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An Essential Role for Death Receptor 3 in Experimental Autoimmune Uveoretinitis

Claudia J. Calder, Bsc (Hons), PhD and Eddie C. Y. Wang, MA, PhD

Institute of Infection and Immunity, School of Medicine, Heath Park, Cardiff University, Cardiff, Wales, UK

Death receptor 3 (DR3, TRAMP, LARD, Apo3, Wsl1, TNFRSF25) is a member of the tumor necrosis factor receptor superfamily (TNFRSF) and shows closest homology to TNFR1.¹ Like TNFR1, DR3 contains 4 extracellular cysteine-rich repeats and is capable of signaling both apoptosis via caspase 8 activation and cell survival via the activation of NF κ B.² In the immune system, DR3 has been shown to affect negative selection during thymocyte development³ and can modulate T, NKT, and myeloid cell function. Essential roles for DR3 and its only confirmed ligand TNF-like protein 1A (TL1A) have been demonstrated in murine models of experimental autoimmune encephalomyelitis (EAE),⁴ experimental colitis,⁵ allergic lung inflammation,^{4,6} and inflammatory arthritis,⁷ while the DR3/TL1A axis has been associated with a number of inflammatory diseases in humans, including rheumatoid arthritis.⁸

Experimental autoimmune uveoretinitis (EAU) is an organ-specific, CD4⁺ T-cell-mediated disease that targets the retina.⁹ Studies in EAU have provided abundant evidence that members of the tumor necrosis factor superfamily play pivotal roles in the pathogenesis of intraocular inflammation and retinal tissue destruction.^{10–14} CD4⁺ Th1 cells directed toward retinal antigens, produce cytokines, such as IFN γ and TNF α , which activate resident and infiltrating mononuclear cells to generate inflammatory mediators such as nitric oxide (NO), essential for maximum tissue destruction. As DR3 is essential for the development of other autoimmune and inflammatory diseases, we tested its involvement in the pathogenesis of EAU.

EAU was induced in 6- to 8-week-old C57Bl/6 (DR3^{WT}) or DR3-deficient (DR3^{KO}, generated as previously described and supplied by CRUK³) mice by immunization with a subcutaneous injection of 500 μ g per mouse of interphotoreceptor retinoid-binding protein (IRBP) peptide residues 1–20 (GPTHLFQPSLVLDMAKVLLD; Sigma, Genosys, UK), emulsified in vol/vol complete Freund's adjuvant (supplemented with 2.5 mg/mL *Mycobacterium tuberculosis*¹²), with an additional intraperitoneal injection of 1.5 μ g of *Bordetella pertussis* toxin (Sigma) on day 0. Animals were specific pathogen-free, isolatorreared, and maintained in accordance to Home Office Regulations for Animal Experimentation, UK. Mice were sacrificed by CO₂ asphyxiation on day 21 postimmunization, normally the peak of disease in C57Bl/6 mice. Eyes were enucleated, sectioned, and stained with hematoxylin and eosin (H&E) for histological grading. The disease incidence in the DR3^{KO} mice was significantly reduced (3 of 8) compared with DR3^{WT} mice (6 of 6), implying partial resistance. Disease grading was determined by

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Correspondence: Claudia J. Calder, Institute of Infection and Immunity, Henry Wellcome Research Building, School of Medicine, Heath Park, Cardiff University, Cardiff, Wales CF14 4XN, UK..

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scoring for cellular infiltrate and structural changes (described¹²) and showed that DR3^{KO} mice displayed a significantly reduced mean score of 0.5 ± 0.4 (maximum score 4) compared to age- and sex-matched DR3^{WT} littermates (mean score of 3 ± 0.5 ; $p = .03$) (Figure 1A–C; data shown of two pooled independent experiments; Fisher's exact test used to determine statistical significance).

