of $CD31^+$ vessels was 25.8 (16.2-31.0) vessels/field in the control group and 17.8 (11.6-23.2) vessels/field in the CRP group. That is, the area of $CD31^+$ vessels was significantly larger in the control group than the CRP group ($P = 0.0028$) (Fig. 5). It thus appears CRP administration suppressed angiogenesis within the tumors.

Discussion

In this study, we found that administration of CRP to the host increases the total TAM population within tumors, but significantly reduces the M2 macrophage fraction. We also found that intratumoral expression of CSF-1 mRNA, which is associated with the M2 macrophage phenotype, was significantly lower in the CRP group. In addition, intratumoral angiogenesis indicated by the presence of $CD31^+$ vessels was significantly lower in the CRP group.

It is now becoming clear that the inflammatory cells present within the tumor microenvironment play an indispensable role in cancer progression. Substantial evidence suggests that stromal cells, including fibroblasts and inflammatory cells such as macrophages, neutrophils and lymphocytes, all interact with the cancer cells (Connolly et al. 1987; Fukumura et al. 1998). Among these various cells, macrophages are the dominant inflammatory cells within the tumor stroma. Upon activation, these TAMs release a wide variety of growth factors, proteolytic enzymes, cytokines and other inflammatory mediators. Above all, however, the immunosuppressive cytokine IL-10 reportedly induces TAMs to express the M2 phenotype (Mantovani et al. 2002). M2 macrophages suppress immune responses through down-regulation of T cell immunity. They also produce endothelial growth factor (EGF), VEGFs and CXCR4, which promote tumor cell invasion and tumoral angiogenesis. At the same time, they lack many of the functional features of M1 macrophages, such as phagocytosis, antigen presentation and activation of immune responses. TAMs are thus involved in initiating tumor inflammation, negatively regulating immune responses, and promoting tumor cell invasion, intravasation and angiogenesis (Sica et al. 2006; Qian & Pollard 2010).

Macrophages are classified into several types, though mainly two phenotypes, M1 and M2, have been characterized. In the present study, we demonstrated that CRP increases the total TAM population. However, because the number of M2 macrophages did not increase as compared to control, the fractional size of the M2 population was smaller in the CRP group. On the other hand, we were unable to demonstrate the increment in the M1 macrophage fraction directly. Furthermore, it is well known that M1 and M2 macrophages are extremes in a spectrum of phenotypes, and that there are macrophages that are neither M1 nor M2 (Gleissner 2012).

In vitro, monocytes isolated from human peripheral blood and incubated in the presence of CRP differentiate into M1 macrophages more frequently than M2 macrophages. Moreover, injecting CRP into Wistar rats increased the M1 macrophage fraction among their peritoneal macrophages (Devaraj & Jialal 2012). In the present study, the macrophage numbers within tumors were significantly larger in the CRP group than the control group, and there was a correspondingly larger number of M1 macrophages in the CRP group and a smaller number of M2 macrophages. To shed light on the mechanism underlying the CRP-related shift in the TAM population, we examined the tumoral expression of IFN-γ, TNF, IL-4, IL-10 and CSF-1 mRNA. IFN-γ mediates differentiation of monocytes into M1 macrophages, and TNF is produced by M1 macrophages. Given the larger M1 macrophage population in the CRP group, levels of TNF mRNA expression were expected to be higher in the CRP group, but we detected no significant increase. Expression of IL-4, IL-10 and CSF-1 mediates monocyte differentiation into M2 macrophages (Verreck et al. 2004). Expression of CSF-1 was significantly weaker in the CRP group. Only tumor cells produce CSF-1, which in turn promotes expression of EGF in TAMs. The expressed EGF then promotes further production of CSF-1 by tumor cells, creating an EGF-CSF-1 paracrine loop (Qian & Pollard 2010), which is an important mechanism underlying the

differentiation of M2 macrophages and is essential for tumor progression. Our findings suggest CRP suppresses the differentiation of M2 macrophages and the establishment of the EGF-CSF-1 paracrine loop. Further studies will be needed to test that idea, however.

Substantial clinical evidence indicates that there is a positive correlation between macrophage density and prognosis in cancer. For example, Ohri et al. reported that in non-small cell lung carcinoma (NSCLC) patients, the macrophage density is markedly higher among patients that survive for extended periods than among those that do not (Ohri et al. 2009). The same group also found that among the extended survival group, M1 macrophage density is significantly higher than the M2 density. Similarly, Ma et al. reported that tumoral M1 macrophage density is positively associated with survival time and is an independent predictor of survival time in NSCLC patients (Ma et al. 2010). Conversely, Zhang et al. reported that M2 macrophages accelerate lymphangiogenesis and lymph node metastasis in lung adenocarcinoma and are associated with poor prognoses (Zhang et al. 2011).

