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The Orchestrated Functions of Innate Leukocytes and T Cell Subsets Contribute to Humoral Immunity, Virus Control, and Recovery from Secondary Poxvirus Challenge

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A pivotal role for antigen-specific recall responses to secondary virus infection is well established, but the contribution of innate immune cells to this process is unknown. Recovery of mice from a primary orthopoxvirus (ectromelia virus [ECTV]) infection requires the function of natural killer (NK) cells, granulocytes, plasmacytoid dendritic cells (pDC), T cells, and B cells. However, during a secondary challenge, resolution of infection is thought to be dependent on antibody but not T cell function. We investigated the contribution of NK cells, granulocytes, and pDC to virus control during a secondary virus challenge in mice that had been primed with an avirulent, mutant strain of ECTV. Mice depleted of NK cells, granulocytes, or pDC effectively controlled virus, as did mice depleted of both CD4 and CD8 T cell subsets. However, mice concurrently depleted of all three innate cell subsets had elevated virus load, but this was significantly exacerbated in mice also depleted of CD4 and/or CD8 T cells. Increased viral replication in mice lacking innate cells plus CD4 T cells was associated with a significant reduction in neutralizing antibody. Importantly, in addition to T-dependent neutralizing antibody responses, the function of CD8 T cells was also clearly important for virus control. The data indicate that in the absence of innate cell subsets, a critical role for both CD4 and CD8 T cells becomes apparent and, conversely, in the absence of T cell subsets, innate immune cells help contain infection.

Smallpox, caused by variola virus, was considered among the deadliest scourges of humankind. It was eradicated more than 30 years ago through one of the most successful immunization campaigns, which employed a vaccine containing the closely related vaccinia virus (VACV). Although the VACV strain used in the smallpox vaccine is not considered safe by current standards, it was potent in inducing long-lived memory and offered a high degree of protection. Much of our current understanding of protection following vaccination and recall responses to secondary challenge has been inferred from animal studies of closely related poxvirus infections, including mousepox (a disease caused by ectromelia virus [ECTV] in mice), VACV, and monkeypox.

We have shown previously that neutralizing antibody, but not the function of CD4 or CD8 T cell subsets, is required to control virus replication during the acute phase of a secondary ECTV challenge (1). In a separate study on monkeypox, depletion of CD4 or CD8 T cells also had no significant effect on virus clearance or on neutralizing antibody production during the acute phase of a secondary challenge in macaques vaccinated with VACV vaccine 6 months previously (2). In both studies, neutralizing antibody produced in the absence of CD4 T cell help (attributed to extrafollicular plasma cells) was sufficient for virus control in immune animals. A number of other studies have found that in vaccinated individuals, humoral immunity to smallpox is stable and lasts longer than memory CD4 and CD8 T cell responses (3, 4). Thus, the current paradigm is that antibody responses are necessary and sufficient for recovery from secondary orthopoxvirus challenge and that T cell subsets do not play a significant role.

The contribution of adaptive immune response during a secondary virus challenge has been well studied in many models of infection, but the role of innate immunity in this process is still poorly understood. In the mousepox and monkeypox studies (1, 2), the contribution of innate immune cells to virus control during the acute phase of a secondary challenge was not considered. However, it is known that NK cells are critical for recovery of mice from a primary ECTV infection (5–8), and recent evidence indicates that memory NK cells can be generated following a primary viral infection and that these cells can respond more rapidly to reinfection with the same pathogen (9–11). Although it is not entirely correct to categorize NK cells as innate cells, since they exert biological functions that have attributes of both innate and adaptive immunity, for simplicity, we will refer to them as innate cells in this study. In addition, we present evidence that granulocytes (Gr-1$^+$) and plasmacytoid dendritic cells (pDC) are also essential for recovery of mice from primary ECTV infection. We hypothesize that memory or naïve NK cells, granulocytes, and/or pDC, which individually play crucial roles in the host response to a primary infection, also contribute to virus control during a secondary ECTV or monkeypox virus infection.

Indeed, it may be speculated that in the absence of T cells or T cell function, as in the case of the monkeypox and mousepox studies (1, 2), these innate immune cell subsets play a compensatory role(s) and are vital for virus control. The antiviral function of these innate immune cells may involve not only direct cytolysis, phagocytosis, and cytokine secretion but also antibody, particularly during secondary challenge. Each of these cell types expresses Fc receptors that can bind to antibody-coated cells and mediate antibody-dependent cellular cytotoxicity (12, 13).

We undertook experiments to determine whether NK cells, granulocytes, and/or pDC contribute to control of virus replica-

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tion during the acute phase (first 8 days postchallenge [p.c.]) of a secondary ECTV challenge. In addition, although T cell subsets do not appear to play a role in recovery from a secondary poxvirus challenge in an otherwise immunocompetent host, in this study we investigated whether T cells contribute to virus control if NK cells, granulocytes, and pDC are absent. We show here that when these innate immune cells are depleted, T cells compensate and play an important role in protection, and conversely, when the T cell subsets are eliminated, the innate immune cells help contain the infection.

MATERIALS AND METHODS

Viruses. ECTV Moscow strain (ATCC VR 1374), here referred to as ECTV wild type (WT), and the thymidine kinase-deficient strain of ECTV-WT, designated ECTV-TK$^{\Delta}$ (14), were propagated as previously described (15) in BS-C-1 cells.

Cell lines. MC57G (ATCC CRL-2295), YAC-1 (ATCC TIB160), and BS-C-1 (ATCC CCL-26) cells were maintained in Eagle's minimum essential medium (Gibco, Invitrogen Corp., Carlsbad, CA) with 2 mM L-glutamine, antibiotics, and 5 to 10% fetal calf serum (FCS).

Mice. Female, specific-pathogen-free C57BL/6J (B6) mice were bred at the Australian National University Bioscience Services Facility, Canberra, Australia, and used at 6 to 12 weeks of age. Animal experiments were reviewed and approved by the Australian National University Animal Experimentation and Ethics Committee.

Infection. Mice were inoculated with 10^3 PFU of ECTV-WT subcutaneously (s.c.) for a primary infection. For a secondary challenge, mice were first immunized with 10^5 PFU of ECTV-TK$^{\Delta}$ given intraperitoneally (i.p.) and 5 weeks later challenged s.c. with 10^3 PFU of ECTV-WT. All animals were monitored daily for clinical signs of disease and euthanized when they had lost 25% of their original body weight and were recorded as dead the following day.

