Published in final edited form as:

Immunogenetics. 2016 August ; 68(8): 525–536. doi:10.1007/s00251-016-0929-7.

Evolution of innate-like T cells and their selection by MHC class I-like molecules

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Abstract

Until recently, major histocompatibility complex (MHC) class I-like-restricted innate-like $\alpha\beta$ T (iT) cells expressing an invariant or semi-invariant T cell receptor (TCR) repertoire were thought to be a recent evolutionary acquisition restricted to mammals. However, molecular and functional studies in *Xenopus laevis* have demonstrated that iT cells, defined as MHC class I-like-restricted innate-like $\alpha\beta$ T cells with a semi-invariant TCR, are evolutionarily conserved and prominent from early development in amphibians. As these iT cells lack the specificity conferred by conventional $\alpha\beta$ TCRs, it is generally considered that they are specialized to recognize conserved antigens equivalent to pathogen-associated molecular patterns. Thus, one advantage offered by the MHC class I-like iTcell-based recognition system is that it can be adapted to a common pathogen and function on the basis of a relatively small number of T cells. Although iT cells have only been functionally described in mammals and amphibians, the identification of non-classical MHC/MHC class I-like genes in other groups of endothermic and ectothermic vertebrates suggests that iT cells have a broader phylogenetic distribution than previously envisioned. In this review, we discuss the possible role of iT cells during the emergence of the jawed vertebrate adaptive immune system.

Keywords

Evolution; Comparative immunology; Amphibians; Unconventional Tcells; iTcells; MHC class I-like; XNC

Introduction

Adaptive immunity, as defined in humans and mice, is common to all jawed vertebrates and is based on broad repertoires of somatically rearranged antigen-binding receptors, immunoglobulins (Ig), and T cell receptors (TCRs) that are expressed on B and T cells, respectively. These receptors provide an anticipatory and evolutionarily advantageous repertoire capable of recognizing a plethora of possible pathogen-derived antigens. Igs and the majority of $\gamma\delta$ TCRs recognize antigens independent of antigen processing and major histocompatibility complex (MHC I and II) presentation (Chien et al. 1996; Ferreira 2013; Schild and Chien 1994; Schild et al. 1994), whereas conventional $\alpha\beta$ TCRs typically recognize processed peptide antigens presented by either MHC I or II molecules (Bluestone

et al. 1992; Jorgensen et al. 1992). In addition, specialized populations of innate-like $\alpha\beta$ T (iT) cells that express restricted or semi-invariant antigen-binding receptors with features similar to innate immune receptors have been described in mammals (Bendelac 1995; Bendelac et al. 1995; Tilloy et al. 1999; Porcelli et al. 1993) and amphibians (Edholm et al. 2013). These iT cells lack the exquisite specificity conferred by conventional $\alpha\beta$ TCRs, and they are generally considered to recognize antigens on the basis of more conserved pathogen-associated patterns in a classical MHC-unrestricted manner reviewed in Lanier (2013)). Despite the relative genetic simplicity of their semi-invariant TCRs and the obvious limitations in their recognition repertoires, iT cells have been discovered to be key regulators of multiple aspects of both innate and adaptive immune responses, recently reviewed in Godfrey et al. (2015)) and various chapters within this special issue. In mammals, semi-invariant $\alpha\beta$ TCRs are expressed by invariant natural killer T (iNKT) cells and mucosal-associated invariant T cells (MAIT) reviewed in Chandra and Kronenberg (2015) and Salio et al. (2014)). Unlike conventional T cells, these two cell subsets do not depend on classical MHC molecules for their education and function but are restricted by MHC class I-like molecules (e.g., CD1d (Bendelac et al. 1995; Gapin et al. 2001) and MR1, respectively (Treiner et al. 2003)). MHC class I-like genes are located outside the MHC locus, and although structurally similar to classical MHC class Ia genes, these genes are less polymorphic, less ubiquitously expressed and can present non-peptide antigens (Adams and Luoma 2013). iNKT cells have been shown to respond mostly to glycolipids presented in the context of CD1d (Kinjo et al. 2011; Kinjo et al. 2006), whereas MAIT cells can recognize riboflavin metabolites as well as folic acid derivatives in a MR1-restricted manner (Corbett et al. 2014; Kjer-Nielsen et al. 2012).

Recently, we described a significant population of invariant (iV α 6) T cells in the amphibian *Xenopus laevis*. Briefly, similar to their mammalian counterparts, *X. laevis* iV α 6T cells express a unique invariant TCR α chain (V α 6-J α 1.43 with no junctional diversity) paired with a limited TCR β chain usage (Edholm et al. 2013). These iV α 6 T cells function as early responders that are critical for eliciting efficient anti-viral immunity and that, unlike conventional T cell, are dependent on the *Xenopus* MHC class I-like molecule XNC10 for their development and function (Edholm et al. 2015). In addition, strong molecular evidence indicates the presence of a classical MHC class Ia-independent iT cell-mediated immune response based on multiple distinct iT cell subsets in *X. laevis* tadpoles (Edholm et al. 2013). The identification of distinct iT cell populations in a species as genetically and evolutionary distant from mouse and humans as *Xenopus* strongly supports the biological relevance of these cells.