It is noteworthy that CRP administration to host mice did not affect tumor size per se; instead, it reduced angiogenesis within tumors, which is a key factor contributing to cancer metastasis. Taken together, our findings highlight the importance of the cancer stroma in mediating cancer metastasis, and suggest CRP has therapeutic potential against cancer through its actions to increase total TAM counts and reduce the M2 macrophage population.

Acknowledgements

This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Science, Sports and the Japan Science and Technology Agency.

Conflict of Interest

The authors declare no conflict of interest.

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Figure Legends

Fig. 1: Tumor size

Tumor weights and sectional areas did not significantly differ between the CRP and control groups.

Fig. 2: Macrophage identification

Photomicrographs of F4/80+ cells, which represent the total macrophage population in the CRP group (A) and the control group (B). CD206+ cells represent the M2 macrophage population in the CRP group (C) and the control group (D).

Fig. 3: Changes in the size of the macrophage fractions

Group data showing that tumors from CRP-treated mice contain significantly larger numbers of F4/80+ macrophages (A), whereas there is no difference in the CD206+ macrophage counts between CRP-treated and control mice (B). Consequently, tumors from CRP-treated mice showed significantly smaller M2 macrophage fractions (C).

Fig. 4: mRNA expression in tumors

Expression levels of IFN-γ, TNF, IL-4, IL-10 and CSF-1 mRNA in tumor samples were normalized to those of β-actin and shown as the IFN-γ, TNF, IL-4, IL-10 and CSF-1 mRNA / β-actin mRNA. Levels of CSF-1 mRNA were significantly lower in tumors from CRP-treated mice than control mice.

Fig. 5: Intratumoral angiogenesis

Numbers of intratumoral CD31+ vessels were significantly smaller in the CRP group than the control group.

Fig. 1

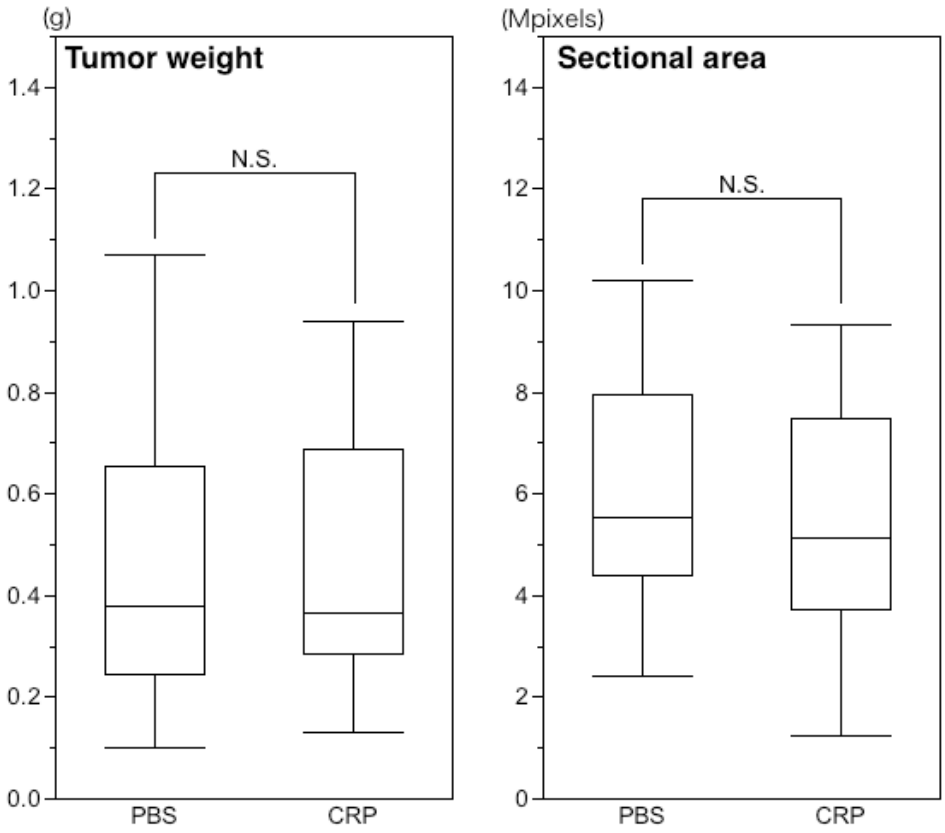
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Fig. 2

