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Elevated non-specific immunity and normal *Listeria* clearance in young and old vitamin D receptor knockout mice

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Abstract

1,25-Dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] and the vitamin D receptor (VDR) are important regulators of autoimmunity. The effect of the VDR on the ability of mice to fight a primary or secondary infection has not been determined. Young and old VDR knockout (KO) mice were able to clear both primary and secondary infections with *Listeria monocytogenes*. However, the kinetics of clearance was somewhat delayed in the absence of the VDR. Memory T cell development was not different in young VDR KO and wild-type (WT) mice; however, old VDR KO mice had significantly less memory T cells than their WT counterparts but still mounted an adequate immune response as determined by the complete clearance of *L. monocytogenes*. Although the primary and secondary immune responses were largely intact in the VDR KO mice, the old VDR KO mice had increased cytokines and antibody responses compared with the old WT mice. In particular, old VDR KO mice had elevated antigen non-specific antibodies; however, these magnified immune responses did not correspond to more effective *Listeria* clearance. The increased antibody and cytokine responses in the old VDR KO mice are consistent with the increased susceptibility of these mice to autoimmunity.

Introduction

The active form of vitamin D, 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$], has been shown to have an important role in regulating the immune system. The effects of vitamin D can be observed in many cells of the immune system and the vitamin D receptor (VDR) is found in myeloid and lymphoid cells in resting and activated states (1). It has been shown that 1,25$(OH)_2D_3$ suppresses T cell proliferation and decreases the production of the T_h1 cytokines IFN-γ, IL-2 and tumor necrosis factor (TNF)-α (2, 3). T_h1-driven autoimmune diseases including experimental multiple sclerosis and experimental inflammatory bowel disease are suppressed by *in vivo* treatment with 1,25$(OH)_2D_3$ (4, 5) and 1,25$(OH)_2D_3$ inhibits the listeriacidal activity and oxidative burst of IFN-γ-stimulated macrophage *in vitro* (6), indicating that 1,25$(OH)_2D_3$ is an important regulator of T_h1-mediated immune responses.

In the absence of the VDR, the immune system develops normal numbers and subsets of T cells and B cells. NKT cell development, however, is blocked in the VDR knockout (KO) mice (7). VDR KO mice have heightened IFN-γ-mediated immune responses and decreased induction of T_h2 response. For example, VDR KO mice are more susceptible to experimental inflammatory bowel disease, have heightened IFN-γ responses and decreased IL-4 responses and are more resistant to the T_h2 disease experimental allergic asthma (8, 9). VDR KO mice develop larger granulomas when infected with *Shistosoma mansoni* [T_h2 dominated, (8)] and have decreased *Leishmania major* (T_h1 dominated) parasite burdens compared with wild-type (WT) controls (10). However, *in vitro* killing of *Listeria* by infected macrophages is not affected by deletion of the VDR (6). The VDR KO mice thus have heightened IFN-γ-mediated immune responses and decreased induction of T_h2 responses.

Listeria monocytogenes is a gram-positive intracellular bacterium that causes infections when ingested in contaminated food. This bacterium is commonly used as a model

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for studying immune responses to intracellular pathogens. IFN-γ, IL-12 and CD8 T cells are critical for clearance of *L. monocytogenes* infections (11). Both MHC class I and class II are required for the response to *Listeria* since deletion of either gene leads to more severe infection, reduced IFN-γ production and increased severity of granulomatous lesions (12). While IL-10 is required for the immune response to *Listeria*, reduction in this cytokine early during infection leads to more rapid clearance of the bacteria (13, 14). $CD8^+$ T cell memory is reduced in the absence of IL-10, accompanied by increased susceptibility to secondary infections, supporting an important role for IL-10 in the response to this disease (15). During secondary *L. monocytogenes* infection, $CD8^+$/$CD44^{high}$ T cells are responsible for early onset of specific immune responses (16). IFN-γ is required for sterilizing immunity to secondary infection and this is partially dependent on IL-12 production, and while IFN-γ-deficient mice are extremely susceptible to primary infections, they can produce $CD8^+$ T cell-mediated responses during secondary infection (17, 18). In addition, IL-12 deficiency leads to decreased effector responses during primary infection and increased memory following immunization (17). Protection from *L. monocytogenes* is dependent on classical T_h1-driven cytokine responses with induction of memory CD8 T cells for sterilizing immunity.

Aging results in a decrease in antigen-specific immunity to new antigens. The elderly are more susceptible to infectious diseases including *L. monocytogenes* infection due to a decline in their ability to specifically generate anti-bacterial immunity (19). Increased susceptibility to infections in the elderly has been attributed to reduced T cell proliferation and decreased IL-2 production (20, 21). The number of mature splenic B cells also decreases with age, and reduced titers of antibodies are made following infection (21, 22). Although the aged immune response is reduced in response to new antigenic challenges, there is an increase in self-reactivity (23).

As $1,25(OH)_2D_3$, and in particular the VDR, is an important regulator of T_h1-mediated immune responses, we have determined the role of the VDR on the ability of mice to mount an immune response to *L. monocytogenes*. Our experiments show that young VDR KO mice exhibited a lag in the clearance of *L. monocytogenes* during primary challenge of the mice. The lag in clearance occurred early post-infection when the VDR KO mice produced significantly higher levels of IL-10 and IFN-γ compared with WT mice. Memory CD8 and CD4 T cell responses, as well as antigen-specific IgG1 and IgG2c developed normally in the young VDR KO and WT mice. The aged VDR KO mouse also effectively cleared *L. monocytogenes* from the spleen with largely the same kinetics of the aged WT mouse. However, following a primary infection, aged VDR KO mice had slower kinetics of clearance in the liver compared with WT mice. By contrast, antigen-specific as well as total IgG1 and IgG2c were significantly higher following secondary infection in aged VDR KO mice. The aged VDR KO mice produced more IFN-γ despite the fact that the number of memory CD4 and CD8 T cells producing IFN-γ were lower in the mice compared with WT. These results indicate that expression of the VDR is not required for host resistance to *L. monocytogenes* in either young or old mice. Instead, old VDR KO mice have heightened cytokine and antibody responses that did not correlate with the ability to clear the infection.