The focus of this review is to evaluate the benefits of specialized populations of iT cells expressing a restricted TCR repertoire from an evolutionary viewpoint. Furthermore, in the context of the large expansion and multiple species-specific adaptations reported for MHC class I-like and non-classical MHC genes in both endothermic and ectothermic vertebrates, we also discuss the likelihood and potential for a more widespread use of MHC class I-like-restricted iT cells in jawed vertebrates as well as the possible role that this system may have had during the emergence of the adaptive immune system.

Adaptive immunity of jawed vertebrates

Origin and evolution of the diversified adaptive immune system in jawed vertebrates

Since one of the hallmarks of adaptive immunity is the generation of vast Ig and TCR repertoires by Ig and TCR gene segment rearrangements occurring early in development, the existence of adaptive T and B cells with limited antigen-binding repertoires is, from an evolutionary standpoint, somewhat puzzling. However, over the past decades, it has become clear that lymphocytes expressing these types of innate-like adaptive receptors such as canonical $\gamma\delta$ T cells, B1 B cells, and invariant $\alpha\beta$ T cells are critically involved in various aspects of the immune responses. In parallel with the conventional generation of multiple highly specific receptors, the invariant receptors reflect an ancillary mechanism of evolutionary specialization. Notably, these types of invariant lymphocytes often arise early during ontogeny and may provide a way to deal with infection using a limited set of cells and receptors. Over many years, comparative studies have accumulated evidence of diversified antigen recognition receptors, immune effector cell populations, and immunity in a wide range of vertebrate species (reviewed in Boehm and Swann 2014; Cooper and Alder 2006; Du Pasquier 1992; Flajnik and Kasahara 2010; Litman et al. 2010). This large body of data has provided insight into the evolutionary acquisition of immune complexity and revealed salient features conserved among all vertebrates. These studies have also highlighted alternative and species-specific adaptations for combating pathogens underpinning a dynamic capacity for revision, diversification, and specialization within this system. Across all jawed vertebrates (a group consisting of cartilaginous fishes, bony fishes, amphibians, reptiles, birds, and mammals), the adaptive immune system is critically dependent on somatically diversified Igs and TCRs generated by recombinase-activating gene (RAG1) and RAG2 enzyme-mediated recombination of variable (V), diversity (D), and joining (J) gene segments (Tonegawa 1983) followed by splicing to a constant (C) region. This V(D)J recombination occurs in B and T cell progenitors in a highly ordered process directed by recombination signal sequences (RSSs) flanking the segmental elements (Sakano et al. 1979). In addition, during the assembly process, non-template nucleotides are introduced at the V, D, and J junctions resulting in a impressive combinatorial power estimated, for the human $\alpha\beta$ TCR to be in the order of 10^{20} possible unique receptors (Murugan et al. 2012; Robins et al. 2010).

This elaborate defense system is considered to have arisen approximately 400 million years ago in jawed fish ancestors, whose actual living descendants are found among sharks and skates (reviewed in Boehm and Swann 2014; Cooper and Alder 2006; Flajnik and Kasahara 2010; Litman et al. 2010). It is remarkable that RAG1/2, Igs, TCRs, and MHC genes have remained conserved and maintained over the long time of jawed vertebrate evolution. Indeed, all these genes are present in cartilaginous fish, the most evolutionary ancestral of the currently living jawed vertebrates. In contrast, until recently, very few remnants if any of these elements were identified in the genomes of non-jawed vertebrate chordates. This observation has prompted the “big bang theory” postulating that a large number of genes, including those encoding Ig, TCRs, MHC, and the cellular processes and organs mediating adaptive immunity were acquired over a short period of evolutionary time by the now extinct placoderm, the common ancestor of jawed vertebrates (Marchalonis et al. 1998; Thompson

1995). This rapid emergence of adaptive immunity is hypothesized to have occurred as a result of two macroevolutionary events: (i) the introduction of a RAG-like transposon element via lateral gene transfer into an ancestral Ig domain-containing gene locus and (ii) two rounds of whole-genome duplication occurring after the early vertebrate divergence from urochordates but before the radiation of jawed vertebrates (reviewed in Flajnik and Kasahara 2010). It has further been suggested that the first integration of a RAG gene took place in a locus encoding for an innate immune receptor followed by tandem element duplications and selective pressure-driven diversification resulting in the Ig and TCR loci (Litman et al. 2010). Thus, an ancestral, putatively immune-related receptor with single specificity would have given rise to a family of highly diverse receptors. It is of note that RAG1 and RAG2-like gene clusters as well as sequence elements with similarities to RAG1 have been identified in the echinoderm purple sea urchin (*Strongylocentrotus purpuratus*) and the amphioxus (*Branchiostoma floridae*), respectively, suggesting that the genetic mechanisms associated with V(D)J recombination might be more ancient than previously thought (Fugmann et al. 2006; Holland et al. 2008). However, even with information gathered from whole-genome sequencing and genome-wide analyses of various chordates including agnathans (jawless vertebrates; lampreys and hagfish), urochordates (sea squirts), and cephalochordates (lancets), no evidence for the presence of Igs, TCR, or MHC homologs has been identified beyond jawed vertebrates. Notably, these non-jawed vertebrate taxa have evolved other systems of immune receptor diversification, distinct from that of jawed vertebrates. For example, agnathans rely on a unique adaptive immune system based on somatically derived diversified leucine-zipper-type receptors termed variable lymphocyte receptors (VLRs) generated by assembling individual leucine-rich repeat elements in a gene conversion-like process (Pancer et al. 2004) and reviewed in Boehm et al. (2012)).