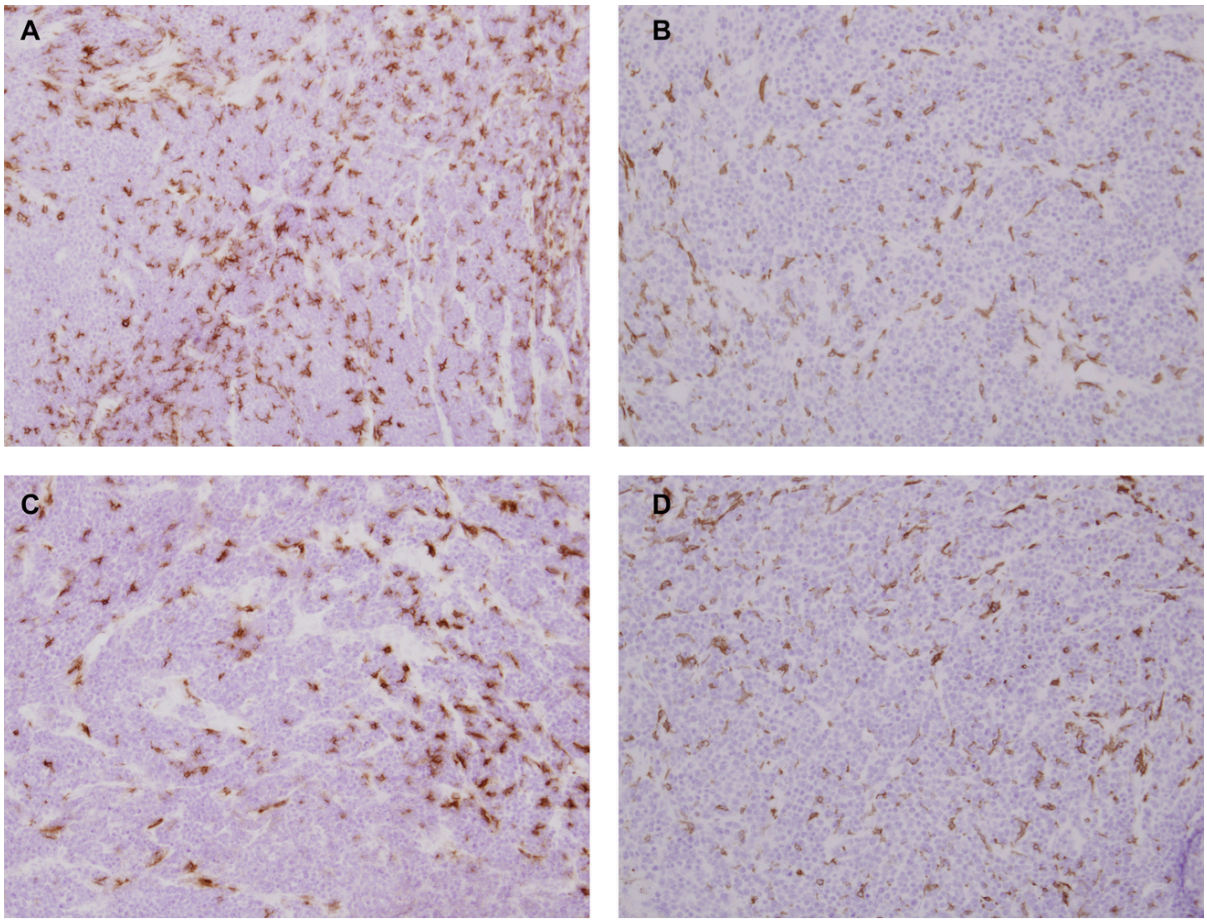

Fig. 3

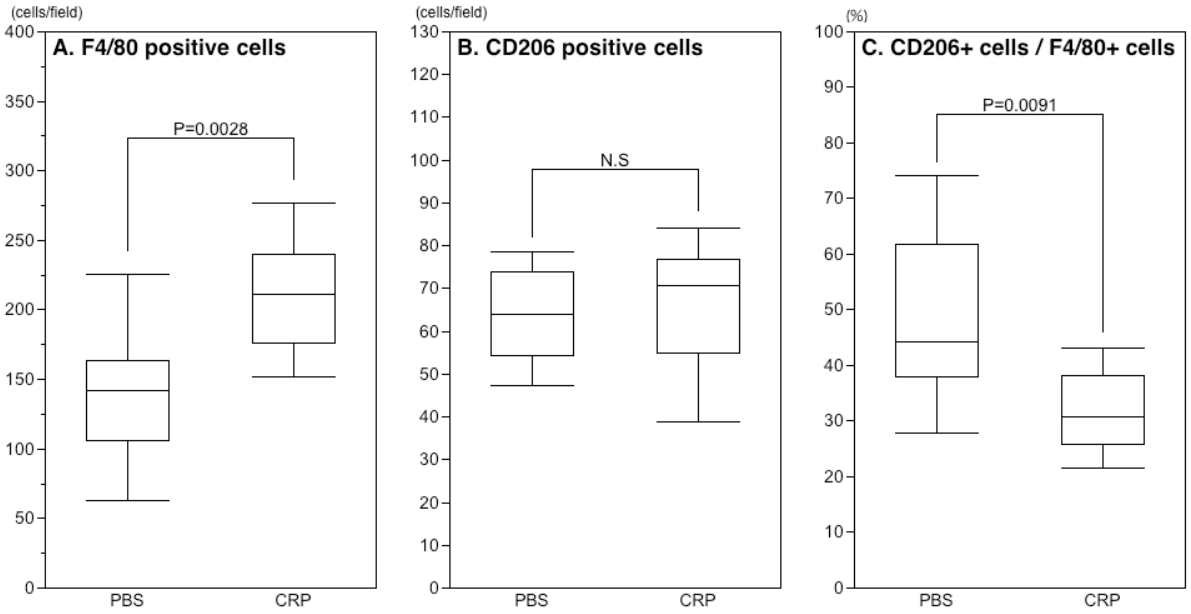
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Fig. 4