Materials and methods

Mice

Female C57BL/6 (WT) and VDR KO mice were bred and maintained at the Pennsylvania State University (University Park, PA, USA). The mice were 2 months (young) or 19 months (old) at the initiation of the experiments. All experimental procedures were approved by the Office of Research Protection's Institutional Animal Care and Use Committee (Pennsylvania State University).

Listeria monocytogenes and immunization

Transgenic recombinant *L. monocytogenes* expressing ovalbumin (OVA) [gift from Hao Shen, University of Pennsylvania, PA, USA (15)] was incubated overnight in blood heart infusion (BHI) broth. For primary infections, mice received 5×10^6 colony-forming unit (CFU) intra-peritoneal injections of *Listeria* and were euthanized 2, 4 and 10 days later. For secondary challenge, mice received a low dose of 1×10^4 CFU for primary exposure, and then 30 days later were challenged with 5×10^6 CFU and euthanized 1, 2 and 4 days later.

Assessment of bacterial burden

Spleens and livers of infected animals were harvested and weighed and the tissue was homogenized in 1 ml sterile PBS. CFUs were determined by plating 10-fold dilutions on BHI agar plates and the colonies were counted 48–72 h later.

Flow cytometry

Splenocytes were collected and after lysis of the RBCs were stimulated 10–12 h with phorbol 12-myristate 13-acetate (0.1 μg ml^{-1}) and ionomycin (0.5 μg ml^{-1}); brefeldin A (10 μg ml^{-1}) was added for the final 6 h. Cells were stained with anti-CD44 (PE), anti-CD4 (ECD) and anti-CD8 (PE–Cy5), fixed with 4% PFA, permeabilized with 0.1% saponin and stained with anti-IFN-γ (FITC), all from BD PharMingen (San Diego, CA, USA). Analyses were performed on a FC500 bench top cytometer (Beckman Coulter, Miami, FL, USA). Data were evaluated with WinMDI 2.9 software (Scripps Institute, La Jolla, CA, USA).

Cytokine production

Splenocytes (5×10^6) were incubated 72 h in RPMI-1640 complete media alone or with additional stimulation with OVA (100 μg ml^{-1}) and supernatants were collected 72 h later. ELISAs were performed for cytokines IFN-γ, IL-10, IL-12 and TNF-α (kits purchased from BD Biosciences). The limits of detection were 40 pg ml^{-1} IFN-γ, 30 pg ml^{-1} IL-10, 35 pg ml^{-1} IL-12 and 100 pg ml^{-1} TNF-α.

Antibody titers

Serum from infected mice was serially diluted and incubated in 96-well plates coated with 1×10^6 *Listeria* for 2 h. Plates were washed three times, probed with HRP-conjugated anti-IgG2c or anti-IgG1 (BD PharMingen) followed by

3.3′,5.5′-tetramethylbenzidine substrate (BD Biosciences) and analyzed on a HTS 7000 BioAssay Reader (PerkinElmer, Norwalk, CT, USA).

Statistical analysis

Results are expressed as the mean ± standard error of the mean. Experiments in young mice were performed twice with four mice per group and at each time point. Representative data from one experiment are shown. Experiments in old mice were performed once due to the high costs associated with housing mice for 19 months. Analysis was performed using unpaired *t*-tests and analysis of variances (Prism 4, GraphPad software, San Diego, CA, USA). Statistical significance is considered to be a value of $P \leqslant 0.05$.

Results

Listeria monocytogenes is cleared from young VDR KO mice following both primary and secondary infections

The clearance of *L. monocytogenes* was tracked in the spleen and liver of young VDR KO and WT mice. Except for day 2, post-infection weights of the spleen and liver of VDR KO and WT mice following primary infection were not significantly different (Supplementary Table S1, available at *International Immunology* Online). Two days following primary infection, WT and VDR KO mice had equal numbers of bacteria in the spleen (even when the organ weights are taken into account, Supplementary Table S1, available at *International Immunology* Online, and Fig. 1A). The kinetics of bacterial clearance in the spleen was faster in the WT compared with the VDR KO mice (Fig. 1A). In the liver, VDR KO mice had more *Listeria* than WT mice at day 4 (Fig. 1A). All VDR KO and WT mice had completely cleared the bacteria in the liver by day 10 post-infection and both groups of mice completely cleared the primary infection by day 14 post-infection (data not shown).

To determine if there were any differences in the recall response, young WT and VDR KO mice were infected with 10^4 *Listeria* organisms and then re-challenged 30 days later with 5×10^6 *L. monocytogenes*. The spleens of the VDR KO mice were significantly heavier at 1 and 2 days post-infection than their WT counterparts (Supplementary Table S1, available at *International Immunology* Online). The liver was also heavier at day 2 post-infection (Supplementary Table S1, available at *International Immunology* Online). As expected, clearance of the secondary challenge was faster than clearance of the primary infection in the WT mice (compare Fig. 1A and B). However, as in the primary infection, the kinetics of the response in VDR KO mice was delayed, with more *L. monocytogenes* recoverable from the spleen (significant) and liver (not significant) of VDR KO mice at 2 days post-infection than WT mice (Fig. 1B). Since the bacterial numbers are calculated per gram of tissue and as noted the spleen and liver of the VDR KO mice were larger than the WT at day 2 post-infection, the differences in bacterial burden at this time point between WT and VDR KO mice are amplified.

