A Study of the Progenitor Potential and Function of Thymic Nurse Cells Using pH91 a TNC- Specific Monoclonal Antibody

By

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Abstract

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Using scanning electron microscopy (SEM), transmission electron microscopy (TEM) and confocal microscopic analyses, we show that TNCs create an intimate association with thymocytes. Thymocytes become trapped within unique extra-cytoplasmic spaces generated by the TNCs. The membrane-derived honeycomb-like fenestrae allow visualization of trapped thymocyte movement into and out of these fenestrae, a process that facilitates interactions between the two cell types. Further, we have data confirming an interaction between the αβTCR expressed on trapped thymocytes and MHC class II antigen expressed on the

surfaces of the TNCs. We also observe lipid-raft accumulation around the contact point between the thymocytes and TNCs.

When we costained freshly isolated thymic nurse cells with TNC-specific monoclonal antibody pH91 and with K5 and K8 cytokeratin antibodies, we observed a subset of TNC that were $K5^{+}K8^{+}$ and $pH91^{+}$. Previous studies have suggested that $k5^{+}k8^{+}$ thymic TECs were thymic epithelial progenitor cells. The studies presented here show that TNCs express the transcription factors Foxn1 and p63 both of which play critical role in the thymic determination as well as maintaining a proliferative subpopulation of TECs. Interestingly, when we co-stained embryonic day 11.5 (E11.5) thymic sections with pH91 and Foxn1 antibodies, their expressions were detected in this phase of early thymic organogenesis. The expression of p63 was detected a day later at E12.5. Also, we have results confirming the expression of pH91 antigen as early as E7.5 along with a stem cell marker Oct4.

Finally using confocal analysis and TNC specific mAb, pH91, we show that the classical complex morphology of TNCs first appears at E17.5 stage of development. However, analyses of major histocompatibility complex (MHC) class II expression on embryonic TNCs cell surfaces show its onset from E13.5. The results show a marked increase in the expression of MHC class II, from 36.2% at E13.5 to 69.1% at E18. 5 stage of development.

Taken together, these data suggest that thymic nurse cells play a significant role in the murine thymus; they create membranous spaces that facilitate MHC restriction and express markers implicating them as possessing progenitor ability.

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Last but not least the almighty who gave me this life and all the best I could have

Jai Mata Di

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restriction and express markers implicating them as possessing progenitor ability.

INTRODUCTION

Thymic Organogenesis

Studies related to the understanding of thymic organogenesis have been of great interest recently. Establishment of proper thymic microenvironment and the related molecular mechanisms that regulate them are still not fully understood. The major cells types that comprise a fully functional thymus include the keratin expressing cells, primarily medullary epithelial cells and cortical epithelial cells. The non-keratin expressing components that make up the thymus are the capsule, septae forming the outer connective tissue, endothelium contributing to the vasculature of the thymus, fibroblast and non-fibroblast mesenchyme (Gray, Tull et al. 2007; Rodewald 2008). All aforementioned thymic components are negative for cluster of differentiation marker, CD45, a marker that labels a wide range of hematopoietic cells. The $CD45^{+}$ component of the thymus includes more dynamic cell types namely thymocytes, dendritic cells, macrophages and B-cells (Raviola and Karnovsky 1972; Muller, Terszowski et al. 2005; Gray, Tull et al. 2007). All of the above mentioned components barring thymocytes and B-cells are addressed as the thymic stroma. The stroma essentially provides the basic architecture for thymocyte development. The stromal architecture of the thymus changes with age and under severe conditions, such as treatment with steroids,

or irradiation or injury (Steinmann, Klaus et al. 1985; Savino 2002; Rodewald 2008).

Development of the murine thymus can be divided into steps with different molecules involved in the process. The germ layer contribution in the origin of thymus has been a controversial. Is the thymus a derivative of ectoderm and endodermal germ layers together or endoderm only? Thymic ontogeny in mice begins with positioning, wherein by day 9.5 (of embryonic development, E9.5) cells of the third pharyngeal pouch with a double layer of cells composed of ectoderm and endodermal sheet fuse together (Cordier and Haumont 1980; Paul 1999; Rodewald 2008). Subsequent studies carried-out in chick quail chimera experiments (Le Douarin and Jotereau 1975) have demonstrated that the cells from pharyngeal pouch endoderm are sufficient to form the thymic rudiment. In another study, Gordon et al., (2005) demonstrated that isolated pharyngeal endoderm can generate a functional thymus with organized cortical and medullary regions (Manley 2000; Gordon, Wilson et al. 2005). It is believed that the fused ecto-endodermal layers would not form a compound structure; instead the cells at the point of fusion undergo apoptosis resulting in death of the ectodermal cells (Manley 2000; Gordon, Wilson et al. 2004). The budding and outgrowth of the thymus from the third pharyngeal pouch is initiated by E11, an event where cells divide to bud from the pharynx (Manley 2000). The detached

thymus moves to its final location. Detachment of thymic rudiment from the pharynx occurs by E11.5 in mice. Further development of the thymus requires interaction of thymic epithelium and hematopoietic progenitors. The migration of the thymic rudiment results in a joining at the midline above the heart by E12.5 (Manley 2000).

Lymphoid progenitor cells (LPCs) arrive at the thymic anlagen from the fetal liver around E11, well before vascularization with capillaries (Owen and Ritter 1969; Fontaine-Perus, Calman et al. 1981). These lymphoid cells have been shown to arrive and accumulate in the mesenchymal layer lining the thymic anlagen and later by E12 enter the thymic epithelium and begin to proliferate (Jotereau, Heuze et al. 1987). In *Foxn1* mutant mice accumulated lymphoid progenitor cells (LPCs) do not gain access into the thymus and are only found in the mesenchymal layer (Nehls, Kyewski et al. 1996). LPCs that enter the primordium by E11-E13 rapidly differentiate to express the Thy1 marker on their surfaces (Jotereau, Heuze et al. 1987). The interaction of thymocytes and the thymic epithelial cell (TEC) population help in differentiation of epithelial cells into cortical and medullary regions. The LPCs that enter after E13 undergo complete development 6-7 days after birth (Jotereau, Heuze et al. 1987). Chemo-attractants released by fetal thymic epithelial cells expressing MHC class II are

involved in the arrival of LPCs to the thymic rudiment (Itoi, Kawamoto et al. 2001; Tsukamoto, Itoi et al. 2005; Itoi, Tsukamoto et al. 2007).

Neural crest cells (NCCs) also play a significant role in thymic organogenesis. Some of the mesenchymal cells found in the pharyngeal pouches are derived from the neural crest expansion (Rodewald and Fehling 1998). Neural crest cells (NCCs) contribute to mesenchymal cells of the third, fourth and fifth pharyngeal pouches and give rise to different structures, including the connective tissues of the thymus and parathyroid (Rodewald and Fehling 1998). The role of these cells was again demonstrated by chick/quail chimeras wherein, ablation of NCCs resulted in defects causing the formation of a small thymus, which is barely competent to promote thymocyte development (Le Lievre and Le Douarin 1975). NCCs were suggested to form a mesenchymal capsule associated with blood vessels penetrating the thymic lobes. The functional significance of NCCs was demonstrated using Splotch (Pax3) mutant with ablation in NCC migration. These mice demonstrated moderate to severe defects in thymus formation (Anderson, Anderson et al. 1997; Conway, Henderson et al. 1997). Mesenchymal cells were also suggested to be required for epithelial cell growth and differentiation, and play a role in the induction of MHC class II expression by providing necessary secreted factors and extracellular matrix (Anderson,

Anderson et al. 1997; Shinohara and Honjo 1997; Suniara, Jenkinson et al. 2000). Studies have shown that mesenchyme can support thymic epithelial development in organ culture (Auerbach 1960). *In vitro* experiments demonstrated that when E12.5 thymi were stripped of their mesenchyme, the thymus failed to develop (Shinohara and Honjo 1996; Shinohara and Honjo 1997; Suniara, Jenkinson et al. 2000). In mice, NCC derived mesenchyme seems to contribute to development as early as E11.5, primarily in the formation of outer the lining of the organ capsule, the interlobular septae and the stromal network (Le Lievre and Le Douarin 1975; Jiang, Rowitch et al. 2000; Yamazaki, Sakata et al. 2005).

Studies suggest that almost a third of the thymic epithelial cells at E13.5 are derived from NCC. Most of the NCCs are not inter-epithelial but just surrounding the thymic primordium (Jiang, Rowitch et al. 2000). By E17.5 a substantial decrease in number of NCC derived mesenchyme is observed. The number of these cells that end up in the thymus is still unknown (Jiang, Rowitch et al. 2000). These studies have suggested that mesenchymal cells receive signals from endoderm, which then translate the signals for further development of thymic epithelial rudiment (Manley 2000). The reciprocal epithelial-mesenchymal interaction in thymic organogenesis is probably mediated through

factors such as epidermal growth factor (Egf), insulin-like factors I and II, retinoic acid and fibroblast growth factors (Fgf) (Shinohara and Honjo 1996; Rodewald and Fehling 1998). The role of NCC derived mesenchyme is not required for the initial endodermal specification, but needed for subsequent developmental steps.

Molecular Significance in Thymic Organogenesis

Tbox1 transcription factor (Tbx1) plays a role in the development of pharyngeal pouches and its derivatives (Lindsay, Botta et al. 1999; Jerome and Papaioannou 2001; Lindsay, Vitelli et al. 2001; Merscher, Funke et al. 2001). Tbx1 role in thymic development has been implicated by its expression in the 3rd pharyngeal pouch and the underlying mesenchyme. However, Tbx1 expression is absent from NCCs (Yamagishi, Maeda et al. 2003). Mutants of *Tbx1* exhibit thymic hypoplasia (Liao, Kochilas et al. 2004). Tbx1 deficiency by E10.5 results in formation of the third pharyngeal pouch but still ends-up being hypoplastic. The loss of Tbx1 by E11.5 or later has minimal effects on the morphogenesis of the thymus suggesting that Tbx1 function is spatial and temporal in terms of pharyngeal pouch formation (Xu, Cerrato et al. 2005). Tbx1 is regulated by forkhead/winged helix transcription factors Foxa2, Foxc1 and Foxc2. These transcription factors are expressed in developing endodermal and mesodermal tissue (Yamagishi, Maeda et al. 2003). Fox and Shh regulate Tbx1 expression, this

regulation is independent of Fox and Shh pathways when Tbx1 is regulated by retinoic acid. Retinoic acid acts via retinoic acid response elements lying within target gene promoters. Retinoic acid is also known to modulate the expression of Pax1, Pax9, Hoxa3, and Fgf8, molecules that have also been known to play a prime role in thymic organogenesis (Manley and Capecchi 1995; Wallin, Eibel et al. 1996; Mulder, Manley et al. 1998; Su, Ellis et al. 2001; Abu-Issa, Smyth et al. 2002).

Bone morphogenic proteins (BMPs), constituting a large sub-group of transforming growth factor-β, have also been suggested in regulation of Tbx1 expression. Bone morphogenetic proteins are also known to play a significant role in the developmental processes (Yamada, Revelli et al. 2000; Yamamoto and Oelgeschlager 2004). BMPs are regulated by their antagonist chordin. Chordin is expressed in the pharyngeal endoderm (Bachiller, Klingensmith et al. 2003). Tbx1 expression in chordin mutants is reduced by E7.5 (Bachiller, Klingensmith et al. 2003). Chordin mutants have abnormal NCCs affecting the patterning of pharyngeal pouches 3, 4 and 6 (Bachiller, Klingensmith et al. 2003). Targets of Tbx1 include Fgf8 and Fgf10. Fgf10 is implicated to be cell autonomously regulated by Tbx1. Consequently mice deficient of Fgf10 or its receptor FgfRIIIb develop a hypoplastic thymus (Revest, Suniara et al. 2001), suggesting the

importance of Fgf10 in thymic epithelial proliferation. Fgf10 has also been implicated in differentiation of immature precursor cytokeratin 5^- cells into mature cytokeratin 5^- cortical epithelial cells (Revest, Suniara et al. 2001). Targets of Tbx1 include paired box transcription factors member *Pax9*. Mutants of *Pax9* have been shown to develop ectopic thymus but are still able to develop T-cell that express αβTCR but not γδ TCR (Peters, Neubuser et al. 1998; Hetzer-Egger, Schorpp et al. 2002). Pax9 is suggested to be downstream of Tbx1 (Hollander, Gill et al. 2006) and has been known to play a role in epithelial and mesenchymal interaction. Tbx1 expression in pharyngeal mesoderm and endoderm is a prerequisite for thymus development.

Homeobox (Hox) genes are involved in regulating morphogenesis and tissue modeling during development. Hoxa3 is the key member of the Hox family, known to play a key role in patterning of the third pharyngeal pouch and its derivatives. Hypoplastic and defective pharyngeal derivatives are seen in *Hoxa3* mutants, including athymia (Manley and Capecchi 1995; Ivins, Lammerts van Beuren et al. 2005). Hoxa3 is expressed in third pharyngeal pouch endoderm and neural crest mesenchyme (Manley and Capecchi 1995). Developmental events prior to gestational stage E11.5 are not affected in *Hoxa3* mutants. Fusion of the second, third and fourth arch is known to occur in wild type mice after

E11.5, during thymi development. This event fails to occur in *Hoxa3* mutants, in addition the third endodermal pouch fails to form the thymic anlagen (Manley and Capecchi 1995). By E11.5 *Hoxa3* mutant epithelial cells of the third pharyngeal pouch show increased apoptosis and constrained proliferation of the mesenchyme (Hollander, Gill et al. 2006) .

Another important observation is that *Pbx1* mutants show abnormal and delayed patterning of the third and fourth pharyngeal pouches, resulting in ectopic or hypoplastic or even a complete loss of thymus formation. Pbx1 is understood to act in tandem with Hox proteins (Su and Manley 2000; Su, Ellis et al. 2001). *Hoxa3* and *Pax1* double mutants are shown to have reduced MHC class II expressing thymic epithelial cells and reduced expression I-A^b in epithelial cells (Su and Manley 2000). Hoxa3 and Pax1 have been shown to regulate the ability of fetal thymic epithelial cells to influence thymocyte development. Double mutants of *Hoxa3* and *Pax1* show increased apoptosis of $CD4^+$ $CD8^+$ thymocytes and $CD44^-25^-$ cells, suggesting theirs role in transition of $CD4^-8^-$ thymocytes in to $CD4^+8^+$ thymocytes (Su and Manley 2000). Pax1 and Pax9 are expressed in the epithelium of the third pharyngeal pouch by E9.5 and are required for the proper development of thymus (Wallin, Eibel et al. 1996; Peters, Neubuser et al. 1998; Wilm, Dahl et al. 1998). During development, epithelial cells express Pax1 at

higher levels, this decreases with development of the thymus. Only a few cortical cells express Pax1 thereafter (Wallin, Eibel et al. 1996). *Pax1* mutants exhibit hypoplastic thymus with development of cysts composed of separate cortical and medullary regions (Dietrich and Gruss 1995). Along with the affects of Pax9 on thymic development explained above, null mutants of *Pax9* show a failure in proper folding of the thymus away from the foregut, instead evaginating into the laryngeal cavity exhibiting a polyp-like morphology at this ectopic location (Peters, Neubuser et al. 1998; Hetzer-Egger, Schorpp et al. 2002).

Eye absent (Eya), has been known to play a role in embryonic organ development. Eya proteins act as phosphatases and are required in regulating genes related to growth (Schedl and Hastie 2000). Eya modulates Six1 (sine oculis 1), an activator of transcription, from being a repressor (Li, Oghi et al. 2003). *Eya* is expressed in NCCs as well as pharyngeal endoderm and co-localizing with Pax and Six (Xu, Woo et al. 1997). Eya deletion mutants do not show the separation of third pharyngeal pouch from the endoderm and the thymus primordium is never formed. Pax1 and Pax9 expression is affected in these mutants but not before the budding of the 3rd pharyngeal pouch by E10.5. This suggests that Hoxa3, Pax1 and Pax9 are located upstream of Eya, and are

regulated independently (Xu, Zheng et al. 2002). All these molecular expressions have a developmental role in thymus specification, localization and formation.