Even though RAG-mediated receptor diversification appears advantageous among jawed vertebrates, it is interesting to note that other complex mechanisms such as expansion of innate pattern recognition types of receptors have been observed in several invertebrates (Zhang et al. 2004) (Watson et al. 2005). For example, the snail (*Biomphalaria glabrata*) produces a somatically diversified (via gene conversion and point mutations) secreted lectin, fibrinogen-related protein 3 (FREP3), that is central to providing resistance to pathogens (Hanington et al. 2010). It is likely that in the absence of conventional adaptive immunity, these sophisticated innate receptors provide the diversity needed to specifically recognize and adequately respond to a variety of different pathogen targets.

MHC-mediated restriction of TCRs

The development and education, as well as antigen recognition and the subsequent elicitation of $\alpha\beta$ T cell effector functions, depend on polygenic, polymorphic MHC class I and class II genes. Like TCRs, MHC class I and class II genes have been described in all taxa of jawed vertebrates. Importantly, these genes provide a selection system to control the TCR repertoire, thereby limiting the maturation of self-reactive T cells that might cause autoimmunity. It is notable that the major features of adaptive immunity in jawed vertebrates, especially the TCR/MHC system, have overall been minimally modified over a long evolutionary time (Flajnik and Kasahara 2010; Litman et al. 1999; 2010; Rast et al. 1997). Homologs of all four TCR chains (α , β , γ , and δ) are present in all major lineages of

jawed vertebrates. These genes, especially those encoding $\alpha\beta$ TCR, have retained a relatively high degree of conservation in terms of structure (heterodimeric $\alpha\beta$ or $\gamma\delta$ transmembrane molecules with each TCR chain made up of a variable-membrane-distal and a constant-membrane-proximal domain), somatic recombination (V/J segments for TCR α and TCR γ ; V/D/J for TCR β and TCR δ), and gene organization (all four TCR loci are in a translocon arrangement with close linkage of α and δ gene segments). This is strong evidence that $\alpha\beta$ T cells and $\gamma\delta$ T cells are common across jawed vertebrates. In fact, it has been shown that a chimeric $\alpha\beta$ TCR, containing amphibian, fish, or shark TCRV β , could recognize antigens presented by mouse MHC class II and CD1 molecules (Scott-Browne et al. 2011). This recognition is dependent on conserved motifs within the TCRV β complementary determining regions (CDR) 2, which implies that features critical for MHC recognition were selected early and have been maintained for a long evolutionary time (Scott-Browne et al. 2011). However, it is difficult to determine if MHC-mediated antigen presentation preceded or succeeded the occurrence of rearranging $\alpha\beta$ TCR receptors, since no MHC class I or class II homologs have been described outside jawed vertebrates. One argument in favor of T cells emerging before the MHC system is based on the ability of certain $\gamma\delta$ TCRs to recognize free antigen in a MHC class I and II unrestricted manner (Chien et al. 1996; O'Brien et al. 1989; Schild and Chien 1994; Schild et al. 1994). This kind of antigen recognition has likely contributed to the higher evolutionary plasticity observed in the $\gamma\delta$ TCR chains and would suggest that non-MHC-restricted antigen recognition represents the more ancestral mode of function.

In addition to classical MHC class I and class II genes, all jawed vertebrates have variable numbers of heterogeneous, typically non-polymorphic non-classical MHC and/or MHC class I-like genes. The evolutionary history of non-classical MHC class I and MHC class I-like genes has been dynamic (Adams and Luoma 2013). Although these genes are present in varying numbers in the genomes of cartilaginous fish, teleost fish, amphibians, reptiles, birds, and mammals, their phylogeny remains unclear and species-specific adaptations are common. Indeed, even among closely related species, non-classical MHC and MHC class I-like genes typically display extensive intraspecies variations in gene composition, numbers, and genomic organization (Adams and Luoma 2013; Adams and Parham 2001; Flajnik and Kasahara 2001). This diversity has been partly attributed to the “birth and death” model of evolution in which new genes arise via gene duplication (Nei et al. 1997). While some of these duplicated genes are maintained mostly unchanged in the genome, others undergo neofunctionalization or degradation (Nei et al. 1997; Nei and Rooney 2005; Piontkivska and Nei 2003). To date, phylogenetic relationships among various non-classical MHC and MHC class I-like genes are not fully understood and only a few unambiguous orthologs or even homologs have been described across different vertebrate orders and families. However, functional analogs spanning divergent taxa have been described, suggesting that at least some of these genes might have been subject to pathogen driven-co-evolution. One salient example of this is the conserved biological role of MHC class I-like molecules (i.e., CD1d, MR1, and XNC10) in the development and function of iT cells (Bendelac 1995; Bendelac et al. 1995; Edholm et al. 2013; Treiner et al. 2003).