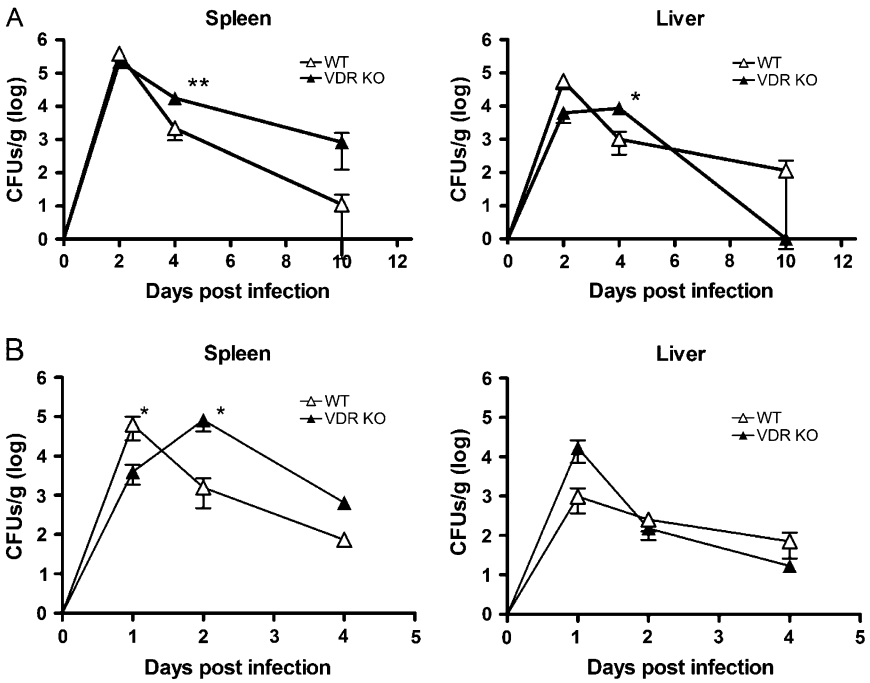

Fig. 1. Bacterial CFU in the spleen and liver of young WT and VDR KO mice following primary and secondary infections with *Listeria monocytogenes*. (A) CFU in the spleen and liver following primary infection with 5×10^6 *L. monocytogenes*. (B) CFU in the spleen and liver following primary infection with 1×10^4 *L. monocytogenes* and secondary infection 30 days later with 5×10^6 *L. monocytogenes*. Values are mean ± standard error of the mean of four individual mice at each time point and one of two representative experiments. VDR KO value is significantly different than the corresponding WT value, $*P \leqslant 0.05$, $**P \leqslant 0.001$.

Cytokine and antibody responses of young VDR KO and WT mice following primary and secondary infection with L. monocytogenes

To examine the cytokine response, splenocytes were removed from VDR KO and WT mice cultured alone or re-stimulated *in vitro* with OVA, following a primary infection with *L. monocytogenes*, since the bacteria carry a transgene expressing OVA (15). In the absence of re-stimulation, splenocytes produced only low levels of cytokines in all groups (data not shown). OVA stimulation of naive splenocytes did not induce cytokine production (data not shown). Because the spleens of both the WT and VDR KO mice still contained *Listeria*, the stimulation with OVA was in effect done in the presence of *Listeria*, therefore inducing production of cytokines from innate immune cells and early T cell responses. Stimulation of the VDR KO and WT splenocytes with OVA induced the same amounts of IFN-γ at all time points except day 4, when VDR KO splenocytes produced significantly higher amounts of IFN-γ than those from WT mice (data not shown for days 2 and 10; Fig. 2A). These early differences may reflect the higher numbers of *Listeria* present in the VDR KO spleen at these time points. Serum IFN-γ levels were also measured following a primary *Listeria* infection. At all time points except day 2 post-infection, the levels of IFN-γ in the serum was below the limits of detection. At day 2 post-infection, WT mice had 1240 ± 64 $pgml^{-1}$ and VDR KO mice had 1665 ± 306 $pgml^{-1}$ IFN-γ in the serum, which was not significantly different. Production of IL-12 was similar in VDR KO and WT cultures, while IL-10 production was significantly increased in VDR KO cultures at all time points (Fig. 2A; day 2 and day 10 not shown). By contrast, the kinetics and the amounts of IFN-γ, IL-12 and IL-10 generated by OVA-stimulated splenocytes of young WT and VDR KO mice after secondary challenge were comparable at all time points (Fig. 2B). Low levels of IFN-γ were detected in the sera at day 1 post-secondary infection but were not different between WT and VDR KO mice with *Listeria* (data not shown). We also determined *Listeria*-specific IgG1 and IgG2c antibody titers in the sera of young VDR KO and WT mice 4 days after the secondary infection and there was no significant difference in the titers of *Listeria*-specific IgG1 and IgG2c antibodies between the two groups (Fig. 2C).