Role of Fork head transcription factor 1 (Foxn1) in thymic organogenisis:

Fork head transcription factor 1 (Foxn1) is an extensively studied transcription factor known to play a major role in initial stages of thymic organogenesis. In humans the gene is located on the chromosome 17 and the protein product is about 648 amino acids in size (Janes, Ofstad et al. 2004). In mice *Foxn1* gene is located on chromosome 11 and the protein product is also 648 amino acids with almost 85% identity to its human homolog (Schorpp, Hofmann et al. 1997). The *Foxn1* gene is expressed in a range of epithelial cells such as skin and thymus (Nehls, Pfeifer et al. 1994). Foxn1 is indispensable for normal differentiation of hair follicles and thymic epithelia (Flanagan 1966; Kopf-Maier, Mboneko et al. 1990). The Foxn1 protein has both an N-terminal DNA binding domain and a C-terminal transcriptional activation domain. Phosphorylation of Foxn1 at multiple regions results in its nuclear localization, thus promoting transcription (Biggs, Meisenhelder et al. 1999; Kops, de Ruiter et al. 1999). Mice recessive for *Foxn1* show a nude phenotype characterized by hairlessness and athymia (Nehls, Pfeifer et al. 1994). Neither the formation of third pharyngeal pouch nor the initial patterning is affected by Foxn1 deficiency (Nehls, Kyewski et al. 1996).

However, lymphoid progenitor entrance into the thymic anlagen is influenced greatly. *Foxn1* mutants do not show the normal mesh-work like architecture of thymic epithelia, instead they show epithelial morphology similar to the one seen in the respiratory tract (Dooley, Erickson et al. 2005). These studies support Foxn1's role in thymic lineage of epithelial cells, suppressing Foxn1 related signals lead to a default respiratory epithelial fate of pharyngeal pouch (Dooley, Erickson et al. 2005; Dooley, Erickson et al. 2005). Functional analysis of Foxn1 demonstrated that thymocyte independent development of thymic epithelial cells initially is not influenced by Foxn1, but is required during thymocyte cross-talk related thymic epithelial differentiation (Nehls, Kyewski et al. 1996). Foxn1 expression is known to be regulated by Bone Morphogenic Protein (BMP4) and Wnt proteins, (Balciunaite, Keller et al. 2002; Tsai, Lee et al. 2003) with BMP directly up regulating Foxn1 expression in thymic stroma (Nehls, Kyewski et al. 1996; Tsai, Lee et al. 2003). Wnts are expressed in third pharyngeal pouch by E10.5 and have been shown to induce Foxn1 expression. As stated above, *Foxn1* mutants show failure of hematopoietic cell entry into thymic anlagen. This is due to lack of chemokines CCL25 (TECK) and SDF-1 expression in the thymic epithelia (Bleul and Boehm 2000). Although not shown directly, TECK, SDF-1, Dll-1 and Dll-4 have been suggested to be downstream targets of Foxn1 (Itoi, Tsukamoto et al. 2007; Rodewald 2008) along with their role in keratin expression

of course (Schorpp, Hofmann et al. 1997). By embryonic day E11.5 expression of *Foxn1* has been observed in the caudal region of the thymic primordium of the third pharyngeal pouch (Gordon, Bennett et al. 2001; Itoi, Tsukamoto et al. 2007). Glial cell missing (*Gcm*) another transcription factor is expressed in the cranial and dorsal portion of the primordium, *Gcm* expression was observed by E9.5 (Gordon, Bennett et al. 2001). Studies showed that *Gcm* and *Foxn1* expression is required for the development of parathyroid and thymus respectively (Gordon, Bennett et al. 2001). *Foxn1* expression is crucial at a stage when pharyngeal pouches are still the common primordium for both parathyroid and thymus. This is because the region of the pouch that express Foxn1 specifically is destined to be the thymus (Gordon, Bennett et al. 2001). *Foxn1* mutant mice show aberrant accumulation of *Gcm* regulated precursors into the third pharyngeal pouch that develops into parathyroid (Gordon, Bennett et al. 2001; Itoi, Tsukamoto et al. 2007). Studies have also shown that Foxn1 expression varies in thymic epithelial cells (TECs). By embryonic age E13, cells that are $Foxn1^{+}Keratin^{+}$ and $Foxn1^{-}$ and $Keratin^{+}$ are already observed, suggesting that Foxn1 probably is expressed in TEC progenitors (Itoi, Tsukamoto et al. 2007) or in a subset that transiently expresses the transcription factor. Expression of Foxn1 by genetic activation in a single TEC has led to formation of productive thymi in nude mice (Bleul, Corbeaux et al. 2006). Recent studies have shown that thymopoiesis is dependent

on the TECs expressing Foxn1 (Corbeaux, Hess et al. 2010). These studies have reported that bi-potent epithelial progenitors express Foxn1. Studies carried out by Corbeaux et al., have shown that Foxn1 negative TECs in the thymus are actually derived from previously Foxn1 expressing cells, also their studies have demonstrated that TECs that were Foxn1 negative to begin with were not capable of promoting thymopoiesis. Foxn1 is one transcription factor that plays a significant role in thymic development. All the above-discussed molecules are involved in thymic organogenesis, directly or indirectly. However, many of these molecules are required developmentally in different organ systems as well. None of them is a molecule that identifies a progenitor that is directly involved in thymic organogenesis. An exception is Foxn1. However, again Foxn1 is not considered to be stem cell marker yet.

Proliferative Markers Expressed by Thymic Epithelial Cells:

Not many studies have been carried out to show the expression of stem cell markers by the thymic epithelial cells. Although a list of stem cell markers were shown to be expressed developmentally by the embryonic thymic primordium, none of them were very specific to the thymus. However their expression in the development of thymus is indispensable. TECs were shown to express markers relating to stemness such as oct4, Sox2, and Nanog (Gillard and Farr 2006). These markers were shown to be expressed in medullary thymic epithelial cells along with other markers known to be related in

maintaining pluripotentiality in cells, namely Stella , Dppa3, Ehox, FoxD3,(Hanna, Foreman et al. 2002; Gillard and Farr 2006). However these markers were shown to be expressed in medullary epithelial cells in *Aire*- deficient mice only and the cortical epithelial cells were not addressed. Since both medullary and cortical epithelial cells are believed to be derived from a common pool of TEC progenitors, expression of markers that maintain stemness is understandably required for formation of differentiated TECs.

Relevance of the p63 Transcription Factor in Thymic Maintenance

P63 is a transcription factor and a homolog of the tumor suppressor, p53 and p73 (Brinck, Ruschenburg et al. 2002; Flores, Tsai et al. 2002; McKeon 2004). Structurally, p63 is comprised of a central DNA binding domain, a tetramerization domain and an N-terminal activation domain. The p63 gene uses two different transcription initiation sites and alternative splicing at the 3' end to generate two distinct proteins: a transactivating (TA)- TAp63 isoform and a non-transactivating ΔNp63 isoform. The ΔN isoform lacks the N terminal transactivating domain (Fig 1) (McKeon 2004). These isoforms are understood to have separate functions in the cells that express them. Studies have shown that the non-transactivating ΔNp63 isoform is expressed in the progenitor cell layers of skin, breast, and prostate with almost no expression of the TAp63 isoform (Signoretti, Waltregny et al. 2000; Ince, Cviko et al. 2002; Yang, Kaghad et al.

2002). ΔNp63 has been suggested to act as a functional repressor of TAp63 and p53 and to also inactivate genes in order to establish the correct genetic environment in epithelial progenitor cells (Yang, Schweitzer et al. 1999). P63 deficient mice die perinatally due to incomplete development of the skin epithelium and of structures including breast, prostate, and urothelia (Mills, Zheng et al. 1999; Yang, Schweitzer et al. 1999; Finlan and Hupp 2007). These mice have been also shown to have small thymi (Senoo, Pinto et al. 2007). ΔNp63*α* is highly expressed in the nuclei of basal regenerative cells of many epithelial cells like skin, breast epithelium, oral epithelium, prostrate and urothelia (Finlan and Hupp 2007). In mouse, the ΔNp63*α* isoform is expressed in the basal layer of the stratified epithelium (Pellegrini, Dellambra et al. 2001; McKeon 2004; Blanpain and Fuchs 2007). Other reports suggest that ΔNp63 governs basal-epidermal gene expression, whereas ΔNp63, possibly together with TAp63, functions in an additional step to promote terminal differentiation (Candi, Rufini et al. 2006; Blanpain and Fuchs 2007). Experiments performed with continuous culture of thymic epithelium demonstrated that cells grown in *in vitro* expanded and expressed p63 (Senoo, Pinto et al. 2007). Knock-down of p63 in these cells reduced the colony size, supporting the argument that p63 is important in TEC or TEC stem cell maintenance (Senoo, Pinto et al. 2007). Reports emphasizing the importance of p63 demonstrated that the expansion of

$K5^+$ $K8^+$ TECs result in recovery from injury and drug treatment, this was proceeded by up regulated expression of p63 (Popa, Zubkova et al. 2007). These findings regarding the role of p63 highlight its significance in maintaining proliferative potential of TECs in the thymus. Expression of p63 is indispensable for thymic establishment (Senoo, Pinto et al. 2007). Senoo et al., have suggested that the expression of p63 would help regulate the expression of other stem cell markers that maintain epithelial cells in a given organ. Studies have shown that p63 is also expressed in different tumors, suggesting its antagonistic role to p53, and also as a marker that maintains proliferative potential of cells. Studies have shown that the ΔNp63 isoform regulates the expression of FgfR2 and Jag2 in the thymus thus playing a role in regulation the thymic development (Candi, Rufini et al. 2007). Expression of the Aire transcription factor has been implicated in mTEC differentiation and has been suggested to regulate p63 expression. Direct evidence to this is still unclear. However, the isoform of p63 that is understood to be down regulated in $Aire^{-/-}$ mice is the ΔNp63 isoform and not the TAp63 isoform (Dooley, Erickson et al. 2008). Together, these data suggest that p63 plays a critical role in regulating TEC differentiation and in maintaining the proliferative potential of TECs from very early stage of development to the later stages of organ maintenance.

Expression Patterns of Different Thymic Epithelial Markers During Ontogeny, and the Significance of Cytokeratin Expression

Cytokeratins are a family of intermediate filament proteins, found within the intra-cytoplasmic cytoskeleton of epithelial tissue. Cytokeratin heterodimers are classified into acidic type I cytokeratins and basic or neutral type II cytokeratins. There are about 20 identifiable cytokeratins known to date. Tissues express different cytokeratins, and their expression pattern are interpreted as biochemical markers that help differentiate various types of epithelial cells (Franke, Schiller et al. 1981). Thymic epithelial cell subsets can be identified by their keratin expression, enabling researchers to study the role of a specific subset in thymic organogenesis. TECs express cytokeratin 8 (K8) and cytokeratin 5 (K5). A majority of cortical thymic epithelial cells are $K8^+$ with a minor subset being $K5^+$. Medullary epithelial cells express cytokeratin 5 (K5) with a minor subset being $K8^+$ (Klug, Carter et al. 1998). The onset of keratin expression by the thymic anlage was initially reported to occur by E11.5 with K8 being expressed first, followed by $K5^+$ a day later (Klug, Carter et al. 1998; Klug, Carter et al. 2002). However, other findings have suggested the presence of $K5^+$ cells by E11.5 (Gill, Malin et al. 2002).

Keratins have played a major role in studies relating to thymic ontogeny. The $K5^+ K8^+$ double positive epithelial cells have been speculated to be markers of the epithelial progenitor cell population (Klug, Carter et al. 1998; Klug, Carter et al. 2002). This was shown by studies performed using MTS24, a controversial TEC progenitor marker (Gill, Malin et al. 2002) discussed later in this proposal. Cortical and medullary TECs are known to exhibit differential expression of keratins namely K8 and K18 and K5 and K14, respectively. K8 forms a heterodimer with K18, and are the first intermediate filament proteins expressed during mouse embryogenesis (Baribault, Price et al. 1993; Boyd, Tucek et al. 1993).

Thymic cortical epithelial cells express K8 and K18, TECs in the cortex and subcapsular region also express K5, demonstrating that the cortex does not contain a homogenous population of TECs (Nabarra and Andrianarison 1987; Klug, Carter et al. 1998). K5 heterodimerizes with K14 and is expressed in the proliferating compartment of stratified squamous epithelia. These two keratin types are reported to be down regulated during epithelial differentiation, as other varieties of cytokeratins are up regulated (Baribault, Price et al. 1993). K5 is expressed by the medullary TEC subsets that are randomly distributed in the thymus and addressed as medullary islets. The K5 and K14 expression pattern is

not identical. Although K5 and K14 are both expressed by medullary TECs, K14 is not found to co-localize with $K5^+$ TECs in the cortex or at the cortico-medullary junction (Klug, Carter et al. 1998). Immunostaining for both K5 and K8 has shown that $K5^+$ TECs in the cortex and cortico-medullary junction(CMJ) also express K8. The $K8^+K5^+$ (double positive) TECs found in the cortex are very few in numbers, but are seen in larger numbers at the cortico-medullary junction (Fig 2). In contrast, the medullary subset expresses a $K5^+K8^-$ phenotype (Klug, Carter et al. 1998). These double positive TECs are suggested to be progenitor of thymic epithelial cells (Bennett, Farley et al. 2002; Gill, Malin et al. 2002) (Klug, Carter et al. 1998).

Ulex Europaeus Agglutinin (UEA-1) is a lectin commonly used to lable medullary epithelial cells (Godfrey, Izon et al. 1990; Penit, Lucas et al. 1996). Epithelial cell subsets stained with antibodies against K5, K8 and UEA-1 have shown a varied pattern of expression. TECs in the medulla co-express K5 and UEA-1. Interestingly, UEA-1 also identifies a distinct $K5^-$ medullary TEC subset that has a globular morphology suggestive of the $K8^+$ medullary subset. UEA-1 and K8 double-staining confirms this phenotype (Klug, Carter et al. 1998). A majority of sub-capsular and cortical TECs express $K8^+K18^+K5^-UEA\text{-}1^-$, a smaller subset express $K8^+K18^+K5^+UEA\text{-}1^-$ and is heavily distributed at the cortico-medullary

junction. Medullary TECs expressing $K8^{-}K18^{-}K5^{+}$ $UEA\text{-}1^{-}$ phenotype are localized to the inner medullary region, and a globular $K8^{+}K18^{+}K5^{-}UEA\text{-}1^{+}$ subset is known to be localizing towards the outer region of the medullary compartment (Klug, Carter et al. 1998). The later is believed to be the mature population of mTECs.

The $K8^{+}K5^{+}$ TEC subset can generate a major cortical TEC $K8^{+}K5^{-}$ subset (Klug, Carter et al. 1998). Lineage relationship experiments between the $K8^{+}$ $K5^{+}$ and $K8^{+}K5^{-}$ cortical TEC subsets were performed using hCD3ε and *RAG*$^{-/-}$ mice. Transgenic hCD3ε mice have human CD3ε gene (*hCD3ε*) and the thymocytes in these mice show a developmental block at $CD4^{-}CD8^{-}CD44^{+}CD25^{-}$ stage of development (Hollander, Wang et al. 1995). Consequently, these mice develop hypoplastic thymi with a derangement of the cTECs, and lack a proper medulla (Hollander, Wang et al. 1995). It was shown that the thymi in these mice predominantly have TECs expressing the $K8^{+}K5^{+}$ phenotype (Klug, Carter et al. 1998). Mice having a mutation in recombination activating gene (RAG) *RAG*$^{-/-}$ mice show a properly organized cortex but, exhibit a block in T-cell development at $CD44^{-}CD25^{-}$ differentiation stage (Shores, Van Ewijk et al. 1994; Hollander, Wang et al. 1995; Penit, Lucas et al. 1996). These animals show a TEC phenotype that is mainly comprised of $K8^{+}K5^{+}$ subset compared to a C57BL/6 mice, and a

smaller subset of $K8^{+}K5^{-}$ that is not seen in hCD3ε mice. When thymi obtained from newborn hCD3ε transgenic mice were grafted under the kidney capsule of either adult hCD3ε transgenic or RAG-$1^{-/-}$ mice, hCD3ε transgenic thymi recovered from RAG-$1^{-/-}$ recipients not only were reconstituted with $CD25^{+}$ thymocytes but also show both $K8^{+}K5^{+}$ and $K8^{+}K5^{-}$ cortical TEC subsets. This finding demonstrates not only a direct lineage relationship between $K8^{+}K5^{+}$ TEC precursors and $K8^{+}K5^{-}$ TEC progeny, but also their dependence on interaction with early thymocyte that are in early developmental stages (Klug, Carter et al. 1998). Findings from our laboratory have shown a subset of Thymic Nurse cells TNCs to be double positive for keratin expression ($K8^{+}$ $K5^{+}$). Like the expression of p63 as discussed above the expression of both K5 and K8 cytokeratins add strength to our proposal that a subset of TNCs is thymic epithelial progenitor cells.