Insight into the evolution of MHC class I-like-restricted iT cells using *Xenopus* as a model for genome and gene evolution

Expansion and conservation of MHC class I-like genes in the Xenopodinae

The subfamily Xenopodinae, belonging to the family Pipida, is one of a few extant vertebrate animal taxa that contain natural polyploid species, ranging from diploid (2n) to dodecaploid (12n) species (Evans 2008; Evans et al. 2015). In the Xenopodinae subfamily, this polyploidization is inferred to have occurred multiple times during the course of evolution spanning a period of 50–80 million years with speciation occurring through a combination of bifurcating speciation (one ancestor giving rise to two descendants with the same number of chromosomes) and reticulate speciation (interspecies hybridization and fusion of the two genomes resulting in a new descendant with, at least in the early stages of evolution, a duplicated genome). Representative species from two separate genera belonging to the Xenopodinae subfamily, the allotetraploid *X. laevis* and the diploid *X. (Silurana) tropicalis*, have been used to study the phylogeny of immune-related genes and have provided valuable insight into the evolution of MHC class I-like genes (Edholm et al. 2014a; Flajnik et al. 1993; Goyos et al. 2011). Both *X. laevis* and *X. tropicalis* possess a single MHC class Ia gene per genome and a large number of *Xenopus* non-classical MHC class I (*Xenopus/Silurana* non-classical (*XNC/SNC*)) genes, including 21 expressed *XNC* genes in *X. laevis* and 29 *SNC* genes in *X. tropicalis*. Both the *XNC* and *SNC* loci are located distally from but on the same chromosome as their respective MHC locus. Comparative genomic analysis of these two species has revealed two phylogenetic patterns among *XNC/SNC* genes; some gene lineages display a high degree of species-specific expansion and contraction, whereas other *XNC/SNC* gene lineages have been maintained as monogenic subfamilies with few changes in their nucleotide sequence across divergent species (Edholm et al. 2014a; Goyos et al. 2011). The *XNC10* gene, encoding the restricting element for iVa6T cells, belongs to the second category with 62 % identity to its *X. tropicalis* ortholog. Indeed, the *XNC/SNC10* gene lineage is highly conserved in all species of the Xenopodinae subfamily examined (Edholm et al. 2014a; Goyos et al. 2011). Furthermore, *XNC10* and *SNC10* share the same expression pattern with a predominant and early developmental thymocyte expression, from the onset of thymic organogenesis, suggesting that these genes have similar functions. Another salient feature that emerged from these comparative studies was that unlike mammals, the majority of the different *XNC/SNC* gene lineages have been preserved between these two divergent species. Such a conservation of *XNC/SNC* gene lineages is even more striking when considering that, regardless of their superficial morphological similarities, *X. laevis* and *X. tropicalis* are estimated to have split from a common ancestor more than 65 million years ago, roughly corresponding to the evolutionary time separating primates and rodents.

In comparison to *Xenopus*, two of the most conserved mammalian MHC class I-like genes, CD1 and MR1, are involved in iT cell development. Conserved CD1d gene homologs specifically involved in iNKTcell development and function have, to date, been described in humans, primates, rodents, and ruminants with ~60 % identity between human and mice CD1d (Dascher 2007). In addition, two divergent CD1 genes have been described in chickens (Miller et al. 2005; Salomonsen et al. 2005), and recently, CD1 gene homologs

were described in the green anole lizard and Crocodylia (Yang et al. 2015), demonstrating that CD1 is ubiquitous across mammals, birds, and reptiles. To date, MR1 has only been described within mammals, but it is highly conserved within this clade and MR1 homolog has been identified in eutherian as well as placental mammals (Huang et al. 2009). It is also worth mentioning that a MHC class I-like molecule YF1*7.1 that may represent an avian MR1-like gene has been identified (Hee et al. 2010). Based on these studies, it has been hypothesized that both MR1 and CD1 are phylogenetically old and; while retaining a general role as antigen-presenting molecules, they have become specialized in presenting non-peptide antigens to distinct subsets of iT cells. Although several ectothermic vertebrate genomes have been fully sequenced and annotated, to date, no CD1 or MR1 homologs have been identified. However, like endothermic vertebrates, amphibians, teleostean, and cartilaginous fish species possess varying numbers of divergent non-classical MHC class I and MHC class I-like genes that are heterogeneous, display lower polymorphism, and have a more tissue-restricted expression pattern when compared to their MHC class Ia counterparts (see “Phylogeny of MHC class I-like genes” section).