Development of memory CD4 and CD8 T cells in young VDR KO mice and WT mice

The memory T cell responses of young VDR KO and WT mice were measured following secondary infection with *L. monocytogenes*. Splenocytes from WT and VDR KO mice were stained for CD4, CD8 and the memory marker CD44. There were similar percentages of memory and naive $CD4^+$

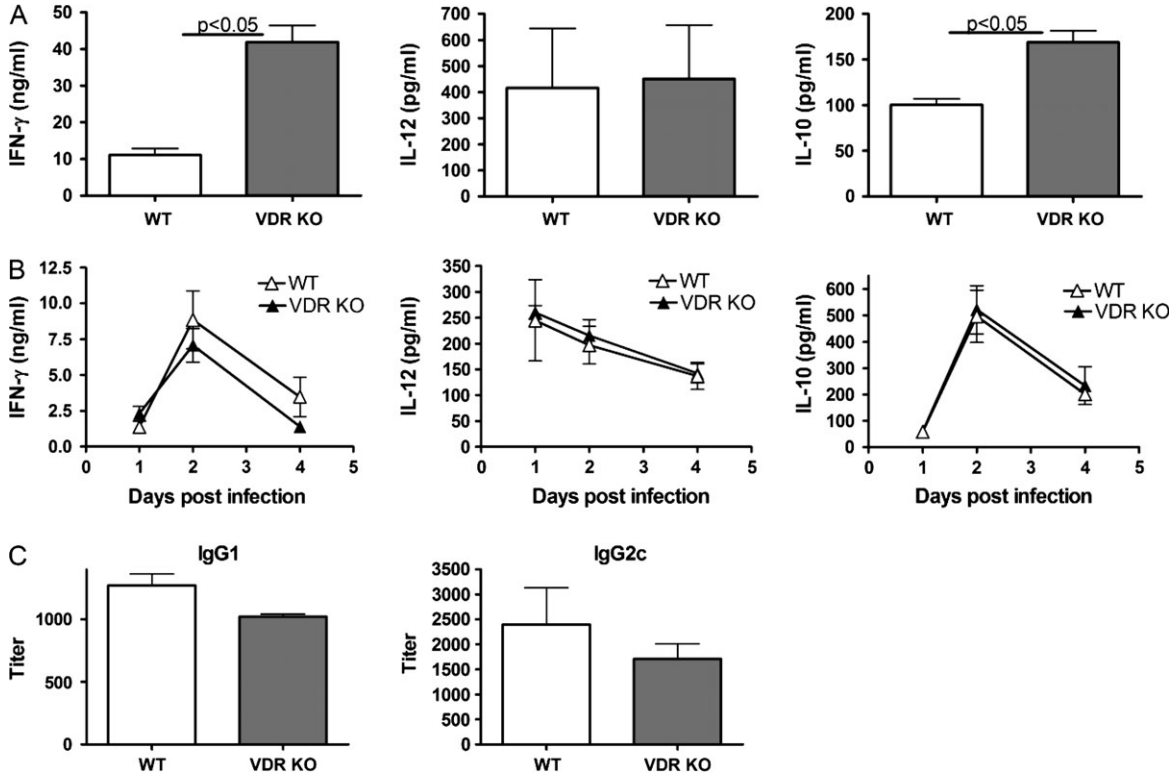

Fig. 2. Peripheral immune responses following primary and secondary infection of young WT and VDR KO mice with *Listeria monocytogenes*. (A) IFN-γ, IL-12 and IL-10 production from whole splenocytes following primary infection and *in vitro* stimulation with OVA at 4 days post-infection. (B) IFN-γ, IL-12 and IL-10 production following secondary *L. monocytogenes* infection and *in vitro* stimulation with OVA. (C) *Listeria*-specific antibody responses in young VDR KO and WT mice following secondary *L. monocytogenes* infection. Values are mean ± standard error of the mean of four individual mice and one of two representative experiments. IFN-γ and IL-10 values from VDR KO mice were significantly higher than WT mice, $P < 0.05$.

and $CD8^+$ T cells in WT and VDR KO mice (Table 1). The percentage of IFN-γ-producing cells was determined in $CD8^+/CD44^{high}$ and $CD4^+/CD44^{high}$ T cells (Fig. 3). The percentages of IFN-γ-secreting memory $CD8^+$ and $CD4^+$ T cells were similar at all time points post-infection in the VDR KO and WT mice (Fig. 3B).

Old VDR KO and WT mice clear L. monocytogenes infection

To determine if age affects the ability of VDR null mice to clear *Listeria* infection, 19-month-old female (old) VDR KO and WT mice were used for the next set of infections. Similar experiments were performed where old WT and VDR KO mice were infected with *Listeria* as done for the young mice. The spleens and livers of old VDR KO mice infected with *Listeria* weighed significantly more than their old WT counterparts at the early time points (day 2 and day 4, Supplementary Table S2, available at *International Immunology* Online). The CFUs per gram of spleen were the same in the old VDR KO and WT mice at day 2 and day 4 post-infection. However, old VDR KO mice had spleens that were twice as big as the old WT spleens and therefore had larger bacterial burdens at day 2 and day 4 post-infection (Fig. 4A and Supplementary Table S2, available at *International Immunology* Online). By day 10 post-infection, old WT and VDR KO mice cleared *L. monocytogenes* primary infections from the spleen (Fig. 4A). This was in contrast to the young VDR KO and WT mice, where *L. monocytogenes* was recoverable at day 10 post-primary infection (Fig. 1A). However, old WT and VDR KO mice showed persistent low grade colonization of the liver following a primary infection (Fig. 4A). Further analysis revealed that the liver of the old VDR KO mice was larger and had significantly more *L. monocytogenes* at day 2 and day 4 following primary infection, but not at day 10, than the old WT mice (Fig. 4A). Old VDR KO and old WT mice were able to completely clear the primary infection from the liver by 21 days post-infection (data not shown).

As described previously for young mice, additional old VDR KO and WT mice were challenged twice, first with a low dose and then with the challenge dose of *L. monocytogenes*. We found that in contrast to the results from young mice, secondary challenge of old WT and VDR KO mice did not result in faster clearance of *L. monocytogenes* compared

Table 1. Memory T cells in young and old VDR KO and WT mice

	WT (%)	VDR KO (%)
Young		
$CD8^+/CD44^{high}$	50 ± 5	42 ± 6
$CD4^+/CD44^{high}$	61 ± 4	63 ± 5
Old		
$CD8^+/CD44^{high}$	26 ± 5	31 ± 16
$CD4^+/CD44^{high}$	42 ± 15	33 ± 7

Values are the mean ± standard error of the mean of $n = 4$.