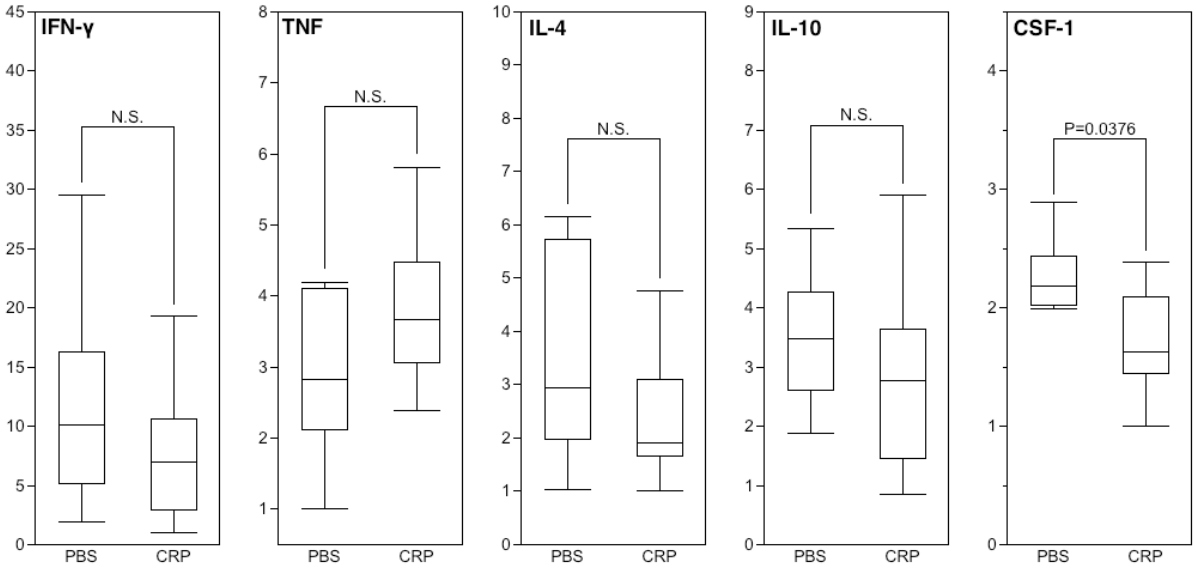
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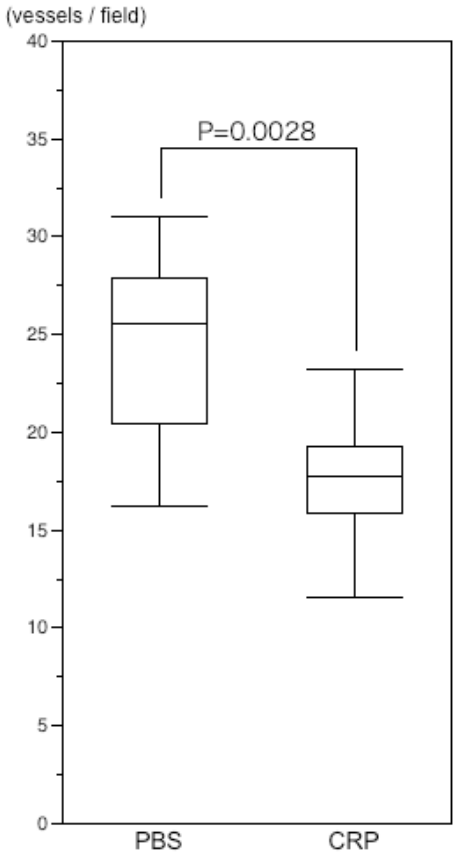
Fig. 5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