Thymocyte-stromal cell cross-talk as a prerequisite for TEC development has also been in question. Experiments performed using CD3ε26tg transgenic animals have shown that an aberrant population of B-cells influences improper TEC differentiation, in addition to thymocyte and stromal interaction (Tokoro, Sugawara et al. 1998). Studies show that TEC differentiation is independent of thymocyte presence in and around thymic anlage until E11.5. With the arrival of

the lymphoid progenitors, TECs differentiate further and assume a three-dimensional architecture (van Ewijk, Hollander et al. 2000; Itoi, Kawamoto et al. 2001).

Thymocyte interaction with thymic epithelial cells has been shown by many studies to be essential for proper maturation of thymocytes (van Ewijk, Hollander et al. 2000). Studies demonstrating cortical epithelial cell development as a means of cross-talk have been very limited. The role of pro-T cells in development of cortical epithelial cells was demonstrated in ε26tg mice. As explained above, a block in cortical epithelial development can be reverted by wild-type thymocytes in the fetal or early postnatal stages of development, but not in the adult thymus. (Hollander, Wang et al. 1995; van Ewijk, Hollander et al. 2000), suggesting that a critical window of time exists for cortical epithelial development.

Many studies have focused on thymocyte interaction with mTECs. Interaction of lymphotoxin-β (LTβ) expressed on thymocytes and its receptor LTβR expressed on mTEC is required for proper development of mTECs (Boehm, Scheu et al. 2003). This interaction is also mediated by nuclear factor–B-inducing kinase (Nik) on thymic epithelial cells. Mice mutant for LTβR ligand, LTβR receptor,

and mutations in *Nik* exhibit aberrant mTECs. Deficient LTβR signaling results in retention of mature T cells in the thymic medulla, resulting in possibly an incomplete induction of central tolerance by TECs (Boehm, Scheu et al. 2003). Medullary TEC maturation in the fetal thymus is linked with induction of RANKL signals by Lyphotoxin Inducer (LTi) cells that promote the maturation of RANK-expressing $CD80^{-}Aire^{-}$ mTEC progenitors into $CD80^{+}Aire^{+}$ mTECs, (Rossi, Kim et al. 2007). However this regulation of mTECs requires prior proper lymphoid cell development induced by cortical epithelial cells. Some studies have shown that Aire (Auto immune regulator) expression is essential for the differentiation of mTECs, these studies have shown that expression of certain transcription factors required to maintain stemness i.e Nanog, Oct4 and Sox2 in mTECs are regulated by Aire (Gillard, Dooley et al. 2007). These studies have demonstrated that the organization of the medullary thymic epithelial component is severely affected by knocking out *Aire*. These studies might be limited to mTECs and does not necessarily address all the epithelial component of the thymus.

Interestingly, studies using thymic cell aggregate cultures demonstrated that lympho-stromal interaction is not required for TEC differentiation, at least initially. Jenkinson et al.2005, have shown that thymic epithelial aggregates

alone, whether from wild type or from CDεtg26 (earlier described to have a block in TEC development at $K5^+K8^+$ stage) mice are able to both differentiate into $K5^-K8^+$ or $K5^+K8^-$ subsets. These studies showed proper T-cell development from the double negative stage to the double positive stage (Jenkinson, Rossi et al. 2005). Studies have tried to define different pathways and molecules involved in TEC development and differentiation, however there is no specific evidence of signals induced in TECs resulting in TECs exhibiting a specific phenotype. As we analyze these studies we begin to ask, is there a marker that could follow the development of these epithelial cells from beginning to end? Identifying thymic epithelial cells has not been a stringent process. Markers are used to identify the same TEC subsets vary from one research group to the other. Segregating TEC phenotypes to explain an event has always been a secondary issue. For example, medullary epithelial cells are identified using K5 or by using UEA-1, as explained above K5 identifies a subset that is not $UEA\text{-}1^+$, and the vice versa. UEA-1 is believed to identify a more mature population of mTECs, interestingly both K5 and UEA-1 are co expressed with K8 mutually exclusive of each other. As these discrepancies are still to be resolved one factor remains intact that is, $K5^+K8^+$ TECs are immature cells. Are these the epithelial progenitor cell subset? Is there a marker that identifies these cells?

Thymic Epithelial Progenitors

Studies in understanding thymic epithelial progenitors, their frequencies and identifying phenotypic markers that label them have been of interest to researchers in the recent past. Identifying the TEC progenitors is essential for a number of reasons primarily: 1) If discovered they could allow the replacement of stromal cells that are affected under stress or malnutrition resulting in thymic atrophy (Maamouri, Khadraoui et al. 1976; Tarcic, Ovadia et al. 1998; Savino, Dardenne et al. 2007), 2) the thymus is known to be affected upon irradiation or due to various drug treatments (chemotherapy) (Yamada, Muramatsu et al. 1990). It is understood that the thymus shrinks in size and also in its efficiency in T-cell development with age (Chidgey and Boyd 2006) and also, it is improper TEC architecture and functional failure of TECs that result in almost all autoimmune phenotypes (Huston, Smathers et al. 1983; Takeoka, Taguchi et al. 1999). The one possible answer that could help overcome all of the above mentioned incongruities is identifying a bona-fide TEC progenitor, and developing therapeutic applications for this population of cells. The thymus, as explained above has been suggested to develop from a common bi-potent progenitor for cortical and medullary epithelium (Rossi, Jenkinson et al. 2006). Epithelial cells isolated from yellow fluorescent protein (YFP) expressing embryonic E12.5 thymus were injected into a non-fluorescent host thymus, these

thymi were transplanted into recipient mice and allowed to develop into a functional thymus. Analysis showed that the single embryonic epithelial cell produced both mTEC and cTEC. The drawback of this study was that cells used for transplantation were sorted from the E12 embryo using EpCAM1, a pan-epithelial marker, and the sorting was not narrowed down to any particular keratin profile. This resulted in sorting a large pool of epithelial cells with undefined progenitor identifier (Rossi, Jenkinson et al. 2006). Thus the question of a single progenitor cells is still elusive.

Subsequent studies perform by Bluel et al., (2006) used a recombinant technique, the Cre-recombinase system under the influence of K14 cre promoter that switches the expression of YFP in a single cell. Further analysis of TECs generated in these animals showed the expression of YFP in cTECs and mTECs, and also persisted in the subsequent mTEC and cTEC progenies. These results not only suggest the origin of TECs from a common progenitor but also suggest the existence of progenitors that could persist in mice (Bleul, Corbeaux et al. 2006). A study proposed by Rossi et al.(2006), showed that a single cell is sufficient to generate an entire thymus, their purification technique were questioned. However, studies relating to the expression of Foxn1, in nude mice using the Cre-recombinase method, demonstrated that expression of Foxn1 in a

single cell postnatally was sufficient to revert the nude phenotype and generate a functional thymus with a normal thymic TEC architecture (Bleul, Corbeaux et al. 2006). This particular result suggested the possibility of two scenarios; 1) a single progenitor cell is sufficient to generate an entire thymus or, 2) the existence of progenitor cells in the nude mice that are switched on to overcome developmental blocks and, further activate cells to differentiate and develop into a functional thymus.

Generation of mTECs from a population of TECs was feasible with more than one method of purification. Studies performed using reaggregate fetal thymic organ culture (RFTOC) with cells isolated from embryonic thymic showed development into diverse TECs but, could not form the defined architecture that exists in the thymus (Anderson, Jenkinson et al. 1993). However when these RFTOC cultured cells were grafted under the kidney capsule, they were able to generate a complete thymus with defined cortical and medullary regions, these grafts could also promote thymocyte development (Rodewald 2000; Rodewald 2008). Studies performed by purifying cells, based on expression of UEA-1 and claudins (Cld) have also demonstrated sorting methods capable of generating mTECs exclusively. Some immature epithelial cells that are UEA-1$^-$ and Aire$^-$ were also shown to be cld3,4$^+$(Guo, Rahman et al 2011.).These cells have been

shown to give rise to only mTECs but not cTECs in RFTOC or in grafts performed in nude mice. However when grafts carried out in nude mice with cld3,4^{low}Aire^{-}UEA-1^{-} TECs resulted in production of both cTECs and mTECs^{+} (Guo, Rahman et al.). These studies suggest that lineage commitment occurs in the thymic anlage as early as E13.3, with some cells retaining the ability to differentiate further based on the signals received by the cells. The above studies provoke questions in understanding the development of mTECs, and the signals required by mTEC progenitors to differentiate into mature mTECs expressing Aire. As explained above probable signal induce by RANK ligand expressing lymphoid cells are required for maturation of mTECs although they express claudins (mature tight junction markers).

Identifying markers that could label TEC progenitors, and help in sorting progenitors exclusively has been of major focus of some laboratories. Markers were suggested to identify cells that have the ability to generate both cortical and medullary cells. A monoclonal antibody 4F1 (mAb 4F1) which marks mature murine cortical epithelium was suggested to stain all epithelial cells at E14 (Bennett, Farley et al. 2002). Monoclonal antibody A2B5, a marker for human thymic medulla, stained 7 week old human fetal thymi (Bennett, Farley et al. 2002), suggesting the existence of markers that could recognize later developed

TECs. One controversial marker that took center stage is MTS24 a monoclonal antibody that binds to surface antigens expressed on TECs. Biochemical analysis showed that MTS24 is a glycoprotein with a MW of 80-90 kDa and was found to co-express on $K8^{+}K5^{+}$ TECs (Gill, Malin et al. 2002). Initial results showed only $MTS24^{+}$ cells to have the potential to generate both thymic cortex and medulla. Similar results were observed when a marker similar to MTS24 described as MTS20 was used. MTS24 and MTS20 (which actually were understood to bind to the extracellular determinants on the same epithelial cells) used together were shown to mark $K5^{+}$ $K8^{+}$ double positive TECs, and exhibited similar pattern of distribution in the thymic epithelium from embryonic to adult stages (Bennett, Farley et al. 2002; Gill, Malin et al. 2002). However, subsequent studies revealed that $MTS24^{+}$ (along with MTS20) as well as $MTS24^{-}$ cells when grafted ectopically under the kidney capsule were able to generate a functional thymus (Rossi, Chidgey et al. 2007). Rossi et al 2007, confirmed that 95% of the cells used in these experiments were keratin positive. The donor cells were characterized using the pan-TEC marker EpCAM1, which does not distinguish a specific epithelial subset or track defined keratin subsets. Moreover, $MTS24^{+}$ cells retained the ability to generate a viable thymus only when isolated by E12, they lose their ability to do so by E15 (Rossi, Chidgey et al. 2007). On the contrary, MTS^{-} cells isolated even at a stage later to E15 still retain the ability to

reaggregate, and were able to generate a thymus upon grafting under the kidney capsule (Rossi, Chidgey et al. 2007). In summary, either MTS24^{+} or MTS24^{-} cells give rise to the entire thymus when derived form E12- E14 mice thymi, and the keratin profiles of the donor cells used in these experiments were not determined. These analyses suggest that MTS24 may not be a candidate marker to distinguish the epithelial progenitor subset.

To date, the best data in terms of defining TEC progenitors comes from indirect evidence obtained through the use of transgenic animals expressing the immature thymus phenotype. Placenta expressed transcript-1 (Plet-1) has been described as a specific marker for early thymic epithelial progenitor cells (Depreter, Blair et al. 2008). Plet-1 was shown to be expressed in various tissues including prostrate and mammary epithelium. Both MTS20 and MTS24 have been shown to recapitulate the Plet-1 staining pattern. Further analysis showed that Plet-1 is the antigen to which markers- MTS20 and MTS24 bind (Depreter, Blair et al. 2008). The identification of epithelial progenitor cells thus far has been inconclusive. An alternative to the current approaches may involve clonal analysis. That is, identifying a unique cell type that expresses a discrete marker for analysis of their progenitor potential. The best way to understand the progenitor potential of an epithelial cell is to carry out clonal analysis that

defines the bi-potent potential of a single cell. Bi-potent nature of TECs with ability to give raise to cTECs and mTECs has been speculated. The question remains, are these bi-potent TECs identifiable? Is there a marker that would exclusively identify progenitors along with the keratin expression? Some studies have suggested that the EpCAM1^{+} CD205^{+} CD40^{-} MHC class II^{low} cTECs my represent cTEC progenitors (Shakib, Desanti et al. 2009). However, the possibility of mixing up population seems inevitable in this case. If TEC cytokeratin expression pattern varies between mature and immature, and if regeneration can occur at any age, there must be a persistent population of progenitors that exist in the thymus. If this is the case we should be able to identify this particular subset within the thymic tissue.

Thymic Nurse Cells (TNCs)

Thymic nurse cells are a subset of epithelial cells distributed in the cortex of the thymus (Fig 3). TNCs exhibit a unique multi-cellular structure with thymocytes internalized into their cytoplasmic vacuoles (Wekerle, Ketelsen et al. 1980). Internalized thymocytes are known to either proliferate or undergo apoptosis within these complexes. Since their discovery by Wekerle and Ketelson in 1980 TNCs have been an enigma. TNCs have been isolated from humans (Ritter, Sauvage et al. 1981), mice (Wekerle, Ketelsen et al. 1980), chickens (Boyd,

Oberhuber et al. 1984), and teleost, yet their function is not fully defined. TNCs are keratin expressing cells and are understood to express MHC class I and class II on their cell surfaces (de Waal Malefijt, Leene et al. 1986; Cordes, Pedersen et al. 1997). The class II MHC antigen expression is uncharacteristic of cortical thymic epithelial cells (cTECs) and is generally restricted to medullary epithelial cells (mTECs), and to cells of the immune system namely dendritic cells or macrophages. *In vitro* studies performed extensively on TNCs were focused on understanding the interaction of TNCs with the thymocytes. *In vivo* studies on TNCs have been limited because, after their isolation from the thymus, TNCs release all internalized thymocytes and lose the ability to uptake any thymocyte subsequently. Studies performed using TNC cells-lines developed in our laboratory have demonstrated the ability of TNCs to bind and internalize $\alpha\beta TCR^{lo}CD4^{+}CD8^{+}CD69^{-}$ thymocyte subset (Philp, Pezzano et al. 1993; Pezzano, Li et al. 1995). Of the internalized thymocytes, a subset gets rescued and is released as mature thymocytes at $\alpha\beta TCR^{hi}CD69^{+}$ stage of development (Philp, Pezzano et al. 1993). Rescue of $CD4^{+}CD8^{+}\alpha\beta TCR^{low}$ triple positive thymocytes from apoptosis by TNCs can be prevented by treating the cells with antibodies against MHC class I and MHC class II, suggesting that the rescue was a function of MHC/TCR interaction. Another subset has been shown to undergo apoptosis within specialized vacuoles of the TNCs. The apoptotic thymocytes are degraded

by lysosomal fusion (Samms, Philp et al. 1999). In another study, Samms et al., (2001) showed circulating macrophages interacting with internalized thymocytes in the cytoplasmic vacuoles of the TNCs. They inferred from these data that macrophages were involved either in antigen presentation or in clearing the cellular debris derived from the apoptotic thymocytes (Samms, Martinez et al. 2001). Also this internalization performed by TNCs has been shown to involve cytoskeletal elements of the TNCs (Webb, Kelly et al. 2004). Furthermore involvement of TNCs in thymocyte development was also demonstrated using monoclonal anybody pH91, the TNC-specific antibody developed in our laboratory. Our experiments show that anti-pH91 recognizes a 43 kDa protein on the surface of TNCs by binding to a discrete surface antigen unrelated to the keratin-family cytoskeletal proteins (Pezzano, King et al. 1998) (Fig 4). To confirm the specificity of pH91 to TNCs we have isolated TNCs from the thymi of C57BL/6 mice and immunostained with anti-pH91 antibody. TNCs are visibly recognized as multi-cellular complexes containing internalized cytoplasmic thymocytes (Fig 5A). In addition, our earlier studies showed that TNCs internalize viable double positive thymocytes and, were visualized by CD4, CD8 staining (Fig 5B)

Thymic nurse cells treated with pH91 mAb fail to bind and internalize thymocytes. In fetal thymic organ culture (FTOC), pH91 was shown to inhibit the development of thymocytes by 70% with the largest reduction found in the $\alpha\beta TCR^{hi}CD69^{+}$ thymocyte subset (Pezzano, King et al. 1998). These data are important because they define a subset of epithelial cells that provide a specific immunological function in promoting T-cell development.

Recent reports published from our laboratory have implicated a role for TNCs in thymocyte selection. The HY-TCR transgenic mouse model was used to demonstrate the role of TNCs in MHC restriction. In the HY-TCR mice, the entire complement of thymocytes express $\alpha\beta$TCR on their surface that recognizes male specific H-Y antigens, hence all the thymocytes are negatively selected and are deleted. In female HY-TCR mice, thymocytes are positively selected and are arrested at the double positive stage of their development. Results have shown that female HY-TCR mice contain TNCs 17 times more TNCs than males per milligram of thymic tissue, and have much larger TNCs (8-11μm larger), and also contain greater numbers of internalized thymocytes (Martinez, Samms et al. 2007). These data concluded that TNCs participate in both positive as well as negative selection.