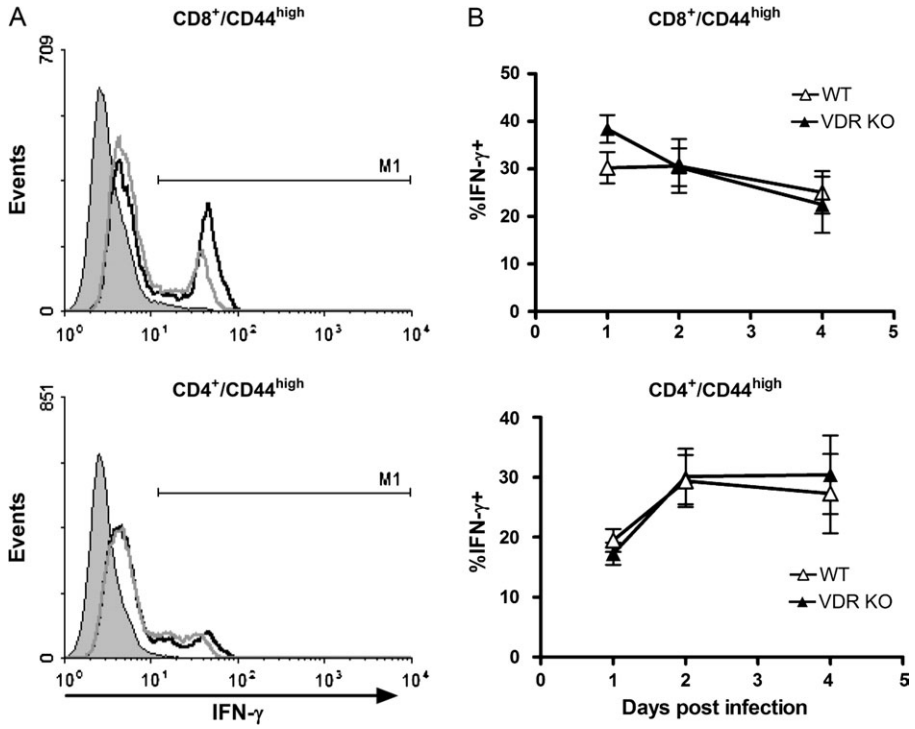

Fig. 3. IFN-γ secretion by memory T cells in young VDR KO and WT mice after secondary exposure to *Listeria monocytogenes*. Splenocytes were stained for IFN-γ, CD8, CD4 and CD44 following 1–4 days post-secondary infection. (A) $CD8^+/CD44^{high}$ (top) or $CD4^+/CD44^{high}$ (bottom) T cells were gated and the histograms showing staining for IFN-γ-producing cells presented. The solid peak shows the isotype control staining and IFN-γ expression of VDR KO (black line) and WT (gray line). (B) Average percentage of IFN-γ-producing $CD8/CD44^{high}$ (top) and $CD4/CD44^{high}$ (lower) T cells in young VDR KO and WT mice. Values are mean ± standard error of the mean of four individual mice. Experiments were repeated twice.

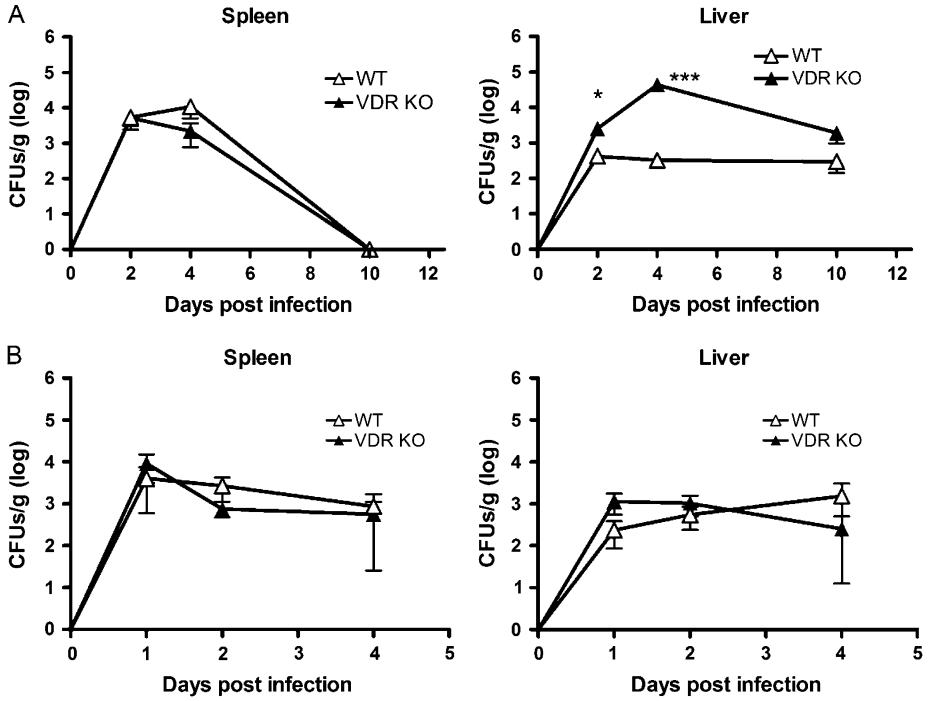

Fig. 4. Bacterial CFU in the spleen and liver of old WT and VDR KO mice following primary and secondary infections with *Listeria monocytogenes*. (A) CFU in the spleen and liver following primary infection with 5×10^6 *L. monocytogenes*. (B) CFU in the spleen and liver following primary infection with 1×10^4 *L. monocytogenes* and secondary infections 30 days later with 5×10^6 *L. monocytogenes*. Values are mean ± standard error of the mean of three to four individual mice at each time point. VDR KO value is significantly different than the corresponding WT value, $*P \leqslant 0.05$, $**P \leqslant 0.001$, $***P \leqslant 0.0001$.

