# Transient complement inhibition promotes a tumor-specific immune response through the implication of natural killer cells.

Valérie Janelle[1,2], Marie-Pierre Langlois[1], Esther Tarrab[1], Pascal Lapierre[1], Laurent Poliquin[2] and Alain Lamarre[1,2]

[1]Immunovirology Laboratory, Institut national de la recherche scientifique, INRS-Institut Armand-Frappier, Laval, Québec, Canada

[2]Biomed Research Center, Department of Biology, Université du Québec à Montréal, Montréal, Québec, Canada

Correspondence should be addressed to:

A. Lamarre: Immunovirology Laboratory, Institut national de la recherche scientifique, INRS-Institut Armand-Frappier, Laval, Québec, Canada, H7V 1B7

Phone: 450-687-5010 ext: 4262,

Fax: 450-686-5501,

E-mail: alain.lamarre@iaf.inrs.ca

**Running title**: Anti-tumor immune response induced by decomplementation

**Keywords**: cancer; complement; NK cell; immunotherapy; melanoma

**Diclosure**: The authors disclose no potential conflicts of interest.

Word count: 2594

Figures: 4

## ABSTRACT

While the role of the complement system in cancer development has been studied, its involvement in the development of an anti-tumoral immune response remains poorly understood. Using cobra venom factor to inhibit the complement cascade via C3 molecule exhaustion in immunocompetent mice bearing B16gp33 melanoma tumors, we show that transient inhibition of the complement system allowed for the development of a more robust gp33-specific anti-tumoral $CD8^+$ T cell response. This immune response proved to be NK-dependent suggesting an interaction of complement proteins with this cellular subset leading to T lymphocyte activation and enhanced cytotoxic T cell activity against tumor cells. This study demonstrates for the first time the implication of the complement system in the development of NK-mediated cytotoxic T cell-dependent anti-tumoral immune responses. The complement pathway could therefore be a potent therapeutic target in cancer patients to improve NK-dependent anti-tumoral immune responses.

# INTRODUCTION

Historically, the complement system has been considered a 'complementing' player in the innate immune response that mediated clearance of various pathogens or dead cells and could induce inflammation (1). In cancer, activated complement proteins were primarily described for their role in tumor defense either through complement-dependent cytotoxicity (2) or antibody-dependent cell-mediated cytotoxicity (3). However, the complement anaphylatoxins C3a and C5a can increase the activation of phosphatidylinositol 3-kinase (PI3K), Akt and mammalian target of rapamycin (mTOR) (4), proteins strongly associated with neoplasia when overexpressed, suggesting a more complex role for complement proteins in tumorogenicity. Furthermore, mice deficient in C3 or C4 show significantly decreased TC-I subcutaneous tumor proliferation compared with wild-type mice (5) while the C5a protein can be involved in $CD8^+$ T cell inhibition (6).

NK cells are large granular lymphocytes part of the innate immune system implicated in host defense against tumors and pathogens (7). NK cells express FcγR, which interacts with immunoglobulin bound to antigen on target cells, leading to NK cell activation (8) and production of IFNγ and TNFα (9). NK cells can also complement cytotoxic T lymphocytes (CTLs) by killing MHC-I-deficient cells that failed to be recognize by T cells (7). NK cells have been shown to cross-talk with dendritic cells (DCs); their effector functions being stimulated through direct contact with activated DCs (10). However, DC/NK-cell interactions are bi-directional, resulting not only in NK-cell activation but also DC maturation depending on the activation status of both cells (11). Soluble factors such as TNFα and IFNγ, as well as cell-to-cell contact, are required for NK-mediated DC activation (12).

Little is known about the link between complement and NK cells but evidence suggests that complement receptor CR3, a receptor implicated in phagocytosis mediated by complement fragment iC3b-opsonized targets (13), is expressed by NK cells and cytotoxic T cells displaying similar functions as CR3 expressed on phagocytes (14). Given that NK cells can be involved in tumor control and that proteins from the complement pathway may interact with NK cells to suppress or limit their functions, we hypothesized that proteins in the complement pathway may favor tumor growth through the impediment of NK cells. Herein, we found that transient inhibition of complement increased NK cell numbers in spleen and tumor leading to the induction of an increased anti-tumoral CTL response.

## MATERIAL & METHODS

### *Cell lines*

Murine B16 and B16gp33 melanoma cells were obtained from Dr. A. Ochsenbein (Bern, Switzerland). The B16gp33 cell (H2-$D^b$) were derived from B16.F10 cells transfected with a DNA minigene encoding the immunodominant $CD8^+$ T cell epitope of the lymphocytic choriomeningitis virus (glycoprotein aa 33-41) (15). Cells were grown in Dulbecco's modified Eagle's medium (Life Technologies, Rockville, MD) supplemented with 10% fetal bovine serum (FBS) and 200 μg/ml of G418 to select for retention of the gp33 minigene.

### ***Enzyme-linked immunosorbent assay*.**

C3 molecule concentration was tested in mice sera at the indicated time points by ELISA. Briefly, blood was harvested and incubated at 37°C to coagulate. Samples were centrifuged and the serum fraction was used. Serial dilutions were added onto an IgM-coated 96-well plate (5μg/ml; Sigma-Aldrich, Oakville, ON). Mouse anti-C3 coupled to biotin (Cedarlane, Burlington, ON) followed with streptavidin-horseradish peroxidase (Southern Biotech, Birmingham, AL) were incubated with samples. A solution of *o*-Phenylenediamine dihydrochloride (Sigma-Aldrich, Oakville, ON) was then used for the detection of C3 molecules. Optical density was measured by spectrophotometry at 490 nm.

