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Research Article

Immunology

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# C3d regulates immune checkpoint blockade and enhances antitumor immunity

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Despite expression of immunogenic polypeptides, tumors escape immune surveillance by engaging T cell checkpoint regulators and expanding Tregs, among other mechanisms. What orchestrates these controls is unknown. We report that free C3d, a fragment of the third component of complement, inside tumor cells – or associated with irradiated tumor cells and unattached to antigen – recruits, accelerates, and amplifies antitumor T cell responses, allowing immunity to reverse or even to prevent tumor growth. C3d enhances antitumor immunity independently of B cells, NK cells, or antibodies, but it does so by increasing tumor infiltrating CD8<sup>+</sup> lymphocytes, by depleting Tregs, and by suppressing expression of programmed cell death protein 1 (PD-1) by T cells. These properties of C3d appear specific for the tumor and dependent on complement receptor 2, and they incur no obvious systemic toxicity. The heretofore unrecognized properties of free C3d suggest that protein might determine the effectiveness of immune surveillance and that increasing availability of the protein might prove advantageous in the treatment or prevention of cancer and premalignant conditions.

## Introduction

Recent reports showing that immune checkpoint inhibitors can facilitate the emergence of protective immunity and control of malignancies (1, 2) confirm the idea that immune surveillance can provide a primary defense against malignant transformation and that abrogation of immune surveillance might be a key step in the development of cancer. Immune surveillance of cancer (and normal) cells is abrogated at certain checkpoints by activation of the PD-1/PD-L1 pathway and by the actions of Tregs and regulatory B cells, among other mechanisms (3). Still unclear, however, is how tumor cells and normal cells determine whether to engage these regulatory mechanisms. Here, we report observations suggesting that complement proteins could have a pivotal role in favoring immune surveillance over immune regulation.

The complement system consists of more than 50 proteins — some circulating in the blood, others bound to cell membranes. The complement system is activated by antibodies, lectins, and the surface of damaged or foreign cells, but also spontaneously owing to intrinsic instability of the C3 component. C3 contains an internal thioester bond that undergoes spontaneous hydrolysis, increasing susceptibility of C3 to cleavage to form C3b. The exposed carbonyl group of C3b forms a bond with neighboring nucleophiles. The properties of the surface to which C3b binds, among other factors, determines whether C3b will form a catalytic complex with another protein, generating downstream effectors, or whether it will be degraded to a catalytically inactive fragment, C3d. Although C3d cannot further activate complement, it can bind to complement receptor 2 (CR2) expressed mainly on follicular DCs (FDCs) and B cells (4). Interaction of C3d with CR2 enhances antibody responses by reducing the threshold for B cell activation (5) and facilitates retention and internalization of antigens (6). For example, conjugation of 3 molecules of C3d to antigens decreases by  $1 \times 10^4$  the amount of antigen needed to elicit antibody responses.

**Conflict of interest:** MC and JLP are inventors on a pending patent for use of C3d as a cancer therapeutic.

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We made the unanticipated observation that the presence of C3d as a free peptide in living tumor cells or in a killed tumor cell vaccine vitiates restraints on immune surveillance, allowing rapid and robust control of lethal cancers in mice. We show that this function of C3d is completely independent of B cells or mature FDCs and, hence, represents a heretofore unrecognized function of C3d in the governance of protective cellular immunity.

## Results

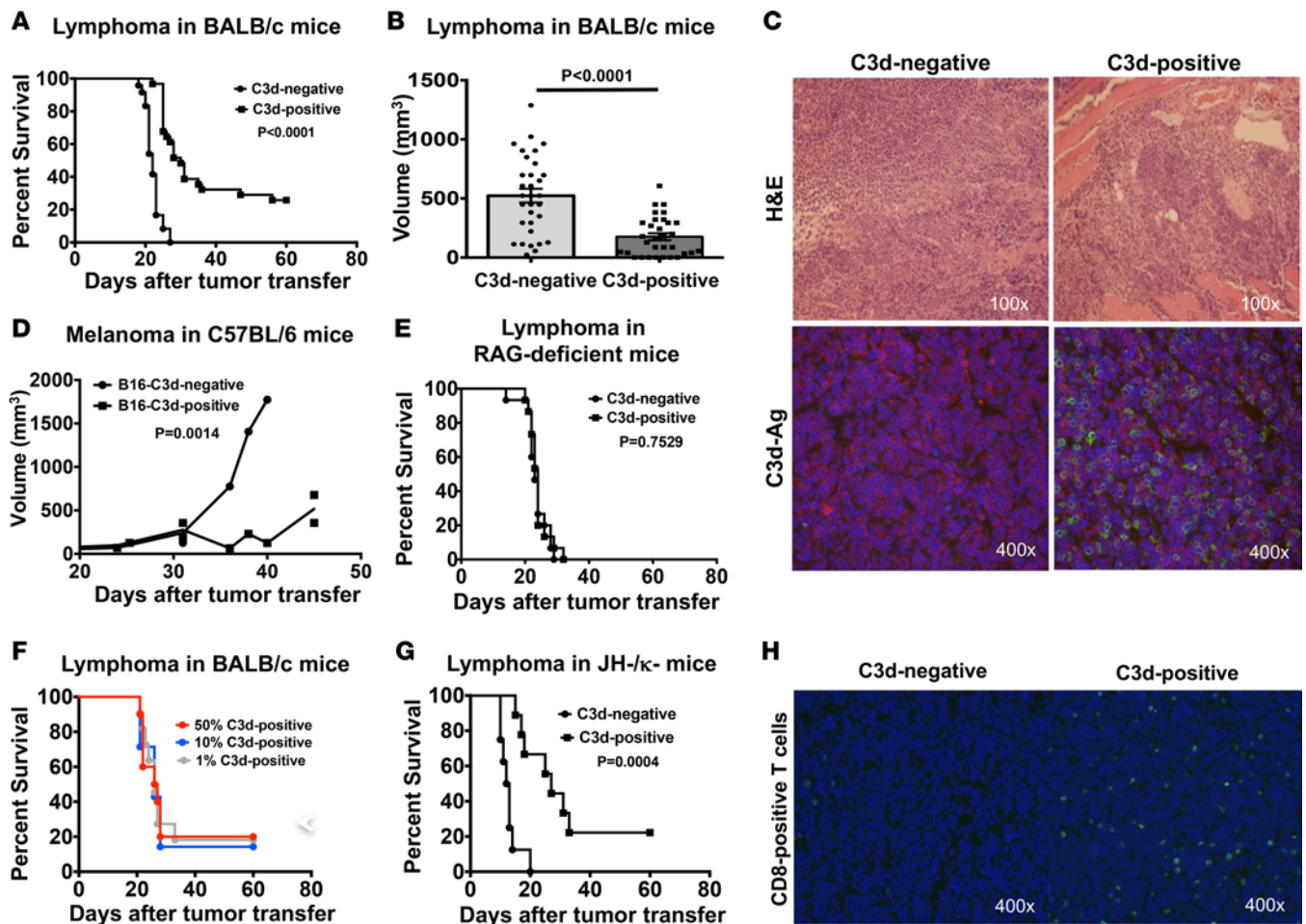
Our observations emerged in the course of testing a vaccine designed to evoke broadly neutralizing antibodies against HIV. Because antigen with covalently attached C3d profoundly decreases the threshold for antibody responses (5), we developed DNA constructs that encoded murine C3d attached to a viral antigen, as previously described (7, 8). To test expression and immunogenicity, the constructs were expressed in 18-81 pre-B lymphoma cells originated by Abelson transformation (9, 10), and transfected lymphoma cells were introduced into isogenic mice. Protective immunity was readily discerned, as transfected lymphoma cells generated slowly growing tumors while mock transfected lymphoma cells generated rapidly growing tumors that invaded spleen and muscle, causing death within 28 days (Figure 1, A–C, and Supplemental Figure 1, A–D; supplemental material available online with this article; <https://doi.org/10.1172/jci.insight.90201DS1>). What we did not anticipate, however, was that control lymphoma cells expressing only C3d as a free polypeptide and no viral antigen grew slower than mock-transfected lymphoma cells in isogenic mice ( $P < 0.0001$ ), and 25% of the lymphomas spontaneously resolved (Figure 1, A–C). The impact of free C3d was not limited to lymphomas, as expression of C3d in B16 melanoma cells also slowed tumor growth and prolonged survival of mice in which the cells were introduced (Figure 1D). C3d, expressed as a free peptide, thus appeared to evoke resistance to tumors.