with the primary infection (compare Fig. 4A and B). There were no differences in the weights of the spleens and livers from VDR KO and WT mice at any time points post-infection (Supplementary Table S2, available at *International Immunology* Online). The number of *L. monocytogenes* in the spleen of old mice at day 4 post-secondary infection was not different than at day 4 post-primary infection (Fig. 4). A similar picture was apparent in the livers of old WT mice where the numbers of *L. monocytogenes* were the same after 1, 2 or 4 days post-secondary and primary infection (Fig. 4). In addition, old VDR KO mice had fewer CFUs in the liver following a secondary *Listeria* infection compared with the primary infection (Fig. 4). However, there was 100% clearance of *L. monocytogenes* in the spleens and livers of all old VDR KO and WT mice by 21 days post-infection (data not shown).

Old VDR KO mice have heightened cytokine responses compared with old WT mice

The cytokine response of the old mice was determined following culture alone or re-stimulation *in vitro* with OVA. Cultures not stimulated with OVA produced undetectable amounts of cytokine. In addition to the OVA, all cultures contained *Listeria* organisms that had not yet been cleared from the spleens of either the WT or the VDR KO mice prior to the day 10 time point. Splenocytes from old WT and VDR KO mice produced similar levels of IFN-γ and IL-10 at all time points following primary infection. However, the IFN-γ response of the old mice (maximum of 6 ng ml^{-1}) was less than that of young mice (maximum of 40 ng ml^{-1}) following a primary *L. monocytogenes* infection (compare Figs 2A and 5A). At day 2 post-infection, old WT mice had 206 ± 56 pg ml^{-1} and old VDR KO mice had 176 ± 90 pg ml^{-1} IFN-γ in the serum, which was not significantly different. At other times post-infection, IFN-γ was undetectable. At 10 days post-infection, the IL-12 response of VDR KO mice remained high while the WT IL-12 response decreased significantly (Fig. 5A).

Secondary infection of old WT mice with *L. monocytogenes* resulted in a blunted IFN-γ response that was also reflected in the IL-12 response (Fig. 5B). IL-10 responses of stimulated splenocytes from old WT mice were extremely variable but also appeared to show little change following secondary challenge (Fig. 5B). The levels of IL-12 and IL-10 were not significantly different between the two groups although at 2 days post-infection, the VDR KO splenocytes trended toward secreting higher levels of these cytokines. Low levels of IFN-γ were detected in the sera at day 1 post-secondary infection but were not different between WT and VDR KO mice with *Listeria* (data not shown). Old VDR KO mice secreted significantly more IFN-γ at 2 days post-infection than old WT mice (Fig. 5B). In addition, the old VDR KO mice produced more IFN-γ 2 days post-infection than young VDR KO mice (compare Figs 2B and 5B).

Old VDR KO mice produce high levels of antibody

Antibody responses following secondary *L. monocytogenes* infection were measured in the old mice. Consistent with the literature, old WT mice showed a decreased ability to mount

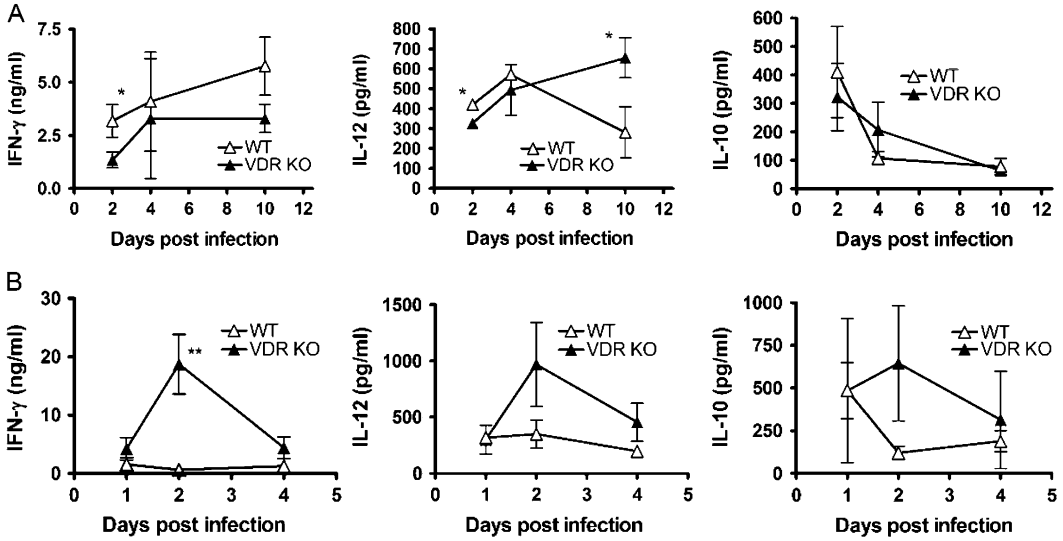

Fig. 5. Cytokine production following primary and secondary infection of old WT and VDR KO mice with *Listeria monocytogenes*. Old WT and VDR KO mice were evaluated for cytokine production following primary and secondary *L. monocytogenes* infection. (A) IFN-γ, IL-12 and IL-10 production from whole splenocytes following primary infection and *in vitro* stimulation with OVA. (B) IFN-γ, IL-12 and IL-10 production following secondary *L. monocytogenes* infection and *in vitro* stimulation with OVA. Values are the mean ± standard error of the mean of $n = 3$–4 individual mice at each time point. VDR KO value is significantly different than the corresponding WT value, $*P \leq 0.05$, $**P \leq 0.001$.

a recall response and produced less antigen-specific IgG1 and IgG2c than young WT mice (compare Figs 2C and 6A; $P = 0.0002$ and $P = 0.052$, respectively). Similarly, old VDR KO mice produced significantly less IgG1 than young VDR KO mice (compare Figs 2C and 6A; $P = 0.0001$). However, at all time points tested, old VDR KO mice produced significantly more *Listeria*-specific IgG1 and IgG2c compared with WT mice (Fig. 6A). To determine if the VDR KO mice had elevated levels of *Listeria* non-specific antibodies as well, the total amounts of IgG1 and IgG2c were also measured in old VDR KO and WT mice. We found that old VDR KO mice produced 2-fold more total IgG1 and 5-fold more total IgG2c than old WT mice (Fig. 6B).