Data increasingly support the role of TNCs in thymocyte selection and maturation. Studies prior to these findings have proposed that TNCs are involved in apoptosis of non functional thymocytes (Aguilar, Aguilar-Cordova et al. 1994). However, a recent report shows TNCs to express Aire, autoimmune regulator known to be expressed primarily by medullary epithelial cells, these studies have shown that Aire is expressed in the cytoplasm of the TNCs (Hansenne, Louis et al. 2009) . The role of TNCs in T-cell development and maturation has gained recent attention. We have shown that TNCs are capable of internalizing triple positive thymocytes. Captured cells survive or are deleted through processes involving interactions between the $\alpha\beta$TCR expressed on thymocytes, and MHC expression on TNCs. The TNC-specific monoclonal antibody pH91 generated in our laboratory has proven to be a valuable tool in understanding the structural and functional aspects of TNCs. Another interesting observation is that TNC like lymphoepithelial complexes have been found in all mammalian species and have been traced back as far as jawless fish (Salkind 1915; Finstad, Papermaster et al. 1964). Differentiation into cortex and medulla does not persist throughout all fish species, but are seen in elasmobranchs (Flano, Alvarez et al. 1996; Romano, Fanelli et al. 1999; Romano, Taverne-Thiele et al. 1999) and in most teleosts (Romano, Taverne-Thiele et al. 1999). The cortex and medulla do not separate in some teleost thymus, yet the

animal still maintains immune function (Castillo, Lopez-Fierro et al. 1991). Studies published in 1915 and 1964 on early vertebrates (lamprey) (Salkind 1915; Finstad and Good 1964) show a clusters of 5 to 20 lymphoid cells within epithelial cells. The existence of these lympho epithelial complexes in various strata of chordates is an interesting observation.

Studies presented in this thesis were focused on further understanding the functional role of thymic nurse cells in T-cell development, here we primarily were focused in understanding the mechanism of interaction of the thymocytes with TNCs. These studies are explained in chapter one. In further understanding the phenotype of TNCs we have made significant observation, these observation suggested a major role for TNCs in the thymus In chapter these new observations were explained with respect to the TNC phenotype and expression of different developmentally related markers. These observations suggest a progenitor potential role for TNCs in thymic epithelial cells development.

Results Section One

Thymic Nurse Cells Generate Unique Extra-Cytoplasmic Membrane Space and Participate in Thymocyte Selection

Note: Information presented in chapter one is already published in Journal of Cellular Immunology, Hendrix et al. 2010)

Internalization of viable cells by another cell has been described for quite a few biological phenomenon. Cellular internalization has received special attention only recently and has been termed entosis (Overholtzer and Brugge 2008). Phagocytosis is a well described process wherein macrophages take up dead or dying cells. However, it has been difficult to advance the idea that a viable cell can internalize another viable cell, and in some cases release the trapped cell from its intra-cytoplasmic space.

Several reports support TNCs ability to engulf another cell, and to participate in shaping the T cell repertoire (Pezzano, King et al. 1998; Samms, Philp et al. 1999; Pezzano, Samms et al. 2001; Samms, Martinez et al. 2001; Webb, Kelly et al. 2004; Martinez, Samms et al. 2007). Most of the evidence about the functionality of TNCs has come from studies using TNC lines. These cell lines have been used to demonstrate the ability of TNCs to internalize thymocytes *in vitro* (Philp, Pezzano et al. 1993; Pezzano, Li et al. 1995). Using co-incubation experiments in

culture, it was shown that only $\alpha\beta TCR^{low}CD4^{+}CD8^{+}$ cells are bound and internalized by TNCs. TNCs were shown to rescue triple positive thymocytes from undergoing apoptosis (Pezzano, Li et al. 1995).

TNCs have been shown to express both class I and class II MHC antigens on their cell surfaces as well as on the surfaces of the vacuoles surrounding internalized thymocytes (Pezzano, Samms el al., 2001) . The expression of membrane class II MHC antigens is not common with reference to epithelial cells i.e., most epithelial cells do not express MHC class II on their surfaces. Class II MHC expression is restricted to cells related to immune function. When antibodies against MHC I and MHC II molecules were used to treat the TNCs prior to interaction with thymocytes, the treatment prevented internalization, suggesting a relationship between the internalization process and MHC restriction (Pezzano, Li et al. 1995).

Until recently, the process of entosis was unrelated to the study of T-cell development. However, current data show thymocytes trapped within TNCs undergo MHC restriction(Hendrix, Chilukuri et al.) Studies from our lab demonstrated that positive as well as negative selection occurs within these cortical multi-cellular complexes (Wekerle, Ketelsen et al. 1980; Pezzano, Samms

et al. 2001). Current opinion suggests that positive selection but not negative selection occurs in the cortex of the mouse thymus (Surh and Sprent 1994; Sprent, Kishimoto et al. 1996). MHC restriction is defined as the positive selection of triple positive thymocytes (rescue from apoptosis), or negative selection (induction of apoptosis) that results from an interaction between the αβTCR on developing thymocytes and MHC molecules on antigen presenting cells (APC)(Robey, Itano et al. 1994; Robey and Fowlkes 1998; Fowlkes and Robey 2002). Auto-Immune Regulator (AIRE) transcription factor has been shown to be required for negative selection (Liston, Lesage et al. 2003). These studies have shown that AIRE regulates the expression of tissue restricted antigens (TRAs) required for antigen presentation (Anderson, Venanzi et al. 2002; Kyewski, Derbinski et al. 2002; Liston, Lesage et al. 2003; Peterson, Org et al. 2008). The process of negative selection has been suggested to be restricted to mTECs where the AIRE transcription factor is detected exclusively. Recently, AIRE expression was detected within the TNC complex (Hansenne, Louis et al. 2009).

As described in our previous studies, TNCs interact and internalize thymocytes with the involvement of cytoskeletal elements (Webb, Kelly et al. 2004). Previous studies from our lab using video microscopic analysis, demonstrated the process of thymocyte internalization into TNCs (Webb, Kelly et al. 2004). These studies

showed the free moment of thymocytes within the TNC, they also show trapped thymocytes to interact with macrophages. The studies reported here were focused on further analyses of the TNC/thymocyte interaction. Scanning Electron Microscopy (SEM), Transmission Electron Microscopy (TEM) and Confocal microscopy were employed to further our investigations. These studies were carried out with a goal of understanding the interaction of thymocytes and TNCs and the role of cytoskeletal elements in this interaction.

Thymocyte Internalization

To visualize the process of thymocyte uptake into TNCs we used scanning electron microscopy (SEM). Temperature sensitive TNC-1 cells (tsTNCs) were co-incubated with thymocytes in Terisaki plates, temperature sensitive TNCs bind and interact with thymocytes at permissive temperature (32^{o}C) and fail to do so at non-permissive temperature (37^{o}C), hence are a good tool to understand the TNCs-thymocyte interaction. Thymic nurse cells displayed a highly ruffled membrane surface (Fig 6A and B). A subset of thymocytes was found trapped within the ruffled extensions of membrane (Fig 6C and D, arrows). Thymocytes were trapped within these unusual membrane folds, which eventually overlapped leading to the capture of thymocytes (Fig 6D, E and F). Thymocytes interacting with TNCs were visible through fenestrae created by overlapping

membrane extensions (Fig 6E, arrow). To recapitulate the event as seen in Figure 6E, we allowed TNCs to interact with thymocytes after labeling thymocytes with CMRA dye and TNCs with carboxyfluorescein diacetate succinimidyl ester CFDA. We co-incubated TNCs and thymocytes in Matrigel growth medium for 10 hours in culture. This was done to retain the three dimensional morphology of the TNC lymphoepithelial complex. Our analysis using confocal microscopy revealed that the thymocytes are visible within the complex. A close examination showed trapped thymocytes to always be located on oneside of the cell, opposite to the nucleus of the TNC (Fig 6G).

Freshly isolated TNCs were exposed in culture medium for 3 hours (Fig 7A and 7G). Membrane unfurling was observed, revealing interactive thymocytes to be trapped within a honeycomb meshwork-like structure containing fenestra. The thymocytes that were trapped were clearly visible (Fig 7B and C). These membrane structures resembled those seen in Figure 6E. With time, thymocytes were observed amidst ruffled membrane extensions (Fig 7D and E) similar to the ones visualized in Figure 6C. A large number of thymocytes seemed to be completely surrounded by the membrane extensions (Fig 7D, inset), whereas other thymocytes were seen partially exposed within a cocoon-like membranous

structure (Fig 7E, inset). We observed within 3 hours that all thymocytes were released, leaving only the membranous structure of the TNC (Fig 7F).

Using TEM we analyzed tsTNC-1 cells co-incubated with thymocytes, as well as freshly isolated TNCs (Fig 8). Upon observation, thymocytes were seen within the freshly isolated TNC (Fig 8A, asterisk). These internalized thymocytes were seen trapped within TNC membrane extension similar to those observed in Figure 6E. The TEM micrographs reveal membrane extensions similar to those seen in scanning electron micrographs. We then observed the thymocyte/tsTNC-1 interaction after co-incubating them for 10 hours using TEM analysis. TsTNC-1 cells produced extensive network of membrane extensions (Fig 8B). Thymocytes within the TNC cytoplasm were visible. Cytoplasmic thymocytes were detected at various stages of apoptosis (Fig 8B, insets).

The meshwork structure created by the aforementioned long membrane extensions was observed in both SEM and TEM micrographs (Fig 9A and B). This membrane meshwork has an extra-cytoplasmic side which creates the extensions and a cytoplasmic side that opens into cytoplasm cytoplasmic vacuole evolving from the meshwork (Fig 9A, panel 2 and 3, 9C). Thymocytes are visibly trapped within these membrane extensions (Fig 9A, panels 1 and 3)(9B, inset).

Figure 9C shows a region TNC vacuole size large enough to facilitate the movement.

MHC restriction within Thymic Nurse Cells

Proper selection of thymocytes requires an interaction between MHC antigen and the αβ TCR. Internalized thymocytes from freshly isolated cells were stained with antibodies against both CD4 and CD8 (Fig 10A). Double positive thymocytes were visible within the TNC complex. Thymocytes were also labeled with Thy1.2. Freshly isolated TNCs with internalized thymocytes (Fig 10B) were labeled with mAbs against the MHC class II antigen and αβTCR. An interaction between the αβTCR and MHC class II molecules was visualized. Images were recreated 3-dimensionally to visualize the MHC-TCR interaction from all angles. An interaction between MHC class II on the surface of a TNC and αβ TCR on bound thymocyte was also visualized (Fig 10B, inset).

Lipid rafts in the plasma membrane play a crucial role in cell signaling mechanisms (Harder and Simons 1997; Simons and Ikonen 1997). These lipid rafts have been suggested to facilitate movement in the membrane proteins during signal transduction (Simons and Ikonen 1997). The TCR-MHC interaction is required for proper T-cell activation and development (Malissen and Schmitt-

Verhulst 1993).The signaling resulting from their interactions requires lipid rafts coalition (Montixi, Langlet et al. 1998). When thymocytes were co-incubated with cells from the TNC cells line FM4, lipid raft accumulation was observed around cell surface $\alpha\beta$TCR (Fig 10C).

Results Section Two

Thymic Nurse Cells as Thymic Epithelial Progenitor TNCs Express

Progenitor Phenotype

Experiments to define an epithelial cell progenitor of the thymus have recently gained the spotlight. The Marker of Thymic Stroma 24 (MTS24) was initially thought to identify thymic epithelial progenitors (Gill, Malin et al. 2002), but later results showed that $MTS24^-$ cells can also produce an entire thymus (Rossi, Chidgey et al. 2007). This initiated a new search for a marker specific to thymic progenitors. Reports demonstrated that by E11.5, all epithelial cells in the third pharyngeal pouch express cytokeratin K8 (Klug, Carter et al. 2002), and double positive $K8^+K5^+$ cells are expressed a day later (Gill, Malin et al. 2002). If this is true, then $K8^+K5^+$ cells represent a progenitor population with the ability to develop into a functional thymus and undergo terminal differentiation into either cTECs or mTECs (Anderson, Lane et al. 2007). Isolation of viable cells via

their keratin profile is not feasible because the methods used to identify and isolate cells by their cytokeratin profile require fixation, which kills the cells. Thus an alternate cell-surface marker is needed to identify thymic epithelial progenitor cells. Thymic nurse cells have been earlier described to be a subset of cortical epithelial cells. To determine their cytokeratin profiles, we stained isolated TNCs with antibodies against cytokeratins K8 and K5. Interestingly, results showed that almost 74% of the TNCs were $K8^+$ only (Fig 11A), and a minor subset of TNCs exhibited the putative TEC progenitor phenotype, $K8^+K5^+$. Twenty four percent of the TNCs were double positive for these cytokeratins (Fig 11-B1). An insignificant number of $K5^+$ cells were detected but we were unable to determine if these were TNCs. We then determined the population of $K5^+K8^+$ double positive cells that were TNCs. Seventy-six percent (76%) of the TNCs ($pH91^+$ cells) exhibited the double positive keratin phenotype. The population counted included single positive cortical as well as medullary epithelial cells. (Fig. Fig11-B2). *Counting criteria*: Thymic nurse cells are known to possess multicellular complex morphology,their size usually ranges between 12-50μm. The average size of a thymocyte is 5μm and the average size of a mature medullay epithelial cells is about 10 μm maximum. Based on the known size criteria, TNC numbers were enumerated and the above stated phenotype was determined, along with the use of the TNC specific antibody pH91.

To show that the $K8^{+}K5^{+}$ double positive cells are TNCs, we co-stained freshly isolated cells with the TNC-specific mAb pH91, anti-K5 and anti-K8 antibodies. The results show a $K8^{+}K5^{+}pH91^{+}$subset. The $K8^{+}K5^{-}$ TNC subset was also $pH91^{+}$ (Fig 12 C). The predominant subset of $K8^{+}K5^{+}$ cells *in vivo* has been reported to be located in the cortico-medullary junction (CMJ) (Fig. 12 A). Cytokeratin 5 and 8 antibody staining of thymic sections show double positive epithelial cells in larger numbers within the CMJ, compared to those found elsewhere in the cortex or medulla (Fig 2 inset). To identify $K5^{+}K8^{+}pH91^{+}$ positive cells *in vivo,* we performed triple staining of C57BL/6 mice thymic sections. Triple positive subsets were readily detectable in these sections (Fig 12A and 12 B).

TNCs Express p63 Transcription Factor

The primary property of stem cells is well defined. These cells usually exhibit the ability to self-renew, divide or proliferate and are believed to maintain these abilities for a substantial length of time. Stem cells, in different organs, maintain self-renewing capabilities. Some of the cells are multipotent that is, differentiating into various cells types that compose the organ. Few are unipotent, giving rise to only one cell type. Small quiescent populations of stem cells are found in organs such as brain, kidney, lungs, liver and pancreas (Zuba-

Surma, Kucia et al. 2008). Almost all major organs in mammals have a small population of stem cells that participate in maintenance and regeneration (Dan and Yeoh 2008; Serafimidis, Rakatzi et al. 2008). Regeneration of the thymus after irradiation and injury, steroid ablation, castration suggests existence of a stem cell population.

Thymic organogenesis is regulated by several transcription factors as discussed above. P63 is a transcription factor that is indispensable in thymic organogenesis and is known to be required to maintain TEC proliferative potential (Senoo et al., 2007). The fact that TNCs were found to be double positive for cytokeratins, and that the regeneration of the thymus upon injury and irradiation was dependent on the $K5^{+}K8^{+}p63^{+}$ double positive epithelial cells (Popa, Zubkova et al. 2007), led to our investigations to determine if TNCs express known transcription factors important for thymic organogenesis and maintenance. Using pH91, we looked at the expression of p63 in the freshly isolated TNCs *ex vivo* using an antibody against Delta-Np63. This isoform of p63 is expressed at high levels within progenitor cells compared to the transactivating isoforms (TAp63)(Fig 1)(Finlan and Hupp 2007). We observed expression of p63 in the thymic primordium beginning at E12.5. Earlier studies have shown the expression of p63 at detectable levels by E12.5 (Senoo, Pinto et al. 2007). Using anti-p63 antibody

along with pH91 antibody, we observed that the thymic anlagen at E12.5 expressed pH91 along with nuclear localization of p63 in almost all the cells examined (Fig 13A). We then studied the expression of p63 and pH91 developmentally at E13.5, E16.5 and E18.5 embryonic thymi and also in adult mice. The expression of p63 along with pH91 was observed in the thymic tissue section. The expression of p63 was observed in most of the cells early in the development at E13.5 (Fig 20), with time TEC expression of p63 started to decrease, but significant number of $pH91^+$ TECs still expressed p63 (67%) (Fig 20). Some of the $pH91^+$ cells were $p63^-$ suggesting a different developmental potential for these cells (Fig 13 E). We further examined the expression of p63 by TNCs upon isolation. The expression of p63 was observed in the nucleus of TNCs (Fig 14 A). Also a subset of TNC complexes that did not express p63 was observed (Fig 14A5, arrow). Experiments were carried out to determine if p63 was expressed in the $K5^+K8^+$ TNC population (Fig 14B). The expression of p63 by pH91 positive cells *in vivo* was also observed, consistent with what was seen in *ex vivo,* a subset of $pH91^+$ cells were expressing p63 but not all of them were expressing the transcription factor (Fig 13 and Fig 14).