Leukocyte cell subset depletion and flow cytometry. B6 mice were immunized and challenged as described above. On days −1, 1, 3, 5, and 7 postchallenge (p.c.), mice were given phosphate-buffered saline (PBS) or 0.02 mg anti-Gr1 (clone RB6-8C5), 1 mg anti-pDC (clone 120G8, a gift from G. Trinchieri), 0.5 mg anti-NK1.1 (clone PK136), 1.0 mg anti-CD4 (clone GK1.5), and/or 1 mg anti-CD8 (clone 2.43.1) monoclonal antibodies (MAb) to investigate the effects of acute leukocyte subset depletion on the outcome of secondary ECTV challenge. Previous studies in our laboratory established that there were no measurable differences in virus replication, the outcome of infection, or the immune response generated when either PBS or an appropriate isotype control rat or mouse MAb was used. Efficiency of cell subset depletion was assessed by flow cytometry and found to be routinely >98% for each population regardless of whether one or more antibodies were used for leukocyte depletion at a given time (data not shown). Treatment with anti-Gr1 MAb at the indicated concentration eliminated cells expressing high levels of Gr-1 (Gr-1^{high}) but not those expressing low levels of Gr-1 (Gr-1^{low}) as assessed by staining with a different clone of MAb (IA8) specific for granulocytes. To assess the depletion of pDC, spleens were first digested with collagenase and DNase as described elsewhere (16) and stained with anti-mPDCA1 (12, 17). All flow cytometry reagents were purchased from BD Biosciences or Biolegend.

Determination of viral load in organs and blood. Virus titers, expressed as $\log_{10}$ PFU/gram of tissue, were determined on BS-C-1 monolayers using the conventional viral plaque assay, as described previously (18). Viral load below the limit of detection by viral plaque assay was measured by quantitative real-time PCR (qRT-PCR) to amplify the target sequence of ECTV-Mos-156, a late gene that encodes the viral hemagglutinin (19). ECTV-Mos-156 oligonucleotide primers used were as follows: forward, CGCTACACCTTATCCTCAGACAC; reverse, GGAATTGGGC TCCTTATACCA. Viral DNA was prepared using the QiaAmp DNA minikit (Qiagen Pty Ltd., Victoria, Australia) as per the manufacturer's instructions. Serial dilutions of a plasmid encoding ECTV-Mos-156 were used as the standard. The qRT-PCR was carried out in SYBR iQ Supermix (Bio-Rad Laboratories) in a total volume of 20 μl using the iQ5 cycler (Bio-Rad Laboratories Pty Ltd., New South Wales, Australia).

Cytotoxicity assays. The standard ^{51}Cr release assay (18) was used to determine *ex vivo* anti-ECTV cytotoxic T-lymphocyte (CTL) activity, using ECTV-infected and uninfected MC57G (H-2b) target cells. YAC-1 cells were used as targets for NK cell cytotoxicity assays.

PRNT. The plaque reduction neutralization test (PRNT), used to determine virus-neutralizing activity of the antibody present in serum samples, is described elsewhere (20). Sera were heat inactivated at 56°C for 30 min prior to use. The neutralization titer was taken as the reciprocal of the dilution of sera that caused a 50% reduction in the number of virus plaques over and above the number of plaques in the samples with sera from naïve mice.

Detection of ECTV-specific antibody-secreting cells (ASC) by ELISPOT assay. ELISPOT was performed using 96-well filter plates (MAHA N4510; Millipore Australia Pty Ltd., Victoria, Australia) that had been coated overnight with partially purified ECTV antigen diluted in PBS. Plates were then washed and blocked with RPMI 1640 containing 10% FCS for 2 to 4 h at 37°C before use. A total of 10^6 splenocytes or bone marrow (BM) cells were then incubated for 4 h at 37°C on 96-well filter plates and developed with an alkaline phosphatase substrate (Astral Scientific, New South Wales, Australia) for 15 to 45 min or when spots were clearly visible without substantial background. Experiments were performed in triplicate for each cell population, and spots were enumerated.

ELISA for determination of anti-ECTV antibodies. ECTV-specific IgG and subtypes were quantified by enzyme-linked immunosorbent assay (ELISA) (1, 18, 20, 21). Briefly, round-bottomed 96-well Immunolon II plates (Thermo Fisher Scientific Australia, Victoria, Australia) were coated with sucrose cushion-purified ECTV-WT at 100 ng/well in a 50-μl volume of coating buffer and incubated at 4°C overnight. The plates were washed 3× with 0.05% Tween 20 in PBS using an ELx405HT microplate washer (BIOTEK, Winooski, VT), and 50 μl of serum samples diluted at 1:200 were added to wells and incubated at 37°C for 1 h. The plates were next washed, and 50 μl of horseradish peroxidase-conjugated goat-anti-mouse total IgG (H+L) or IgG1, IgG2b, IgG2c, or IgG3 detection antibodies (Southern Biotech, Birmingham, AL) diluted at 1:5,000 was added to wells. After incubation at 37°C for 1 h, plates were washed and color developed with 50 μl TMB substrate (DakoCytomation, Carpinteria, CA). Absorbance was read at 650 nm using a SpectraMax 190 microplate spectrophotometer with SOFTmax Pro software (Molecular Devices, Sunnyvale, CA).

Statistical analysis. Statistical analyses of experimental data, employing parametric and nonparametric tests, were performed using GraphPad Prism (GraphPad Software). A P value of <0.05 was taken to be significant.

RESULTS

NK cells, granulocytes, and pDC are essential for virus control during a primary infection. NK cells are critical for recovery of mice from a primary ECTV infection (5–8). To determine whether granulocytes and pDC are necessary for virus control during a primary infection, groups of mice were treated with depleting MAb specific for granulocytes or pDC prior to and after virus infection. Additional groups treated with anti-NK1.1 MAb to eliminate NK cells or left untreated were also included.