Resistance conferred by C3d depended entirely on adaptive immunity and did not have any direct impact on tumor growth, as C3d<sup>+</sup> and C3d<sup>-</sup> lymphomas grew and caused death at the same rate in RAG-deficient mice (Figure 1E). Also, C3d itself did not serve as a target, as mixtures of C3d<sup>+</sup> and C3d<sup>-</sup> tumor cells in ratios as low as 1:100 elicited resistance (Figure 1F). Resistance did require the native C3d structure, as it was vitiated by administration of CR2-Ig, which binds to and blocks C3d interactions with CR2 (11) (Table 1).

Unlike the known actions of C3d on B cell responses (5, 12), the protection against tumors did not depend on B cells or FDCs, as protection was fully manifest in B cell and Ig-deficient JH<sup>-/-</sup> κ<sup>-/-</sup> mice, which lack mature FDCs, as well (13) (Figure 1G). Protection depended on T cells, since a single infusion of anti-CD4 and anti-CD8 antibodies suppressed protection (Supplemental Figure 1E) and since C3d expression by lymphoma cells markedly augmented the number of tumor-infiltrating CD8<sup>+</sup> lymphocytes (Figure 1H). Thus, the presence of C3d polypeptide in even a small fraction of tumor cells hastened and amplified cell-mediated immune surveillance directed against endogenous tumor antigens.

To confirm that free C3d promoted adaptive immunity, we tested the impact of free C3d on the efficacy of killed tumor cell vaccines. As Figure 2A shows, vaccination with killed C3d<sup>+</sup> lymphoma cells protected mice from living lymphoma cells injected 35 days later, regardless of whether the living tumor cells expressed C3d. Vaccination with killed C3d<sup>-</sup> lymphoma cells (Figure 2B) conferred significantly less protection against living C3d<sup>-</sup> lymphomas and provided no incremental protection against living C3d<sup>+</sup> tumor cells. C3d also promoted adaptive immune surveillance of melanoma (Figure 2, C–F). Thus, mice vaccinated once with killed C3d<sup>+</sup> melanoma cells and later injected with living melanoma cells not expressing C3d lived longer and had, on average, 5.7-fold smaller tumors than nonvaccinated mice and 3-fold smaller tumors than mice vaccinated with C3d<sup>-</sup> melanoma cells (Figure 2, D–F). These results confirm that C3d, as a free polypeptide, promotes protective immunity directed at endogenous tumor antigens and not against C3d.

A function for free C3d in adaptive immunity, and particularly cell-mediated immunity, has not been previously reported. However, T cells have been reported to express CR2, the cognate receptor for C3d (14–17). We therefore wondered whether C3d might govern immune surveillance by interacting with CR2. At baseline, < 5% of Tregs in the spleens of naive mice expressed CR2 (Supplemental Figure 2A), consistent with some reports (18). After injection of C3d<sup>+</sup> lymphoma cells, Treg exhibited a striking increase in expression of CR2 mRNA ( $P = 0.01$ ) (Figure 3A and Supplemental Figure 2, A–D). However, the frequency of Treg CR2<sup>+</sup> cells, as measured by FACS, was decreased by almost 2-fold within C3d<sup>+</sup> lymphomas, relative to that frequency in C3d<sup>-</sup> lymphomas ( $P = 0.02$ ) (Figure 3, B and F), suggesting selective loss of CR2<sup>+</sup> Treg in C3d<sup>+</sup> lymphomas. Expression of CR2 on Treg anticipated depletion, as CR2<sup>+</sup> Tregs in C3d<sup>+</sup> lymphomas



**Figure 1. Free C3d in tumor cells enhances resistance of mice to tumors by promoting adaptive immunity.** (A) Survival of BALB/c mice challenged with  $5 \times 10^3$  isogenic lymphoma cells expressing ( $n = 31$ ) or not expressing ( $n = 24$ ) free murine C3d. (B) Lymphoma tumor volumes 10 days after injection of  $1 \times 10^7$  C3d<sup>+</sup> or C3d<sup>-</sup> cells. On average, C3d<sup>+</sup> tumors were 176 mm<sup>3</sup> and C3d<sup>-</sup> tumors 523 mm<sup>3</sup>. Data represent mean  $\pm$  SEM analyzed by Mann-Whitney 2-tailed test. (C) H&E (upper panels) and immunofluorescence staining (lower panels) of lymphoma tissue. C3d<sup>+</sup> and C3d<sup>-</sup> tumors have similar morphology (above). Both are recognized by antibodies against B220 (red; below), but only the transgenic C3d<sup>+</sup> lymphoma stains with antibodies against an ENV fusion construct used to detect expression of C3d (green; below). Images are representative of tissues from 5 mice analyzed per condition and a minimum of 3 images/mouse. (D) Melanoma tumor volumes at various times after s.c. injection of  $4 \times 10^4$  C3d<sup>+</sup> B16 ( $n = 10$ ), or  $4 \times 10^4$  C3d<sup>-</sup> ( $n = 9$ ) melanoma cells in syngeneic mice. (E) Survival of recombinase activating gene 2-deficient (RAG-2-deficient) mice injected with  $5 \times 10^3$  isogenic C3d<sup>+</sup> ( $n = 15$ ) or C3d<sup>-</sup> ( $n = 15$ ) lymphoma cells. (F) Survival of mice injected with  $5 \times 10^3$  lymphoma cells of which 1%–50% were C3d<sup>+</sup>. (G) Survival of B cell-deficient (JH<sup>-/-</sup>  $\kappa$ <sup>-/-</sup>) mice challenged with  $5 \times 10^3$  C3d<sup>-</sup> ( $n = 8$ ) or C3d<sup>+</sup> ( $n = 9$ ) tumor cells. (H) Immunofluorescence analysis of frozen sections of C3d<sup>+</sup> or C3d<sup>-</sup> lymphoid tumors stained with anti-CD8 antibodies (green). Each panel is representative of at least 3 different tumors. CD8<sup>+</sup> T cells were enumerated by flow cytometry, and results are shown on Figure 3H. All survival curves are Kaplan-Meier plots, and differences between curves were analyzed by the log-rank Mantel-Cox test.

expressed caspase 3 and caspase 7 (Figure 3, C and G, and Supplemental Figure 2B) more frequently than CR2<sup>+</sup> Tregs in C3d<sup>-</sup> lymphomas ( $P = 0.0003$ ). Consistent with the possibility that C3d interactions with CR2-depleted Tregs, C3d<sup>+</sup> lymphomas contained few Tregs, while C3d<sup>-</sup> lymphomas contained many ( $P < 0.0001$ ) (Figure 3, D and E). Although CD8<sup>+</sup> T cells did not express CR2 (not shown), CD8<sup>+</sup> T cells were more frequent in C3d<sup>+</sup> lymphomas and rare on C3d<sup>-</sup> lymphomas (Figure 1H), and they were more frequently apoptotic in C3d<sup>-</sup> lymphomas (Figure 3, H and I), suggesting that C3d expressed by tumors might prevent apoptosis of CD8<sup>+</sup> intratumor lymphocytes.