T cell development and selection in the absence of classical MHC molecules

In addition to somatically rearranging antigen receptors, another conserved hallmark of jawed vertebrate adaptive immunity is the essential role of the thymus in T cell differentiation and education. Considering T cell differentiation in *Xenopus*, the T cell compartment is subjected to two distinct temporally separated developmental programs. Similar to mammals, amphibian intrathymic T cell development follows a series of defined molecular and cellular stages that result in T cell lineage-specific differentiation (reviewed in Hansen and Zapata 1998). However, following the first round of T cell education during tadpole ontogeny, a second “adult-type” T cell differentiation and selection take place during metamorphosis (Turpen and Smith 1989). This is preceded by a drastic reduction in the total numbers of thymocytes and splenic lymphocytes and an involution and translocation of the thymus toward the tympanum (Du Pasquier and Weiss 1973; Rollins-Smith et al. 1984). Of particular interest in the context of MHC class Ia-restricted conventional T cells and MHC class I-like-restricted iT cell ontogeny is the differential regulation of MHC class I genes during tadpole and adult life stages. Indeed, no consistent MHC class Ia surface protein expression is detected until the pre-metamorphic developmental stage 56–58 (~4 weeks of age), and its expression subsequently increases during metamorphosis (Flajnik et al. 1986; Rollins-Smith et al. 1997; Salter-Cid et al. 1998). It is noteworthy that unlike in mammals, in which experimental impairment of MHC class Ia expression results in severe immunodeficiency and/or death, *Xenopus* tadpoles, despite their sub-optimal MHC class Ia expression, are immunocompetent and have circulating CD8⁺ T cells (Barlow and Cohen 1983). This observation can at least partly be explained by the expression of several distinct *XNC* genes in the tadpole thymus at the onset of thymic organogenesis ((Goyos et al. 2011); Edholm and Robert, unpublished observation). Many of these XNCs are preferentially expressed on thymocytes, and their gene expression is maintained through metamorphosis as well as in the fully matured adult thymus ((Edholm et al. 2014b); Edholm and Robert, unpublished observation). These expression patterns suggest that tadpoles have a functional and specialized larval T cell compartment that follows a differentiation program distinct from that of the adult. This developmental program may be more dependent on MHC class

I-like selected iT and unconventional T cells than MHC class Ia-restricted conventional $\alpha\beta$ T cells.

Evidence for multiple MHC class I-like-restricted iT cell subpopulations in *Xenopus* and predominance of iT cells during early ontogeny

Using a global TCR α chain targeted deep-sequencing repertoire analysis in combination with MHC class I-like tetramers and RNA interference-mediated loss of function, we demonstrated that iT cells are not only conserved in *Xenopus* but may, in fact, constitute a major component of the amphibian immune system, especially in the tadpoles (Edholm et al. 2013). At the tadpole stage, before the onset of metamorphosis, there is clear evidence for preferential V α and J α segment usage in the CD8⁻ and CD8^{dim} T cell compartments. Notably, six unique invariant TCR α rearrangements represent >80 % of the TCR α repertoire utilized by the CD8^{-/dim} T cell pools (Edholm et al. 2013). In addition, in the tadpole, the various rearrangements display distinct tissue-specific expression patterns indicative of specialized functions (Fig. 1; Edholm and Robert unpublished data). Thus, although the TCR β repertoire remains to be determined and even if it is more diversified, it is probable that T cells in tadpoles have a more limited TCR $\alpha\beta$ repertoire than *Xenopus* adults.

As in all tetrapod species analyzed, to date, the gene segments encoding the TCR α and TCR δ chains in *X. laevis* are tightly clustered with some TCR δ genes nestled among the TCR α genes. As expected, analysis of the *X. laevis* TCR α/δ loci revealed a strong conservation with the previously annotated TCR α/δ loci of *X. tropicalis* (Parra et al. 2010). Using the recently released *X. laevis* genome assembly version 9.1, which includes the attribution of gene models to long (L) and short (S) chromosomes (Xenbase.org; Session et al. submitted), we identified a total of 103 V gene segments within the *X. laevis* TCR α/δ loci (Edholm and Robert, unpublished data). The majority of these genes map to the *X. laevis* chromosome 1L (Fig. 2). Based on nucleotide identity and phylogenetic relationships, 80 of these V segments are V α s and 7 are V δ s, whereas similar to *X. tropicalis*, the locus also contains 9 V segments that are more related to V H genes and are thus designated V $H\delta$ (Parra et al. 2010; Edholm and Robert, unpublished data). In addition, there is a large number of J α segments located upstream of the C α 1 gene on 1L. On the same chromosome, within the V α genes is a second C α gene (C α 2) that shares only 45 % amino acid identity with C α 1. Whether or not C α 2 is expressed remains to be determined. Furthermore, three C δ genes were identified; all of which appear functional with an open reading frame (ORF) and canonical cysteine residues required to form the intrachain disulfide bond. Out of the 103 V segments identified, 19 V α segments were identified on the *X. laevis* chromosome 1S. In addition, 7 V α segments, 9 J α , and a C α that share 77.78 % amino acid identity to C α 1 were identified on a unassigned scaffold (scaffold 131) that, based on sequence gaps in the annotated genome and nucleotide similarities, is likely linked to the V α gene segments identified on *X. laevis* chromosome 1S (Fig. 3a, c; Edholm and Robert, unpublished data). To what extent that the TCR α gene segments on *X. laevis* small chromosome 1 contribute to the overall repertoire is currently unknown, however, transcripts containing the 1S C α have been reported, albeit at a much lower frequency than transcripts containing the 1L C α 1. These observations suggest that a large number of TCR genes, including all δ genes and