### **In vivo *studies***.

All procedures were approved by the INRS Institutional Animal Care and Use Committee. To establish subcutaneous tumors, $5 \times 10^5$ B16 or B16gp33 cells in 100 μl PBS were injected into the flank of C57Bl/6 mice. Six days after tumor implantation, mice were injected with cobra venom factor (CVF; 20 μg/mouse; Quidel, Santa Clara, CA) intraperitoneally. For NK depletion experiments, 200 μg of purified monoclonal anti-NK1.1 antibodies from PK136

hybridoma, kindly provided by Dr. Suzanne Lemieux (Laval, Canada), were injected intraperitoneally two days prior to decomplementation (Fig. S2).

***Flow cytometry***.

Spleen and tumors were recovered from mice and dissociated *in vitro* to achieve single-cell suspensions using nylon 100 μm cell strainers (BD Falcon). Cells were washed, resuspended in phosphate-buffered saline containing 1% bovine serum albumin and 0.1% sodium azide (FACS buffer), and incubated with directly conjugated primary antibodies for 30 minutes at 4°C. Cells were then washed and resuspended in 200 μl FACS buffer containing 1% paraformaldehyde. Samples were acquired on a FACS Fortessa (BD Biosciences, San Diego, CA) and analyzed using Flowjo software (Tree Star, Ashland, OR). Anti-CD45 PE/CF-594, anti-CD25 APC and anti-NKp46 Alexa 700 were purchased from BD Biosciences. Anti-CD4 APC/Cy7, anti-CD4 FITC, anti-CD8 PE/Cy7, anti-CD62L Alexa 700, anti-CD44 PercP/Cy5.5, anti-B220 APC/Cy7, anti-CD11b Pacific Blue, anti-Gr1 PE/Cy5, anti-CD11c FITC and anti-F4/80 APC were purchased from BioLegend (San Diego, CA).

***Dendritic cell activation*.**

Single-cell suspensions were prepared from spleen and draining lymph node harvested 9 days after CVF injection. Red blood cells were lysed and remaining cells were resuspended in FACS buffer prior to CD11c-FITC and CD86-PE labeling (BioLegend, San Diego, CA). Samples were acquired on a FACS Fortessa (BD Biosciences, San Diego, CA) and analyzed using Flowjo software (Tree Star, Ashland, OR).

***NK intracellular staining assay*.**

For IFNγ intracellular staining, single-cell suspensions were prepared from spleen and tumor harvested 9 days after CVF injection. Cells were cocultured with YAC-1 cells (provided

by Dr. Suzanne Lemieux, Laval, Canada; at 10:1 effector-target ratio) with or without PMA-ionomycin. Cytokine production was measured in presence of monensin A (20 μg/ml), brefeldin A (10 μg/ml) and anti-CD107a FITC antibody for 5 hours. Cells were stained for surface markers, fixed, and permeabilized for intracellular staining using fixation and permeabilization buffers from BioLegend according to the manufacturer's instructions. IFNγ-PE and CD107a-FITC were obtained from BioLegend (San Diego, CA.)

***T lymphocyte intracellular staining assay*.**

For IFNγ and TNFα intracellular staining, single-cell suspensions were prepared from spleen (three mice per group) 9 days after CVF injection. Cytokine production in response to tumor antigen, was measured by incubation with the gp33 peptide (KAVYNFATC 1 μg/ml) in the presence of brefeldin A (10 μg/ml) and IL-2 (100 U/ml) for 5 hours. Cells were stained for surface markers then fixed, and permeabilized for intracellular staining using fixation and permeabilization buffers as described above. For degranulation assay and granzyme B intracellular staining, cells were incubated with the peptides in the presence of monensin A (20 μg/ml) and anti-CD107a FITC antibody for 5 hours. Cells were stained for surface markers, then fixed, and permeabilized for intracellular staining. IFNγ-PE and TNFα-APC were obtained from BioLegend (San Diego, CA) and granzyme B eFluor 450 was purchased from eBiosciences (San Diego, CA).

***Statistical analyses***.

A Student T test was used for group comparison and *P* values of less than 0.05 were considered significant.

## RESULTS AND DISCUSSION

### CVF-mediated decomplementation slows down early tumor progression and promotes immune cell infiltration

In recent years, there has been regained interest in the role of complement in various disease conditions. In cancer, the role of complement is unclear since it has been reported to play a role in both tumor clearance and progression (2, 3). Nevertheless, studies have indicated that blocking complement proteins might improve the efficacy of anti-tumor immunotherapy (16). Some complement activation products, like the anaphylatoxins C3a and C5a, are potent proinflammatory mediators and it is now well recognized that inflammation is able to both promote and exacerbate tumor growth.

To establish the role of the complement system in the generation of an anti-tumoral immune response, transient decomplementation was performed using cobra venom factor (CVF) 6 days following tumor administration, at the time when T cell priming is expected to occur. CVF is a structural and functional analog of the C3 molecule, which will act as a C3/C5 convertase. CVF-containing convertases are more stable and resistant to regulatory inhibitors and deplete complement activity by C3 consumption (17). Unlike complement protein knockout mice (eg. $C3^{-/-}$, $C4^{-/-}$ or $C5^{-/-}$), CVF does not affect splenic architecture thus allowing a complete and unaltered immune response to be mounted and analyzed (18). Since CVF-mediated complement depletion lasts approximately three days in treated mice (Fig. S1), we hypothesized that this short period of time could affect T cell priming. CVF-mediated transitory decomplementation soon after tumor implantation efficiently led to a significant slowdown of B16 melanoma tumor growth (Fig. 1A). Even if this was not associated with a significant increase in the percentage of tumor-infiltrating immune cells 9 days following decomplementation (Fig. 1B), it modulated the

composition of infiltrating cells. Strikingly, CVF treatment affected NK cell proportions, but had little to no effect on the proportions of $CD4^+$ T cells, regulatory T cells, $CD8^+$ T cells, B cells, neutrophils, macrophages and DCs (Fig. 1C).