Next, we asked whether vaccination with C3d<sup>+</sup>-killed melanoma cells promoted apoptosis of Treg following tumor challenge. Figure 4A shows that vaccination with C3d<sup>+</sup>-killed melanoma cells promoted apoptosis of Tregs in the draining lymph nodes, while vaccination with C3d<sup>-</sup>-killed melanoma cells did not ( $P = 0.02$ ). As observed in the lymphoma model, C3d<sup>+</sup> tumors had more frequent apoptotic Tregs than C3d<sup>-</sup> tumors ( $P = 0.03$ ) 14 days after tumor challenge (Figure 4A). Vaccination with C3d<sup>+</sup>-killed melanoma cells reduced, by 6-fold, the size of C3d<sup>-</sup> tumors 14 days after tumor challenge and by 5-fold compared

**Table 1. Analysis of survival of mice recipients of tumors containing varying proportions of C3d<sup>+</sup> cells by Kaplan-Meier plots, and differences between curves were analyzed by the log rank Mantel-Cox test**

% C3d <sup>+</sup> tumor cells	0%	1%	10%	50%	100%
<i>P</i> relative to 0%		<i>P</i> = 0.0015	<i>P</i> = 0.0054	<i>P</i> = 0.0035	<i>P</i> < 0.0001
% Survival	0% ( <i>n</i> = 24)	18.2% ( <i>n</i> = 11)	14.3% ( <i>n</i> = 7)	20% ( <i>n</i> = 10)	25.8% ( <i>n</i> = 31)

with vaccination with C3d<sup>-</sup> melanoma cells (Figure 4B). In contrast, C3d<sup>-</sup> melanoma vaccination did not reduce B16 tumor growth, as the average tumor size after vaccination was almost identical to the average tumor size in nonvaccinated mice (Figure 4B). Figure 4, A and B, also show that the frequency of apoptotic Tregs varies reciprocally with tumor size: the smaller the tumors, the more frequent apoptotic Tregs are within each group. Supplemental Figure 3 shows that expression of C3d by melanoma cells yielded smaller tumors with larger necrotic areas, greater infiltration by CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes and fewer Foxp3<sup>+</sup> cells (Supplemental Figure 3, B, F, J, and N) compared with C3d<sup>-</sup> melanomas (Supplemental Figure 3, A, E, I, and M). Supplemental Figure 3 shows that vaccination with C3d<sup>+</sup>-killed melanoma cells was more effective at causing involution of C3d<sup>-</sup> melanomas (generated by injection of tumor cells 35 days after vaccination), at promoting infiltration of CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes, and at decreasing Foxp3<sup>+</sup> cells (Supplemental Figure 3, D, H, L, and P) than vaccination with C3d<sup>-</sup>-killed melanoma cells (Supplemental Figure 3, C, G, K, and O).

As expected, both C3d<sup>+</sup> and C3d<sup>-</sup> vaccines decreased the frequency of CR2<sup>+</sup> Tregs in lymph nodes draining melanomas compared with the frequencies in nonvaccinated mice (Figure 4, C and D). Treg CR2 expression was independent of vaccination, since unvaccinated mice expressed the highest frequency of CR2<sup>+</sup> Tregs, and Treg CR2 expression increased with the size of the tumor (Figure 4, C and D). In mice given C3d<sup>-</sup> tumor vaccines, the frequency of CR2<sup>+</sup> Tregs was independent of the size of the tumor (Figure 4D), while in mice given C3d<sup>+</sup> vaccines, the frequency of CR2<sup>+</sup> Tregs and tumor size were decreased (Figure 4D). Figure 4E shows that C3d<sup>+</sup> vaccines, but not C3d<sup>-</sup> vaccines, reduced the frequency of Tregs in the tumor-draining lymph nodes, suggesting C3d<sup>+</sup> vaccines selectively promote apoptosis of CR2<sup>+</sup> Tregs, in agreement with results shown above. Consistent with this concept, tumor size increased in parallel with the frequency of Tregs (Figure 4F).

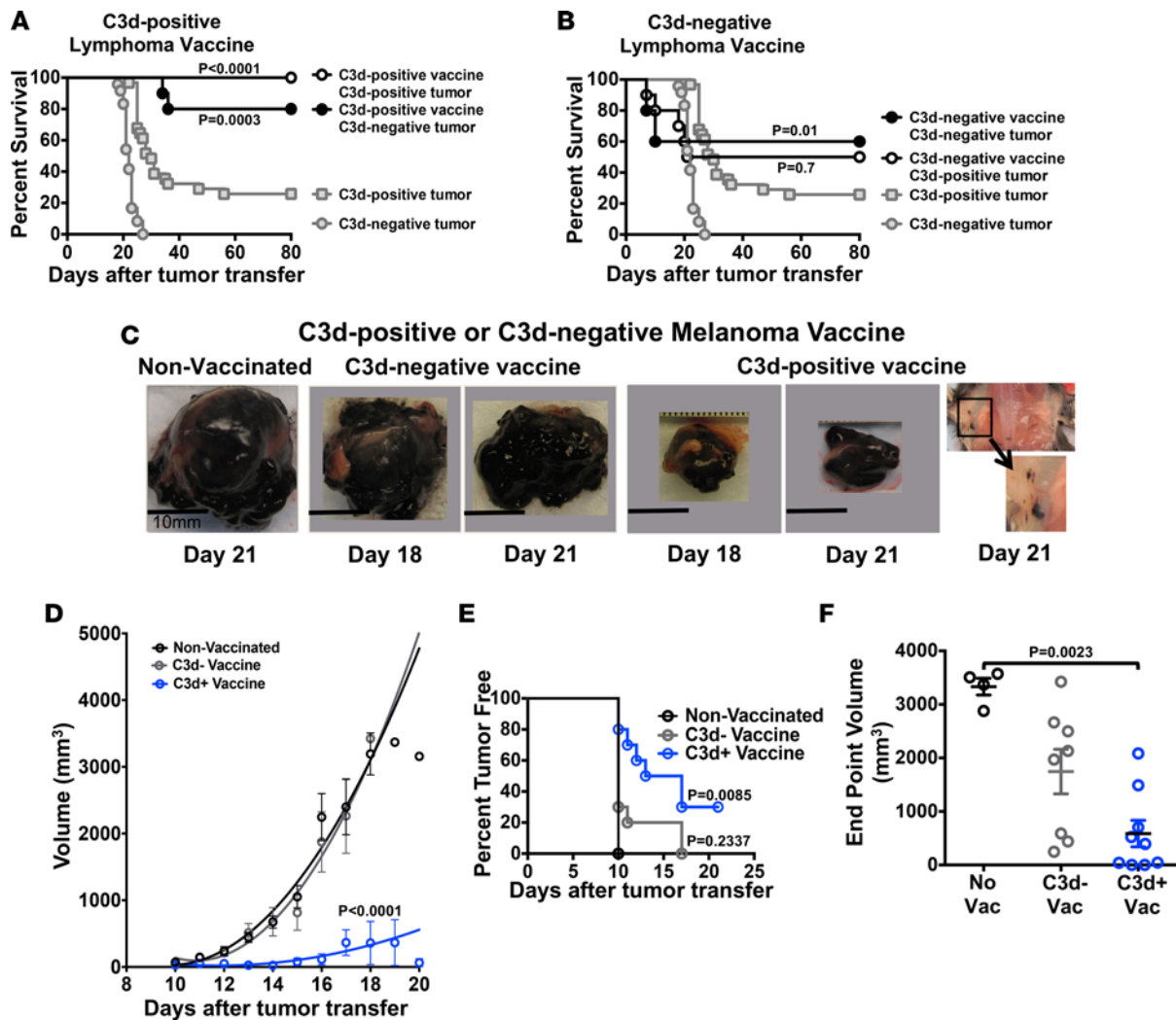
Interaction of C3d with CR2 had another potentially important function. Increased expression of CR2 correlated with dramatically decreased expression of PD-1 (Figure 5, A and B), which silences effector T cells by engaging PD ligand-1 (PD-L1) (19). Consistent with an impact of C3d on this pathway, C3d<sup>+</sup> tumors had increased frequencies of infiltrating T cells producing perforin, TNF $\alpha$ , and IFN $\gamma$  (Figure 5, C and D), and T cells isolated from C3d<sup>+</sup> tumors exhibited increased cytotoxicity against C3d<sup>-</sup> tumor cells (Supplemental Figure 2E).