many of the α genes in *X. laevis*, have been diploidized and are now present as single-copy genes (Edholm and Robert, unpublished data).

Notably, the invariant V α and J α segments are all located on chromosome 1L where they are interspersed within the α/δ loci and, based on nucleotide and phylogenetic analysis, cluster in different V α subfamilies. Thus, there is no apparent positional effect explaining the dominant usage of these specific gene segments (see map of the TCR α/δ loci in Fig. 2). This observation, consistent with the specific requirement of *XNC10* for development and function of one specific iT cell population without significantly affecting the expression of the other invariant TCR α rearrangements, suggests that the dominant and invariant TCR α chain usage in tadpoles (and in the absence of MHC class Ia) is the result of multiple XNC-mediated thymic selection processes.

Insight into the function of iT cells in amphibians

In *X. laevis*, iV α 6T cells are critical for efficient anti-viral immunity against the ecologically relevant ranavirus frog virus 3 (FV3) in both MHC class Ia-deficient tadpoles and MHC class Ia-competent adults (Edholm et al. 2013; Edholm et al. 2015). However, the absence of the iV α 6T cell subset has different consequences in tadpoles and adults. In tadpoles that typically fail to control FV3 infection and die within 1 to 2 months post infection, the lack of iV α 6 T cells markedly accelerated death during the early stages of infection (Edholm et al. 2013). In adults that typically clear FV3 infection within 1–2 weeks, the loss of iV α 6T cells considerably delayed the onset of the anti-viral immune response, resulting in impaired viral clearance both at the site of initial infection and in the kidney (the main target organ for FV3) and markedly more pronounced FV3-induced kidney damage (Edholm et al. 2015). However, the delay in anti-viral immune induction was presumably compensated by the infiltration of NK cells and anti-FV3-specific MHC class Ia-restricted conventional CD8⁺ T cells, as these animals did eventually clear the infection with no increase in mortality (Edholm et al. 2015).

Similar to mammalian iT cells, amphibian iV α 6T cells were shown to be rapid and transient responders. In the adult, within a few hours following intraperitoneal challenge with FV3, iV α 6T cells are recruited from the spleen (note that *Xenopus* lack lymph nodes and, thus, the spleen functions as both a primary and secondary lymphoid organ) to the initial site of infection.

Notably, the specificity of iV α 6T cell response to FV3 pathogens is highlighted by the observation that only iV α 6-J α 1.43 expression, but not any of the other iTCR α rearrangements, was increased following FV3 challenge (Edholm et al. 2015). In addition, iV α 6T cell recruitment is dependent on a fully replicating virus and is impaired with various less virulent knockout FV3 pathogens, which is consistent with an antigen-dependent response (Edholm and Robert, unpublished observations).

Interestingly, a similar rapid infiltration of iV α 6T cells was observed in the peritoneal exudate of tadpoles following intraperitoneal transplantation of a MHC class Ia-negative lymphoid tumor (Haynes-Gilmore et al. 2014). A marked increase in the relative number of infiltrating iV α 6T cells was observed following transplantation with *XNC10*-deficient tumor

cells consistent with a regulatory role of iV α 6T cells, possibly as a result of XNC10-antigen-iTCR interaction-induced activation status (Haynes-Gilmore et al. 2014).

How widespread are MHC class I-like-restricted iT cells across phyla?

Phylogeny of MHC class I-like genes

Amphibians occupy a key position on the evolutionary ladder connecting mammals with vertebrates of more ancient origin (bony and cartilaginous fishes) that shared a common ancestor ~350 million years ago (reviewed in Robert and Ohta 2009). Thus, despite the lack of conserved MHC class I-like gene orthologs and the fact that the functional roles of ectothermic MHC class I-like class I genes remain largely unknown, the identification of MHC class I-like-restricted iT cell populations in the amphibian *Xenopus* raises the possibility that these types of specialized MHC class Ia-unrestricted T cell subsets are more widespread than previously thought. The fact that MHC-class I-like genes are present in all taxa of jawed vertebrates attests to their evolutionary primordial origins, while the high degree of species-specific expansion and contractions might reflect a dynamic adaptation to the antigen micro-environment.