Expression of Foxn1 by Thymic Nurse Cells:

Foxn1 is an important transcription factor for thymic organogenesis (Gordon, Bennett et al. 2001). Foxn1 is expressed by the bi-potent ($K5^{+}K8^{+}$) thymic epithelial cells that can give rise to mature cTCEs or mTECs (Corbeaux, Hess et al.). Studies by Corbeaux et al., have suggested that embryonically and postnatally bi-potent TECs express Foxn1. Mice that are mutant for the *nude* gene are athymic and fail to develop a thymus capable of mounting T-cell development (Nehls, Kyewski et al. 1996; Bleul, Corbeaux et al. 2006). Foxn1 expression is indispensible for attracting the lymphoid progenitors into the thymic anlagen and for the cross-talk initiated differentiation of thymic epithelial cells. This is critical for establishment of a fully functional thymic microenvironment that promotes T-cell development. Expression of Foxn1 by TECs was also suggested to be required for the maintenance of the thymus postnatally and for the proliferation and turnover of postnatal TECs (Cheng, Guo et al. ; Chen, Xiao et al. 2009). These findings suggest that Foxn1 expression is critical for both the prenatal and postnatal thymus. Foxn1 was first observed in the thymic-parathyroid primordium around E11.25 in small subset of epithelial cells and with increased expression by E11.75 (Gordon, Bennett et al. 2001; Manley NR 2010). With TNCs being positive for K5, K8 and Trp63, we looked for the expression of Foxn1 in TNCs. We looked for the expression of pH91 specific antigen in E11.5 thymic primordium along with Foxn1. Interestingly, pH91 was

expressed by the entire thymic-parathyroid primordium at E11.5, along with the expression of Foxn1 (Fig 15). The expression of Foxn1 was consistent with previous published data (seen along the caudal and slightly to the ventral end)(Gordon, Bennett et al. 2001). Expression of Foxn1 was also observed with pH91 throughout the thymic anlagen at E12.5 (Fig 15). We followed Foxn1 expression ontogenically in the thymic section along with pH91 at E13.5, E16.5 and E18.5 (Fig 16A-C). Foxn1 expression pattern was similar to what was observed for p63. Initially a larger percentage of $pH91^+$ cells were observed to express Foxn1 (94%). We were also able to identify $pH91^+$ $Foxn1^-$cells (Fig 17 Arrow Panel C). Foxn1 expression was observed in medullary TECs (Fig 17 Panel C arrow heads). Postnatally, $pH91^+$ cells were observed to express Foxn1; some of the $pH91^+$ cells were $Foxn1^-$. We further examined the expression of Foxn1 in isolated TNCs. Nuclear localization of Foxn1 was observed in freshly isolated TNCs (Fig 18).

Co-Expression of Foxn1 and p63 by pH91 positive TECs

The downstream targets of p63 and Foxn1 are not completely known. Both transcription factors are required for maintenance and growth of the thymus (Cheng, Guo et al. ; Senoo, Pinto et al. 2007). For example, p63 regulates the expression of FgfR2 and Jag2 (Candi, Rufini et al. 2007). Foxn1 was shown to be

required for vascularization of the thymus (Nancy R Manley 2009) and for expression of Notch ligands such as Dll1 and Dll4 (Manley 2009). It is not known if Foxn1 and p63 expression are related. We looked for the expression of Foxn1 and p63 in thymic tissue and also in the isolated TNCs, both in the embryo and in the postnatal TNCs. Expression of both p63 and Foxn1 was observed in the TNC nuclei (Fig 19A-E). There is a significantly larger subset of TNCs that express both the transcription factors. A smaller subset of TNCs expresses only pH91 and Foxn1 but not p63 (Fig 19 F-I).

To determine the frequency of TNCs that express Foxn1 and p63, we manually counted the number of complexes that expressed both markers in the adult mice. Approximately 83.4% of TNCs were found to express both Foxn1 and p63 and 12.35 % of TNCs were found to be $Foxn1^{+}$ $pH91^{+}$ $p63^{-}$ (Graph Fig 20B). During the early stages of development, by E 13.5, the expression of both Foxn1 and p63 is almost at the same levels (94% and 92% respectively) (Fig 20). Studies have shown that Foxn1 expression decreases from embryonic to adult stages and is limited to a very small subset (Corbeaux, Hess et al. 2010). One study suggested that the expression of Foxn1 by keratin expressing cells significantly decreased two weeks after birth (from 80% at E13 to 20% after 2–4 week of birth) (Itoi,

Tsukamoto et al. 2007). Contrary to these findings, a large number of pH91 positive cells expressed Foxn1 (approximately 80%) 2 weeks after birth.

Expression of pH91 early in the embryonic development.We further studied the expression of pH91 in early embryonic development, embryonic day E7.5. Embryonic E 7.5 sections were stained with pH91 and Oct4. Initially, Oct4 (a transcription factor) is expressed during blastulation. In the blastula Oct4 is associated with the establishment of all of the germ layers in mammals (Pan, Chang et al. 2002). During gastrulation Oct4 expression is associated exclusively with the ectodermal layer (Pan, Chang et al. 2002). We used Oct4 as a reference marker in our staining experiments at E7.5 of development. Cells stained for pH91 were seen distributed in regions defined by Oct4 staining (Fig 21). Interestingly, we observed Oct4 expression along with pH91 in the ectoderm. We detected a subset of pH91+ cells that co-expressed Oct4, and also a subset of pH91+ cells that did not express Oct4 (Fig 21,Panel C and F).

The development of TNC multi-cellular complexes and the onset of MHC-Class II Expression

Thymic nurse cells were first identified by Wekerle and Ketelson based on their intimate association with thymocytes. Studies carried out by Van Ewijk et al

2000, have suggested that the cross talk signals received by the TECs during development from thymocytes results in the formation of the thymic nurse cell complex morphology. Using the TNC-specific monoclonal antibody pH91, we have followed the ontogenic development of the multi-cellular complex that defines TNCs in C57Bl/6 mice. Starting from E13.5, we isolated TECs from embryonic thymi by enzymatic digestion. These resulting cells were visualized microscopically after staining with pH91 antibody. Although $pH91^{+}$ cells were detectable, multi-cellular complexes were not evident at developmental stages E13.5 and E14.5. Cells positive for pH91 antibody during development displayed different phenotypes. Some of the cells were bi-nucleated suggesting cellular division (Fig 22, Panel A), while some showed fibrous extensions (Fig 22, Panel B), characteristic of TNCs isolated from adult mice. Bi-nucleated TNCs persist through E17.5. The first multi-cellular complexes were visualized at E17.5. These complexes contained $CD4^{+}$ $CD8^{+}$ double positive thymocytes (Fig 22, panel D). Developmentally we then examined the timeline for the expression of MHC class II on the surface of these $pH91^{+}$ cells (Fig 22E). At the E13.5 stage, approximately 64% of the $pH91^{+}$ cells were MHC class II^{low} and about 36% of the cells were MHC class II^{hi}. By E16.5 $pH91^{+}$ cells showed increase expression of MHC class II on their surfaces, 56% MHC class II^{low} and 44 % MHC class II^{hi}. By embryonic

stage E18.5 almost 62% of pH91^{+} cells expressed MHC class II^{hi} levels and approximately 29% of pH91^{+} cells were MHC class II^{low}.

Discussion:

Part 1: The TNC/ Thymocyte Interactions:

The use of electron microscopy has uncovered new insights into the thymocytes/TNC interaction. Results from our studies have shown that thymocytes are trapped and enclosed by TNC membrane extensions. Thymocytes were seen trapped in web-like structures. These findings are the first of their kind (Fig 6E, 7C and 9B, Panel 1). Using a combination of SEM, TEM and confocal microscopy, we were able to capture the uptake process. Membrane extensions were shown protruding from one side of the TNC. TEM data revealed that below the meshwork lay cytoplasmic vacuoles to which trapped thymocytes are accessible. TEM results showed a couple of membrane network types, one that opens to the outer cellular spaces (Fig 9A, Panel 2), and the other is closed to the outside of the cell (Fig 9B, Panel 2). These structures may represent different stages of the internalization process, with different events happening at different times during the uptake process. Earlier video microscopic and TEM analysis demonstrated that thymocytes play a role in their own internalization by the TNC (Webb, Kelly et al. 2004). Internalized thymocytes have been shown to interact with other thymocytes and macrophages (Samms, Martinez et al. 2001; Webb, Kelly et al. 2004). Movement of thymocytes has been detected within the TNCs fenestrae (Fig 7C). Fenestrae large enough to hold thymocytes were seen

upon close observation (Fig 9B). Collectively our studies demonstrate that thymocytes initially interact with membrane extensions that eventually surround the trapped thymocytes. The spaces between overlapping membranes create a fenestrated honeycomb-like structure with areas large enough for several thymocytes to move around each other. For several reasons, we do not believe these unique spaces to be cytoplasmic. First, macrophages were visualized moving freely within these compartments. Second, thymocyte release could not have occurred from cytoplasmic vacuoles without a complicated series of membrane fusion events, for which no evidence exists. As described above, the membrane meshwork that interacts with thymocytes is associated with classical vacuoles on the cytoplasmic side. This arrangement makes possible for the movement of trapped thymocytes from the membrane meshwork into the cytoplasm. Although we have no microscopic proof of a cytoplasmic entry event, we believe that thymocytes programmed to die through apoptosis become cytoplasmic to facilitate lysosomal fusion.

Part II: TNC as Thymic Progenitors

The thymus is an organ that has been shown by studies to have the ability to undergo regeneration upon injury and irradiation (Popa, Zubkova et al. 2007). These observations suggest that the thymus contains a population of epithelial cells that maintain progenitor potential. These cells are either quiescent or continue to maintain the turnover of TECs. Studies have shown that TECs undergo turnover in the thymus rapidly in the fetal lobes and continue in the adult thymi at a decreased rate (Gray, Seach et al. 2006). Thymic epithelial cells have been shown to undergo turnover at higher rates, almost doubling in number every day until the age of 4 weeks, with respect to cells that express high levels of MHC class II (Gray, Seach et al. 2006). Gray et al 2006., have shown that almost 10% of these cells are generated per day. Studies have shown that when populations of TECs were seen in the proliferative stage, it was the TECs that expressed $K5^+$ and $K8^+$ that differentiated and resulted in mature TEC formation (Gray, Seach et al. 2006). These studies have shown that upon castration or steroid ablation in middle-aged mice, TECs proliferated and the thymic architecture reverted to that of young mice. These studies indicate that populations of TECs that have the ability to proliferate and differentiate exist in the thymus throughout the life of the animal. Our data, with TNCs being double positive for K5 and K8 cytokeratins indicate that they might have the ability to

proliferate and differentiate and mature into cortical and medullary TECs. The fact that almost 75% of TNCs were double positive for cytokeratins K5 and K8 suggests that this is the subset of TNCs may maintain progenitor potential.

Using pH91, a thymic nurse cells specific antibody, we were able to show that TNCs express developmentally significant transcription factors. Expression of p63 with respect to basal epithelium has been implicated in maintaining the proliferative potential of the epithelium and give rise to stratified epithelium (McKeon 2004; Truong, Kretz et al. 2006) (Senoo, Pinto et al. 2007). Studies have shown that p63 expression in the thymus has the role of maintaining the proliferative potential of TECs (Popa, Zubkova et al. 2007; Senoo, Pinto et al. 2007). Data from our experiments demonstrated that within the embryo and also after birth a large number of TNCs express p63 transcription factor (83%)(Fig 20). These results suggest that thymic nurse cells maintain proliferative potential. Also, our data showed that $K5^+$ $K8^+$ TNCs express p63 (Fig 14). Previous studies performed by Popa et al., suggest that $K5^+$ $K8^+$ TECs are the cells that regenerate thymic epithelium upon injury and irradiation. A significant finding here is that pH91 is expressed along with p63 by E12.5, when almost all the thymic TECs express p63. This is interesting since thymic nurse cells complexes were believed to arise by days E17.5, because pH91 is TNC-specific marker it stands reason that

the origin of TNCs or TNC precursors occur earlier but the complex morphology is only seen by E17.5 (van Ewijk, Hollander et al. 2000). Also, data from our studies show that the TNC-specific marker pH91 is expressed along with p63 very early in development (Fig 13). These studies re-enforce the possibility that TNCs maintain their proliferative potential.

Fork head transcription factor (Foxn1) expression occurs early in the development of the thymus. The expression of Foxn1 along with pH91 in the thymic primordium is a very significant finding (Fig 15). This is because the thymus as organ is not yet detached from the pharyngeal pouch by E11.5. It still exists as a thymus/parathyroid common primordium (Gordon, Bennett et al. 2001). The expression of pH91 along the thymic-parathyroid primordium suggests that it might be marker for cells that can give rise to both structures. Developmentally, pH91 expression was observed with Foxn1 and was shown here to continue into neonates and beyond (Fig 15 and 16). This observation was important, Foxn1 is considered to be a significant transcription factor not only in thymic specification but also in the postnatal thymus (Cheng, Guo et al.; Corbeaux, Hess et al. ; Chen, Xiao et al. 2009). These studies have shown that postnatal expression of Foxn1 is required for thymopoiesis. Studies by Corbeaux et al 2010, have shown that Foxn1 positive cells or the cells that were previously

expressing Foxn1 are the only cells capable of promoting thymopoiesis in the postnatal thymus. Their studies have suggested that bi-potent TECs express Foxn1. Studies from Chen et al 2009., have shown that Foxn1 expression is required in a dosage dependent manner for the maintenance and proliferation of TECs. Data from Cheng et al 2010, have also shown that disruption of Foxn1 expression results in acute-atrophy of the thymus. Keratin double positive TECs are localized predominantly in the CMJ region of the thymus, this is believed to be a strategic location for these cells as they may differentiate into either of the TEC subsets without requiring greater movement into either the cortex or the medulla as mature TECs. Also, studies have shown that the Foxn1 expressing cells within the cortico medullary junction are the ones that are primarily lost in induced thymic atrophy. Thymic nurse cells not only make up a major proportion of double positive TECs but also express Foxn1 (Fig 11). The fact that TNCs express Foxn1 not only within the embryo (with respect to pH91) but also to a larger extent in the adult mice is significant (Figs 15, 16 and 20). This observation suggests that these cells have the ability to initiate thymic development and to maintain the thymus as an organ. These cells may also give rise to terminally differentiated cells.

Oct4 has been known to be expressed in the totipotent cells in the mouse embryonic stage (Pan, Chang et al. 2002). Oct4 belongs to the Pit-Oct-Unc family

of transcription factors hence is sometimes referred as Oct4-POU transcription factor (Scholer, Ruppert et al. 1990). Studies have suggested that Oct4 is known as the only transcription factor that is involved in the establishment of the germ layers in mammals (Yeom, Fuhrmann et al. 1996). Expression of Oct4 by day E7.5 in murine embryo was suggested to be seen in the primitive streak region (Yeom, Fuhrmann et al. 1996). Expression of different known stem cells markers by thymic epithelial cells in mice was demonstrated by studies for Andrew Farr's laboratory. Using PCR analysis they showed that TECs express markers such as Nanog, Oct4, and Sox1. Expression of these markers in adult TECs was an interesting observation (Dooley, Erickson et al. 2008). Using pH91 as a marker and Oct4 as stem-cell marker, we have demonstrated that pH91 positive cells express Oct4 very early in development (Fig 21). Oct4 expression is believed to be confined to ectoderm cells. In the blastocyst stage of development Oct4 expression was seen in the inner mass of cells (ICM), these cells give rise to cells of ectodermal, endodermal and mesodermal origins (Pan, Chang et al. 2002). The expression of Oct4 in the parietal endodermal region is probably a transient event and may explain the presence of pH91 positive cells. One study has shown that progenitor cells that give rise to the thymus (endodermal in origin) can give rise to skin cells by microenvironmental reprogramming (Bonfanti, Claudinot et al.) (Skin cells are derivatives of ectodermal germ layer). These studies suggest

that based on the signals, cells derived from different germ layers/organs when reprogrammed can give rise to structures that are not usually derived from the initial germ layer/organ.