An important mechanism used by malignancies to suppress immune responses against tumor antigens is abnormal myelopoiesis and the recruitment of myelomonocytic cells to the tumor microenvironment and peripheral lymphoid organs (19). In concordance with the study by Markiewski *et al*. (5), complement inhibition also significantly decreased myeloid-derived suppressor cells (MDSC) in tumor and spleen (Fig. 1C and Fig. S2). MDSC are a population of intermediately differentiated myeloid cells known to abolish cancer immunosurveillance therefore potentiating neoplastic proliferation (2). These cells express high levels of membrane C5aR (5). C5a/C5aR binding on MDSC promotes their migration and increases their production of immunosuppressive molecules such as reactive oxygen and nitrogen species, consequently inhibiting cytotoxic $CD8^+$ T lymphocytes and NK cells and stimulating tumorigenic cytokine production and angiogenesis (5, 20). MDSC can also be recruited to tumors by anaphylatoxin C5a deposited in the tumor vasculature (5).

**CVF treatment promotes a better NK cell availability**

NK cell proportions were greatly increased following decomplementation both in the tumor (eight-fold) and in the spleen (three-fold) (Fig. 2A). We next wanted to assess whether decomplementation also modulated NK cell function. Tumoral and splenic NK cells harvested 9 days after CVF treatment were not impaired functionally compared to non-treated animals, as determined by degranulation assay (CD107a) and IFNγ production after exposure to YAC-1 target cells. Furthermore, NK cells retained their ability to respond to *ex vivo* stimulation (Fig. 2B). Complement proteins are known to induce the production of transforming growth factor-β

(TGF-β) (21) which may facilitate angiogenesis, invasion and metastasis (22) and downregulation of IL-2Rβ expression as well as IFNγ secretion by NK cells (23). Our results suggest that it is unlikely that this pathway is involved here since NK cells harvested from CVF-treated mice are fully functional and respond normally.

**The complement system limits systemic T cell activation**

It is well recognized that the clearance of apoptotic cells through iC3b opsonisation and CR3 phagocytosis can be accompanied by a reduction in the expression of co-stimulatory molecules and an impaired maturation of dendritic cells (24). The recruitment of large numbers of NK cells may therefore be critical for optimal DC activation and subsequent induction of T cell responses in these low-inflammation conditions. Given that NK cells can cross-talk with DCs to mediate their activation, we wanted to assess their activation status after transient inhibition of the complement system. We did not observe any modulation of CD86 expression levels in splenic or draining lymph node DCs (Fig. 3A). We then sought to determine if the enhanced NK cell proportions at the tumor site could nevertheless lead to an increased T cell activation. The majority of $CD4^+$ and $CD8^+$ T cells present in the tumor were of the effector/effector memory phenotype and their proportions did not change following complement inhibition. However, in the spleen, CVF-mediated depletion of complement led to reduced proportions of naive T cells associated with increased proportions of effector $CD4^+$ and $CD8^+$ T cells (Fig. 3B). It is therefore likely that, in the absence of complement proteins, functional DCs have access to more tumor antigens due to increased NK-mediated cell lysis allowing for a better presentation to $CD8^+$ T lymphocytes.

**The enhancement of tumor-specific CTL responses in decomplemented mice is NK-dependent**

It has been reported that complement components can interfere with the binding of NK cells to monoclonal antibodies (mAbs) used in certain cancer treatments thus limiting NK cell-mediated lysis of antibody-coated target cells (25). It was also showed that this inhibition was dependent on C3b, likely by its binding to CR3 molecules abrogating signaling via Fcγ receptor III (CD16) (25). Furthermore, depletion of complement has been shown to enhance survival rates following mAb therapy in a syngeneic mouse model of lymphoma (26).

To ascertain if the enhanced tumor control we observed in decomplemented mice was a direct consequence of the increased proportions of NK cells in these mice, we depleted NK cells 4 days after tumor implantation. It is of importance to note that *in vivo* depletion with anti-NK1.1 antibody is complement-mediated, thus required prior to CVF treatment. We found that in the absence of NK cells, CVF treatment had no effect on tumor implantation and early development demonstrating that NK cells were necessary for the decreased tumor growth observed following decomplementation (Fig. 4A).

Considering that T cell activation in the spleen is also increased in the absence of complement, we wanted to determine if decomplementation also affected the induction of tumor-specific cytotoxic T lymphocytes. As assessed by intracellular cytokine staining of gp33 peptide-stimulated T cells from decomplemented and control B16gp33-bearing mice, we found that complement inhibition led to higher levels of tumor-specific (gp33) T cells secreting mainly TNFα (Fig. 4B). Accordingly, the enhancement of gp33-specific CTL induction by CVF was also abolished in the absence NK cells indicating that the effect of decomplementation on early tumor growth is largely mediated by CTLs (Fig. 4B). However, CVF-mediated decomplementation did not lead to superior levels of gp33-specific cytotoxicity as assessed by CD107a expression, a marker of recent degranulation (Fig. 4C). Thus, in the B16 melanoma

model, we demonstrate that CVF treatment limits early tumor growth and promotes a better availability of splenic and tumoral NK cells. This suggests that complement components and NK cells interact thus modulating tumor-specific CTL induction.

Therefore, our study has identified a previously unrecognized function for complement in tumor biology. We have shown that complement and NK cells interact and influence tumor growth by limiting tumor-specific CTL induction. Factors from the complement system may restrict NK cell availability in tumor tissue where NK cells would normally participate in the induction of a tumor-specific CTL response via, among other things, tumor cell lysis. Furthermore, complement proteins could also promote infiltration of the tumor by MDSCs that could then suppress lymphocyte function (Fig. 4D).

Many drugs targeting complement proteins are currently in clinical trials and few side effects have been reported (1, 27). CVF has also been used in laboratory animals for the treatment of various diseases. One of the limits of this treatment alternative in the clinic is the high immunogenicity of this compound (28). However, a human C3 hybrid, containing crucial regions of the CVF β chain (so-called humanized CVF or HC3-1496), has been created for this purpose (29). It will be of great interest to test other complement inhibitors currently in development in various solid tumor models to determine if sustained treatment will favour the induction of a better tumor-specific immune response and promote $CD8^+$ T cell expansion. Thus, a better understanding of the link between complement proteins and NK cells in the development of tumors could lead to the design of new therapeutic strategies against cancer.

