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Nonclassical MHC-Restricted Invariant Vα6 T Cells Are Critical for Efficient Early Innate Antiviral Immunity in the Amphibian *Xenopus laevis*

Eva-Stina Edholm, Leon Grayfer, Francisco De Jesús Andino, and Jacques Robert

Nonclassical MHC class Ib–restricted invariant T (iT) cell subsets are attracting interest because of their potential to regulate immune responses against various pathogens. The biological relevance and evolutionary conservation of iT cells have recently been strengthened by the identification of iT cells (invariant Vα6 [iVα6]) restricted by the nonclassical MHC class Ib molecule XNC10 in the amphibian *Xenopus laevis*. These iTαv6 T cells are functionally similar to mammalian CD1d-restricted invariant NKT cells. Using the amphibian pathogen frog virus 3 (FV3) in combination with XNC10 tetramers and RNA interference loss of function by transgenesis, we show that XNC10-restricted iTαv6 T cells are critical for early antiviral immunity in adult *X. laevis*. Within hours following i.p. FV3 infection, iTαv6 T cells were specifically recruited from the spleen into the peritoneum. XNC10 deficiency and concomitant lack of iTαv6 T cells resulted in less effective antiviral and macrophage antimicrobial responses, which led to impaired viral clearance, increased viral dissemination, and more pronounced FV3-induced kidney damage. Together, these findings imply that *X. laevis* XNC10-restricted iTαv6 T cells play important roles in the early anti-FV3 response and that, as has been suggested for mammalian invariant NKT cells, they may serve as immune regulators polarizing macrophage effector functions toward more effective antiviral states. *The Journal of Immunology*, 2015, 195: 000–000.

Human and murine nonclassical MHC class Ib (class Ib)–restricted invariant T (iT) cell subsets, such as CD1d-restricted invariant NKT (iNKT) and MR1-restricted mucosal-associated invariant T (MAIT) cells, are being increasingly appreciated as early innate-like responders and immune regulators (1–4). For example, MAIT cells have antimicrobial activity and respond in an MR1-dependent manner to different microbes consistent with their involvement at early stages of microbial infections (5). Similarly, iNKT cells have been associated with early stages of immune responses against diseases caused by a variety of bacterial, parasitic, and viral pathogens (reviewed in Ref. 6). With regard to viral infections, multiple lines of evidence suggest that iNKT cells are involved in immune surveillance and early clearance of different viruses, including HSV-1 (7), hepatitis B virus (HBV) (8), and influenza A virus (9). In further support of the importance of these cells in antiviral immunity, HIV-1 (10–12), HSV-2 (13), Kaposi sarcoma–associated herpes virus (14), vesicular stomatitis, and vaccinia virus (15) have all developed strategies to interfere with various stages of CD1d intracellular trafficking, thereby evading recognition by iNKT cells (reviewed in Ref. 16). However, despite recent progress in the characterization of human and mouse iT cells, the general relevance of these cells in host defenses and the mechanisms by which iT cells respond to infections still remain poorly understood.

The evolutionary ancestry of iT cells was recently unveiled by the identification of a distinct iT cell subset in the amphibian *Xenopus laevis*, providing compelling evidence of the biological importance of class Ib–restricted iT cells throughout jawed vertebrates (17). Similar to their mammalian counterparts, *X. laevis* iT cells (invariant Vα6 [iVα6] T cells) express a unique semi-invariant TCR comprising an invariant TCRα–chain (Vα6-Jα1.43) in conjunction with a limited TCRβ repertoire. These iTαv6 T cells represent a significant fraction of splenic lymphocytes in healthy *X. laevis* adults (~4%) and tadpoles (~2%). In addition to the more extensively characterized iTαv6 T cells, *X. laevis* also possesses a population of XNC10-reactive CD8dim T cells with a more diverse, albeit iTαv6-Jα1.43 biased, TCR repertoire termed XNC10-restricted type II cells. Currently, it is unclear whether these non-invariant type II cells represent a distinct T cell subset reminiscent of mammalian type II NKT cells that are (like the type I iNKT) CD1d restricted but do not express the canonical TCR α–chain (18). Reminiscent of the CD1d requirement for the development and function of iNKT cells and MR1 for MAIT cells (2, 4), iTαv6 T cells require the *X. laevis* nonclassical gene 10 (XNC10) for their development (17). Unlike most nonclassical MHC genes, XNC10 has remained highly conserved among divergent *Xenopus* species, implying an important and nonredundant function for this gene (19, 20). Indeed, XNC10-deficient transgenic tadpoles lacking iTαv6 T cells are more susceptible to infection with the ranavirus frog virus 3 (FV3), an ecologically relevant amphibian pathogen causing extensive disease and mortalities of wild and cultured amphibian species (21). Specifically, the defect in iTαv6 T cell development resulted in dramatically higher mortality within the first weeks of infection. This critical involvement of iTαv6 T cells during early
antiviral immunity in tadpoles implies that despite a long evolutionary interlude, important and specialized iT cell functions have been conserved (17).