Overall, our data show Oct4 to be expressed along with pH91 during gastrulation. Oct 4 has been shown by many groups to be associated with the ectoderm at this stage of development. On the other hand, our data show that pH91 is specific to TNCs. TNCs are found in the thymus, which is derived from the endoderm. One may attempt to explain these data in several different ways. First, we can go back to the old argument about whether the thymus is derived from both the ectoderm and endoderm or the endoderm exclusively. There have been experiments to show that cells derived exclusively from the endoderm at day E10.5 have the potential to give rise to a functional thymus. Experimentally, ectodermal cells are stripped away from the endoderm layer during the isolation procedure. Now, this is day E10.5, are pH91 positive cells associated with the endoderm at this stage of development? Do they continue to express Oct4? These questions are yet to be answered. Thou no explanation can be given yet, may be pH91 defines the split of endoderm away from the ectoderm. Second as stated above, cells at early stages of development are pluripotent, their developmental potential depends on their niche (microenvironment). We have no way of

defining the developmental potential of cells in this region of the embryo at E7.5. Further the expression of Oct4 in pH91^{+} cells may indicative of their stem cell potential. That is to say, it has been shown that Oct4 is expressed in the blastocyst. Is pH91 expressed there? We have not been able to isolate the blastocyst. This is technically very difficult to do. However we plan to perform this experiment in the near future.

One issue that remains left open for discussion is how pluripotent are pH91 positive cells. The expression of pH91-specific antigen at such early (E7.5) stages of development suggests that this protein may be expressed in tissue other than the thymus. Oct4 positive cells become liver, lungs, nerves and all the organs of ectodermal origin. We propose to analyze other tissues in the mouse for their expression of the pH91 specific antigen. With this in mind and because it is expressed so early during ontogeny, we used the pH91 mAb to study the evolutionary significance of TNCs.

TNCs have been shown to exist in man, (Brelinska, Paczkowska et al. 2002) mice, rats, chicken frogs, fish and sharks (Frommel, Litman et al. 1971; Wekerle and Ketelsen 1980; Romano, Fanelli et al. 1999; Bowden 2008). Sharks represent the first vertebrate species to have a jaw. Jawless vertebrate (lamprey) do not have a

thymus, but a suggestion of a thymic candidate have been made (Bajoghli et al., 2011). Good and Finstad 1964 reported that lamprey do not have the thymus but described the "pro-thymus" as epithelial cells in the pharyngeal gutter that contain 5-20 lymphoid cells. Their description explains that there is no membrane barrier between the lymphoid and epithelial cell. The authors of this study suggest that the multi-cellular complex evolved into the thymus. The evolution of the thymus has become quite controversial because several studies have shown that the immune system in lamprey is very different from that of mammals. It involves a genomic rearrangement of the lucine- rich repeats modules at the Variable Lymphocyte Receptors (VLR) locus (Herrin and Cooper ; Pancer, Amemiya et al. 2004; Guo, Hirano et al. 2009). Rearrangement at this locus creates an immune receptor diversity similar to that of the immunoglobulin (Ig) system (Herrin and Cooper). There have been no suggestions as to how the VLR based immune system evolved into the mammalian Ig system (Herrin and Cooper). Other papers have reported that in the same area of the gut (typhlosole), there are lymphoid cells that express Thy1 and a TCR-like protein (Yu, Ehrhardt et al. 2009). Using our pH91 antibody, we found pH91 positive cells in association with Thy1 expressing cells in the typhlosole of the lamprey. Our results suggest that although immune system of lampreys is VLR based, this type of immune system did not evolve. However, in the typhlosole there are cells

that are pH91^{+} in association with Thy1^{+} lymphoid cells that quite possibly have the potential to evolve into the mammalian thymus (Fig 24).

In summary our studies show that TNCs create an unusual arrangement with its associated thymocytes. Initially, it was suggested by Werkerle and Ketelson that the interactive population of thymocytes was cytoplasmic. For years, our laboratory has supported this theory. However our most recent data indicate that most of the thymocytes trapped within the TNC complex are not cytoplasmic. This study shows the thymocytes to reside within a unique membrane-derived honeycomb-like structure. We propose that this structure results from membrane extensions generated by the TNCs that initiate contact with the thymocytes. Thymocytes become trapped within a space created by the overlapping of numerous membrane extensions. Gaps in the overlapping extensions create fenestrae that allow visualization of trapped thymocytes. When one sees these structures through the lens of a microscope, trapped thymocytes appear to be cytoplasmic. However, it is difficult to believe that thymocytes and macrophages move around in the cytoplasm of another cell. We do find fenestra within this membrane meshwork that are large enough to facilitate cellular mobility. The only evidence of a cytoplasmic thymocyte population is a previous study that revealed the degradation of apoptotic thymocytes within the TNC complex results from lysosomal fusion. Lysosomes are exclusively cytoplasmic.

We have had many questions about the function of this unusual multi-cellular complex in the thymus. Our previous work has shown TNCs to express both class I and class II MHC antigens on their cell surfaces. Previous studies have also shown the initial interactive thymocyte subset to be $\alpha\beta TCR^{low}$ $CD4^+$ $CD8^+$ $CD69^-$ cells. Subset of interactive thymocytes dies through apoptosis, while the other cells mature to the $\alpha\beta TCR^{hi}$ $CD4^+$ $CD8^+$ $CD69^+$ stage of development. However, questions remained about TNC involvement during MHC restriction. In this study, we showed an interaction between the αβTCR expressed on trapped thymocytes and MHC class II antigen expressed on the surface of the TNCs (Fig 10). We also show lipid raft accumulation around this interaction (Fig 10). Taken together, these data suggest that there is an interaction between αβTCR expressed on trapped $CD4^+$ $CD8^+$ and MHC class II antigen expressed on the surface of the TNCs. By definition, this is MHC restriction. We propose that this interaction activates the increased expression of αβTCR and turns on CD69 expression.

Our final studies involved an investigation of the progenitor potential of the TNCs. In these studies we define TNCs by their unique morphology and their ability to bind the monoclonal antibody pH91. By day E11.5, pH91 is seen in association with Foxn1 in the para-thyroid/thymus primordium. The next day,

pH91^{+} cells express both p63 and Foxn1. These associations persist through out development. Foxn1, P63, K5^{+} K8^{+} cells have been proposed to be associated with thymic epithelial progenitor cells. The early expression of the pH91 antigen (E7.5 days) has initiated some interesting ideas. These ideas have not yet been thoroughly investigated in this study but experimental solutions are approachable. Is the expression of pH91 specific antigen a link between the "true thymus" and the "pro-thymus"? We do not have the answers to their question now but what better way to be a thymus without being a "true thymus" then to be an epithelial package that can separate several Thy1^{+} lymphocytes from the general microenvironment of the lamprey gut. Given 500 million years, these isolated packages of lymphocytes have the potential to evolve into a "true thymus". We are currently attempting to isolate pH91^{+} epithelial cells with trapped Thy1^{+} lymphoid cells within a multi-cellular complex from the typhlosole of the lamprey.

This study is the first to show the development of TNCs using pH91 as a marker. Developmentally, larger numbers of TECs express pH91 and these cells are seen in different phases of cell cycle (Fig 22). Expression of MHC class II on these cells increases gradually suggesting, their maturation from progenitors to differentiating/differentiated cells. An interesting observation is that by E18.5, 29% of

pH91^{+} cells are still single positive and do not express MHC class II, this has striking correlation to that fact that 25% of TECs are double positive in a adult mice.

In summary, our findings here have shed light on TNCs in terms of their role in T-cell development and also with respect to them being thymic epithelial progenitors. Further studies are needed to further establish the progenitor potential of TNCs/pH91 positive cells. Nonetheless data presented here only strengthens our hypothesis. We prepare to sort, reaggregate and graft pH91 positive cells under the kidney capsule to establish the progenitor potential of these cells. Future studies will also be directed in understanding the ability of these cells to rescue thymic abnormalities seen in certain autoimmune phenotypes (Takeoka, Chen et al. 1996; Takeoka, Taguchi et al. 1999).

Materials and Methods:

Isolation of TNCs and Thymocytes.

C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) were dissected aseptically and the thymi were removed. Thymi were slightly disrupted with fine needles and subjected to enzymatic digestion in a solution of 0.015% collagenase D (Sigma Aldrich, St Louis, MO), 0.01% DNAse I (Sigma Aldrich), and 25 ml of trypsin (GIBCO, Carlsbad, CA) along with gentle agitation. The solution was changed every 10 minutes until the thymi were completely digested. The resulting cells were subjected to 1xg gradient separation in fetal bovine serum (Atlas Biological, Fort Collins, CO) at 4^{o}C to enrich TNC numbers. Thymocytes were obtained by the mechanical disruption of thymi obtained from 4 to 6 week old C57BL/6 mice. Macrophage depletion was accomplished by negative sorting using CD11b Microbeads (Miltenyi Biotech, Auburn, CA)

Scanning Electron Microscopy.

One million thymocytes were allowed to incubate with 1x10^{5} TNCs from our temperature sensitive cell line, tsTNC-1 (Pezzano, Li et al. 1991), at 37^{o}C in Terisaki culture plates for 0 - 12 hours and transferred to microscope slides. Freshly isolated TNCs were allowed to incubate on microscope slides for 2 hours at 37^{o}C. The samples were fixed with 3.2% gluteraldehyde in 0.1 M sodium cacodylate buffer pH 7.2 (Electron Microscopy Sciences, Hatsfield, PA), and

stored at 4°C for 12 - 24 hours. The cells were then rinsed in distilled water, dehydrated in a graded series of ethanol (Electron Microscopy Sciences), rinsed twice in amyl acetate (Electron Microscopy Sciences) and critical point dried. The cells were then sputter coated with 6-10 nm of gold and observed in a Zeiss DSM 940 Scanning Electron Microscope. Secondary electron images were captured using the Spirit image acquisition system (version 1.07) at a 1024 x 1024 pixel resolution.

Transmission Electron Microscopy.

For studies requiring co-incubation, $5x10^6$ TNCs were incubated with $5x10^7$ thymocytes for 0 - 20 hours in glass petri dishes at 37°C. Co-incubated cells and isolated TNCs were fixed in 0.1 M cacodylate, 2% gluteraldehyde, and 1% osmium tetroxide (Electron Microscopy Sciences), pH 7.4 at 4°C for 30 minutes. Cells were then dehydrated in ascending concentrations of acetone (Electron Microscopy Sciences). After dehydration, cells were embedded in Embed 812 (Electron Microscopy Sciences). Ultra thin sections were made on a LKB Ultrotome III and stained with uranyl acetate followed by lead citrate (Electron Microscopy Sciences). Cells were viewed on a Zeiss EM 902 Electron Microscope using a SIS MegaView III digital camera at a resolution of 1376 x 1032 pixels.

Video Microscopy.

Phase contrast videography of $1x10^4$ TNCs co-incubated with $2x10^6$ thymocytes was viewed using a Nikon Diaphat Microscope with a Hoffman Modulation Contrast System. The microscope was attached to a Nikon CCD-72 camera. The samples were visualized on a color monitor coupled to a VCR. Videography using a light microscope was observed using an IX70 Olympus microscope attached to a DP11 Olympus camera. Video images were captured in real time and immediately digitized. All video microscopy was performed at 37°C.

Timed Pregnancy and Thymic Sections

C57Bl/6 mice were mated overnight; Females were separated the next day on detection of vaginal plug. Day of separation of females was designated as day 0.5 Fetal pups/thymi were dissected aseptically from C57BL/6 mice. For early timed animals (E7.5, E11.5 and E12.5 sections) entire fetus was embedded in OTC medium. Form E13.5 onwards individual lobes were embedded in OTC medium (Richard Allan Scientific, Kalamazoo, MI). Thymic sections 10μm in thickness were made using a Leica CM1950 Cryostat. Sections were mounted on Bond-Rite microscope slides (Richard Allan Scientific) for immunostaining.

Immunostaining of TNCs and Thymic Sections.

Isolated TNCs were deposited onto glass slides using a Thermo Scientific Shandon Cytospin 4. Thymic sections or isolated TNCs were fixed in 2%

paraformaldehyde (Baker, Phillipsburg, PA) for 30 minutes followed by 3 washes with PBS (GIBCO). Sections were blocked and permeabilized in 3% BSA (Fisher Scientific, Pittsburg, PA), 0.1% Triton-X (Fisher Scientific) in PBS. Samples were incubated with primary and secondary antibodies at 37^{o}C for 1 hour each. Samples were mounted in ProLongGold antifade with DAPI (Molecular Probes, Carlsbad, CA). Images were acquired using the Zeiss LSM510 Confocal Microscope.

Primary antibodies used were as follows: rat anti-mouse pH91 monoclonal antibody (IgG2a), cytokeratin 8 (K8) - TROMA-I (IgG2a) (Developmental Studies Hybridoma Bank, Iowa City, IA), chicken anti-mouse K8 polyclonal antibody (IgY) (Abcam, Cambridge, MA), goat anti-rabbit cytokeratin 5 (K5) polyclonal antibody PRB-160B (IgG) (Covance, Princeton, NJ), rabbit anti-goat ΔNp63 (N-16): sc-8609 (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-Foxn1 polyclonal antibody (IgG) H-270 (Santa Cruz Biotechnology, Santa Cruz, CA), goat-anti Oct3/4 polyclonal antibody N-19, (Santa-Cruz Biotechnology, Santa Cruz, CA) antibody FITC-conjugated anti-mouse MHC class II (Miltenyi Biotech), biotinylated anti-mouse $\alpha\beta$TCR (BD Pharmingen, San Jose, CA), APC-conjugated CD4 (BD Pharmingen), PE-conjugated CD8 (BD Pharmingen), FITC-conjugated Thy 1.2 (BD Pharmingen), FITC-conjugated rat IgG2a isotype control

(BD Pharmingen), and TRITC-conjugated rabbit IgG2a isotype control (BD Pharmingen). Lipid rafts were visualized using Alexa Fluor 647-conjugated cholera toxin subunit B (Invitrogen, Carlsbad, CA). Secondary antibodies used are as follows: FITC-conjugated mouse anti-rat IgG2a (BD Pharmingen), APC-conjugated donkey anti-chicken IgY (Jackson ImmunoResearch Laboratories, West Grove, PA), TRITC-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories), APC-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories), TRITC-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories), TRITC-conjugated donkey anti-goat IgG (Jackson ImmunoResearch Laboratories), TRITC-conjugated rabbit anti-goat IgG (Jackson ImmunoResearch Laboratories), and TRITC-conjugated streptavidin (BD Pharmingen). CMRA and CFSE dyes were obtained for Invitrogen (Molecular probes) and concentration were prepared as per manufacturer recommendations.

Figure Legends

Figure 1: **Schematic representation of different isoform of p63**: These isoforms are produced from each gene, resulting from use of second promoter, from internal translation and from alternative splicing events. The TA isoform is homologous to p53 in function; this isoform is involved in the trans-activation p53 gene. The dnP63 isoform regulates activities antagonistic to Tap63 isofom. The Amino acid numbers refer to full-length isoforms TAp63, murine variants.SAM (sterile-alpha motifs).

Figure 1.