## ACKNOWLEDGMENTS

The authors thank Simon Janelle for his help on the schematic illustration of the proposed model. This work was supported by the Jeanne and J.-Louis Lévesque Chair in Immunovirology to A.L. from the J.-Louis Lévesque Foundation. V.J. and M.-P.L. acknowledge studentship support from the Fondation Armand-Frappier. P.L. holds a Fonds de recherche du Québec - Santé (FRQS) postdoctoral award and a Thomas F. Nealon, III Postdoctoral Research Fellowship from the American Liver foundation.

## REFERENCES


1. Ricklin D, Lambris JD. Complement-targeted therapeutics. Nat Biotechnol. 2007;25(11):1265-75.
2. Ostrand-Rosenberg S. Cancer and complement. Nat Biotechnol. 2008;26(12):1348-9.
3. Gelderman KA, Tomlinson S, Ross GD, Gorter A. Complement function in mAb-mediated cancer immunotherapy. Trends Immunol. 2004;25(3):158-64.
4. Venkatesha RT, Thangam EB, Zaidi AK, Ali H. Distinct regulation of C3a-induced MCP-1/CCL2 and RANTES/CCL5 production in human mast cells by extracellular signal regulated kinase and PI3 kinase. Mol Immunol. 2005;42(5):581-7.
5. Markiewski MM, DeAngelis RA, Benencia F, Ricklin-Lichtsteiner SK, Koutoulaki A, Gerard C, et al. Modulation of the antitumor immune response by complement. Nat Immunol. 2008;9(11):1225-35.
6. Rutkowski MJ, Sughrue ME, Kane AJ, Mills SA, Parsa AT. Cancer and the Complement Cascade. Mol Cancer Res. 2010;8(11):1453-65.
7. Moretta A, Bottino C, Mingari MC, Biassoni R, Moretta L. What is a natural killer cell? Nat Immunol. 2002;3(1):6-8.
8. Arase N, Arase H, Park SY, Ohno H, Ra C, Saito T. Association with FcRγ Is Essential for Activation Signal through NKR-P1 (CD161) in Natural Killer (NK) Cells and NK1.1+ T Cells. The Journal of Experimental Medicine. 1997;186(12):1957-63.
9. Roda JM, Parihar R, Lehman A, Mani A, Tridandapani S, Carson WE. Interleukin-21 Enhances NK Cell Activation in Response to Antibody-Coated Targets. The Journal of Immunology. 2006;177(1):120-9.

10. Fernandez NC, Lozier A, Flament C, Ricciardi-Castagnoli P, Bellet D, Suter M, et al. Dendritic cells directly trigger NK cell functions: Cross-talk relevant in innate anti-tumor immune responses in vivo. Nat Med. 1999;5(4):405-11.

11. Vitale M, Chiesa MD, Carlomagno S, Pende D, Aricò M, Moretta L, et al. NK-dependent DC maturation is mediated by TNFα and IFNγ released upon engagement of the NKp30 triggering receptor. Blood. 2005;106(2):566-71.

12. Piccioli D, Sbrana S, Melandri E, Valiante NM. Contact-dependent Stimulation and Inhibition of Dendritic Cells by Natural Killer Cells. The Journal of Experimental Medicine. 2002;195(3):335-41.

13. Caron E, Hall A. Identification of Two Distinct Mechanisms of Phagocytosis Controlled by Different Rho GTPases. Science. 1998;282(5394):1717-21.

14. Muto S, Větvička V, Ross G. CR3 (CD11b/CD18) expressed by cytotoxic T cells and natural killer cells is upregulated in a manner similar to neutrophil CR3 following stimulation with various activating agents. J Clin Immunol. 1993;13(3):175-84.

15. Prevost-Blondel A, Zimmermann C, Stemmer C, Kulmburg P, Rosenthal FM, Pircher H. Tumor-infiltrating lymphocytes exhibiting high ex vivo cytolytic activity fail to prevent murine melanoma tumor growth in vivo. J Immunol. 1998;161(5):2187-94.

16. Macor P, Tedesco F. Complement as effector system in cancer immunotherapy. Immunol Lett. 2007;111(1):6-13.

17. Vogel CW, Smith CA, Müller-Eberhard HJ. Cobra venom factor: structural homology with the third component of human complement. The Journal of Immunology. 1984;133(6):3235-41.

18. JE M, CK F, S J, Y W, MC C, SH. S. The allogeneic T and B cell response is strongly dependent on complement components C3 and C4. Transplantation. 2001;72(7):1310-8.
19. Gallina G, Dolcetti L, Serafini P, Santo CD, Marigo I, Colombo MP, et al. Tumors induce a subset of inflammatory monocytes with immunosuppressive activity on CD8+ T cells. The Journal of Clinical Investigation. 2006;116(10):2777-90.
20. Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. Nature. 2008;454(7203):436-44.
21. Gionanlis L, Alexopoulos E, Papagianni A, Leontsini M, Memmos D. Fibrotic Mechanisms in Idiopathic Rapidly Progressive Glomerulonephritis: The Role of TGF-β1 and C5b-9. Ren Fail. 2008;30(2):239-46.
22. Derynck R, Akhurst RJ, Balmain A. TGF-beta signaling in tumor suppression and cancer progression. Nat Genet. 2001;29(2):117-29.
23. Penafuerte C, Galipeau J. TGFβ secreted by B16 melanoma antagonizes cancer gene immunotherapy bystander effect. Cancer Immunol Immunother. 2008;57(8):1197-206.
24. Morelli AE, Larregina AT, Shufesky WJ, Zahorchak AF, Logar AJ, Papworth GD, et al. Internalization of circulating apoptotic cells by splenic marginal zone dendritic cells: dependence on complement receptors and effect on cytokine production. Blood. 2003;101(2):611-20.
25. Wang S-Y, Racila E, Taylor RP, Weiner GJ. NK-cell activation and antibody-dependent cellular cytotoxicity induced by rituximab-coated target cells is inhibited by the C3b component of complement. Blood. 2008;111(3):1456-63.
26. Wang S-Y, Veeramani S, Racila E, Cagley J, Fritzinger DC, Vogel C-W, et al. Depletion of the C3 component of complement enhances the ability of rituximab-coated target cells

to activate human NK cells and improves the efficacy of monoclonal antibody therapy in an in vivo model. Blood. 2009;114(26):5322-30.