The immune system is, overall, remarkably well conserved between mammals and Xenopus. However, unlike in mammals, X. laevis T cell development and differentiation are subjected to an additional developmental program during metamorphosis, resulting in an adult-type immune system distinct from that of tadpoles (22). Notably, although both tadpoles and adults are immunocompetent and have conventional CD8+ T cells and unconventional iVo6 T cells, tadpoles lack significant class Ia protein expression until metamorphosis (23–25). In the context of FV3 infection, tadpoles exhibit delayed anti-FV3 innate immune responses of lower magnitude compared with adults and typically succumb to infection (26). Conversely, despite incurring greater viral loads compared with tadpoles (27) adults are inherently more resistant to FV3 infection and mount effective anti-FV3 responses. The antiviral response is initiated by a robust recruitment of mononuclear and polymorphonuclear phagocytes to the peritoneal cavity as early as 1 d postinfection (dpi), followed by an increase in NK cells at 3 dpi (28), culminating in CD8+ T cell–mediated viral clearance (28, 29 and reviewed in Ref. 21). Recent findings indicate that macrophage lineage cells are integral to amphibian antiviral immunity against FV3. Indeed, peritoneal macrophages elicited with IL-34 are more resistant to in vitro FV3 infections compared with MCSF-elicited peritoneal macrophages. IL-34–derived macrophages also exhibited a stronger type I IFN gene expression response (30). These data indicate that the two macrophage growth factors polarize peritoneal macrophages for divergent roles (30).

Thus, X. laevis provides an attractive platform for studying iTV T cell involvement in a naturally occurring sublethal infection model as well as a powerful comparative model system to gather insight from an evolutionary perspective into how class Ib–mediated iT cell biology is regulated in response to viral infections.

Materials and Methods

Experimental animals

Outbred and LG-15 strains of X. laevis were from the X. laevis Research Resource for Immunology at the University of Rochester (www.urmc.rochester.edu/mbi/resources/Xenopus/index.html). Transgenic X. laevis was generated using I-Scel meganuclease, as previously described (31). Briefly, the I-Scel-X. laevis nonclassical gene 10 (XNC10) short hairpin RNA (shRNA)–GFP expression vector was constructed by cloning the GFP reporter flanked by the 18-bp I-Scel recognition sites into the I-ScelBSIISK’ vector (provided by R. Grainger, University of Virginia, Charlottesville, VA). Subsequently, the XNC10 shRNA under the control of the hU6 Pol III promoter was cloned into the I-ScelGFP vector. Before microinjection, X. laevis females were primed with human chorionic gonadotropin (Sigma-Aldrich), and eggs were fertilized, dejellied, and injected with 80 pg 1-I-Scel meganuclease–digested XNC10shRNA–GFP expression vector, as previously described (31). After injection, all embryos were incubated at 13°C for 4 h to delay cell division, transferred to 0.3 × modified Barth’s saline supplemented with 5 μg/ml gentamycin, and reared at 18°C until hatching. Transgenic larvae were screened for GFP expression at developmental stage 56 (32) using an SMZ1500 Nikon stereomicroscope. All animals were handled under strict laboratory and University Committee on Animal Resources regulations (100577/2003–151), and discomfort was minimized at all times.

Flow cytometry analysis

All anti–X. laevis mAbs were from the X. laevis Research Resource (www.urmc.rochester.edu/mbi/resources/Xenopus/). XNC10-tetramers (XNC10-T) were generated as previously described (17). Briefly, β2-microglobulin (β2m) linked via a 23 aa Gly-rich C-terminal flexible linker to α1–α3 domains of XNC10 containing a BirA site–specific biotinylation site at the end of the α3 domain and cloned into the pM6BV5-HisA expression vector (Invitrogen). The β-m–linker–XNC10 construct was expressed in S09 insect cells, and monomeric β2m–linker–XNC10 was purified by Ni-NTA–Agarose (Qiagen) chromatography and concentrated to 1 μg/μl using Amicon Ultra Centrifugal Filter (Millipore). BirA enzymatic biotinylation was performed for 18 h at 30°C according to the manufacturer’s protocol (Avidity), and the purified biotylated proteins were extensively dialyzed against amphibian PBS, pH 7.5, to remove any unbound biotin. XNC10-T were generated by incubating β2m–linker–XNC10 with fluorochrome–labeled streptavidin at a 5:1 ratio at room temperature for 30 min at 4°C, followed by incubation with X. laevis anti-CD8 (AM22) and FITC–conjugated goat anti–mouse secondary Abs (Southern Biotech). For analysis of peritoneal leukocytes, cells were isolated by peritoneal lavage, and 0.25 × 106 cells were stained using Xenopus anti-CD8 (AM22), MHC class II (AM20), or MHC class I (TB17) mAbs, followed by allophycocyanin–conjugated goat anti–mouse secondary Abs (Southern Biotech). Dead cells were excluded using propidium iodide (BD Pharmingen), and 50,000 events were collected with BD Accuri C6 (BD Biosciences) and analyzed using FlowJo software (TreeStar). Two-color (CD8 and XNC10–T) cell sorting by flow cytometry was performed with peritoneal leukocytes from FV3–infected animals and splenocytes from uninfected animals using an F-Aria-18 (BD Biosciences).