Memory T cell response in old WT and VDR KO mice

Analysis of the percentage of memory $CD4^+$ and $CD8^+$ T cells ($CD8^+/CD44^{high}$ or $CD4^+/CD44^{high}$) in the spleens of old WT and VDR KO mice revealed that there was no significant difference (Table 1). In addition, there were no differences in the number of splenocytes, $CD4^+$ or $CD8^+$ T cells in WT and VDR KO mice (data not shown). However, old WT mice had higher percentages of $CD8^+$ and $CD4^+$ memory T cells that produced IFN-γ compared with old VDR KO mice (Fig. 6C). Old WT mice had higher percentages of IFN-γ-producing $CD44^{high}$-expressing T cells than their younger counterparts (compare Figs 3B and 6C at all time points except day 4 in $CD4^+$ T cell compartment). Old VDR KO mice had higher percentages than young VDR KO mice early but not later post-infection (compare Figs 3B and 6C).

Discussion

VDR KO mice mount a strong primary and secondary immune response to *L. monocytogenes* infection that is adequate to completely clear infection in both young and old VDR KO mice. However, young VDR KO mice exhibited delayed *L. monocytogenes* clearance (compared with young WT mice) that was likely the result of higher levels of both IL-10 and IFN-γ early post-infection. IL-10 and IFN-γ have opposing effects on growth and clearance of *L. monocytogenes* (13, 18, 24). The early secretion of IL-10 (likely due to macrophage or other innate cells) in the VDR KO host likely reduced the efficacy of the IFN-γ response, leading to delayed kinetics in clearing infections in the spleen and liver. Interestingly, the generation of memory T and B cell responses occurred unimpeded in the VDR KO mice and therefore the observed delayed kinetics of clearance in young VDR KO versus young WT mice was less pronounced in the secondary challenge.

Acquired immunity decreases with age and although old VDR KO and WT mice took longer than young mice to clear a primary and secondary *L. monocytogenes* infection, they eventually resolved the infections. The age-related delay in clearance of a primary infection was associated with reduced amounts of IFN-γ secreted by the old mice. The increased secretion of IFN-γ by the VDR KO mice following secondary infection may explain the ability of these mice to clear the infection with kinetics that were more similar to WT mice. In addition, although the VDR KO mice had a lower frequency of memory cells secreting IFN-γ, they were able to secrete significantly more IFN-γ following stimulation, suggesting that the VDR KO memory T cells secreted more IFN-γ per cell.

Antibody responses, both antigen specific and non-specific, were higher in old VDR KO than WT mice. However, the high amounts of antibody produced in the old VDR KO mice did not correspond to improvements in host resistance to *L. monocytogenes*. Overall, the primary and secondary immune response of old VDR KO mice was as effective as that of old WT mice for clearance of *L. monocytogenes*. The