As expected, mice not depleted of any leukocyte subset effectively controlled a primary infection and all animals recovered (Fig. 1A). However, mice in groups depleted of NK cells, granulocytes, or pDC all succumbed to mousepox within the first 10 days of infection (Fig. 1A). In mice depleted of NK cells, granulocytes, or pDC, ECTV titers in liver and spleen were significantly higher than those in nondepleted controls on day 8 postinfection (p.i.) (Fig. 1B to D), indicating that death was a direct consequence of

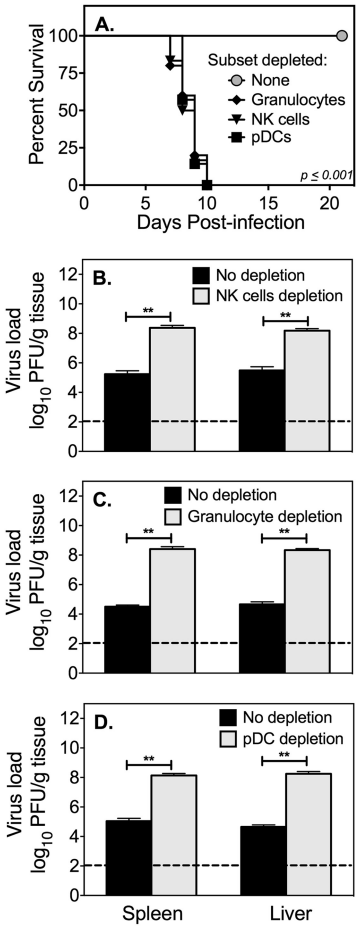

FIG 1 Outcome of primary ECTV infection and viral load in mice depleted of NK cells, granulocytes or pDC. Groups of 5 female B6 naïve mice were left untreated (None) or depleted of granulocytes, NK cells, or pDC by treatment with the appropriate MAb, infected s.c. with 10^3 PFU of ECTV, and monitored for 21 days p.i. (A). Survival rates in all groups depleted of any of the subsets were significantly different compared to those of the nondepleted group ($P \leq 0.01$; log-rank Mantel-Cox test). In a separate experiment, viral titers were measured in the spleen and liver of infected mice ($n = 5$) treated with MAb to deplete NK cells (B), granulocytes (C), or pDC (D) and sacrificed at day 8 p.i. Control mice were left untreated (None). Viral titers are presented as means of $\log_{10}$ PFU ± standard deviations (SD). *, $P \leq 0.05$; **, $P \leq 0.01$ (Mann-Whitney nonparametric test). Data shown are from one of 3 separate experiments with similar outcomes.

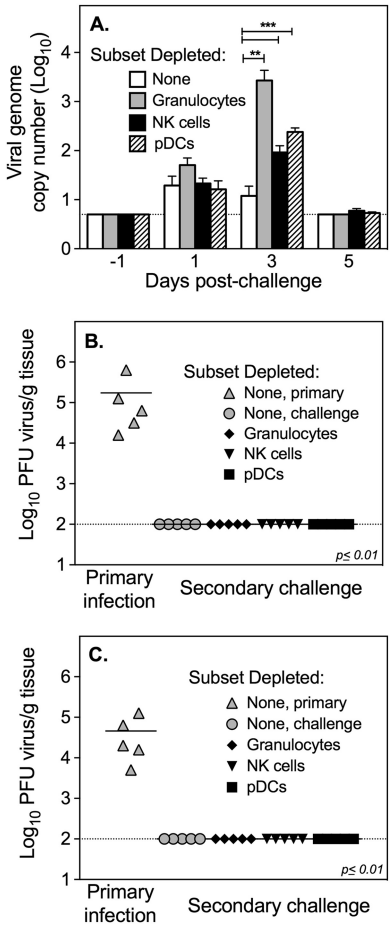

FIG 2 Viral titers in B6 mice depleted of NK cells, granulocytes, or pDC during secondary challenge. Groups of 5 female B6 mice immunized with 10^5 PFU of ECTV-TK$^{\Delta}$ 35 days previously were left untreated (None) or treated with MAb to deplete granulocytes, NK cells, or pDC. A control group of naïve mice was also infected for determination of viral load in organs (Primary infection). Mice were challenged with 10^3 PFU of ECTV-WT 1 day after the start of MAb treatment. Viral load in blood samples (A) was measured by qRT-PCR and presented as means ± standard deviations (SD) of $\log_{10}$ viral genome copy numbers. Mice were sacrificed on day 8 p.c., and organs were collected for determination of viral titers in spleen (B) and liver (C). Data are presented as means of $\log_{10}$PFU/g tissue ± SD. **, $P \leq 0.01$; ***, $P \leq 0.001$ (Mann-Whitney nonparametric test).

significantly elevated viral titers. Although the viral loads in organs of mice depleted of NK cells, granulocytes, or pDC were similar at day 8 p.i., it is conceivable that differences between groups may have been evident if titers had been measured at earlier time points p.i. Nevertheless, the similarity of increases in viral load in treated groups at day 8 p.i. is consistent with the mice in these groups succumbing to mousepox at about the same time (Fig. 1A). The important finding here is that in the absence of granulocytes or pDC, the normally resistant C57BL/6 mice become highly susceptible to ECTV infection.

The contribution of NK cells, granulocytes, and pDC to virus control during a secondary virus challenge is different from that during a primary infection. To ascertain the role of NK cells, granulocytes, and pDC in virus control during a secondary virus challenge, mice were first primed with the avirulent ECTV-TK$^{\Delta}$ (14), which primes the animals for potent primary and recall cell-mediated and antibody responses (1, 20, 21). Five weeks later, when memory was established, mice were challenged with virulent ECTV-WT. Just prior to and after virus challenge, mice were treated with MAb to deplete specific leukocyte subsets.

Strikingly and in contrast to results found in a primary infection, elimination of NK cells, granulocytes, or pDC did not affect the ability of B6 mice to effectively control virus replication during a secondary challenge. Although an increase in viral load was detected by qRT-PCR on day 3 postchallenge (p.c.) in the blood of mice depleted of granulocytes, virus was contained and titers were at or below the level of detection by day 5 (Fig. 2A). Consistent with the absence of virus in the blood at day 5 p.c., no virus was detected in the spleen or liver at day 8 in any group by viral plaque assay (Fig. 2B and C). Virus was also effectively cleared from these organs regardless of whether NK cells, granulocytes, or pDC were