Quantitative PCR and genomic PCR

RNA and genomic DNA were prepared using TRIzol Reagent (Invitrogen). RNA was further treated with DNase (Ambion; Life Technologies) according to the manufacturer’s protocol; 1 μg total RNA was transcribed into cDNA with iScript reverse transcriptase using oligo–dT primers (Bio-Rad). Quantitative PCR parameters were as follows: 2 min at 95°C followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Relative quantitative PCR (qPCR) gene expression analysis (XNC10, Ivo45-J11.14, Ivo41-J11.40, Ivo40-J11.22, Ivo23-J13.1, Ivo22-J13.2, Ivo66-J11.43, GCSFR, MCSFR, MCSF, IL-34, iNOS, type I INF, TNF-α, IL-1β, IL-10, and vDNA pol II) was performed using the ΔΔCT method. Expression of the different genes was examined relative to the endogenous GAPDH control and normalized against the lowest observed tissue expression. Absolute qPCR was performed to measure FV3 viral loads in isolated genomic DNA, using a serially diluted standard curve, as previously described (27). Experiments were performed using the ABI 7300 real-time PCR system and Perfecta Taq SYBR Green FastMix, ROX (Quanta Biocience). Expression analysis was performed using ABI Sequence Detection System software. Rearrangements of specific TCR Vb and Jα genes in the genome were detected by PCR, using 50 ng genomic DNA and primers specific for Ivo41-J11.40, Ivo66-J11.43, and EF1-α. PCR parameters were as follows: 5 min at 95°C followed by 40 cycles of 95°C for 30 s, 58°C for 40 s, and 72°C for 1 min followed by a final extension at 72°C. All primers were validated prior to use. Primer sequences are available upon request from the X. laevis Research Resource (www.urmc.rochester.edu/mbi/resources/Xenopus/).

FV3 infections, plaque assays, and histology

FV3 (Iridoviridae) was grown as previously described. Briefly, FV3 was grown by a single passage on BHK cells, purified by ultracentrifugation on a 30% sucrose cushion, and quantified by plaque assay on a BHK cell monolayer under a 1% methylcellulose overlay (28). All infections were performed by i.p. injection of 1 × 108 PFUs of FV3 in 100 μl amphibian PBS. At the indicated times, peritoneal leukocytes were collected by peritoneal lavage; alternatively, animals were euthanized by immersion in 0.5% tricaine methane sulfonate (MS-222), and tissues were removed and processed for RNA and DNA isolation. Kidneys to be assayed for infectious FV3 loads were homogenized and subjected to three rounds of freeze/thaw lysis. All plaque assays were performed on BHK cell monolayers under a 1% methylcellulose overlay, as previously described (28). For histological analysis, kidneys were isolated, immersed in 8% sucrose for 16 h at 4°C, embedded in Tissue-Tek O.C.T., frozen on dry ice, sectioned (5-μm sections), and H&E stained. The resulting slides were examined using a Nikon Eclipse E200 phase contrast microscope, and images were taken using Nikon SPOT Idea digital camera and analyzed using SPOT Imaging software.

Statistical analysis

All quantitative data were analyzed by a one-way test of variance (ANOVA) and the Vassar Stat software (http://faculty.vassar.edu/lowry/anovalu.html). A p value > 0.05 was considered significant.
Results

Changes in frequency and tissue localization of XNC10-restricted iVα6 T cells during FV3 challenge

Intraperitoneal infection of *X. laevis* adults with the natural amphibian ranavirus pathogen FV3 elicits a stepwise antiviral immune response involving both innate and adaptive immune effector cells (28, 29). To delineate the involvement of unconventional class Ib–restricted iT cells during FV3 infection in MHC class Ia competent adult *X. laevis*, we sought to examine changes in location and frequency of XNC10-restricted iT cells using XNC10-T (17). Given that mammalian iT cells often have specialized functions early in immune responses, we anticipated that upon FV3 infection *Xenopus* iVαT cells would be recruited to the site of inoculation prior to the influx of NK and conventional T cells. Accordingly, we i.p. infected adult *Xenopus* with FV3 at different times: 0, 6, 24, and 72 h postinfection (hpi). We then determined the frequency and number of XNC10-T+ cells in the spleen (the central amphibian draining lymphoid organ) and in peritoneal leukocytes (the first cells to come in contact with the virus) (Fig. 1). Prior to FV3 infection, XNC10-restricted iVα6 T cells were principally located in the spleen, where they represented, on average, 4% of the total lymphocyte population (Fig. 1D). No XNC10-T+ cells were detected in peritoneal leukocytes isolated from uninfected animals (Fig. 1A). Comparably, flow cytometry analysis of peritoneal leukocytes revealed a significant increase in the relative fraction of XNC10-T+ cells (1.3% ± 0.2) at 24, but not 72, hpi, which could suggest that once recruited to the infection site these cells undergo activation-induced cell death or alternatively down-regulated their TCRs. Of interest, we observed a concomitant, albeit not statistically significant, decrease in the total number of splenic XNC10-T+ cells as early as 6 hpi, consistent with the rapid mobilization and recruitment of XNC10-restricted iVα6 T cells to other tissues, including the peritoneal cavity, within hours following FV3 infection (Fig. 1C, 1D). XNC10-T+ cells detected in the peritoneal

![Figure 1](http://www.jimmunol.org/)