27. Kohl J. Drug evaluation: the C5a receptor antagonist PMX-53. Curr Opin Mol Ther. 2006;8:529-38.

28. Gowda DC, Petrella EC, Raj TT, Bredehorst R, Vogel CW. Immunoreactivity and function of oligosaccharides in cobra venom factor. The Journal of Immunology. 1994;152(6):2977-86.

29. Fritzinger DC, Hew BE, Thorne M, Pangburn MK, Janssen BJC, Gros P, et al. Functional characterization of human C3/cobra venom factor hybrid proteins for therapeutic complement depletion. Developmental & Comparative Immunology. 2009;33(1):105-16.

## FIGURE LEGENDS

**Figure 1.** Effect of CVF treatment on B16 tumor growth and immune cell infiltration. C57Bl/6 mice were injected subcutaneously with B16 (day 0) and CVF was administered by intra-peritoneal injection on day 6. **A,** Tumor growth was measured at indicated time points. Data show the mean ± SEM and are representative of two independent experiments. $^{**}P < 0.01$. **B,** Nine days following CVF treatment, tumor and spleen were harvested and total tumor $CD45^+$ cells and **C,** immune cell subpopulation proportions of CD4+ T cells ($CD4^+CD25^-$), regulatory T cells ($CD4^+CD25^+$), CD8+ T cells, B cells ($B220^+$), NK ($NKp46^+$), MDSC ($Gr1^+CD11b^+$), neutrophils ($Gr1^+CD11b^-$), dendritic cells ($Gr1^-CD11b^+CD11c^+$) and macrophages ($Gr1^-CD11b^+F4/80^+$) were determined.

**Figure 2.** Decomplementation increases NK cell availability without affecting their effector function. B16gp33-bearing mice were treated or not with CVF. On day 9 following treatment, spleen and tumor were harvested. **A,** Percentage of NK cells among $CD45^+$ cells. Mean ± SEM are shown (n=9). $^{***}P < 0.001$. **B**, NK degranulation assay in YAC-1 co-cultures (10:1 Effector-target ratio) with or without PMA-ionomycin. Data are the mean ± SEM of three independent experiments (n=9).

**Figure 3.** Increased systemic T cell activation following CVF treatment. **A**, Spleen and draining lymph nodes were harvested 9 days following CVF treatment and DC activation was assessed by CD11c and CD86 staining. Data are the mean ± SEM of two independent experiments (n=6). **B,** T cell activation was assessed by CD62L and CD44 staining on spleen and tumor. Naïve ($CD62L^+CD44^-$), effector/effector memory ($CD62L^-$) and central memory T cells

364 ($CD62L^+CD44^+$) are shown. Data are the mean ± SEM of three independent experiments (n=9).
365 $*P < 0.05$, $**P < 0.01$
366
367 **Figure 4.** Influence of NK cells on CVF therapy. **A,** C57Bl/6 mice were injected subcutaneously
368 with B16 cells (n=6-8), NK-depleted or not at day 4 and treated or not with CVF at day 6. Tumor
369 growth was measured at indicated time points. Data show the mean ± SEM and are
370 representative of two independent experiments. $*P < 0.05$, $**P < 0.01$. **B**, C57Bl/6 mice were
371 injected subcutaneously with B16gp33 cells, NK-depleted or not at day 4 and treated or not with
372 CVF at day 6. Nine days following CVF treatment, spleen was harvested and an *ex vivo*
373 stimulation with the gp33 (KAVYNFATC) peptide was performed to analyze cytokine secretion
374 and **C,** degranulation. Data are the mean ± SEM of two independent experiments (n=6). **D,**
375 Proposed model for the influence of complement proteins and NK cells in the B16 melanoma
376 model. Inhibition of the complement system by CVF allows for increased NK cell availability in
377 tumor tissue. This expanded proportion of NK cells leads to increased tumor cell lysis and
378 antigen release thus facilitating activation of tumor-specific $CD8^+$ T lymphocytes. Inhibition of
379 complement proteins also acts on MDSC and limits the immunosuppressive tumor
380 microenvironment leading to increased CTL activation. Abbreviations: CTL, cytotoxic T
381 lymphocyte; CVF, cobra venom factor; IFNγ, interferon gamma; iDC, immature dendritic cell;
382 mDC, mature dendritic cell; MDSC, myeloid-derived suppressor cell; NK, natural killer cell;
383 TNFα, tumor-necrosis factor alpha.
384
385 **Supplemental Figure 1.** Complement system inhibition by CVF. C57Bl/6 mice (n=3) were
386 injected intraperitoneally with CVF (20 μg) at day 0. C3 molecule concentration in sera was

measured every following day and ratios over non-treated animals are shown. Data show the mean ± SEM and are representative of three independent experiments.

**Supplemental Figure 2.** MDSC proportions following CVF treatment. B16-bearing mice were treated or not with CVF. On day 9 following treatment, spleen and tumor were harvested. Percentage of MDSC among $CD45^+$ cells is shown. Mean ± SEM (n=9). ***$P < 0.001$.