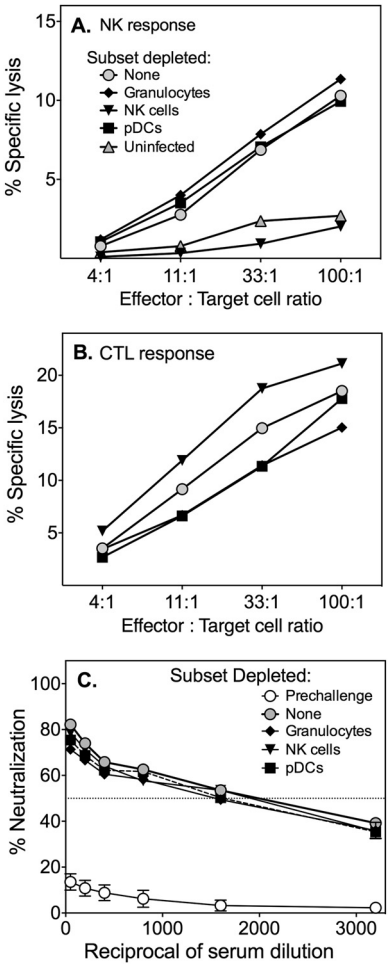

FIG 3 Effect of depletion of NK cells, granulocytes, or pDC on NK, CTL, and neutralizing antibody responses during secondary ECTV infection in mice. Groups of 5 female B6 mice, primed with ECTV-TK$^{\Delta}$ 35 days previously, were left untreated (None) or depleted of granulocytes, NK cells, or pDC and challenged with ECTV-WT. Separate groups were sacrificed on day 5 to measure NK cell responses (A) and on day 8 p.c. to measure CTL responses (B). Data shown are from one of two experiments with similar results. Virus-neutralizing activity (C) was performed by the PRNT using serum samples collected before virus challenge (day −1) and at day 8 p.c. There were no significant differences in neutralizing activity between the nondepleted group and groups depleted of specific leukocyte subsets. The reciprocal dilution of serum was log transformed and normalized before using sigmoidal (four-parameter) curve fitting on GraphPad Prism to determine 50% neutralization titers (NT_{50}) and perform sum-of-squares F test comparison. There was no statistical difference in log NT_{50} between the nondepleted group and any of the groups depleted of specific leukocyte subsets. There is a significant difference ($P \leq 0.05$) between results for the prechallenged and challenged groups.

depleted in mice that were challenged with a 100-fold-higher dose of ECTV-WT (data not shown).

As expected, depletion of NK cells in primed mice abrogated the splenic NK cell response, but elimination of pDC or granulocytes had minimal effects on NK cell-mediated cytolytic activity (Fig. 3A). In addition, the elimination of any one of these subsets did not affect the anti-ECTV CTL response on day 8 p.c. (Fig. 3B) or the virus-neutralizing antibody response (Fig. 3C). These data indicate that although NK cells, granulocytes, and pDC each play a crucial role in the control of virus replication during primary infection, their function during secondary ECTV challenge is expendable.

The combined functions of NK cells, granulocytes, pDC, and T cell subsets are required for virus control during virulent virus challenge of immune animals. Data from the preceding experiments suggested at least 3 likely reasons why elimination of NK cells, granulocytes, or pDC individually did not influence virus control during a secondary challenge. First, it is possible that the innate cell types do not play a role during a secondary infection. Second, it is possible that when the function of one innate leukocyte subset is absent, other innate cell subsets compensate and contribute to virus control. Third, in the absence of one or more innate leukocyte subsets, the T cell subsets may be sufficient to keep viral titers under check. The combined elimination of NK cells, granulocytes, and pDC (triple innate cells) together with CD4 and/or CD8 T cells should help elucidate the extent to which innate cells and/or T cell subsets contribute to virus control during the acute phase of a secondary response.

ECTV-immune B6 mice, primed in the presence of the full complement of leukocyte subsets, were therefore depleted of combinations of specific leukocyte subsets just prior to and after virulent virus challenge. Groups of mice were depleted of NK cells, granulocytes, and pDC (triple innate cells), CD4 and CD8 T cells, or triple innate cells plus CD4 and/or CD8 T cells.

Figure 4A shows that by day 5 p.c. all groups of mice that had been depleted of leukocyte subsets exhibited significantly increased viral titers compared to those of mice that were not depleted of any subset. However, by day 8 p.c., mice in the control group (not depleted of any subset) and in the groups depleted of either triple innate cells or CD4 and CD8 T cells had restricted virus replication and exhibited titers below the level of detection (Fig. 4B and C). In sharp contrast, ECTV titers in groups of mice depleted of triple innate cells plus CD4 T cells or triple innate cells plus CD8 T cells were significantly higher ($P \leq 0.01$) by about 4 to 6 $\log_{10}$ PFU in both the spleen and liver (Fig. 4B and C). Further, the viral load in mice depleted of triple innate cells plus both CD4 and CD8 T cell subsets was also significantly higher ($P \leq 0.01$), by about 5.5 to 6.5 $\log_{10}$ PFU, than those in groups depleted of triple innate cells plus just one of the T cell subsets (Fig. 4B and C). The data are consistent with the idea that in the absence of the 3 innate cell subsets, both the T cell subsets contribute to virus control whereas in the absence of T cells, the innate cell populations keep virus replication under check.

Recall B cell responses in the absence of innate immune subsets and/or T cell subsets. As antibody is considered to be of paramount importance for recovery from secondary poxvirus infection (1, 2), we investigated whether the substantial increases in viral load in immune mice depleted of specific innate and T cell subsets at the time of challenge were related to changes in recall B cell responses in those animals. The gating strategy and definitions used for phenotyping B cell subsets are shown in Fig. 5.

We found significant decreases in the total numbers of naïve B cells ($B220^+$ IgM^+ IgD^+) (Fig. 6A), memory B cells, including cells expressing low levels of peanut agglutinin (PNA^{low}) ($B220^+$ $CD38^+$ PNA^{low} IgG^+) (Fig. 6B), total-antibody-secreting cells (ASC) ($B220^-$ $CD138^+$) (Fig. 6C), and plasmablasts ($B220^+$ $CD138^+$) (Fig. 6D) in the bone marrow (BM) of mice depleted of some combinations of leukocyte subsets compared with numbers in the nondepleted control mice. While depletion of innate cells had only minimal effects on the number of ECTV-specific ASC