**FIGURE 1.** Rapid egress of XNC10-restricted iVα6 T cells from the spleen to the peritoneal cavity following i.p. FV3 inoculation. One-year-old outbred *X. laevis* were infected i.p. with $1 \times 10^6$ PFU of FV3; spleen and peritoneal leukocytes were collected at the indicated times and analyzed by flow cytometry. (A) Representative flow cytometry of live peritoneal leukocytes isolated by peritoneal lavage from either uninfected or i.p. FV3-infected adults at 6, 24, and 72 h postinfection (hpi). We then determined the frequency and number of XNC10-T+ cells in the spleen and peritoneal leukocytes isolated from either uninfected or FV3-infected adults at 24 hpi. PCR was performed on 50 ng genomic DNA (40 cycles) using primers specific for iVα41-Jα1.40, iVα6-Jα1.43, and EF1-α. The total number of live XNC10-T+–staining cells from either peritoneal leukocytes (C) or spleen leukocytes (D) collected at the indicated times. Data are means (±SE) of seven individuals/indicated time point (*n* = 7). *p* < 0.05 above bars denotes statistical significance relative to respective uninfected controls, and *p* < 0.05 above the line denotes significant differences between the indicated groups (Student t test). (E) Representative flow cytometry analysis showing forward scatter versus XNC10-T staining for spleen leukocytes and peritoneal leukocytes isolated from either an uninfected frog or 24 h following FV3 infection, respectively.
cavity following FV3 infection were uniformly CD8<sup>neg</sup>, CD5<sup>neg</sup>, and MHC class II<sup>low+</sup> (data not shown), corresponding to the type I iVo6 T cell subset previously reported (17). In contrast, both type I and type II XNC10-T<sup>T</sup> cells were detected in the spleen of the same animal (1.47% ± 1.10 for type I CD8<sup>neg</sup>/XNC10-T<sup>T</sup> and 1.35% ± 1.227 for type II CD8<sup>pos</sup>/XNC10-T<sup>T</sup>). In addition, XNC10-T<sup>T</sup> cells in the peritoneal cavity showed evidence of activation with a relative size ≥2 times larger (as determined by forward scatter) compared with XNC10-T-reactive cells isolated from the spleen (Fig. 1E). To further control for the specificity of our XNC10-T and the identity of XNC10-T<sup>+</sup> cells, we sorted peritoneal and splenic XNC10-T<sup>+</sup> as well as CD8<sup>+</sup> and XNC10-T<sup>2</sup>/CD8<sup>2</sup> subsets from FV3-infected (24 hpi) and uninfected animals. We determined TCR<sub>a</sub> rearrangements by PCR on genomic DNA. As expected, a strong canonical V<sub>a</sub>6-J<sub>a</sub>1.43 signal was amplified from splenic XNC10-T<sup>+</sup> cells (either CD8<sup>+</sup> or CD8<sup>neg</sup>), whereas no V<sub>a</sub>6-J<sub>a</sub>1.43 signal was detected in XNC10-T<sup>2</sup>/XNC10-T<sup>2</sup> cells and not from XNC10-T<sup>T</sup> cells. Importantly, XNC10-T<sup>+</sup>, but not CD8<sup>+</sup>, cells isolated from the peritoneal cavity were positive for the V<sub>a</sub>6-J<sub>a</sub>1.43 rearrangement. Of note, the V<sub>a</sub>6-J<sub>a</sub>1.43 rearrangement was also detected in a fraction of peritoneal leukocytes from infected animals negatively stained for CD8 and XNC10-T. This finding suggests that a fraction of iVo6 T cells infiltrating the peritoneum during infection might have downregulated their surface TCR expression.

To obtain additional evidence of the splenic egress and consequent transitory influx of iVo6 T cells into the peritoneal cavity, we quantified transcript levels of the specific invariant V<sub>a</sub>6-J<sub>a</sub>1.43 rearrangement in the spleen, peritoneal leukocytes, and kidney (the main site of FV3 replication) at different time points following FV3 infection (Fig. 2A, 2D, 2G). Because iVo6 T cells interact with the class Ib molecule XNC10, we also examined its gene expression profile (Fig. 2B, 2E, 2H). To evaluate viral loads and dissemination, we determined the FV3 genome copy number in different tissues using absolute qPCR (Fig 2C, 2F, 2I). Consistent with the loss of splenic XNC10-T<sup>T</sup> cells following FV3 infection, expression of the iVo6-J<sub>a</sub>1.43 transcript was markedly reduced by 1 dpi, remaining low at 3 dpi and increasing again at 6 dpi (Fig. 2A). This finding suggests the replenishment of the iVo6 T cell pool, presumably via influx of naive iVo6 T cells from the thymus. Conversely, peritoneal leukocyte iVo6-J<sub>a</sub>1.43 expression was undetectable in uninfected animals, became significantly elevated at 6 hpi, markedly peaked at 1 dpi, and then returned to low but detectable levels from 2 to 6 dpi, indicating a rapid and transitory influx of iVo6 T cells to

![FIGURE 2](http://www.jimmunol.org/)
the peritoneal cavity upon i.p. FV3 inoculation (Fig. 2D). It is noteworthy that the increase in iVo6-Jx1.43 transcript levels observed at 6 hpi likely reflects an initial low-frequency iVo6 T cell infiltration that was below detection levels using XNC10-T flow cytometry analysis. In kidneys, iVo6-Jx1.43 expression was modest and highly variable, with no significant increase in iVo6-Jx1.43 mRNA levels until 6 dpi (Fig. 2G). As expected, high viral loads were detected in peritoneal leukocytes as early as 6 hpi, whereas splenic FV3 loads were either undetected or modest, with an average of 2 logs lower viral loads than in peritoneal leukocytes (Fig. 2C, 2F). Congruent with the kidney being the main site of FV3 replication, viral loads in this tissue were higher and increased from 1 to 3 dpi, followed by a decline at 6 dpi, consistent with the previously described influx of CD8+ T cells and the onset of viral clearance (29).