**Supplemental Figure 3.** Effectiveness of NK cell depletion. C57Bl/6 and $RAG^{-/-}$ mice (which are known to possess high numbers of NK and NKT cells) were injected intra-peritoneally with either 100 or 200µg of anti-NK1.1 antibody (clone PK136). Spleen was harvested two days later and analyzed. NKp46 and NK1.1 staining of $CD3^-$ cells are shown. Complete depletion was achieved with 200µg in both mouse strains.

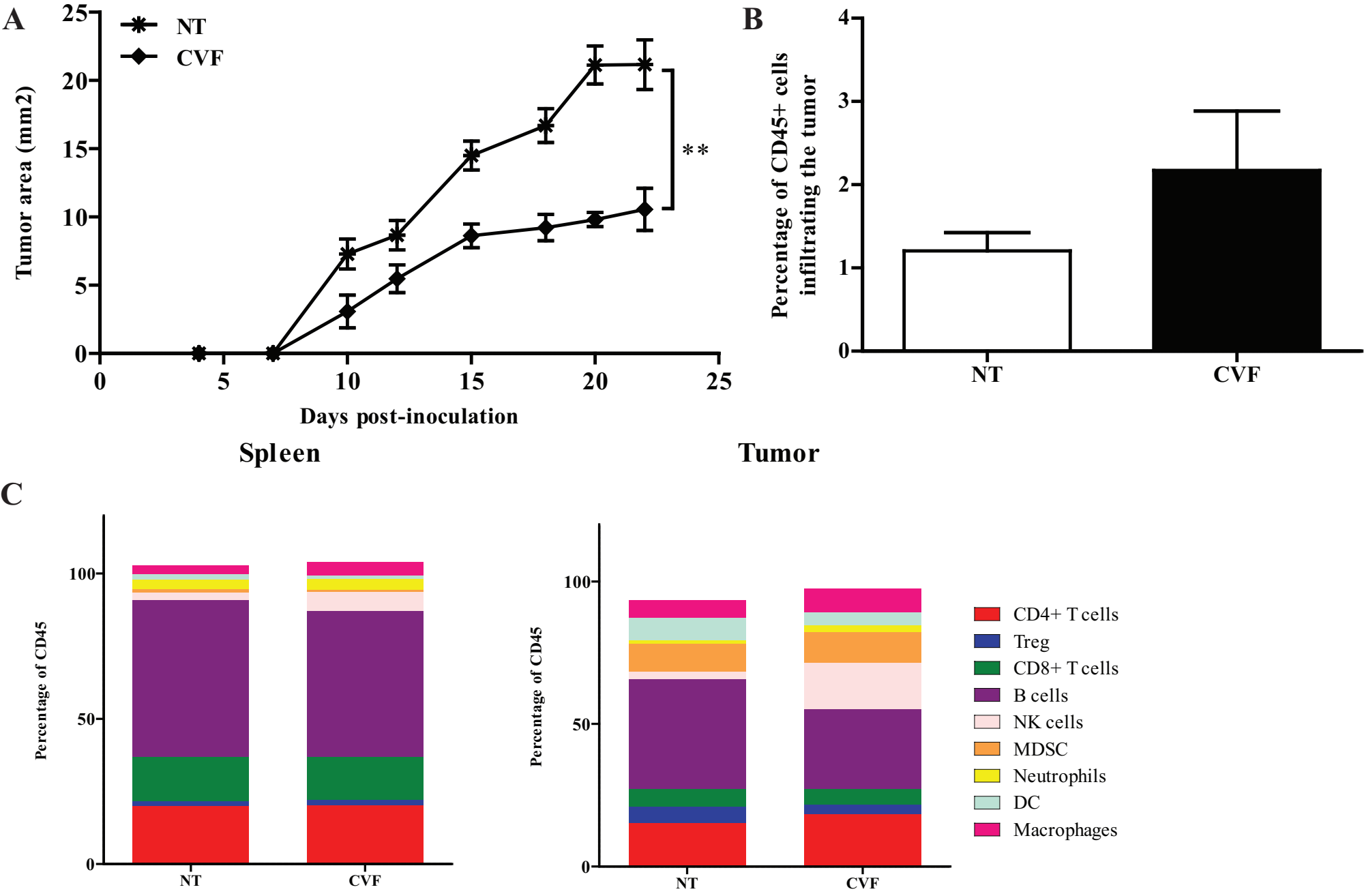
A
NT
CVF
Tumor area (mm2)
Days post-inoculation
**
B
Percentage of CD45+ cells infiltrating the tumor
NT
CVF
Spleen
Tumor
C
Percentage of CD45
NT
CVF
CD4+ T cells
Treg
CD8+ T cells
B cells
NK cells
MDSC
Neutrophils
DC
Macrophages