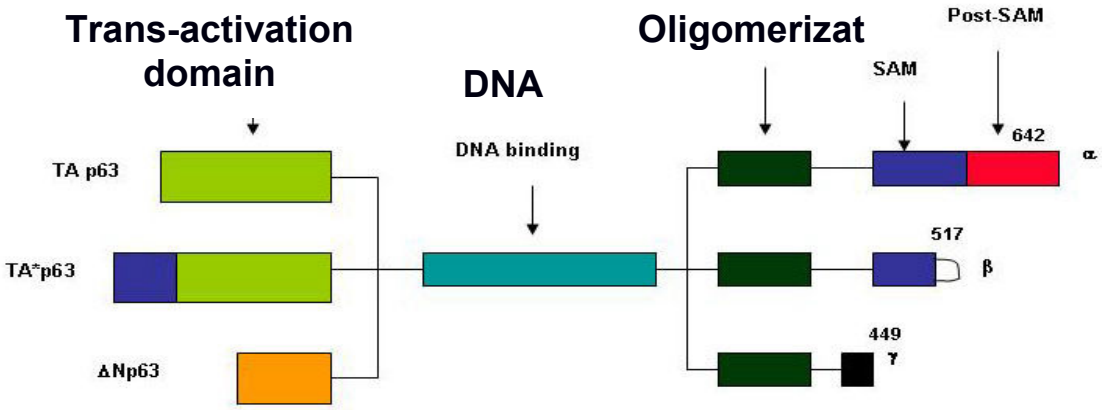

Trans-activation domain
DNA
Oligomerizat
Post-SAM
SAM
642
α
DNA binding
TA p63
TA*p63
517
β
ΔNp63
449
γ

Figure 2: Cytokeratins K5 and K8 expression pattern in a mouse thymus: Thymic sections of 4-week-old C57Bl/6 were stained with antibodies against cytokeratins K8 and K5 medullary islets (red) seen surrounded by cortical cells (green). Inset shows magnified CMJ area with $K8^{+}K5^{+}$ double positive TECs (yellow)

Figure 2.

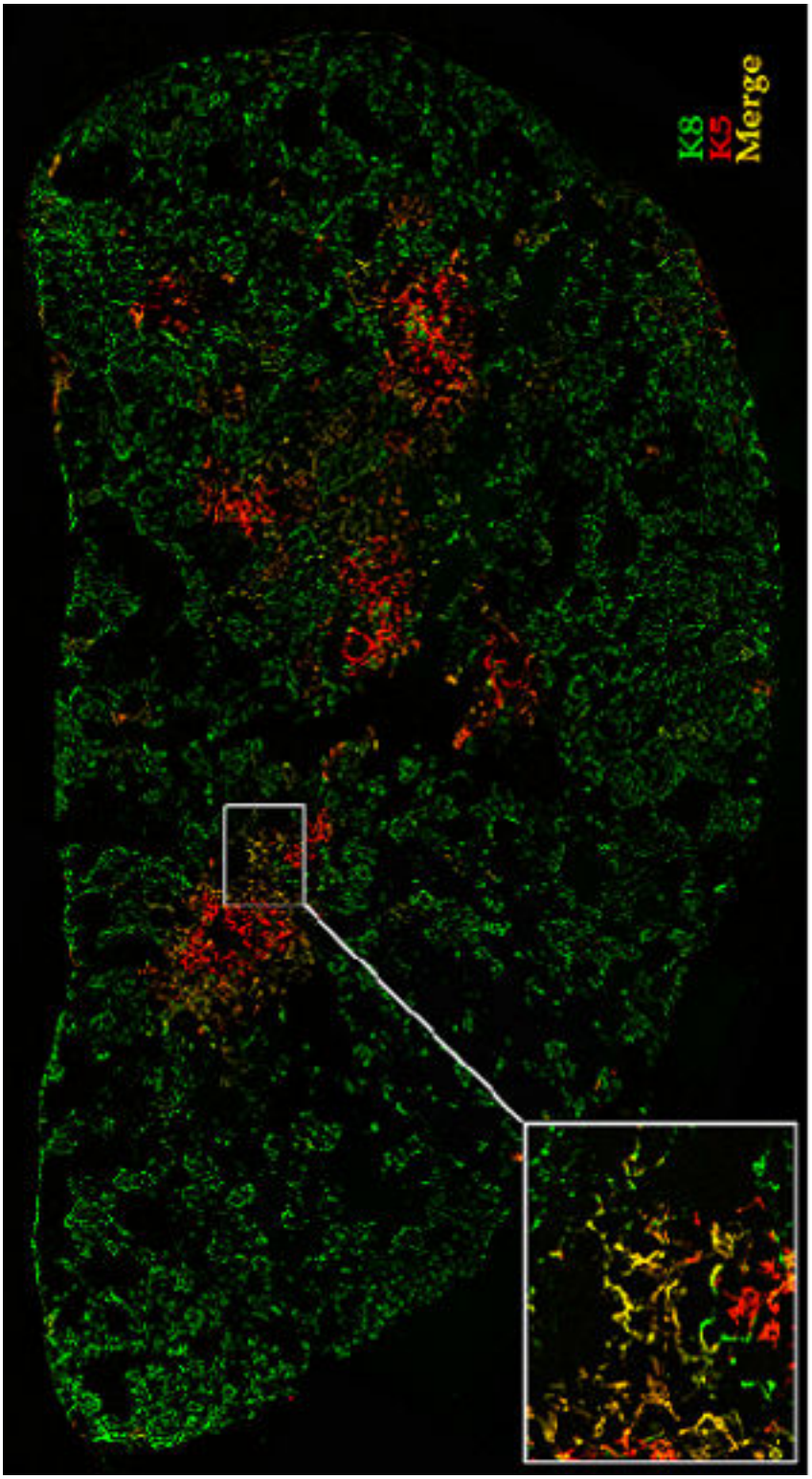


Figure 3: **Distribution of TNCs in the thymus**: Immunostaining of thymic section of C57BL/6 mice with anti-bodies against pH91 and cytokeratin K5. Distribution of pH91 positive TNCs (green) can be seen in the cortex (C), Medullary epithelial cells (red) can be seen as an islet (M). Also dispersed $K5^{+}$ cells are visible in the cortex

Figure 3.

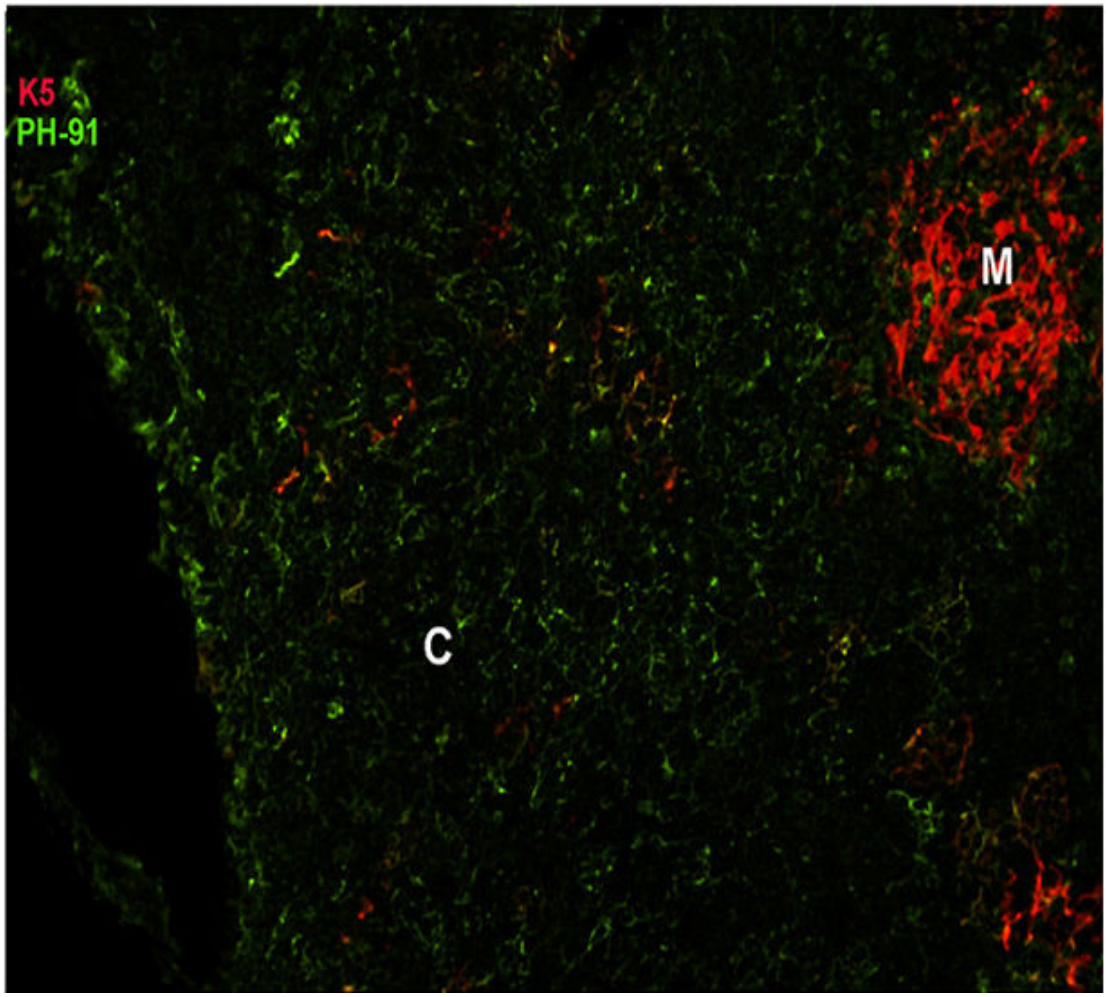


Figure 4: Expression of pH91 by TNCs *ex vivo*: Panel A shows phase image of TNC; Panel B TNC stained with pH91 antibody; Panel C shows DAPI nuclear staining; Panel D shows a merged image. Panel E and F show pH91 to be a 47kDa glycoprotein expressed on the surface of the TNCs. *(Ref: Pezzano et al 2001. PhD thesis 1998)*

Figure 4.

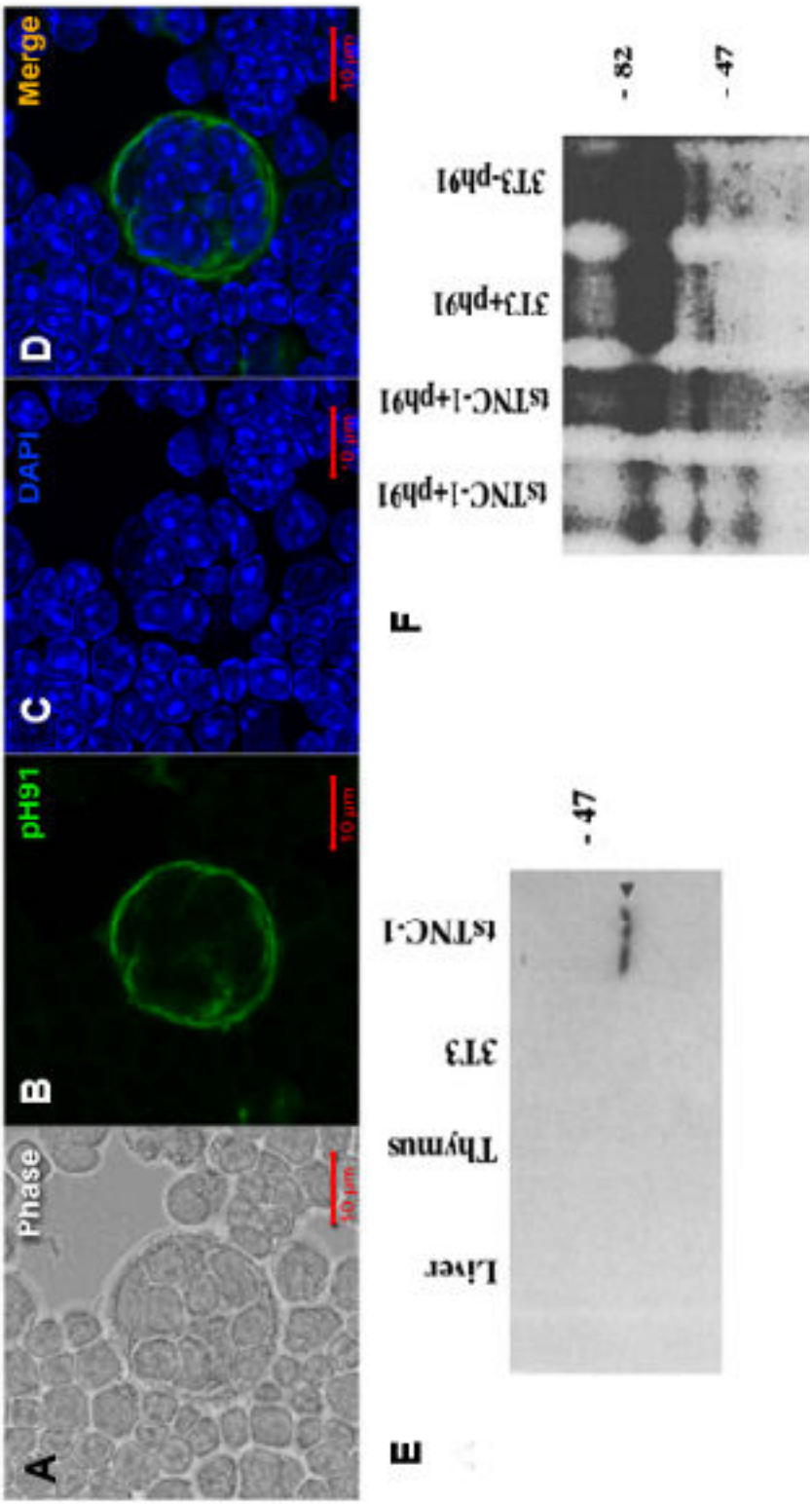

A
Phase
B
pH91
C
DAPI
D
Merge
E
Liver
Thymus
3T3
sTNC-1
- 47
F
3T3-ph91
3T3+ph91
sTNC-1+ph91
sTNC-1+ph91
- 82
- 47

Fig. 5: TNC internalization of viable thymocytes: Panel A shows phase image of TNC; Panel B shows internalized thymocytes expressing Thy 1.2 staining (red), Panel C shows DAPI nuclear staining (blue), and Panel D shows an overlay. Scale bar represents 5 μm. Panel G-H show freshly isolated TNCs stained with CD8 (F), CD4 (G) and Merge (H), Phase (E).Magnification 40 X. Images represent three individual repeats.

Figure 5.

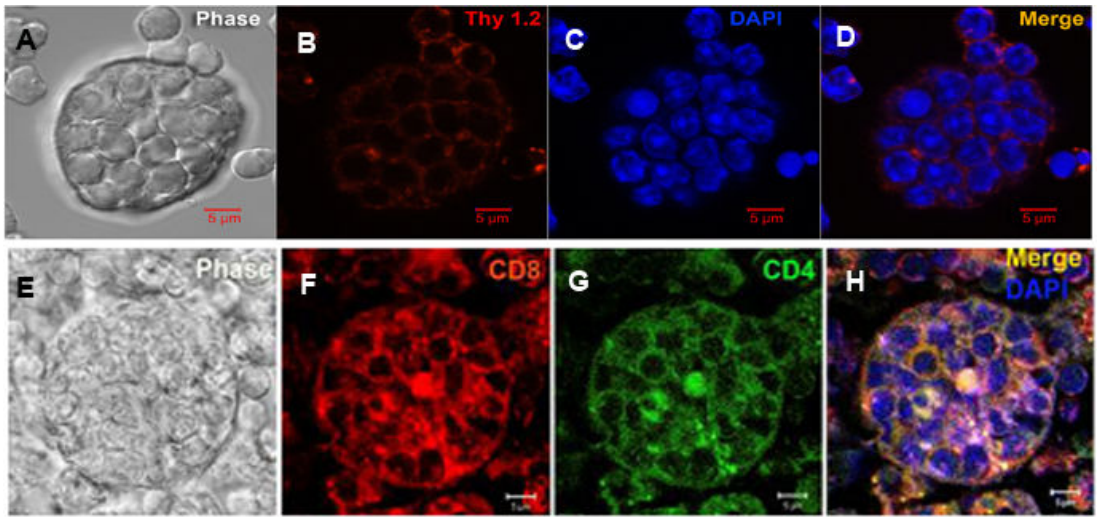

A
Phase
5 µm
B
Thy 1.2
5 µm
C
DAPI
5 µm
D
Merge
5 µm
E
Phase
F
CD8
G
CD4
H
Merge
DAPI

Figure 6. Internalization of thymocytes by tsTNC-1 in culture: SEM of tsTNC-1 cells co-incubated with thymocytes for up to 10 hours. (A) An individual TNC displaying membrane extensions polarized to one side of the cell. The body of the cell is indicated by "*b". (Time of incubation = 0 hour) (B) TNCs co-incubated with thymocytes for 1 hours display larger areas of membrane extensions. (C) Thymocytes are trapped within the folds of the membrane extensions after 2 hours of incubation (arrow). (D) Membrane extensions of TNCs wrapped around the surface bound thymocytes (arrow), seen after 2 hours of co-incubation. (E) After 4 hours of incubation, TNC no longer displays prolific membrane extensions but does contain internalized thymocytes. An arrow points to a partially internalized thymocyte. (F) Large TNC complex contains internalized thymocytes. (G) Confocal micrograph of tsTNC co-incubated with thymocytes for 10 hours. Prior to co-incubation the TNCs were labeled with CFDA (green) and the thymocytes were labeled with CMRA (red). The TNC nucleus (N) is visible as are internalized thymocytes (T).