During the course of infection, the most dramatic change in XNC10 gene expression was observed in peritoneal leukocytes. Notably, unlike spleen, which exhibited high and uniform expression levels regardless of FV3 infection, XNC10 gene expression in peritoneal leukocytes was markedly induced upon FV3 infection, with a significant increase detected as early as 6 hpi followed by a peak at 2 dpi (Fig. 2B, 2E). In kidneys, compared with peritoneal leukocytes, XNC10 gene expression was delayed and modest; it did not significantly increase until 2 dpi, remaining elevated at 3 dpi and then diminishing by 6 dpi (Fig. 2H). Collectively, these data indicate that XNC10-restricted iVo6 T cells are rapidly mobilized upon FV3 infection, leaving the spleen and migrating to the peritoneal cavity several days prior to the influx of NK and conventional T cells.

Relative expression of other putative invariant TCRα gene rearrangements in response to FV3 infection

Although the Ag (or Ags) recognized by iVo6 T cells has not yet been identified, we sought to determine whether the influx of iVo6 T cells into the peritoneal cavity following FV3 infection reflected the recruitment of only this particular iT cell subset. Accordingly, we determined the mRNA levels of the five additional predominant putative iTCRα rearrangements previously identified (Vo45-Jx1.14, Vo41-Jx1.40, Vo40-Jx1.22, Vo23-Jx1.3Vo22-Jx1.32, and iVo6-Jx1.43) (17) in peritoneal leukocytes following FV3 infection (Fig. 3A). Remarkably, iVo6-Jx1.43 was the only rearrangement significantly elevated in expression upon FV3 infection, which indicates a specific recruitment of iVo6 T cells in response to FV3 challenge. We also noted a relative decrease in the expression of the Vo41-Jx1.40 rearrangement post FV3 infection, suggesting that the particular iT cell subset expressing this rearrangement is predominant in the peritoneal cavity in naive animals. It is noteworthy that no significant increase in gene expression in any of the six iTCRα rearrangements, including the iVo6-Jx1.43, was observed following i.p. challenge with heat-killed E. coli (Fig. 3B).

This type of bacterial stimulation has been shown to induce a strong nonspecific inflammatory response accompanied by a robust recruitment and accumulation of innate leukocytes in the peritoneal cavity (28). These results argue against a general infiltration of iT cells owing to inflammation and provide compelling evidence that iVo6 T cells are specifically recruited to the initial site of FV3 infection.

**XNC10 loss of function by RNA interference in vivo results in compromised viral clearance in the peritoneal cavity**

Based on our results indicating that iVo6 T cells are rapidly (within hours) recruited to the peritoneal cavity upon FV3 infection, we hypothesized that these cells are critical for eliciting an efficient antiviral immune response. As such, we anticipated that iVo6 T cell deficiency would result in weakened host resistance to FV3. To address this hypothesis, we used a XNC10 loss-of-function approach shown to result in impaired iVo6 T cell development (17, 31). One-year-old F0 animals deficient for XNC10 were generated by combining RNA interference with I-SecI meganuclease-mediated transgenesis. Transgenic and age-matched control animals (reared from fertilized eggs of the same batch that were dejellied but not injected with the XNC10shRNA) were i.p. infected with FV3, and levels of XNC10 knockdown in the spleen were assessed by qPCR at 6 dpi. Only transgenic animals that showed >75% XNC10 knockdown (n = 5) compared with the average XNC10 gene expression in control animals (n = 10, Fig. 4A) were used in further analysis.

Consistent with our published data (17), XNC10 silencing in transgenic animals resulted in a drastically decreased expression of the invariant iVo6-Jx1.43 rearrangement in the spleen (p = 0.00655) compared with age-matched controls (Fig. 4B). Upon FV3 infection, the sharp increase of both XNC10 and iVo6-Jx1.43 transcript levels observed at 1 dpi in peritoneal leukocytes of control animals was ablated in transgenic animals (Fig. 4C, 4D). Importantly, XNC10 and iVo6 T cell deficiency affected FV3 viral replication, as evidenced by significantly higher viral loads in transgenic animals at both 1 dpi (p = 0.01903) and 6 dpi (p = 0.02332) compared with control animals (Fig. 4C). Moreover,
7 of 10 control *X. laevis* displayed 1–2 log units lower FV3 loads on day 6 compared with day 1 postinfection, indicative of onset of viral clearance. In contrast, the transgenic animals displayed either the same (3/5) or even 1 log higher (2/5) viral loads at 6 dpi compared with 1 dpi, further suggesting that XNC10-deficient transgenic *X. laevis* have an impaired ability to control FV3 replication.