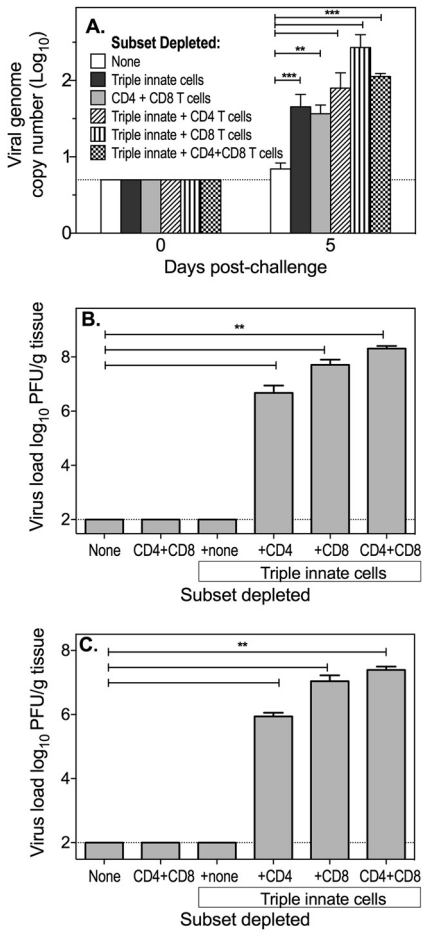

FIG 4 Viral load in blood and organs of primed mice depleted of innate and/or T cell subsets and challenged with ECTV-WT. Groups of 5 female B6 mice immunized with ECTV-TK$^{\Delta}$ 35 days previously were left untreated (None) or treated with MAb to deplete triple innate cells (NK cells, granulocytes, and pDC), CD4 plus CD8 T cells, triple innate plus CD4 T cells, triple innate plus CD8 T cells, or triple innate plus CD4 plus CD8 T cells and challenged with 10^3 PFU of ECTV-WT. Viral load in blood samples (A) was measured by qRT-PCR and presented as means ± standard deviations (SD) of $\log_{10}$ viral genome copy numbers. The dotted line represents the limit of detection of assay. Viral loads in spleen (B) and liver (C) at day 8 p.c. are presented as means ± SD of $\log_{10}$ PFU/g tissue. **, $P \leq 0.01$; ***, $P \leq 0.001$ (Mann-Whitney nonparametric test). Data shown are from one of two independent experiments with similar results.

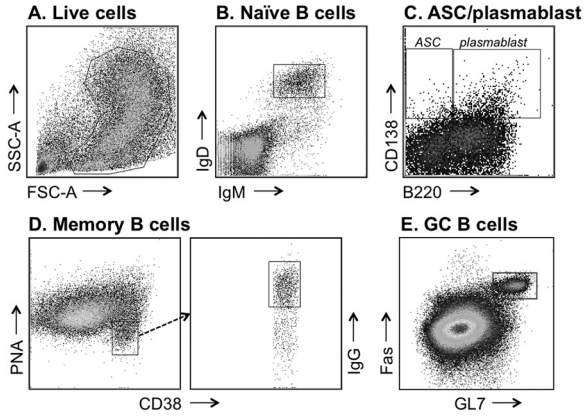

FIG 5 Gating strategy for BM and spleen B cell populations. Flow cytometry was used to phenotype B cell subsets in the spleen and BM by gating on live cells (live gate) based on forward scatter (FSC-A) and side scatter (SSC-A) plots (A). Naïve B cells (B) were gated on B220-, IgM-, and IgD-positive cells ($B220^+$ IgM^+ IgD^+), total ASC (C) were gated on B220-negative and CD138-positive cells ($B220^-$ $CD138^+$), plasmablasts (C) were gated on B220-positive and CD138-positive cells ($B220^+$ $CD138^+$), and memory B cells (D) were gated on B220-positive, CD38-positive, and IgG-positive cells and cells with low levels of peanut agglutinin (PNA) expression ($B220^+$ $CD38^+$ PNA^{low} IgG^+). Splenic GC B cells (E) were gated on B220-positive, FAS (CD95)-positive, and GL7-positive cells ($B220^+$ FAS^+ $GL7^+$).

(Fig. 6E), the elimination of both T cell subsets significantly ($P \leq 0.05$) affected the numbers. Further, the combined depletion of innate cells plus one or both T cell subsets further reduced the numbers of ECTV-specific ASC. In contrast to results in the BM, there were only minimal changes in numbers of B cell subsets in the spleen following leukocyte subset depletion. While no changes in numbers of naïve B cells (Fig. 7A) or ASC (Fig. 7B) were noted, memory B cell (Fig. 7C) numbers increased in the group depleted of innate cells and CD8 T cells. The elimination of CD4 and CD8 T cells resulted in a reduction in germinal center (GC) B cell ($B220^+$ FAS^+ $GL7^+$) (Fig. 7D) numbers, while depletion of only innate cells resulted in reduced numbers of plasmablasts (Fig. 7E). Interestingly, while no reduction in ECTV-specific ASC numbers in spleens of mice depleted of any combination of leukocyte subsets was evident, elimination of innate cells or innate cells plus CD8 T cells resulted in significantly increased numbers (Fig. 7F).

Diminished serum antiviral IgG and neutralizing antibody responses are associated with reduced B cell responses in the BM. The decreases in specific B cell subsets, particularly ECTV-specific ASC, in the BM correlated with a significant reduction ($P \leq 0.0001$) in serum virus-neutralizing activity at day 5 in groups depleted of triple innate cells plus CD4 T cells, triple innate plus CD8 T cells, or triple innate cells plus both CD4 and CD8 T cell subsets, compared with results for the other groups (Fig. 8A). However, by day 8 p.c., while virus-neutralizing activity was still significantly lower ($P \leq 0.0001$) in groups depleted of triple innate cells plus CD4 T cells or triple innate cells plus both CD4 and CD8 T cell subsets, the neutralizing activity in the group depleted of triple innate plus CD8 T cells had rebounded and titers were comparable with those of the untreated group (Fig. 8B). Contemporaneous with reduced ECTV-specific ASC in the BM and a significant reduction in virus-neutralizing activity, serum levels of ECTV-specific total IgG (Fig. 8C and D) and IgG subtypes (Fig. 8E and F) were also decreased to various degrees in groups depleted of innate cells plus one T cell subset at days 5 and 8 p.c. In contrast to these results and unlike results in the BM, increases in numbers of ECTV-specific ASC in the spleens of mice as a consequence of depletion of innate cells or innate cells plus CD8 T cells were not reflected by any increases in ECTV-specific IgG or the virus-neutralizing antibody titers (Fig. 8).