With regard to ectothermic vertebrates, the urodele amphibian *Ambystoma mexicanum* displays a large expansion of MHC class I-like genes suggesting a diversified and biologically relevant role for these genes (Sammut et al. 1997). In bony fish, to date, five different MHC class I lineages have been described U, Z, S, L, and P (reviewed in Grimholt 2016). In the absence of functional data, the teleost classical and non-classical/MHC class I-like lineages have mainly been distinguished based on allelic polymorphism, phylogenetic analysis, tissue expression, and conservation of canonical peptide-binding residues in the MHC α 1- α 2 antigen-binding groove. Classical MHC class I genes all belong to the U lineage, which have been identified in all teleost species studied to date. In addition, non-classical U lineages with more restricted expression patterns and limited polymorphism have been described (Dijkstra et al. 2006; Lukacs et al. 2010; Miller et al. 2006). A striking example of U-lineage expansion was reported in the teleost fish Atlantic cod (*Gadus morhua*). Analysis of the cod genome has revealed a surprising remodeling of the immune genome. While most genes involved in the conventional jawed vertebrate immune response are present in this species, the MHC II, CD4, and invariant chain (Ii) genes have been lost (Star et al. 2011). In contrast, the Atlantic cod genome contains as many as 100 MHC I loci, which is a highly expanded number compared to other teleosts, suggesting that compensatory mechanisms have evolved to deal with immunity against bacterial and parasitic infections in the absence of a MHC class II/CD4-mediated recognition response. To date, the level of polymorphism and expression patterns of these genes is unknown. Thus, it is premature to classify this group of expanded MHC class I genes as classical or non-classical/MHC class I-like. The unusual specialization and reliance on innate immunity and, perhaps, cross talk with iT cells are further suggested by the marked expansion of TLRs recognizing nucleic acids. With regard to other bony fish, while the number of Z lineage genes is highly variable ranging from 1 to 18, at least one typical Z lineage gene is expressed in all species studied (Grimholt et al. 2015). In contrast, the S, L, and P lineages are differentially expressed among species and lack conservation of the canonical peptide-

binding residues conserved in classical MHC sequences, suggesting that these molecules possibly bind non-peptide ligands. Furthermore, depending on the species considered, there is a marked difference in expansion of the various lineages. For example, zebrafish (*Danio rerio*, a member of the cypriniformes) and Atlantic salmon (*Salmo salar*, a salmonids) have relatively large numbers of L lineage genes, 16 and 10 genes, respectively. In the Tetraodontiforme Fugu (*Takifugu rubripes*), the L lineage is absent, and instead, the fugu genome consists of a large number of P lineage genes (Grimholt et al. 2015) and reviewed in (2016)). Last but not least, variable numbers of highly divergent species-specific MHC class I-like genes have also been described in elasmobranchs (Bartl et al. 1997; Wang et al. 2003). Overall, this diversification of MHC class I-like/non-classical genes in all major cold-blooded vertebrates is intriguing and strongly suggests that different species have evolved different approaches to antigen presentation and immune surveillance.

iT cells in early development

The MHC class I-like iT cell-based recognition system has several advantages including the ability to function with a relative small number of T cells and to adapt to a particular or common pathogen. The potential benefit of this stratagem becomes apparent when examining *Xenopus* development. Unlike mammals, *Xenopus* tadpoles, like most other ectothermic vertebrates, hatch in the surrounding antigen-rich water and develop in the absence of maternal protective influences. As such, the tadpole needs a way to recognize a broad array of antigens using relatively few lymphocytes (15–20,000 T cells). Thus, a system relying on recognition of conserved antigens equivalent to pathogen-associated molecular patterns coupled with a direct activation of specialized T cell effectors exhibiting limited TCR diversity is clearly beneficial.

An interesting example highlighting the use of invariant TCRs during early development has been described in marsupials. Marsupial possesses a unique TCR gene, termed TCR μ (Parra et al. 2007). Unlike most TCR loci, TCR μ is typically organized in tandem clusters with each cluster containing either a single or few V μ , D μ , and J μ segments located upstream of the exons encoding the C μ domain. Unique to TCR μ , there is also a germline VJ (V μ j) located between the most 3' J μ and the C μ encoding exons. The resulting TCR μ gene can be transcribed as two distinct isoforms, TCR μ 1.0 and TCR μ 2.0. TCR μ 1.0, which exclusively uses the germline-joined invariant V segment, is detectable as early as 1 day after birth when the marsupial thymus is still primarily undifferentiated epithelium (Parra et al. 2009). In contrast, the TCR μ 2.0, which contains a somatically diversified V domain located membrane distal to the V μ j and C μ domains and thus consists of an unusual two V configuration, is not detected until day 13 when the thymus is histologically mature. In contrast to most eutherian mammals, which have a thymus and other lymphoid tissues that develop in utero, the marsupial thymus is primarily undifferentiated epithelium at birth, and there are few or no circulating lymphocytes. T cell-dependent responses in marsupials are correspondingly absent or delayed in the first week of life. Therefore, it is interesting to speculate that while the function of TCR μ 1.0⁺ T cells is still unknown, the invariant nature of the V μ j gene suggests a conserved recognition role for the TCR μ 1.0 receptor possibly mediated in a MHC class Ia-unrestricted manner.