**XNC10 deficiency has little effect on the overall recruitment of leukocytes into the peritoneal cavity following FV3 infection**

To assess for a possible difference in the peritoneal leukocyte composition resulting from XNC10 deficiency, we performed flow cytometry analysis with XNC10-deficient transgenic and genetically identical nontransgenic isogenic LG-15 cloned animals with a defined MHC a/c haplotype. In several independent experiments (one representative shown in Fig. 5), we found comparable compositions of MHC class II and MHC class I surface-positive cells in uninfected animals as well as similar frequencies of CD8⁺ T cells (Fig. 5A). Moreover, both control and XNC10-deficient transgenic animals showed a robust influx of large, highly internally complex MHC class II⁺⁺⁺-staining cells at 1 dpi with overall similar MHC class II⁺⁺⁺ mean fluorescent intensity, 1002 ± 275 for transgenic and 724 ± 58 for control animals. (Fig. 5B). We noted, however, that peritoneal leukocytes isolated from infected XNC10-deficient transgenic animals consistently contained a population of small, agranular MHC class II⁺⁻⁻-staining cells (indicated by arrow) constituting between 9 and 11% of the total population that was absent in control animals (Fig. 5B).

**Effects of XNC10 deficiency and lack of iVα6 cells on antiviral peritoneal leukocyte response**

To delineate the putative functional roles of XNC10-restricted iVα6 T cells during FV3 infection, we examined peritoneal leukocytes isolated at the peak of iVα6 T cell infiltration (1 dpi) and at the peak of the conventional CD8⁺ T cell response (6 dpi; Refs. 25, 27). Although the total number of leukocytes infiltrating the peritoneum at 1 dpi was slightly decreased in infected XNC10-deficient transgenic animals compared with infected controls, it was not statistically significant (Fig. 6A). Using GCSFR and MCSFR gene expression determined by qPCR to monitor polymorphonuclear granulocytes and mononuclear phagocytes, we observed similar gene response kinetics in control and transgenic animals. There was a similar early increase of GCSFR at 1 dpi and a later increase of MCSFR at 6 dpi in peritoneal leukocytes of both control and XNC10-deficient transgens, consistent with an early infiltration of polymorphonuclear granulocytes followed by an influx of mononuclear phagocytes (Fig. 6A–C). We have previously shown that *X. laevis* macrophage-lineage cells are integral to immune responses against FV3 (33, 34) and that the two macrophage growth factors MCSF (35) and IL-34 (30) elicit distinct macrophage effector functions (30). In particular, IL-34–derived peritoneal macrophages exhibit more robust type I IFN and inducible NO synthase (iNOS) expression responses, as well as more potent anti-FV3 activity. Therefore, we sought to determine whether part of the delayed anti-FV3 immune response in XNC10 loss of function and lack of iVα6 T cells could reflect a change in macrophage effector functions. To address this possibility, we monitored the expression profiles of MCSF, IL-34, and iNOS genes in peritoneal leukocytes isolated from uninfected, 1 dpi (early innate response), and 6 dpi (peak of conventional CD8⁺ T cell response) XNC10-deficient transgenic and control *X. laevis*. Notably, the early infiltration and accumulation of leukocytes at 1 dpi in XNC10-deficient transgenic animals was accompanied by significantly lower gene expression for IL-34 but not MCSF (Fig. 6D–F). In addition, the iNOS gene expression response was delayed in FV3-infected XNC10-deficient transgenic animals and significantly increased only at 6 dpi, in contrast to 1 dpi in control animals. This finding suggests that iVα6 T cells have a notable role in promoting macrophage antimicrobial immunity.

Given that type I IFN in *X. laevis*, as in mammals, is critical in controlling viral replication (27), we postulated that the delayed IL-34 gene expression upon FV3 infection in peritoneal leukocytes of XNC10-deficient animals should affect type I IFN gene
expression. Indeed, unlike control animals, type I IFN gene expression was magnitudes lower at 1 dpi in peritoneal leukocytes of XNC10-deficient animals, whereas at 6 dpi both control and transgenic animals exhibited high transcript levels of type I IFN (Fig. 7A). The defect in antiviral gene expression response in transgenic animals was not general because FV3 infection induced similar expression profiles for the proinflammatory gene IL-1β (28) and the anti-inflammatory gene IL-10 between control and XNC10-deficient animals. Notably, the TNF-α expression remained elevated at 6 dpi in transgenic animals, correlating with the higher viral loads observed in these animals (Fig. 7B–D). Overall, these findings suggest that XNC10-deficient transgenic animals lacking iVα6 T cells mount delayed anti-FV3 responses that, in part, result from a defect in macrophage effector functions.

Effects of XNC10 deficiency and lack of iVα6 cells on viral replication and pathogenesis in kidneys

Given our findings that the onset of type I INF antiviral response was delayed in peritoneal leukocytes of XNC10-deficient transgenic animals, we next examined the impact of this deficiency on viral replication and pathogenesis in the kidneys, the main site of FV3 replication. Notably, kidneys isolated from XNC10-deficient transgenic animals showed significantly higher FV3 genome copy numbers compared with controls, as assessed by qPCR detecting
the FV3 vDNA Pol II gene (Fig. 8A, \(p = 0.05\)). Furthermore, compared with controls, kidney homogenates from transgenic animals contained greater levels of infectious viral particles (plaque-forming units per milliliter; Fig. 8B, \(p = 0.02543\)), clearly indicating a less efficient control of viral replication. To determine whether this increased viral replication resulted in increased pathological changes, we conducted a histological examination of kidneys from the different treatment groups. Most strikingly, in the absence of iV\(\alpha\) T cells, we observed dramatically more pronounced tissue damage in FV3-infected kidneys compared with FV3-infected controls, including a marked loss of tissue architecture and destruction of the characteristic proximal tubular epithelia in XNC10-deficient transgenic animals (Fig. 8E, 8F). We also assessed the degree of viral dissemination in other tissues, including spleen, liver, and intestine of transgenic and control \(X.\ laevis\), at 6 dpi but did not see significant difference at these secondary sites (Fig. 8G–I). Collectively, our findings suggest that loss of the class Ib MHC molecule XNC10 and the XNC10-restricted iV\(\alpha\) T cell population leads to an impaired anti-FV3 response in the class Ia competent adult \(X.\ laevis\) that is in part due to less efficient antiviral immunity and macrophage effector functions.