We next asked whether the free C3d polypeptide exerts a discernable impact on responses of isolated T cells. Addition of C3d to naive CD4<sup>+</sup> T cells caused a modest increase in proliferation, and this increase was greatly potentiated by anti-CR2 antibodies with agonist properties (20) (Supplemental Figures 4, A and B). C3d and anti-CR2 also inhibited Treg differentiation (Supplemental Figure 4A). These

**Table 2. Analysis of survival of mice recipients of C3d<sup>+</sup> or C3d<sup>-</sup> tumors injected or not with CR2-Ig to block C3d**

	C3d <sup>+</sup>	C3d <sup>+</sup> + CR2Ig	C3d <sup>-</sup>	C3d <sup>-</sup> + CR2Ig
% Survival after 15 days	60% ( <i>n</i> = 5)	0% ( <i>n</i> = 5)	0% ( <i>n</i> = 5)	0% ( <i>n</i> = 5)
<i>P</i> value (Mantel-Cox test)		<i>P</i> = 0.001		<i>P</i> = 0.119

Table shows that CR2-Ig enhances tumor growth and abolishes the advantage in survival brought by C3d expression, indicating that enhancement of survival of recipient mice with C3d<sup>+</sup> tumors relative to survival of mice with C3d<sup>-</sup> tumors depends on C3d. Data were analyzed on Kaplan-Meier plots, and differences between curves were measured by the log rank Mantel-Cox test.

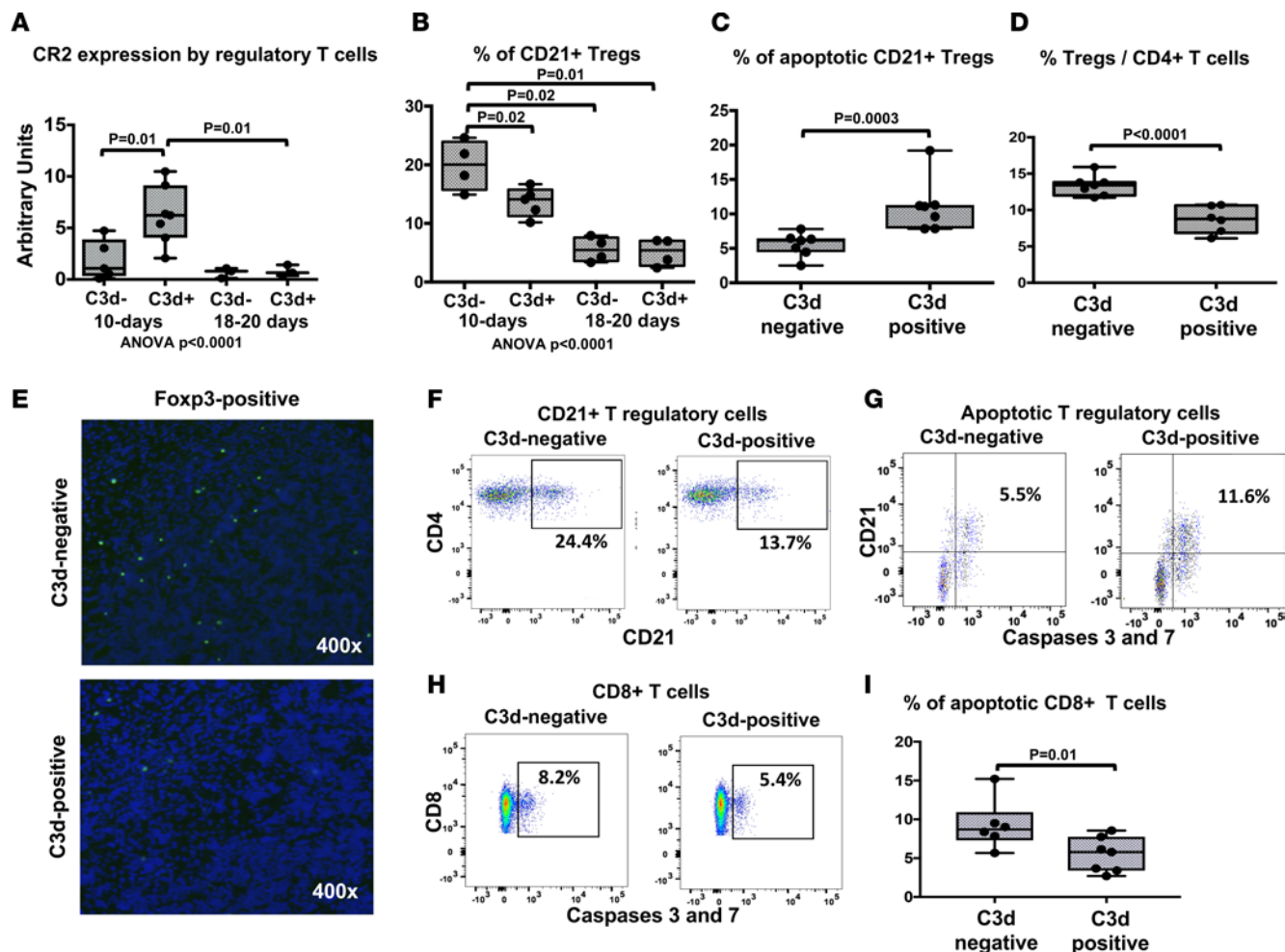


**Figure 2. Adaptive immunity evoked by vaccination with killed C3d<sup>+</sup> tumor cells protects against lymphoma and melanoma.** Mice were vaccinated with  $1 \times 10^7$  C3d<sup>+</sup> ( $n = 10$ ) or C3d<sup>-</sup> ( $n = 10$ ) killed lymphoma followed by administration of living  $5 \times 10^3$  C3d<sup>+</sup> or C3d<sup>-</sup> lymphoma cells 35 days later (**A** and **B**), or with  $1 \times 10^7$  C3d<sup>+</sup> ( $n = 10$ ) or C3d<sup>-</sup> ( $n = 10$ ) killed melanoma cells followed by transfer of  $2 \times 10^5$  live melanoma cells 35 days later (**C–F**). (**A**) Survival of mice vaccinated with killed C3d<sup>+</sup> lymphoma cells. (**B**) Survival of mice vaccinated with killed C3d<sup>-</sup> lymphoma cells. Note, the C3d-positive tumor and C3d-negative tumor groups in (**A**) and (**B**) are the same data as presented in Figure 1A. The data were not collected contemporaneously as the other groups in (**A**) and (**B**), but are shown here for reference. (**C**) Photographs of C3d<sup>-</sup> melanoma tumors excised at indicated days after tumor inoculation in mice vaccinated with C3d<sup>+</sup> or C3d<sup>-</sup> irradiated melanoma cells. Shown also is a photograph of an incipient tumor growing s.c. (inset) in mice vaccinated with C3d<sup>+</sup> melanoma cells, 21 days after tumor inoculation. (**D**) Impact of C3d<sup>+</sup> or C3d<sup>-</sup> vaccine on growth of C3d<sup>-</sup> melanoma. Data represent mean  $\pm$  SEM. Analysis was by Mann Whitney 2-tailed test. (**E**) Prevention of C3d<sup>-</sup> melanoma by vaccination with killed C3d<sup>+</sup> or C3d<sup>-</sup> melanoma cells. Shown are Kaplan-Meier plots, and differences between curves were analyzed by the log rank Mantel-Cox test. (**F**) Impact of vaccination on growth of C3d<sup>-</sup> melanoma estimated by tumor size at death (sacrifice) or at 21 days in mice that were alive at 21 days. All nonvaccinated mice and 8 of 10 mice vaccinated with C3d<sup>-</sup> melanoma died or were sacrificed at 18 or 19 days for humane reasons. Four of 10 C3d<sup>+</sup>-vaccinated mice either had no apparent tumors or smaller tumors at sacrifice (21 days). Data represent mean  $\pm$  SEM. Analysis was by Mann Whitney 2-tailed test.

results appear to represent at least some facets of the impact of C3d on immune surveillance because mice given anti-CR2 antibodies resisted C3d<sup>-</sup> tumors to the same extent as mice given C3d<sup>+</sup> tumor cells but no antibody (Figure 5E and Table 2).