Conclusions and perspective

In the absence of functional data, it is difficult to obtain clear evidence of the presence of iT cells in ectothermic vertebrates (Fig. 4). However, with the rapid advance in genomics and transcriptomics, increasing amounts of data are becoming available that should allow investigators to identify potential invariant TCR rearrangement, as well as tissue-specific expression and low polymorphism of potential MHC class I-like genes. In addition, the progress in genome editing systems, such as CRISPR-Cas9, that have been successfully adapted to non-model species should permit direct investigation (Harrison et al. 2014).

The existence of non-classical MHC and MHC class I-like genes and their presumed involvement in unconventional T cell development, selection, and function raise an interesting possibility with regard to the evolution of the adaptive immune system. One issue concerning the view that the adaptive immune system has emerged as a “big bang” initiated by the horizontal transfer of RAG1/2 genes mediating somatic diversification of TCRs is the concurrent need for a selection system. In order to avoid autoimmunity through the generation of self-reactive T cell clones, a rapid and intricate selection system (MHC) must have been acquired at the same time as the generation of TCR diversification. It has previously been suggested that the MHC class I-like-restricted iT cell type of restriction may have represented a primordial recognition system. Thus, non-classical MHC and MHC class I-like genes positively selecting T cells of limited TCR diversity may have permitted a transitional phase during which a more elaborate system of positive/negative selection and more specific antigen presentation using rich allelic polymorphism took place.

Acknowledgments

We would like to thank Dr. Edith Lord for critical reading of the manuscript. This work was supported by an R24-AI-059830 grant from the National Institute of Allergy and Infectious Diseases (NIH/NIAID). M.B. was supported by a predoctoral fellowship Ruth L. Kirschstein Predoctoral F31 (F31CA192664) from the National Cancer Institute (NIH/NCI). E-S.E. was supported by the National Science Foundation IOS-1456213 and a 2015 Career in Immunology Fellowship from the American Association of Immunologists.

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Fig. 1.

Tissue-specific expression of invariant TCR α rearrangements in pre-metamorphic stage 50–53 (~2 weeks of age) tadpoles. Gene expression of iVa6-Ja1.43, iVa22-Ja1.32, iVa40-Ja1.22, iVa41-Ja1.40, iVa45-Ja1.14, and iVa23-Ja1.1.3 in the thymus, kidney, intestine, liver, skin, and gill is shown. Results are normalized to an endogenous control and presented as fold change in expression compared with the lowest observed expression. Data are presented as mean $\pm$ SE ($n = 4$); *nd* below detection level. All animals were from the *X. laevis* Research Resource for Immunology at the University of Rochester (<http://www.urmc.rochester.edu/smd/mbi/xenopus/index.htm>)

Fig. 2.

Schematic representation of the *X. laevis* TCRα/δ locus on chromosome 1L. Vα (light blue) Vα utilized in invariant TCRα rearrangements (dark blue), Vδ (yellow), VHδ (light red), J (black), and three Cα (green) and three Cδ (orange) were numbered according to their position in the locus or, as in the case of Cα, to prior description. Transcriptional orientation is demonstrated by an arrow above each gene segment. Syntenic genes between the 1L and 1S are shown in white. Olfactory receptors (OR) are shown in gray. For Vα segments on 1S and scaffold 131, the percent amino acid identity to the closest V relation on 1L is denoted above the respective gene (Color figure online)

Fig. 3.

Schematic representation of the *X. laevis* TCR α genes identified on chromosome 1S (**a**) and scaffold 131 (**b**). V α (light blue), J (black), and C α (green). Transcriptional orientation is indicated by an arrow above each gene segment. Syntenic genes between the 1L and SL are shown in white. Gap in sequence is denoted by dashed boxes. For V α segments, the percent amino acid identity to the closest Vrelation on 1L is denoted above the respective gene (Color figure online)

MYA	Vertebrate taxa	MHC class I				T cells		
		Class Ia	MHC-like	CD1	MR1	$\gamma\delta$ T	$\alpha\beta$ T	iT
100	Mammals	+	+	+	+	+	+	+
200	Birds	+	+	+	?	+	+	?
250	Reptiles	+	+	+	-	+	+	?
360	Amphibians	+	+	-	-	+	+	+
400	Bony fish	+	+	-	-	+	+	?
460	Cartilaginous fish	+	+	-	-	+	+	?
550	Jawless vertebrates	No MHC or TCR homologs						

Fig. 4. Summary of the currently known distribution of MHC class Ia, MHC class I-like, CD1, MR1, $\gamma\delta$ T, $\alpha\beta$ T, and iT cells across jawed vertebrate taxa. Note that iT cells are defined as MHC class I-like-restricted $\alpha\beta$ T cells with a semi-invariant TCR. *MYA*: millions years ago