DISCUSSION

Previous work from our laboratory established that recovery from an ECTV infection in vaccinated mice required antibody but not $CD8^+$ CTL function and/or $CD4^+$ helper T cell responses during the acute phase of a secondary infection (1). Similarly, in a separate study, elimination of $CD4^+$ T cells with anti-CD4 MAb was found not to affect monkeypox virus titers or neutralizing anti-

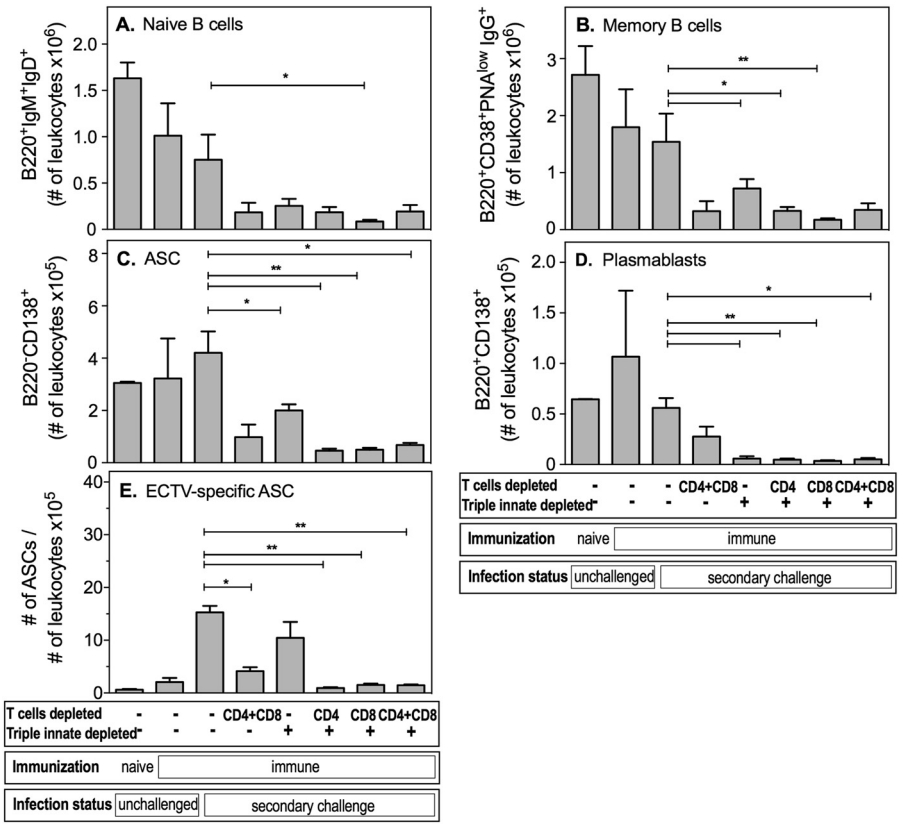

FIG 6 Effect of leukocyte subset depletion on naïve B cell, memory B cell, ASC, plasmablast, and ECTV-specific ASC numbers in the BM. Groups of 5 female WT mice previously immunized with ECTV-TK$^{\Delta}$ were left untreated (immunized, nondepleted) or depleted of leukocyte subsets and challenged with ECTV-WT as described for Fig. 4 and sacrificed on day 8 p.c. Groups of unimmunized (naïve), immunized, and unchallenged mice were included as controls. Flow cytometry was used to quantify naïve B cells (B220^{+} IgM^{+} IgD^{+}) (A), memory B cells (B220^{+} CD38^{+} PNA^{low} IgG^{+}) (B), ASC (B220^{-} CD138^{+}) (C), and plasmablasts (B220^{+} CD138^{+}) (D) in the BM of mice. ECTV-specific ASCs (E) were quantified by ELISPOT assay. *, $P \leq 0.05$; **, $P \leq 0.01$ (Mann-Whitney nonparametric test). Data shown are from one of two independent experiments with similar results.

body responses during the acute phase of a secondary monkeypox challenge in macaques vaccinated with VACV 6 months previously (2). However, the role of leukocytes that form part of the innate immune system in virus control in the absence of T cell subsets was not considered in either of these studies.

While an important role for NK cells in ECTV control is well established (5–8), this is the first demonstration that pDC and granulocytes are necessary for recovery of mice from a primary poxvirus infection. pDC produce high levels of type I interferon (IFN) and interleukin 6 (IL-6), and both of these cytokines are essential for recovery from primary ECTV infection (20, 22–24). A number of studies have demonstrated an important role for granulocytes in the control of other viral infections (25–27); hence the finding reported here for ECTV is not entirely unexpected. Granulocytes can phagocytose virus-infected cells or virus particles in addition to their ability to produce cytokines and chemokines, which can activate and recruit other effector cells to the sites of virus replication to combat the infection.

In contrast to results in a primary infection, the elimination of NK cells, granulocytes, or pDC individually did not affect virus control or the recall anti-ECTV immune response during the acute phase of a secondary virus challenge. In addition, although the elimination of all 3 innate leukocyte subsets or both CD4 and CD8 T cell subsets resulted in marginal increases in viral load at day 5 p.c., titers were below the level of detection by day 8 p.c. The lack of any effect on virus control following depletion of both T cell subsets during secondary virus challenge is consistent with our previous finding (1). It remained possible that in vaccinated mice depleted of NK cells, granulocytes, and pDC, the memory T cell subsets compensate for and control virus replication. Furthermore, it was also possible that in the absence of both T cell subsets, the innate cell populations may contribute to virus control. Our data indeed established that viral titers in groups of mice depleted of triple innate cells and CD4 T cells or triple innate cells and CD8 T cells were significantly higher than those of groups depleted of only innate cells or CD4 and CD8 T cells. Viral load in mice depleted of triple innate cells plus both CD4 and CD8 T cell subsets was further elevated compared to those in groups depleted of triple innate cells plus just one of the T cell subsets.

The grossly elevated viral load at day 8 p.c. in mice depleted of triple innate cells plus only CD4 T cells along with the group depleted of triple innate cells plus both CD4 and CD8 T cell subsets directly correlated with a reduction in virus-neutralizing activity and antigen-specific antibody responses. This finding suggested that the significant drop in the neutralizing activity of antibody was likely responsible for the increase in viral load. However, it was clear that in addition to T-dependent neutralizing antibody responses, the effector function of CD8 T cells is also