**Discussion**

To our knowledge, this study shows, for the first time outside mammals, the decisive role of class Ib-restricted iT cells in mounting timely and efficient antiviral immune responses. This finding in turn provides compelling evidence of the evolutionary conserved roles of class Ib-restricted iT cells in antiviral immunity, reinforcing the biological conservation of these cells throughout jawed vertebrates. Using a loss-of-function approach by transgenesis, we showed that silencing XNC10 in vivo resulted in a delayed antiviral immune response to FV3, permitting more robust viral replication and dissemination during the early phase of infection, leading to increased tissue damage in the kidney. This impaired ability to control early FV3 replication in class Ia competent

**FIGURE 6.** XNC10 deficiency results in a delayed antiviral peritoneal leukocyte response. One-year-old XNC10-deficient transgenic (\(n = 5\), black bar) or age-matched dejellied control (\(n = 8\), white bar) \(X.\ laevis\) were i.p. infected with \(1 \times 10^6\) PFU FV3. (A) Total number of FV3-induced peritoneal leukocytes. Quantitative gene expression analysis of markers for polymorphonuclear monocyte receptor, GCSFR (B); macrophage growth factor receptor, MCSFR (C); MCSF (D); IL-34 (E); and iNOS (F). Gene expression was determined relative to an endogenous control (GAPDH) and normalized against respective uninfected control gene expression. All results are presented as mean ±SE. *\(p < 0.05\) denotes statistical significance relative to respective uninfected controls, and \(*p < 0.05\), when over bars, denotes significant differences between wild-type and XNC10-deficient transgenic groups.

**FIGURE 7.** XNC10-deficient transgenic \(X.\ laevis\) have delayed type I IFN response. Quantitative gene expression analysis of antiviral (type I) IFN (A), TNF-\(\alpha\) (B), IL-1\(\beta\) (C), and IL-10 (D) cytokines in peritoneal leukocytes isolated from either uninfected or FV3 (\(1 \times 10^6\) PFU) infected XNC10-deficient transgenic animals (black bar, \(n = 6\)) and the XNC10-restricted iV\(\alpha\) T cell population leads to an impaired anti-FV3 response in the class Ia competent adult \(X.\ laevis\) that is in part due to less efficient antiviral immunity and macrophage effector functions.
before the peak of the conventional CD8+ T cell response (6 dpi; the detection of NK cell infiltration (3 dpi; Ref. 28) and well
the iV6a1.43 transcript levels in peritoneal leukocytes by 1 dpi. It is noteworthy that
sorted XNC10-T+ but also in CD8/XNC10 double negative peri-
the spleen and the peritoneum following FV3 infection is con-
observed increase in size and complexity of these cells between
and later to the kidney. These changes correlate
well with the dissemination of infection. The rapid decrease in
X. laevis state. Infection studies in
and other amphibian species
have shown that macrophage-lineage cells are integral to amphibian
immunity against FV3 (30, 33, 34). We have recently demonstrated
following FV3 infection are predominantly, if not exclusively, type I
iV6a T cells. Although the involvement of type II XNC10-restricted T cells expressing low levels of CD8 and a more
diverse, albeit iV6a-Jo1.43 biased, TCR repertoire, remains possi-
ble, this subset will first need more precise characterization with the
help of specific reagents (e.g., Abs).
Although the effector functions of iV6a T cells are likely to be
mediated by multiple mechanisms that remain to be characterized in
detail, our data suggest that iV6a T cells promote timely and ef-
fective type I IFN-mediated anti-FV3 responses and influence the
polarization of peritoneal macrophages into a more robust antiviral
state. Infection studies in X. laevis and other amphibian species
are prone to FV3 infection in vitro and exhibit more robust type I IFN and
anti-viral properties (30). Compared with MCSF-elicited peritoneal
macrophages, IL-34-derived peritoneal leukocytes were more resis-
tant to FV3 infection in vitro and exhibit more robust type I IFN and
iNOS gene expression responses (30). Consistent with this idea,
XNC10-deficient transgenic animals exhibited delayed IL-34, iNOS,
and type I IFN gene expression responses during the first stages of
FV3 infection. Thus, the modulation of macrophage antiviral effector
functions by iT cells suggested by our findings provides novel and
promising avenues of research.
During the time span of our study, XNC10 deficiency and lack of
iV6a T cells did not increase FV3-induced mortality (data not shown).
However, transgenic animals displayed a weaker ability to
control FV3 replication in the kidney by 6 dpi, at the peak of
CD8 a T cell infiltration and the onset of viral clearance, resulting
in dramatic kidney pathological changes. Although the long-term