**Figure 1.**

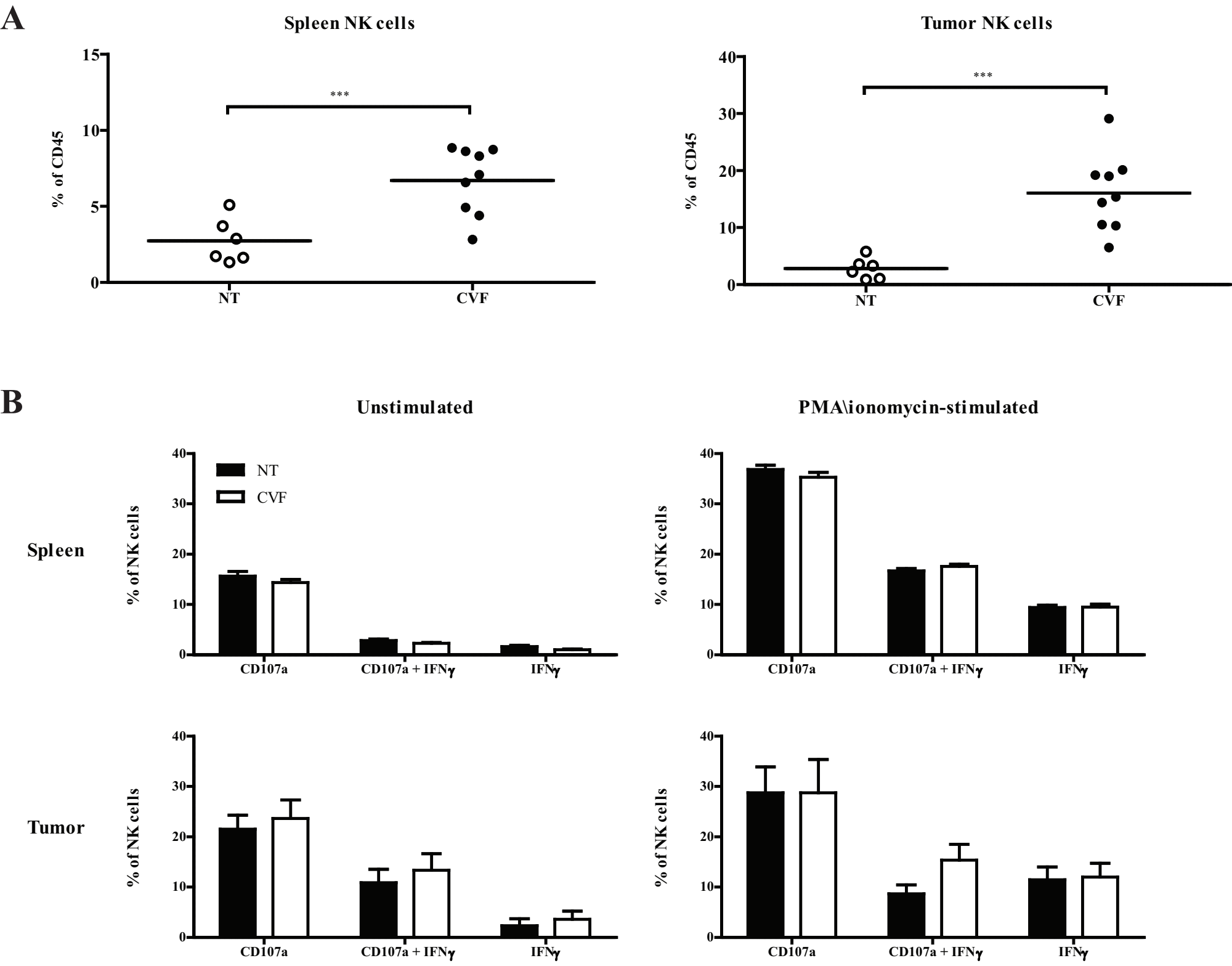
A
Spleen NK cells
% of CD45
15
10
5
0
***
NT
CVF
Tumor NK cells
% of CD45
40
30
20
10
0
***
NT
CVF
B
Unstimulated
PMA\ionomycin-stimulated
Spleen
Tumor
NT
CVF
% of NK cells
40
30
20
10
0
CD107a
CD107a + IFNγ
IFNγ

**Figure 2.**

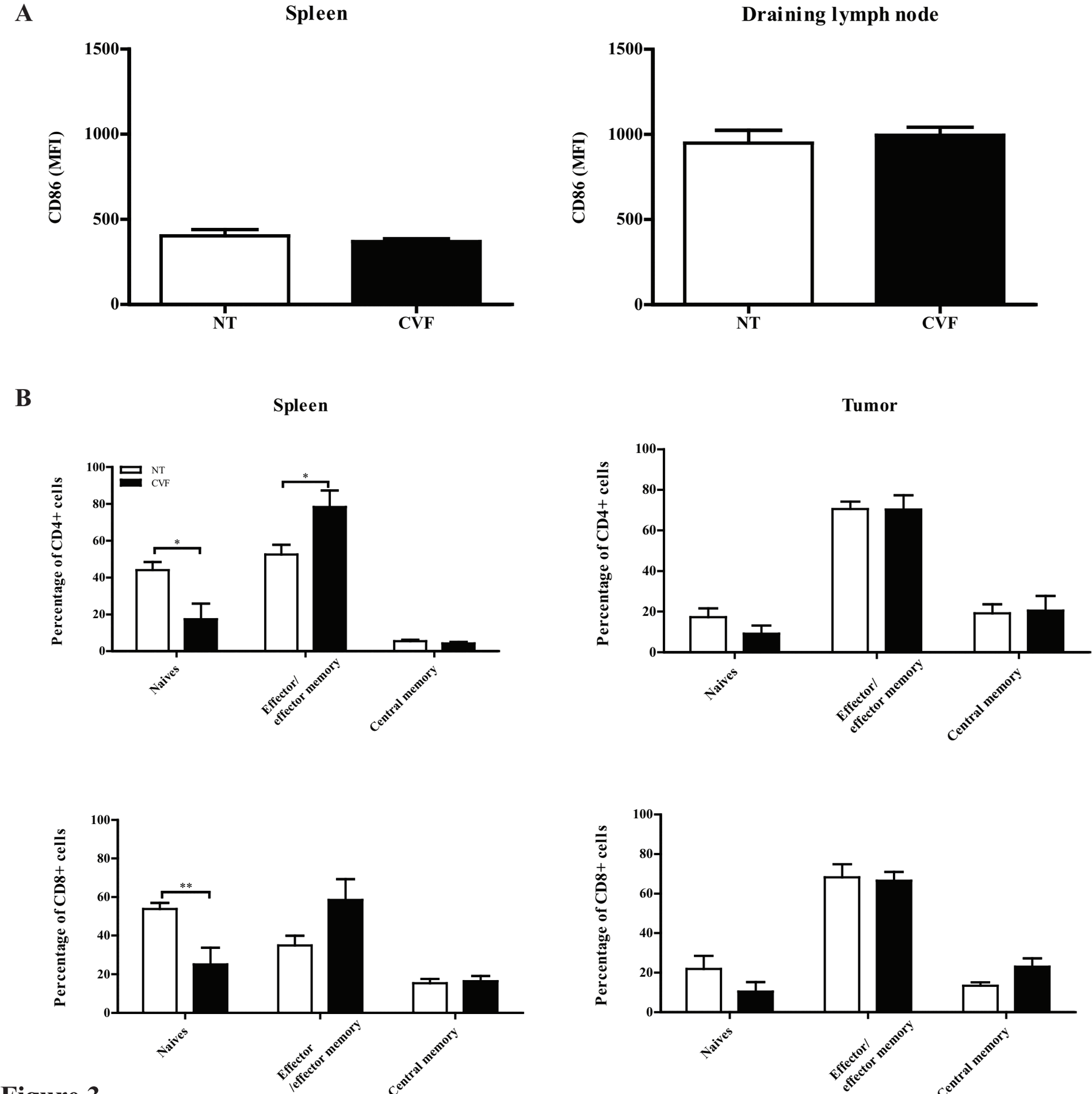
A
Spleen
Draining lymph node
CD86 (MFI)
1500
1000
500
0
NT
CVF
B
Spleen
Tumor
Percentage of CD4+ cells
Percentage of CD8+ cells
NT
CVF
*
**
100
80
60
40
20
0
Naives
Effector/
effector memory
Effector
/effector memory
Central memory