### Discussion

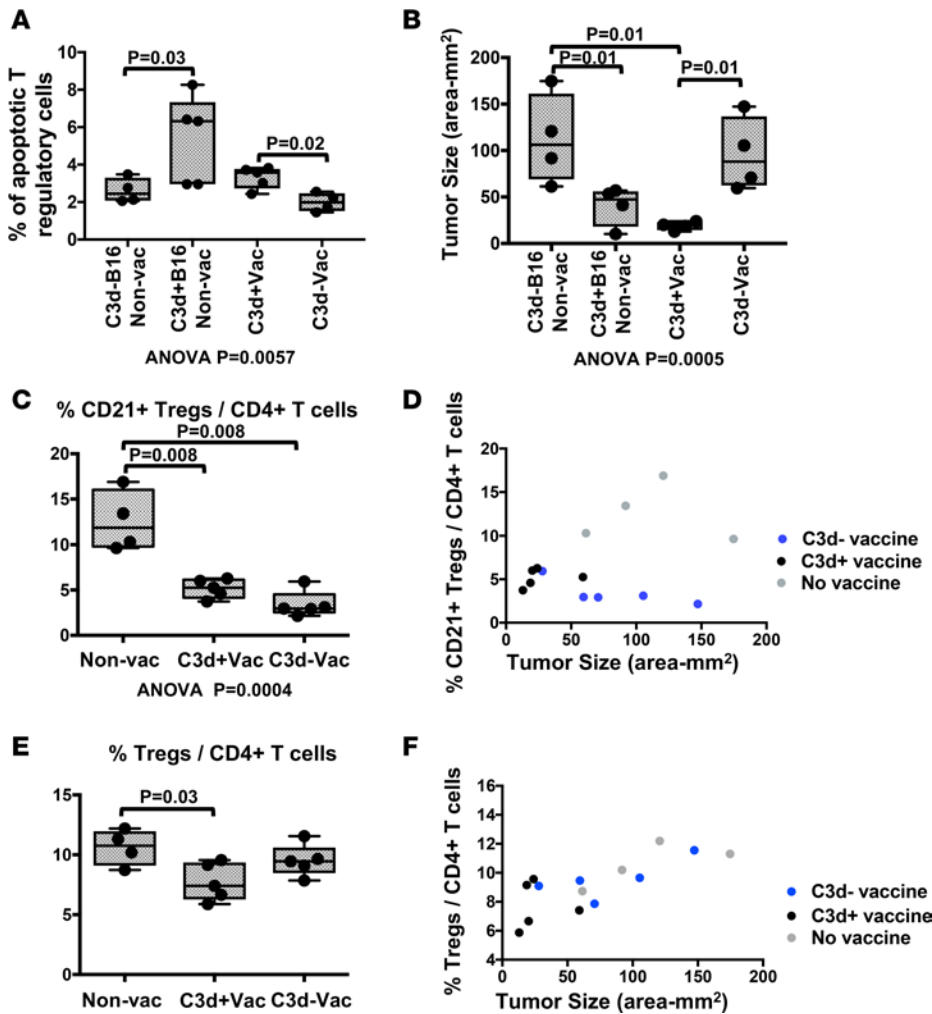
Our findings, to our knowledge, reveal hitherto unrecognized involvement of the complement system in regulation of immune surveillance. Complement is well known to amplify B cell responses, particularly against C3d-tagged antigen, and to facilitate T cell activation by activating antigen-presenting cells (21, 22). However, a function of free C3d in the governance of cellular immunity to endogenous tumor antigens has not been reported. The dramatic impact of therapies directed at immune checkpoints



**Figure 3. Impact of C3d on expression of CR2 (CD21) by Tregs and on apoptosis of Tregs and CD8<sup>+</sup> cells in lymphomas.** (A) CR2 expression by Tregs after introduction of C3d<sup>+</sup> or C3d<sup>-</sup> lymphoma cells. Expression was determined by qPCR relative to GAPDH; CR2 expression by naive Tregs was subtracted. Graphs reflect analysis of 3–7 mice per time point, in triplicate. (B and F) Frequency of CD21<sup>+</sup> Tregs determined by flow cytometry analysis of splenocytes. (C and G) Expression of CR2 (CD21) and caspase-3 and -7 in Tregs (CD4<sup>+</sup>, Foxp3<sup>+</sup>), assayed by flow cytometry, in mice injected with C3d<sup>+</sup> or C3d<sup>-</sup> lymphoma cells 10 days before analysis. The graphs depict frequencies of apoptotic Tregs in tumor-recipient mice. (D) Frequency of Tregs (CD4<sup>+</sup>, Foxp3<sup>+</sup>) determined by flow cytometry, 18 days after tumor transfer. (E) Immunofluorescence analysis of frozen sections of C3d<sup>+</sup> or C3d<sup>-</sup> lymphoid tumors stained with anti-Foxp3 antibodies (green). Each panel is representative of at least 3 different tumors. (H and I) Expression of caspase-3 and -7 in CD8<sup>+</sup> T cells assayed by flow cytometry, in mice injected with C3d<sup>+</sup> or C3d<sup>-</sup> lymphoma cells 10 days before analysis. Flow cytometry graphs reflect analysis of splenocytes in 4–5 mice per condition. Boxes represent distribution of data between the 25th and the 75th percentiles. The mean is indicated by a horizontal line, and whiskers represent maximum and minimum values. Statistical analysis in A and B was by the Kruskal Wallis test followed by Dunn's multiple comparison test. Analysis in C, D, and I was by the Mann Whitney 2-tailed test.

suggests that such governance, rather than initiation of immunity, limits immune surveillance of cancer — and perhaps more generally. Work in complement-deficient mice indicates that C3 acting in part on antigen-presenting cells promotes the priming of T cells at initiation of cellular immunity (23, 24). However, the actions of free C3d we report are exerted later, at regulatory checkpoints in WT mice, which manifestly fail to exert effective immune surveillance of tumors. Thus, we show for the first time to our knowledge that complement, particularly C3d, may be a key factor in determining whether immune surveillance or immune suppression prevails.