Splenocytes from DR3^{KO} and DR3^{WT} mice were assessed for proliferative ability to priming antigen at different time points following induction of EAU. Spleens were removed and splenocytes were extracted, seeded at $1 \times 10^6/\text{mL}$, and stimulated with a range of concentrations of IRBP₁₋₂₀ ($0.01\text{--}1 \mu\text{g}/\text{mL}$) for 72 h. For the last 16 h, cells were pulsed with $0.5 \mu\text{Ci } ^3\text{H}$ thymidine (Amersham Biosciences) and uptake was measured using a beta counter (Wallac, Perkinelmer). PBS/2% DMSO and $2.5 \mu\text{g}/\text{mL}$ Con A (Concavalin A, Sigma) were used as negative and positive controls, respectively. Results were calculated as the geometric mean counts per minute (cpm) $\pm$ SEM of quadruplicate cultures and converted into a stimulation index (SI). The SI was calculated by dividing stimulation-induced proliferation by the negative control. There were no significant changes in proliferative ability of DR3^{KO} and DR3^{WT} splenocytes in response to IRBP₁₋₂₀ 10 days following EAU induction (data not shown). However, by day 21 (peak day of disease) significant differences between DR3^{WT} and DR3^{KO} IRBP₁₋₂₀ stimulated splenocyte cultures were observed (Figure 1D; $*p > .05$ and $**p > .005$); DR3^{KO} splenocytes demonstrated reduced peptide (IRBP₁₋₂₀)-specific proliferation compared to their DR3^{WT} counterparts. Concomitant production of IFN γ and IL-17 were measured by ELISA (OptEIA, Pharmingen, UK and R&D, UK, respectively). DR3^{KO} splenocytes produced equivalent amounts of IL-17 (Figure 1E), but significantly reduced IFN γ (Figure 1F) compared with DR3^{WT} splenocytes. These data indicate that T-cell priming is intact in DR3^{KO} mice, but sustained splenic T-cell responses seem impaired. The exact mechanisms underlying this phenomenon remain an area of further study.

Macrophages are believed the main mediator for tissue damage in EAU, because of their ability to cause peroxidation of the lipid membrane by the production of nitric oxide (NO). In the absence of TNFR1, macrophages are unable to produce NO in response to IFN γ , leading to minimal tissue destruction and resistance to EAU. As retinal destruction was markedly reduced in DR3^{KO} mice, we wanted to determine whether DR3^{KO} macrophage activation was impaired, as has been previously observed in TNFR1^{KO} mice.¹² We generated bone marrow macrophages (BM-M ϕ) in Teflon bags as previously described¹²; these F4/80-positive macrophages were nonactivated and exhibited a resting phenotype (MHCII^{low}, CD11b^{low}, and CD86^{low}; data not shown). Both DR3^{WT} and DR3^{KO} macrophages were able to produce NO in response to IFN γ stimulation (248 ± 29 and 287 ± 12 nM, respectively) compared with unstimulated controls (media alone; 127 ± 10 and 156 ± 40 nM, respectively) (Figure 1E).

Overall, these results are completely consistent with previous studies of DR3^{KO} mice in inflammatory diseases, although it is clear that DR3 function is very diverse, acting on stromal as well as immune cells. In renal inflammation, TL1A acts in a DR3-dependent fashion on tubular epithelial cells within kidney organ cultures, inducing NF κ B and caspases with the potential of both promoting and protecting against injury.¹⁵ In inflammatory arthritis,⁷ DR3 helps promote the differentiation of macrophages to bone-damaging osteoclasts. We clearly show that intrinsic production of NO by macrophages in response to IFN γ is not impaired in DR3^{KO} mice and this is unlikely to be the primary mechanism through which the absence of DR3 confers resistance to EAU. Instead, the impaired development of IFN γ -producing effector CD4⁺ T cells in the absence of DR3 is a more likely explanation, although the impact of DR3 signaling on other cytokines and chemokines cannot be ruled out. This would be in keeping with observations in allergic lung

inflammation, neuroinflammation,⁴ and colitis,⁵ where DR3 drives the accumulation of effector T cells, whether they are Th1, Th2, or Th17.

Perhaps of greatest significance, all of our findings highlight the differences between DR3 and other members of the TNFR superfamily in the induction of ocular inflammation. Apart from the role of TNFR1 described above, Fas,¹¹ CD40L,¹³ and 4-1BBL¹⁴ have also been implicated in the pathogenesis of EAU. Mice deficient in 4-1BB (TNFRSF9) are susceptible to EAU, however, treatment with agonistic anti-4-1BB mAb alleviated disease through the expansion of IFN γ -producing CD11c⁺CD8⁺ T cells. IFN γ produced by these cells induces indoleamine 2,3-dioxygenase (IDO) in dendritic cells, and the induced IDO then becomes a suppressor of antigen-specific CD4⁺ T cells.¹⁴ Disruption of CD40/CD40L (TNFRSF5/TNFSF5) interactions by administration of an anti-CD40L mAb completely protected against EAU and suppressed most related immunological responses, including DTH and cellular responses. CD40L blockade inhibited innate responses to immunization and reduced priming.¹³ Fas (*gld*; TNFRSF6) or FasL (*lpr*) deficient mice developed lower incidence and severity following EAU induction compared with wild-type controls. The underlying mechanisms for this resistance were not defined, but bone marrow chimaeras demonstrated that normal expression of Fas and FasL on hemopoietic cells of the immune system, but not on ocular tissue, was required for the induction.¹¹

It is clear that although DR3 is the closest structural relative to TNFR1 and has similar signaling properties to other TNFRSF members, its biological functions are distinct and should represent an area of increasing interest in inflammatory research.

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FIGURE 1.