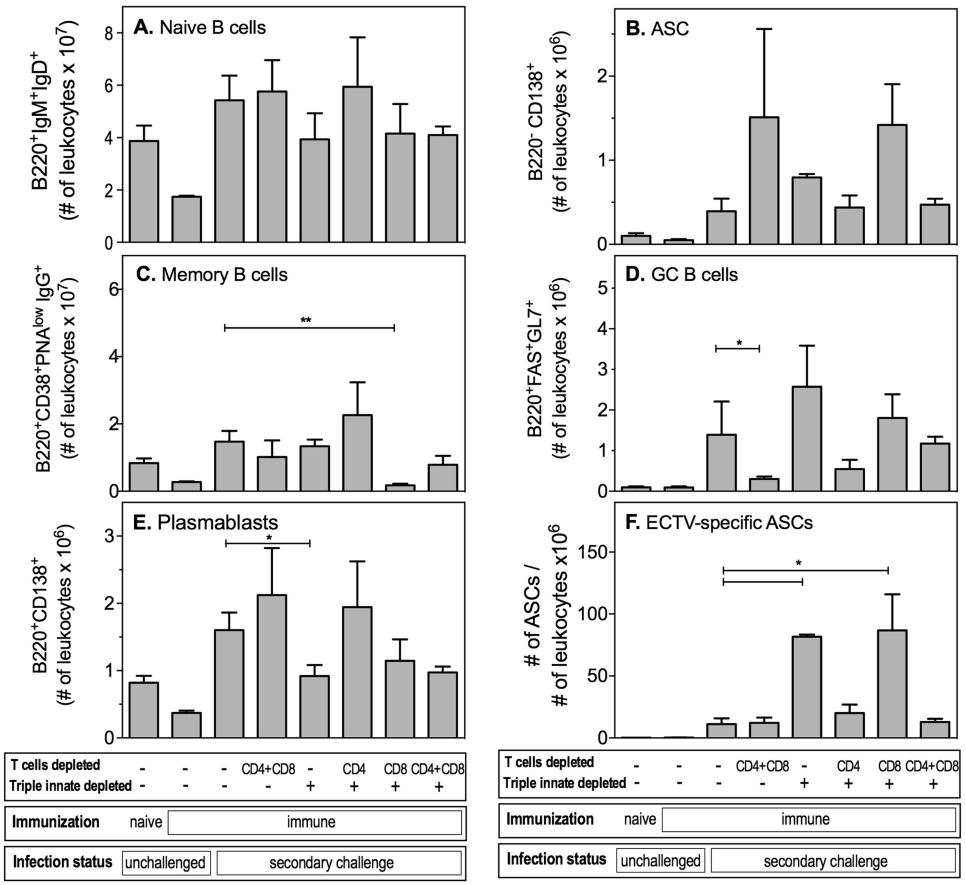

FIG 7 Effect of leukocyte subset depletion on naïve B cell, memory B cell, ASC, plasmablast, GC B cell, and ECTV-specific ASC numbers in the spleen. Groups of 5 female WT mice previously immunized with ECTV-TK$^{\Delta}$ were left untreated (immunized, nondepleted) or depleted of leukocyte subsets and challenged with ECTV-WT as described for Fig. 4 and sacrificed on day 8 p.c. Groups of unimmunized (naïve), immunized, and unchallenged mice were included as controls. Flow cytometry was used to quantify naïve B cells ($B220^+$ IgM^+ IgD^+) (A), memory B cells ($B220^+$ $CD38^+$ PNA^{low} IgG^+) (B), ASC ($B220^-$ $CD138^+$) (C), plasmablasts ($B220^+$ $CD138^+$) (D), and GC B cells (E) in the spleen of mice. ECTV-specific ASCs (F) were quantified by ELISPOT assay. *, $P \leq 0.05$; **, $P \leq 0.01$ (Mann-Whitney nonparametric test). Data shown are from one of two independent experiments with similar results.

clearly important for virus control. Viral loads in the organs of mice depleted of triple innate cells plus CD8 T cells were significantly increased by 5.5 to 6.5 $\log_{10}$ PFU (Fig. 4B and C) despite these animals having neutralizing antibody titers similar to those of the nondepleted control group at day 8 p.c. (Fig. 8B). In the absence of the innate cells, a nonredundant but critical role for each of the T cell subsets became evident.

Interestingly, while the depletion of the three innate cell subsets or CD4 and CD8 T cells had only marginal effects on the neutralizing antibody titers, the combined elimination of innate cells and CD4 T cells or innate cells and both CD4 and CD8 T cells resulted in much lower levels of neutralizing antibody responses. Curiously, elimination of the innate cells also resulted in significant reductions in specific B cell subsets in the BM and serum antibody levels. It appears, therefore, that these innate cells may have an important role to play in optimizing the antibody and B cell response necessary for the dominant protective role of antibodies. The mechanism(s) through which NK cells, granulocytes, or pDC contribute to antibody production is currently not known. Nonetheless, a number of recent studies have provided evidence that leukocytes of the innate immune system contribute significantly to antibody responses. The first study showed that elimination of basophils in mice resulted in a reduced humoral recall response and increased the susceptibility of immunized mice to sepsis in a model of *Streptococcus pneumoniae* infection (28). Antigen-reactive basophils were found to increase specific antibody production, and activated basophils, in combination with CD4 T cells, augmented B cell proliferation and IgG production. In the second study, eosinophils were found to be critical for long-term maintenance and retention of ASC in the BM (29). Eosinophils supported ASC survival through production of a proliferation-inducing ligand (APRIL) and IL-6. In the third study, neutrophils localized to the marginal zone of the spleen were shown to induce IgG class switching, somatic hypermutation, and antibody production (30). B cell help was provided by neutrophils through the cytokines B-cell-activating factor, belonging to the TNF family (BAFF), APRIL, and IL-21. Finally, pDC, through the production of type I IFN and IL-6, can induce human B cells to differentiate into plasma cells and produce IgG (31, 32). It is thus possible that in our model, granulocytes and pDC assist in antibody responses through the production of specific cytokines.

The elimination of innate and/or T cell subsets resulted in a significant reduction in ECTV-specific ASC and some other B cell subsets in the BM. While a reduction in numbers of ECTV-spe-