FIGURE 8. Increased viral replication and tissue damage in kidneys of transgenic X. laevis deficient for XNC10 and iV6a T cells. One-year-old XNC10-deficient transgenic (gray circles, n = 5) or age-matched dejellied controls (white circles, n = 10) X. laevis were i.p. infected with 1 × 10⁶ PFU FV3. Kidneys were collected at 6 dpi and divided into three parts (anterior, middle, and posterior) to determine viral loads (A, middle part) by absolute qPCR (viral DNA polymerase II, of 625 ng total DNA) and plaque assays (B, anterior part). *p < 0.05 denotes statistical significance relative to respective uninfected controls, and *p < 0.05 denotes significant differences between groups denoted by the bars (Student t test). (C–F) Posterior part. Representative histology on cryosections stained with H&E for controls (C), controls at higher magnification (D), XNC10-deficient transgenic (E), and XNC10-deficient transgenic at higher magnification (F). (G–I) XNC10 deficiency has little effect on viral dissemination. Viral loads were assessed at 6 dpi in spleen (G), liver (H), and intestine (I) by qPCR. NS between groups (Student t test).
consequences of such tissue damage for survival and fitness in the wild remain to be determined, this observation reinforces the importance of a functional early iV6α T cell response in the generation of appropriate anti-FV3 immune responses. Thus, our findings strongly suggest that in the context of FV3 infection XNC10 impairment results in inefficient early recruitment of iV6α T cells to the site of infection. The absence of these iT cells in turn prevents the generation of IL-34–derived macrophages, resulting in delayed type 1 IFN and iNOS responses and weakened host control of FV3 replication.

In line with our study in X. laevis, activation of murine iNKT cells by simultaneous administration of αGalCer and infection with a mouse-adapted influenza A strain (PR8) initiated rapid recruitment of iNKT from the liver to the infected lungs, which resulted in enhanced early innate immune response, reduced viral titers in the lungs, and a significantly improved disease outcome (9). Furthermore, adoptive transfer of iNKT cells rescued the survival of Jc18’d ’ mice in a high-pathogenicity model of influenza infection (36). A functional role of iNKT cells during influenza infection was suggested by the observation that iNKT cells suppress virally induced expansion of myeloid-derived suppressor cells, including immature dendritic cells, macrophages, and granulocytes, and reduce the suppressive activity of these cells, thus restoring CD8+ T cell cytotoxic responses and viral clearance (36). However, the involvement of IL-34 in this system was not investigated. In the context of HBV infections, α-GalCer injection profoundly inhibited HBV replication in the liver of HBV transgenic mice through activation of intrahepatic iNKT cells (8). The antiviral effects of α-GalCer were rapid (inhibition of HBV replication was detected within 24 h post injection), mediated by IFN-γ as well as IFN-α/β and associated with a strong induction of 2′,5′-oligoadenylate synthetase, iNOS, and TNF-α gene expression (8). Furthermore, α-GalCer–induced inhibition of HBV replication was T cell independent but did result in an increased infiltration of NK cells into the liver, peaking at 3 d post injection, which suggested that activated iNKT cells trigger the recruitment and activation of NK cells.

Although the nature of the XNC10 ligand is currently unknown, primary and secondary sequence analysis of XNC10 and its cognate invariant TCRs receptor suggested that XNC10 presents a common or conserved antigenic motif to iV6α T cells (17, 19, 20). In support of a specific recruitment of iV6α T cells in response to an FV3-derived Ag presented in the context of XNC10, only the iV6α-Jα1.43 rearrangement, and not the other five invariant TCR arrangements (17), was elevated in response to FV3 infection. Moreover, no increase in the expression of the iV6α-Jα1.43 rearrangement was detected after i.p. stimulation with heat-killed bacteria, arguing against a nonspecific inflammation-induced recruitment of iT cells. However, whether iV6α activation during an FV3 infection results from a direct interaction with XNC10-expressing cells presenting a virus-derived Ag or an endogenous ligand remains to be determined. It is to be noted that FV3 virions contain peptide and lipid components that could in theory be processed by XNC10+ cells, loaded onto XNC10, and presented to iV6α T cells (21). It is also possible that FV3 infection induces synthesis and/or posttranslational modification of an endogenous Ag (or Ags) or products that would enhance XNC10 surface expression, thus facilitating XNC10-iV6α–TCR interaction. Alternatively, iV6α T cell activation could be driven by cytokines produced by innate immune cells in an Ag-independent manner. For example, upon infection with mouse CMV, iNKT cells were activated and produced IFN-γ in response to IL-12 and IFN-α/β stimulation in a CD1d-independent manner (37–39).

In summary, these results provide evidence that in response to a natural viral pathogen XNC10-restricted iV6α T cells are specifically recruited to the site of infection; become activated; and, by enhancing and possibly polarizing the early innate immune response such as macrophages, critically contribute to antiviral immunity and improved disease outcome. This evolutionary functional conservation, highlighting the involvement of class Ib–restricted iT cells in early response and immune modulation against viral challenges, shows the biological importance of iT cells in all jawed vertebrates. In particular, the potential function of iV6α T cells and possibly iNKT cells in polarizing macrophages and other leukocytes promises to be an interesting area of investigation.

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Disclosures

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References