The complement system has manifold functions that facilitate the phagocytosis, killing, and disposal of microorganisms and damaged or foreign cells. The complement system also regulates the physiology of normal cells to circumscribe sites of injury and infection (23, 25) and to recruit adaptive immunity, as mentioned above (5). Whether complement provides an intrinsic defense against cancer (26), as long supposed, or promotes a microenvironment that nurtures the inception and progression of cancer (27–32), as recently proposed, or both is yet undecided; the net impact might well vary from case to case. Similarly, the products



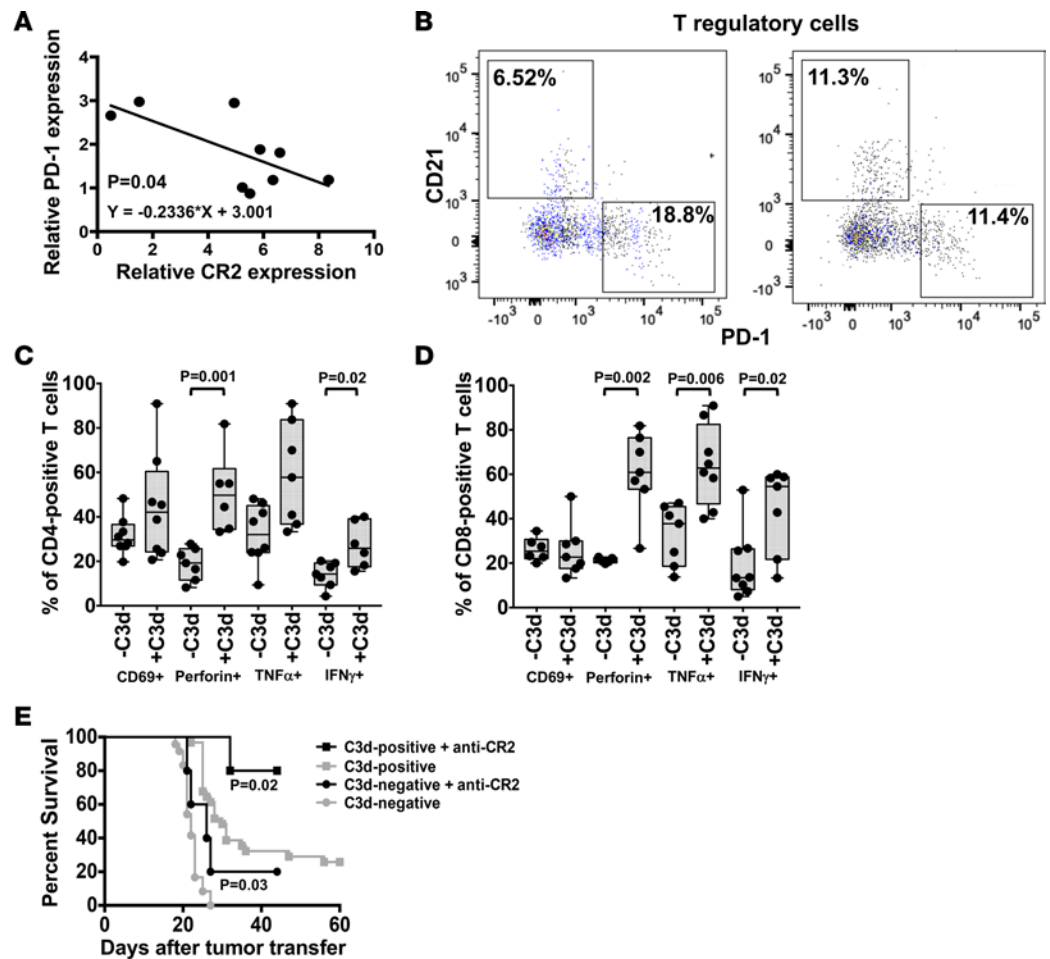
**Figure 4. Impact of C3d on CD21 expression by Tregs, on apoptosis of Tregs, and on Treg frequencies following vaccination against melanoma.** Graphs reflect analysis of lymphocytes obtained from tumor-draining lymph nodes of 4–5 mice/group 14 days after tumor transfer. Mice were vaccinated by injection of  $1 \times 10^7$  killed melanoma cells 35 days prior to transfer of  $2 \times 10^5$  live tumor cells. Vaccinated mice were challenged with C3d<sup>-</sup> tumor cells (expressing empty vector marker). (A) Frequency of apoptotic Treg (CD4<sup>+</sup>, Foxp3<sup>+</sup>, caspase 3<sup>+</sup> and -6<sup>+</sup>/CD4<sup>+</sup>, Foxp3<sup>+</sup> cells  $\times 100$ ) in nonvaccinated or vaccinated mice determined by flow cytometry. (B) Tumor size (measured as areas) in the mice corresponding to the lymphocyte analysis. (C) Frequency of CD21<sup>+</sup> Tregs (CD4<sup>+</sup>, CD21<sup>+</sup>, Foxp3<sup>+</sup>/CD4<sup>+</sup>  $\times 100$ ). (D) Plot compares the frequencies of CD21<sup>+</sup> Tregs with the respective tumor sizes. Each dot represents one mouse. (E) Frequency of Tregs (CD4<sup>+</sup>, Foxp3<sup>+</sup>/CD4<sup>+</sup>  $\times 100$ ) determined by flow cytometry. (F) Plot compares the frequencies of Tregs with the respective tumor sizes. Each dot represents one mouse. Boxes in graphs represent distribution of data between the 25th and the 75th percentiles. The mean is indicated by a horizontal line, and whiskers represent maximum and minimum values. Statistical analysis in A, B, C, and E was by the Kruskal Wallis test followed by Dunn's multiple comparison test.

of complement activation and the impact of complement on malignant cells might promote or regulate adaptive immune responses of B cells and T cells with uncertain net impact on resistance to cancer. However, our experiments reveal a clear, unidirectional protective impact of free C3d in tumor cells that might be exploited to some advantage in advancing the treatment or prophylaxis of cancer. However, since we did not modify the complement system of the mice we used, the impact of C3d should not be taken as favoring or opposing the potential involvement of the complement system in the spontaneous development of or natural resistance to cancer.

The protection against C3d<sup>-</sup> tumor cells conferred by vaccination with killed C3d<sup>+</sup> tumor cells would seem to exclude the possibility that efficacy was owed to immunity targeting C3d-derived peptides. However, Knopf et al. (33) and De Groot et al. (34) propose that presentation of C3d-derived peptides by antigen-presenting cells in other systems might stimulate autoreactive T cells. We cannot exclude entirely the possibility that protective immunity evoked by C3d<sup>+</sup> tumor cells in our experiments reflected cross-reactivity with “normal” peptides and, hence, autoimmunity rather than tumor-specific immunity. However, in experiments using a  $1 \times 10^4$ -fold range of doses of C3d<sup>+</sup> tumor cells, we never observed evidence of autoimmune disease, and only tumor appeared to limit vitality. We interpret this lack of toxicity to suggest that, predominantly, immunity was directly against tumor antigens and consider such selectivity to be potentially a major advantage of this approach. Since systemic suppression of checkpoint controls engender significant toxicity, we speculate that our approach might have averted toxicity in part because C3d was associated with tumor cells and was not introduced systemically. If that is correct, then toxicity of other approaches might be similarly decreased if suppressive agents can be delivered with tumor cells.

Although our findings suggest free C3d predominantly disrupts immune checkpoint suppression, we cannot exclude the possibility of an impact on T cell priming, perhaps shifting ensuing responses away





**Figure 5. C3d modifies antitumor T cell functions.** (A) CR2 and PD-1 expression measured by qPCR on Tregs. Expression of CR2 and PD-1 were relative to that of GAPDH. (B) Flow cytometry analysis of lymphocytes obtained from the spleen of mice 10 days after injection of  $5 \times 10^3$  lymphoma cells. Figure shows reciprocal expression of CD21 and PD-1 by Tregs ( $CD4^+$ ,  $Foxp3^+$ ). (C and D) Figure shows the percent (%) of  $CD4^+$  (C) or  $CD8^+$  (D) T cells isolated from the spleen of recipients of  $C3d^+$  or  $C3d^-$  tumors that express perforin,  $TNF\alpha$ , and  $IFN\gamma$ , 3.5 hours after culture on anti- $CD3^-$  and anti- $CD2^-$ -coated plates. Boxes in graphs represent distribution of data between the 25th and the 75th percentiles. The mean is indicated by a horizontal line, and whiskers represent maximum and minimum values. Statistical analysis was by the Mann Whitney 2-tailed test. (E) Impact of anti-CR2 antibodies on survival of mice given  $C3d^+$  or  $C3d^-$  lymphoma cells. Anti- $CD21/CD35$  (7G6) antibodies were administered at the same time as tumor cells. Figure represents Kaplan-Meier plots obtained with at least 5 mice per condition, and differences between curves were analyzed by the log rank Mantel-Cox test. Note, the  $C3d^+$ -positive tumor and  $C3d^-$ -negative tumor groups in (E) are the same data as presented in Figure 1A. The data were not collected contemporaneously as the other groups in (E), but are shown here for reference.

from suppression. However, the absence of generalized lymphoproliferation and absence of notable differences in sizes of draining lymph nodes between mice vaccinated with  $C3d^+$  versus  $C3d^-$  tumor cells suggests any impact on priming was limited.