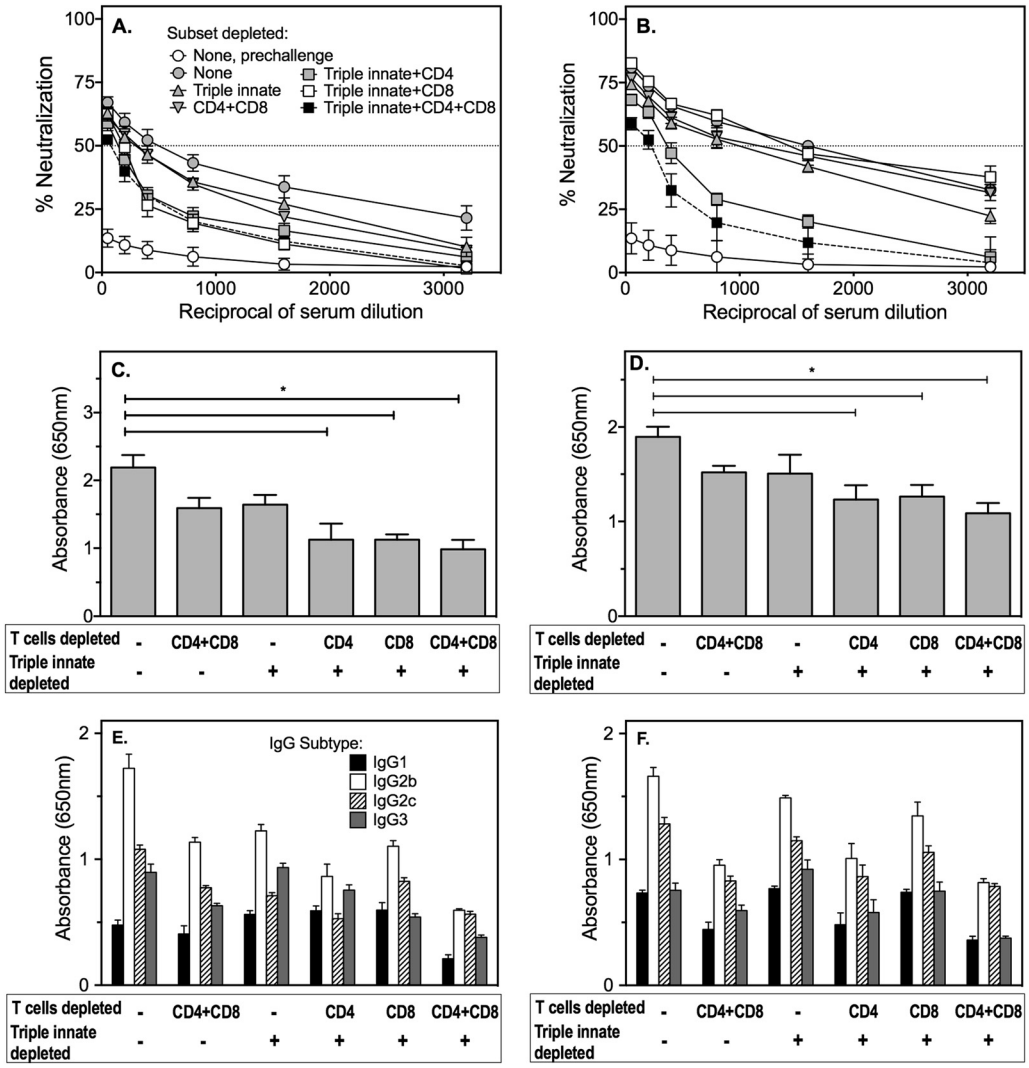

FIG 8 Effect of leukocyte subset depletion during secondary ECTV challenge on virus-neutralizing antibody and anti-ECTV IgG responses. Groups of 4 or 5 WT mice previously primed with ECTV-TK$^{\Delta}$ were left untreated (None) or depleted of leukocyte subsets and infected as described for Fig. 4. Virus-neutralizing activity was determined by the PRNT using serum samples collected before challenge (day −1; prechallenge) and at days 5 (A) and 8 (B) p.c. Statistical analysis was performed as for Fig. 3C. On day 5 p.c., the neutralizing titers in groups depleted of triple innate plus CD4, triple innate plus CD8, or triple innate plus CD4 plus CD8 cells were significantly different ($P \leq 0.0001$) compared with those of the untreated group (None). At day 8 p.c., the neutralizing titers in groups depleted of triple innate plus CD4 or triple innate plus CD4 plus CD8 were significantly different ($P \leq 0.0001$) compared with those of the untreated group. ECTV-specific total IgG (C and D) and ECTV-specific IgG subtypes (E and F) were measured on days 5 (C and E) and 8 (D and F) p.c., at a serum dilution of 1:200 using an ELISA. Shown are means of absorbance values ± standard errors of the means of serum samples from 4 individual mice in each group. *, $P \leq 0.05$; (Mann-Whitney nonparametric test). Data shown are from one of two independent experiments with similar results.

cific ASC may be expected, particularly in the absence of T cell help, it is not clear why numbers of naïve and memory B cells or total ASC and plasmablasts were also decreased when some combinations of leukocyte subsets were depleted. We speculate that given the complex interactions between the various leukocyte subsets in the generation of an immune response, elimination of combinations of specific cell types may alter the numbers or frequencies of particular B cell populations. This happens to a larger extent in the BM compared to the spleen. In contrast to the BM, in the spleen there were only minimal changes in numbers of specific B cell populations. Furthermore, unlike the BM, there was no correlation between the levels of virus-neutralizing serum antibody levels and numbers of ECTV-specific ASC in the spleen.

The finding that the ECTV-specific ASC responses in the spleen were largely unaffected by leukocyte depletion compared to the responses in the BM may, in part, be explained by the fact that the latter is the major niche for ASC (33, 34). BM stromal cells provide a microenvironment that supports the survival of ASC through cell contact-dependent signals and through secretion of specific chemokines and cytokines. Indeed, ECTV-specific ASC were detected in the BM of immune animals, and numbers increased rapidly following secondary challenge. On the other hand, ECTV-specific ASC were below the level of detection in the spleen in immune animals but numbers increased only following a secondary challenge (Fig. 7F). Finally, unlike results in the BM, elimination of CD4 T

cells had no impact on ECTV-specific ASC numbers in the spleen over the first 8 days p.c., suggesting that these were mainly extrafollicular plasma cells.

In summary, although NK cells, granulocytes, and pDC each play a crucial role in the control of virus replication during primary infection, their function during secondary challenge in immune animals seems less critical but becomes apparent in the absence of all three of these subsets. It is possible that either there is a level of redundancy in the contribution of these subsets to a secondary virus challenge or the additive effects of loss of function for each tip the balance in favor of the virus. In either case, collectively NK cells, granulocytes, and pDC play an important role in the control of virus during a secondary poxvirus challenge. Furthermore, when these innate immune cells are absent, both CD4 and CD8 T cell subsets compensate and play an important role in recovery, and conversely, when both these T cell subsets are eliminated, the innate immune cells help contain the infection. This finding challenges previous reports (1, 2) that antibody and B cells alone are necessary and sufficient for protection against secondary orthopoxvirus infections. Although antibody plays a dominant role, both innate immune cells and CD4 and CD8 T cells also contribute to virus control in a recall response to infection.

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