Figure 3.

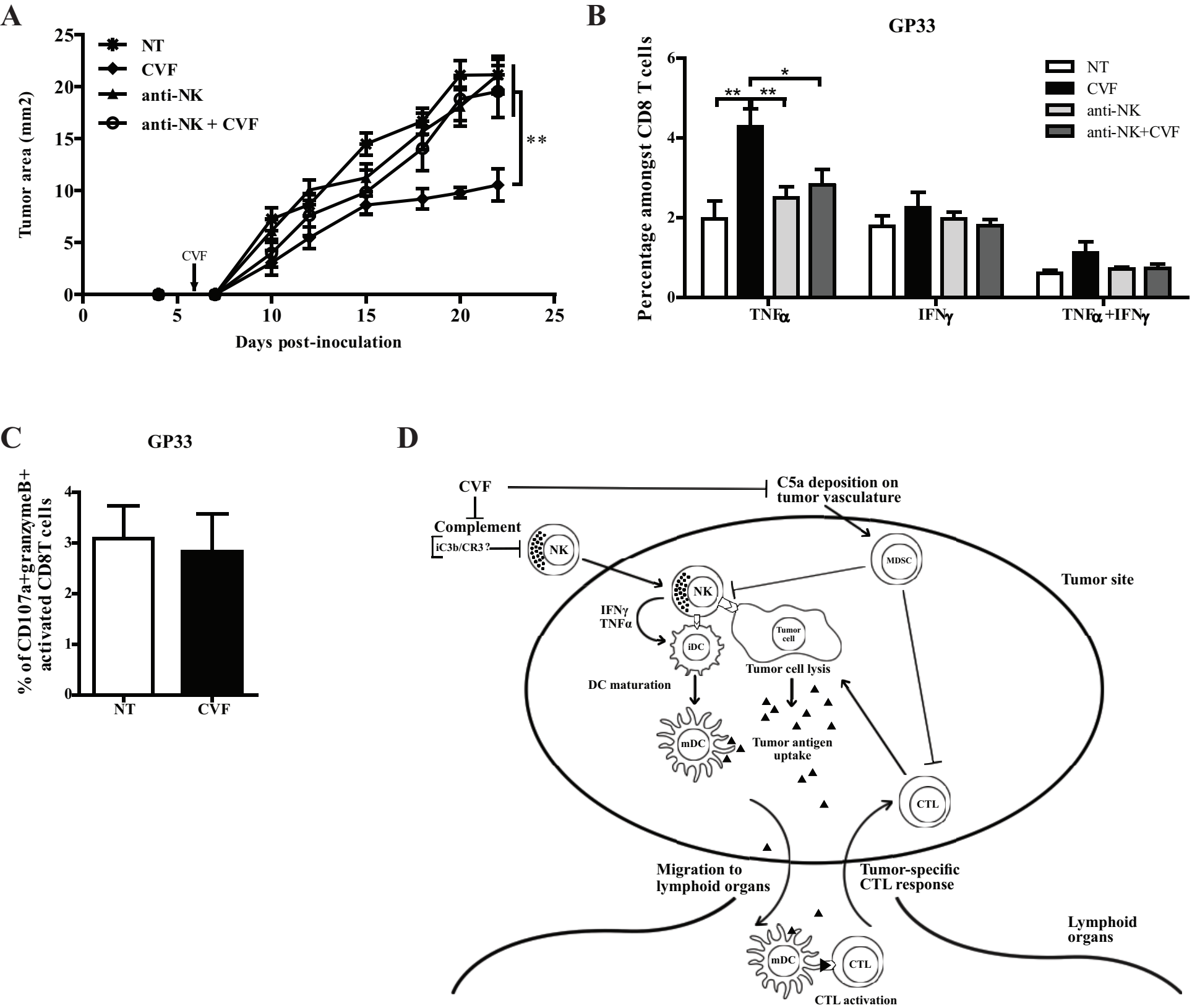
A
NT
CVF
anti-NK
anti-NK + CVF
Tumor area (mm2)
Days post-inoculation
CVF
**
B
GP33
Percentage amongst CD8 T cells
NT
CVF
anti-NK
anti-NK+CVF
TNFα
IFNγ
TNFα+IFNγ
C
GP33
% of CD107a+granzymeB+ activated CD8T cells
NT
CVF
D
CVF
Complement
iC3b/CR3 ?
C5a deposition on tumor vasculature
NK
MDSC
Tumor site
IFNγ
TNFα
iDC
Tumor cell
Tumor cell lysis
DC maturation
mDC
Tumor antigen uptake
CTL
Migration to lymphoid organs
Tumor-specific CTL response
Lymphoid organs
CTL activation

**Figure 4.**