Our observations suggest C3d might prove advantageous for immunotherapies and vaccines. Association of C3d with tumor cells or tumor cell vaccines might allow T cells specific for tumor antigens to generate protective immunity, leaving T cells specific for pleiotropic targets subject to regulation.

## Methods

**Mice, tumor cell lines, and tumor formation.** Female BALB/c ( $H-2^d$ ),  $Rag-2^{-/-}$  ( $H-2^d$ ), and C57BL/6J ( $H-2^b$ ) mice were purchased from the Jackson Laboratory (JAX). BALB/c  $JH^{-/-}\kappa^{-/-}$  ( $H-2^d$ ) mice lack mature B cells and Ig owed to gene-targeted deletion of the JH and Jk segments, and they were bred at the University of Michigan. At the time of vaccination or tumor transfer, mice were between 6 and 10 weeks of age and were sex matched and age matched in each experiment. Lymphoma cells were the murine pre-B cell 18-81 lymphoma, subclone

A3.43 (35, 36). Lymphoid tumors were induced by i.p. adoptive transfer of  $5 \times 10^3$  or  $1 \times 10^7$  cells, as indicated in the text, of either pre-B cell lymphoma cells expressing C3d or not. Whenever tumor diameter exceeded 10 mm, or whenever there were signs of excessive morbidity, animals were euthanized and the event recorded as death from tumor burden. Melanoma tumors were obtained by s.c. injection of  $4 \times 10^4$  or  $5 \times 10^5$  B16-F0 cells, purchased from ATCC (catalog CRL-6322), expressing C3d or not, in the flank region. Tumor volumes were determined by measuring 2 diameters of the dissected tumors, using electronic calipers, calculating the average radius and converting to areas or spherical volumes. Tumors cells purchased from ATCC were free of mycoplasma and free of aerobic or anaerobic bacterial contamination, as per the company's certificate of analysis.

*C3d expression vectors.* To generate the vector-encoding expression of only the C3d gene to transfect lymphoma cells, the pcDNA3.1 vector containing the E $\mu$  enhancers,  $\lambda_1$  promoter, and HIV-ENV-C3d vector was modified (7). Specifically, the completed vector was partially digested with NheI and fully digested with BAM HI. The DNA was then separated by gel electrophoresis, and the 10.7-Kb band representing the pcDNA 3.1 vector without the gp140 sequence— but with the E $\mu$  enhancer,  $\lambda_1$  promoter, and C3d DNA — was purified from the gel. The ends of this DNA were then filled in with Klenow (Thermo Fisher Scientific) and ligated together to form a vector that would drive expression of only C3d by the B cell regulatory elements. The E $\mu$  was a 678-bp sequence (NCBI M12827 position [pos] 7–68, and the  $\lambda_1$ p was a 1,625-bp sequence (NCBI AC140201 pos 32,137–30,517). Both were gifts from Faith Young (University of Rochester, Rochester, New York, USA) (37).

*C3d-vector for B16-melanoma cell transduction.* C3d(x3) fragment was excised from the pcDNA3.1 C3d vector by BAMH1-XHO1 digest and cloned into the BAM-H1 site of the pLentiox 3.7-puro vector obtained from the Vector Core at the University of Michigan. Positive clones were confirmed by PCR and sequencing of the insert. C3d expression is driven by the CMV promoter and enhancer. Lentiviral preps were made by the Vector Core at the University of Michigan from empty vector or from C3d encoding vector. Transduced cells (controls with empty vector or C3d- expressing vector were selected by puromycin resistance, and C3d expression was confirmed by PCR of cDNA and by Western blot.

*Western blot analysis.* C3d protein expression by tumor cells was determined in cell lysates obtained from cells grown to approximately  $1 \times 10^6$  cells per ml in 20 ml media. Cells were lysed with RIPA Lysis Buffer supplemented with a cocktail of protease inhibitors, PMSF in DMSO and sodium orthovanadate in water (Santa Cruz Biotechnology Inc.). Protein amount in the lysates was measured by spectrophotometry using the Pierce Microplate BCA Protein Assay Kit (Pierce Biotechnology, Thermo Fisher Scientific). Cell lysates were precleared by centrifugation (12,000 g, 15 minutes), separated by SDS PAGE of 5–20  $\mu$ g of cell lysate, under reducing conditions, on 7.5% Tris-HCl READY GELS (Bio-Rad) followed by transfer to an Immobilon PVDF membrane (Millipore). On the blots, C3d was detected using goat anti-murine C3d antibody (R&D systems) (1:1,000) followed by rabbit anti-goat IgG-HRP (Novus Biologicals).

*Pathology and immunofluorescence.* Fresh tumors were harvested in linear growth phase ( $\sim 600$  mm<sup>3</sup>) embedded in optimal cutting temperature (OCT) compound (Tissue-Tek) and snap frozen. Cryo-sections (5  $\mu$ m) were processed, and after fixation, the slides were incubated with primary antibodies: goat anti-mouse C3d (15  $\mu$ g/ml, R&D Systems), rat anti-mouse CD4 (GK1.5, 1:100, BD Biosciences), rat anti-mouse CD8 (53-6.7, 1:100, BD Biosciences), and anti-FoxP3 (Rabbit Polyclonal; Novus Biologicals) for 2 hours at room temperature, followed by anti-rat CF488a or anti-goat CF555 (Sigma-Aldrich) donkey secondary anti-goat IgG-AP (1:200, Southern Biotech) incubated for 1 hour at room temperature.

For unequivocal identification of transgenic C3d expression in sections, tumor cells were transfected with a C3d-HIV-ENV chimera (in-house). In tumor sections, the ENV protein was detected by immunofluorescence with human HIV Immunoglobulin (HIVIg) (NIH AIDS Research & Reference Reagent Program Division of AIDS, NIAID, NIH — from Luiz Barbosa; diluted 1:3,000 to 1:5,000) followed by goat anti-human FITC-conjugated antibody (Southern Biotech, catalog 210-02, diluted 1:50). Sections were counterstained with a rat anti-mouse B220 antibody (RA3-6B2, BD biosciences) followed by goat anti-rat IgG F(ab)<sup>2</sup> rhodamine conjugated antibody (Jackson ImmunoResearch, diluted 1:100).

No specific staining was observed in control slides stained only with the secondary antibody. Imaging was performed with a Leica DMI6000B microscope using a MicroPublisher 3.3RTV camera. For each mouse, 3 images were taken at 400 $\times$  magnification, focusing on the tumor and adjacent stroma. Each section was systematically photographed in neighboring 100 $\times$  fields such that 80%–100% of each tumor section was photographed. Total stain area/low power field, as defined by pixel area and hue, was assessed using QCapture Pro Imaging software.

*Antibodies for cell depletion and receptor/ligand blockade.* T cells were depleted with anti-CD4 plus anti-CD8 mAb (Gk1.5 mAb [anti-CD4], 300 µg/kg of body weight plus 53–6.7 mAb [anti-CD8], 600 µg/kg of body weight; eBioscience). Antibodies were given i.p. 1 day before and every day after tumor transfer ( $5 \times 10^3$  cells), for the first 2 weeks, and every other day afterward until euthanasia. Mice in control groups received injections of isotype control immunoglobulin. To test whether C3d and engagement of CR2 potentiate each other in inducing tumor regression, we administered mAb anti-CR2/CR1 (mAb 7G6, eBioscience), 300 µg, 1 day before and every day after tumor transfer ( $5 \times 10^3$  cells) for 2 weeks. C3d blockade was achieved with 800 µg of soluble CR2-IgG1 (sCR2-IgG1) or IgG1 isotype control in PBS, administered i.v. 24 hours before inoculation of  $1 \times 10^7$  tumor cells.