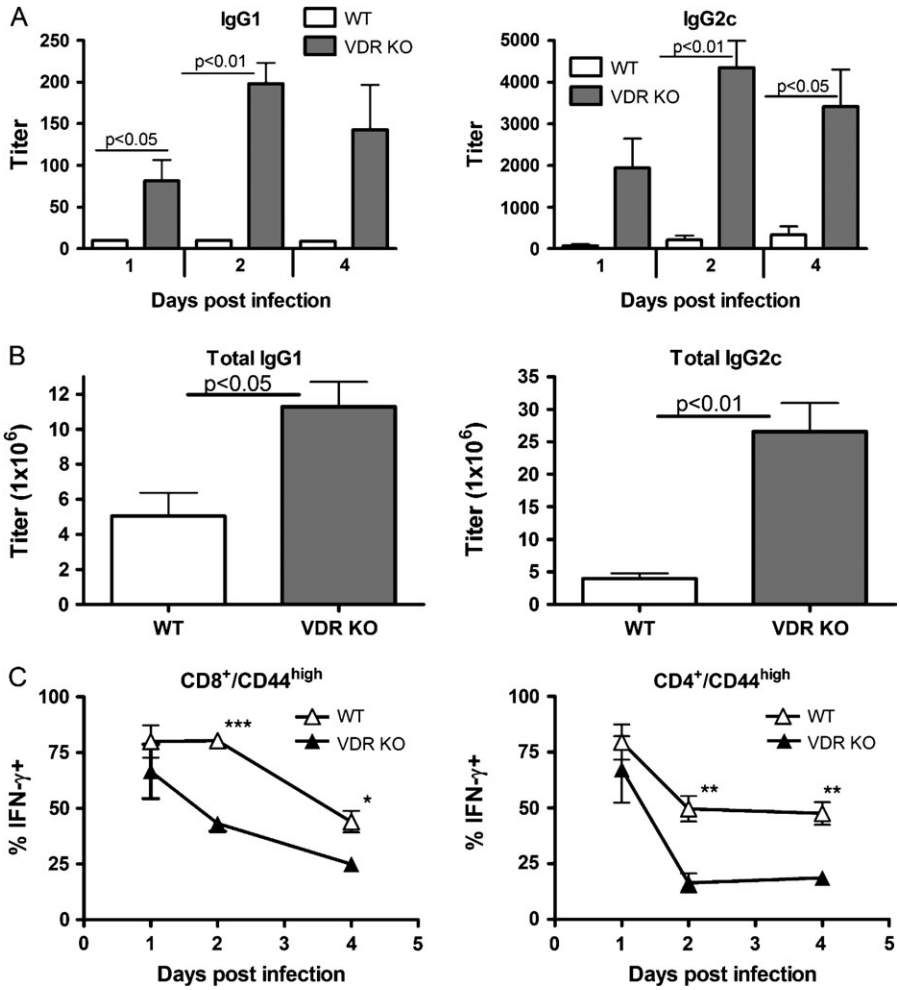

Fig. 6. B cell and T cell memory function in old VDR KO and WT mice following secondary challenge with *Listeria monocytogenes*. (A) *Listeria*-specific IgG1 and IgG2c responses following secondary challenge with *L. monocytogenes*. (B) Total IgG1 and IgG2c in old mice. C) Average percentage of IFN-γ-producing CD8/CD44^{high} and CD4/CD44^{high} T cells in old VDR KO and WT mice. Values are the mean ± standard error of the mean of n = 3–4 individual mice per time point. VDR KO value is significantly different than the corresponding WT value, *$P \leqslant 0.05$, **$P \leqslant 0.001$, ***$P \leqslant 0.0001$.

high amounts of cytokines coupled with the large amounts of antibodies produced in the old VDR KO mice suggest that non-specific immune responses and autoimmunity are higher in the absence of the VDR. The heightened immune responses in old VDR KO mice are consistent with the previously described susceptibility of the VDR KO mice to experimental autoimmunity (8, 25).

Based on the effects of $1,25(OH)_2D_3$ on T_h1-driven immunity and the heightened IFN-γ and IL-12 responses in the VDR KO host, it is surprising that the VDR KO mice cleared *Listeria* infections with slower kinetics than their WT counterparts. NKT cells have been shown to be important innate immune cells during *L. monocytogenes* infection; NKT cell-deficient CD1d KO mice exhibit decreased liver IFN-γ production and increased susceptibility to *Listeria* infection (26). Approximately 30% of the liver mononuclear cells from WT mice are NKT cells, compared with only 6% NKT cells in VDR KO mice (7). In addition, the remaining NKT cells in the VDR KO mice are of an immature phenotype and produce low levels of cytokines including IFN-γ (7). Therefore, VDR KO mice are essentially NKT cell deficient, suggesting that the delayed kinetics of *L. monocytogenes* clearance following primary infection is likely a result of low amounts of NKT cell produced IFN-γ, which would predominately affect the response in the liver. $1,25(OH)_2D_3$ differentially regulates IFN-γ production depending on the cell type that produces it. Paradoxically, $1,25(OH)_2D_3$ enhances IFN-γ production from NKT cells and inhibits IFN-γ production from T_h1 cells (3, 7), indicating a cell type-specific IFN-γ regulation by $1,25(OH)_2D_3$. It appears that early during an immune response, $1,25(OH)_2D_3$ induces IFN-γ production by NKT cells, followed by inhibition of IFN-γ along with other T_h1-mediated immune responses.

The role of vitamin D, $1,25(OH)_2D_3$ and the VDR in B cell development and function has not been extensively studied. B cells express the VDR depending on how they are stimulated and from where they are recovered (27). $1,25(OH)_2D_3$ has been reported to act as a vaccine

adjuvant at mucosal sites and to induce antigen-specific IgA (28, 29). More recently, $1,25(OH)_2D_3$ has been shown to inhibit B cell proliferation *in vitro* depending on when it is added to the culture (27). In addition, memory B cell and plasma cell generation is inhibited by $1,25(OH)_2D_3$ in cultures of human cells (27). Moreover, $1,25(OH)_2D_3$ has been shown to suppress experimental systemic lupus in the MRL/MpJ mouse that is a model of a B cell-mediated autoimmune disorder (30). While we find that B cells from VDR KO mice generated normal amounts of *Listeria*-specific antibody in young mice, old VDR KO mice produced significantly higher levels of the IFN-γ-driven IgG2c and IL-4/IL-5-driven IgG1. In addition, there were high amounts of antigen-specific and total antibodies in the old VDR KO mice. Increased amounts of antibodies in old VDR KO mice suggest a role for the VDR and $1,25(OH)_2D_3$ as a negative regulator of B cell antibody responses, and vitamin D may play a role in limiting the development of self-reactivity that occurs with aging.

Our data indicate that the expression of the VDR is not required for clearance of *L. monocytogenes* following either primary or secondary infection, although young VDR KO mice showed delayed kinetics of clearance that corresponded to early production of IL-10. There was a decline in the percentage of memory cells that developed with the age of the mice and VDR KO mice had fewer IFN-γ-producing memory T cells than WT mice. IFN-γ production, however, was higher in cells from old VDR KO mice than old WT mice. Consistent with the increased susceptibility of the VDR KO mice to various autoimmune diseases, old VDR KO mice over-produced IFN-γ, IL-12 and both antigen-specific and non-specific antibodies. Expression of the VDR is therefore critical for the inhibition of cytokine and antibody production but is not required for clearance of the intracellular pathogen *L. monocytogenes*.

Supplementary data

Supplementary tables S1 and S2 are available at *International Immunology* Online.

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Abbreviations

BHI	blood heart infusion
CFU	colony-forming unit
KO	knockout
OVA	ovalbumin
TNF	tumor necrosis factor
VDR	vitamin D receptor
WT	wild type

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