*Flow cytometry and antibodies.* Single cell suspensions were obtained from spleens and tumors 10 days or 18–20 days after tumor inoculation. Cells were counted and stained with allophycocyanin-conjugated (APC-conjugated) mAbs to either CD4 (GK1.5), CD8 (53-6.7), or CD19 (ID3) (BD Biosciences); phycoerythrin-conjugated (PE-conjugated) mAbs to CD21/CD35 (7G6) and to PD1 (RMPI-30) purchased from BD Biosciences; PE-conjugated mAbs to Foxp3 (FJK-16s) and FITC-conjugated Foxp3 (FJK-16s) purchased from eBioscience; and Prcp/Cy5.5-conjugated CR2/CR1 (7G6) purchased from BioLegend. Intracellular staining followed cell surface staining and was performed using eBioscience Foxp3/Transcription Factor Staining Buffer Set (catalog 00-5523-00), according to the manufacturer's instructions (Thermo Fisher Scientific, eBioscience). Intracytoplasmic cytokines were measured in T cells purified from spleens or tumors and were stained with APC-conjugated mAbs against Perforin (eBiOMAK-D), TNF $\alpha$  (MP6-XT22), and IFN $\gamma$  (XMG1.2) (eBioscience). All antibodies used in flow cytometry analysis were diluted 1:50. Four-color flow cytometric analysis of  $1 \times 10^6$  cells was performed using a FACSCanto II (BD Biosciences).

*T cell isolation.* Spleens were harvested from euthanized animals, and single cell suspensions were obtained by gently dissociating the splenic capsule over a 40-µm filter. Red cells were depleted by incubating with ACK Lysing Buffer (Lonza), and T cells were isolated by negative selection on a LS<sup>+</sup> column, using the Pan T cell isolation kit, on a MiniMACS Separator (Miltenyi Biotec), as per manufacturer's instructions. T cell purity was assessed by flow cytometry and was always greater than 80%.

*Vaccination and tumor challenge.* C3d<sup>+</sup> and C3d<sup>-</sup> lymphoma cells (18-81 pre-B cell line (10, 36) were thawed and grown in vitro in selection medium (RPMI 1640 with 10% FCS;  $\beta$ -mercaptoethanol, 55 mM; penicillin, 100 U/ml; streptomycin, 100 µg/ml; L glutamine, 2mM; Hygromycin, 1mg/ml) for 6 days before use. C3d<sup>+</sup> or C3d<sup>-</sup> B16-F0 cells were grown in RPMI1640 supplemented as detailed above with 5 µg/ml of puromycin instead of hygromycin. On the day of vaccination, cells were washed and suspended in fresh medium, and viable cells were counted using Trypan Blue cell exclusion. Afterward, tumor cells were irradiated with 10 Gy (IC-320 Irradiator, BSRB, University of Michigan) and then washed twice with PBS immediately before inoculation. Vaccine inoculations were performed by i.p. injection of  $5 \times 10^6$  lymphoma cells (twice, a week apart) into mice in the BALB/c genetic background or by a single s.c. injection of  $1 \times 10^7$  B16 melanoma cells into C57BL/6 mice. Lymphoma-vaccinated mice were challenged with i.p. injection of  $5 \times 10^3$  live tumor cells. Mice vaccinated with melanoma cells and their controls were challenged with s.c. injection of  $2 \times 10^5$  live tumor cells 35 days after the last vaccination with  $1 \times 10^7$  cells. Nonvaccinated melanoma mice were challenged with s.c. injection of  $4 \times 10^4$  live tumor cells. Mice were monitored daily.

*Cellular apoptosis assay.* Cellular apoptosis was measured using a FAM-FLICA assay purchased from ImmunoChemistry Technologies, according to the manufacturer's instructions.

*qPCR.* RNA was obtained from Tregs isolated from recipient mice 10 days or 18–20 days following tumor transfer, with a Midi MACS T cell isolation kit (Miltenyi Biotec). Treg purity was verified by flow cytometry and found to be always greater than 90%. Total RNA was prepared using PureLink RNA Mini Kit (Thermo Fisher Scientific). cDNA was produced using the VILO kit (Invitrogen, Thermo Fisher Scientific), according to the manufacturer's instructions. Real-time PCR was performed using TAQMAN primers purchased from Invitrogen. CR2-specific primers, Mm00801681\_m1; CR1-specific primers; HPRT-specific primers, Mm01545399\_m1; PD-1-specific primers, Mm01285676\_m1; and Foxp3-specific primers, Mm00475162\_m1. The PCR conditions were recommended by the manufacturer, 50°C, 2 minutes, 95°C for 10 minutes followed by forty cycles of 95°C for 15seconds and 60°C for 1 minute.

*T cell proliferation assays.* T cells isolated from spleens of naive BALB/c mice were stained with Vibrant CFDA SE (Invitrogen) prior to culture. CFDA SE was reconstituted with DMSO to a final concentration of

5 mM. Cells were resuspended in PBS + 5% FCS (Gibco) at  $5 \times 10^6$ /ml and were incubated with CFDA SE for 5 minutes at room temperature. Incubation was stopped by placing cells on ice for 5 minutes, and cells were washed twice in ice-cold 5% FCS-RPMI and resuspended in medium with final concentration  $2 \times 10^6$  cells/ml prior to distribution into wells ( $1 \times 10^5$  in 100  $\mu$ l/well) coated with 10  $\mu$ g/ml anti-mouse CD3e (clone 145-2C11, BD Biosciences). Cells were stimulated with 1  $\mu$ g/ml anti-mouse CD28 (clone 37.51, BD Biosciences), with 100 ng/ml recombinant human IL-2 (R&D Systems), with or without 1:10 dilution of supernatant from C3d<sup>+</sup> or C3d<sup>-</sup> tumor cell cultures, or with or without 10  $\mu$ g/ml anti-mouse CR1/CR2 (clone 7G6, BioLegend) mAb or 10  $\mu$ g/ml isotype control, as indicated in the manuscript.

**Tumor-specific killing by cytotoxic T cells.** Lymphocytes from the spleens and tumors were harvested from vaccinated mice 10 days after injection of tumors, as explained above. Isolated T cells were cultured with tumor cells (irradiated with 10 Gy) derived from the same clone that originated the tumors, in the presence of IL-2 for 6 days. Cytotoxicity was measured against C3d<sup>+</sup> or C3d<sup>-</sup> targets at various effector/target ratios (E:T ratios) by a standard LDH cytotoxicity assay (Pierce, Thermo Fisher Scientific), according to the manufacturer's instructions.

**Statistics.** All comparisons were done with Prism-Graphpad software (v6). Comparison of survival curves was by the log rank Mantel Cox test. Averages were either compared by unpaired Student's *t* test (2-tailed) or by nonparametric Mann Whitney *U* test, and when appropriate, with correction for multiple comparisons by Dunn's test. We performed contingency analysis, or linear or nonlinear regression analysis, with tools available in the Prism-Graphpad software. A *P* value less than 0.05 was considered significant.

**Study approval.** Animals were maintained in accordance with regulations of the University of Michigan Committee on the Use and Care of Animals under specific pathogen-free condition.

## Author contributions

JLP and MC wrote the manuscript and analyzed results. MC, IS, ARL, EF, and SJB performed experiments and analyzed results. MCC discussed results, critically read the manuscript, helped with reagents, and advised on experimental protocols. TMR provided critical reagents and discussed results.

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