Figure 6.

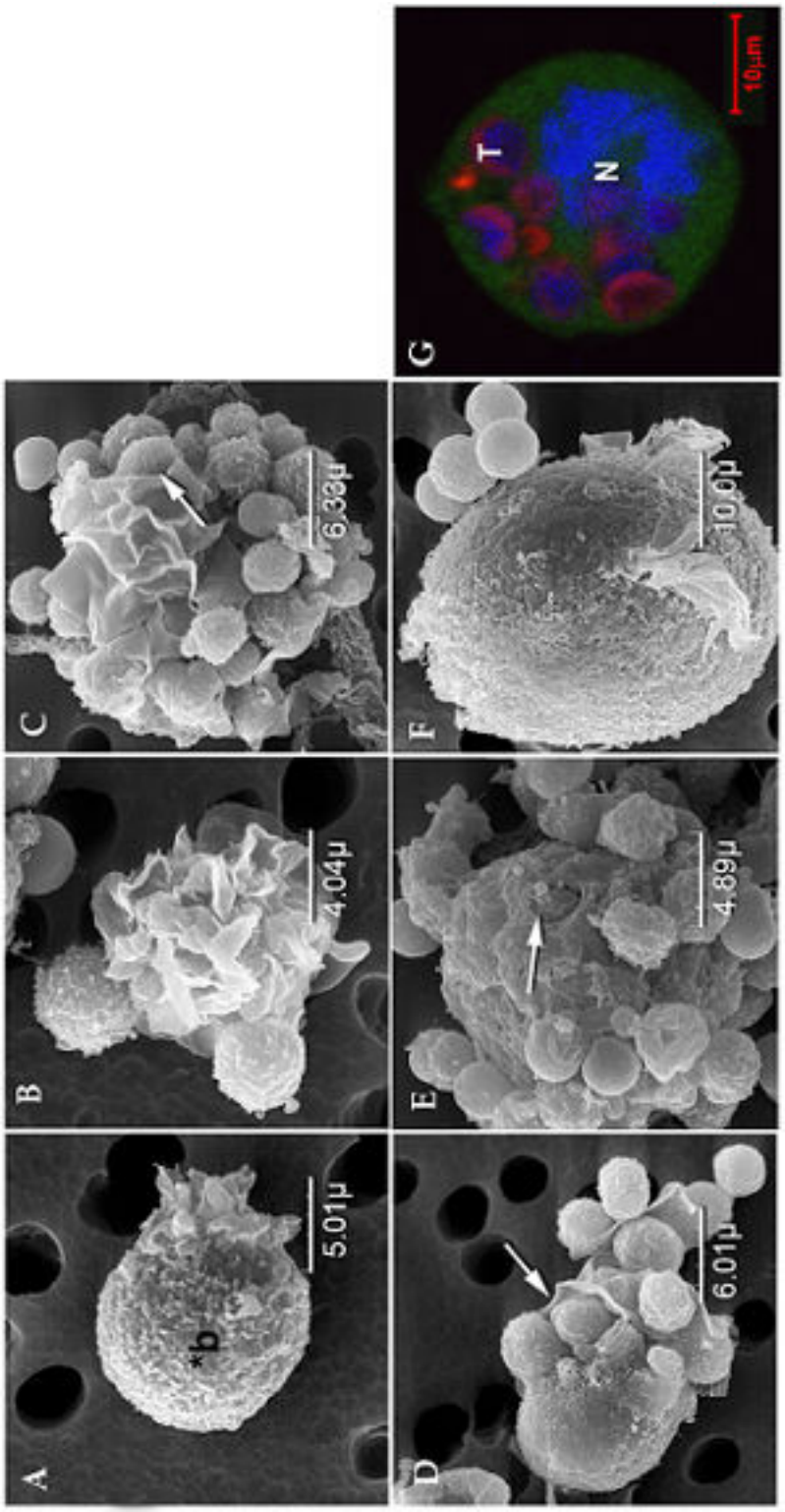


Figure. 7. Release of thymocytes by freshly isolated TNCs *ex vivo*: SEM of TNCs isolated from murine thymus and incubated over a 3 hours time period. (A) Thirty minute after isolation, the cell exhibits a circular morphology and layers of membrane folds (see inset). (B) Membrane begins to unfold and wraps around released thymocytes (see inset) (60 minutes post isolation). (C) Membrane layer is completely unfolds exposing thymocytes located within the fenestrae (inset) (90 minutes post isolation). (D) TNC membrane is completely unfolded (120 minutes post isolation). (E) (150 min) Thymocytes are then gradually released from the TNC. (F) No thymocytes are associated with the TNC. (180 min) (G) TEM image of a freshly isolated TNC corresponds to cell shown in panel A.

Figure 7:

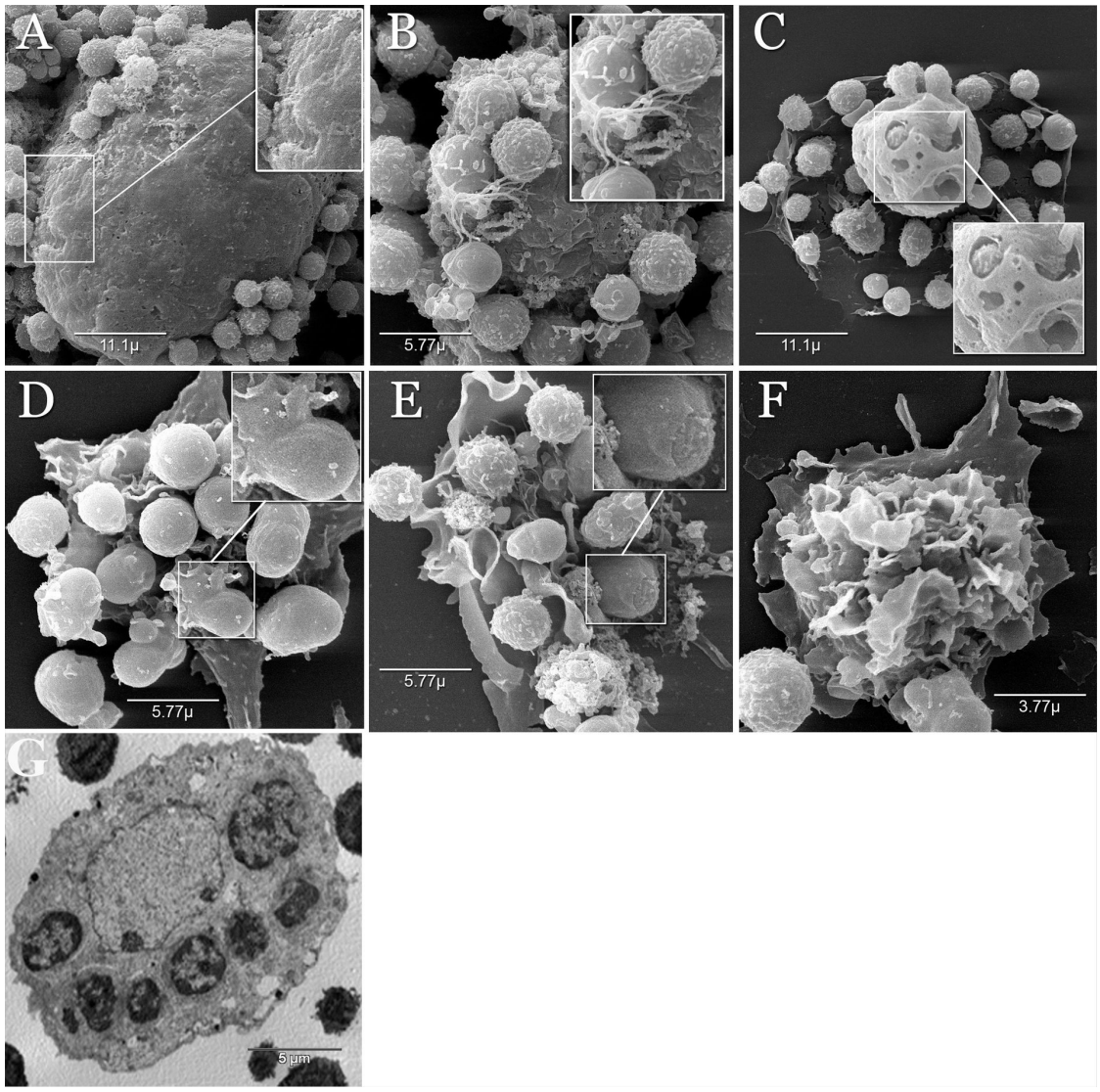


Figure 8. Transmission electron micrographs of TNCs containing internalized. (A) Freshly isolated TNC fixed and prepared for TEM analysis immediately following isolation. Membrane partially encloses a thymocyte (asterisk). (B) Thymocytes and tsTNC-1 cells were incubated for 10 hours and then analyzed by TEM. Thymocytes at different stages of apoptosis (insets 1 and 2) are visible within the cytoplasmic vacuoles

Figure 8:

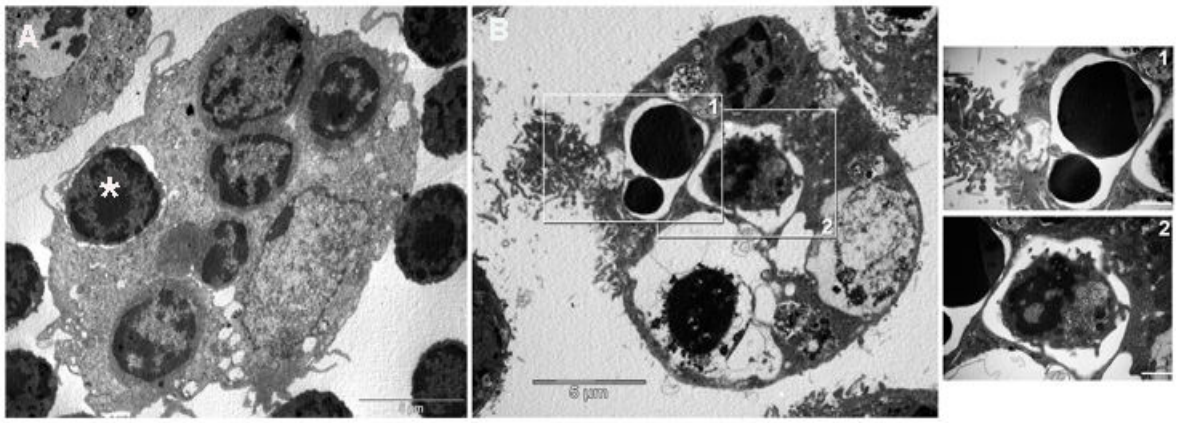


Figure. 9. Microscopic identification of membrane extensions and cage structure of TNCs during thymocyte binding and internalization: (A) Membrane extensions of TNCs visualized with SEM (panel 1) and TEM (panel 2). The circle in panel 2 corresponds to the membrane extensions seen in panel 1. TNC membrane extensions, equivalent to those in panels 1 and 2, are wrapped around a thymocyte (asterisk, panel 3) with a large cytoplasmic vesicle directly beneath (arrow). (B) The cage structure of TNCs visualized with SEM (panel 1), TEM (panel 2), and confocal microscopy of thymic section stained with pH91 (panel 3). The inset in panel 1 shows thymocytes residing within the cage like structure. The circle in panel 2 surrounds the cage as it appears in TEM. The arrow points to its SEM equivalent in panel 1. (C) Phase contrast video microscopy of TNC - thymocyte interaction. Thymocytes bound to the TNC surface are phase bright. Thymocytes trapped within the cage-like structure (circle, B and C) are phase dark. Thymocyte movement within cage was also detected. The arrow shows initial contact of thymocytes by membrane extensions from TNC. Membrane extensions pull thymocytes to the TNC complex.

Figure 9:

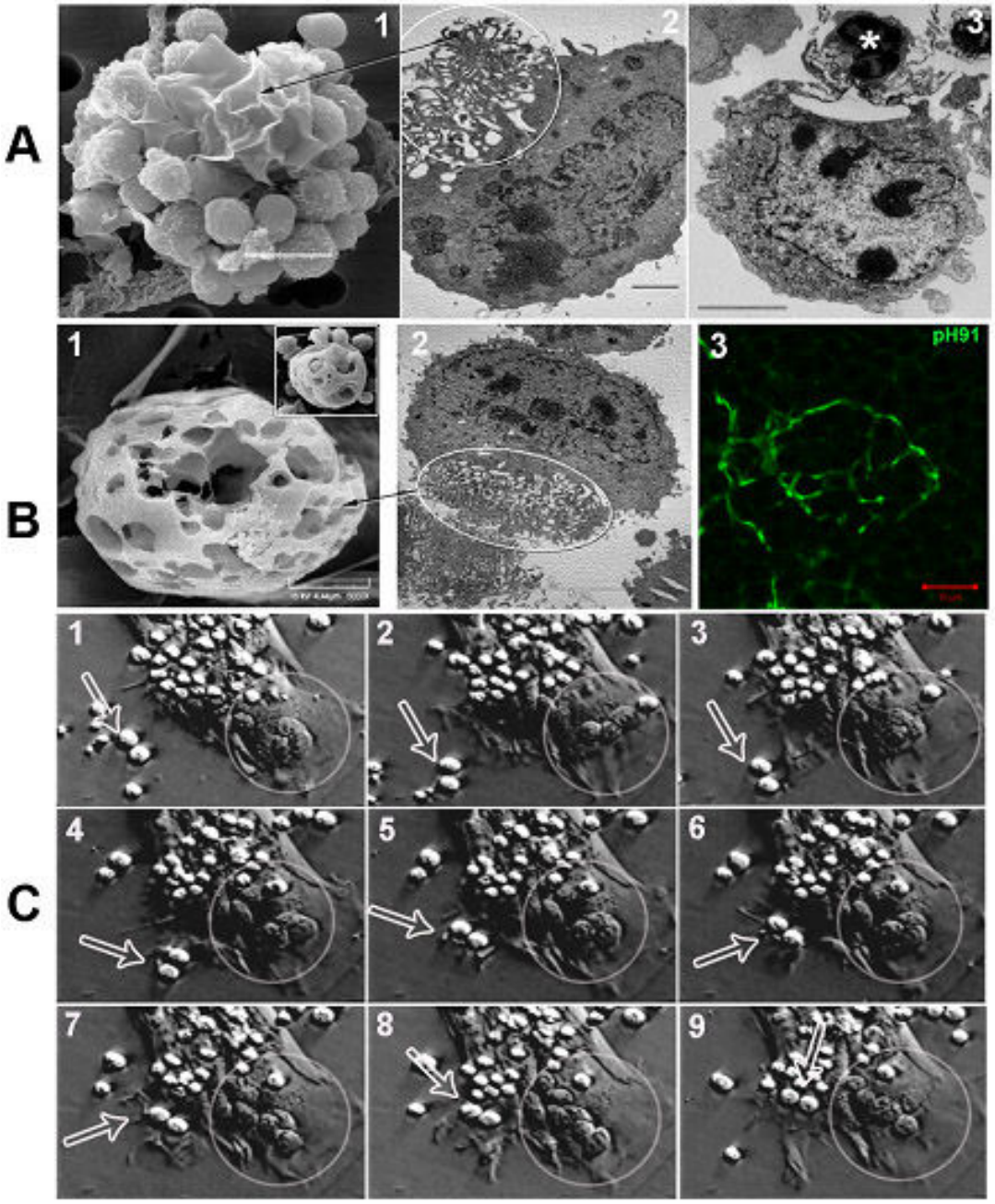


Figure. 10: TNCs participate in the MHC restriction of thymocytes: (A) Freshly isolated TNCs were stained with mAbs against CD8 (red), CD4 (magenta), and Thy 1.2 (green). Merge panel shows captured thymocytes to be $CD4^{+}CD8^{+}$. (B) Freshly isolated TNC stained with mAbs to αβTCR (red) and MHC Class II (green). Thymocyte nuclei are visualized using DAPI staining (blue). Insets show co-localization of MHC and αβTCR (yellow). (C) Co-localization of thymocyte lipid raft with αβTCR. tsTNC-1 cells were co-incubated with freshly isolated thymocytes for 4 hours. Cells were stained with cholera toxin subunit B (red) for presence of lipid rafts and mAbs to αβTCR (green) and pH91 (magenta). Merge images (insets 1 and 2) are orthogonal projections that show the contact areas between αβTCR and lipid raft (yellow). (D) Panels 1, 2, and 3 show controls for CD8, CD4, and Thy 1, respectively. Panels 5 and 6 show mAb controls for αβTCR and MHC class II.

Figure 10:

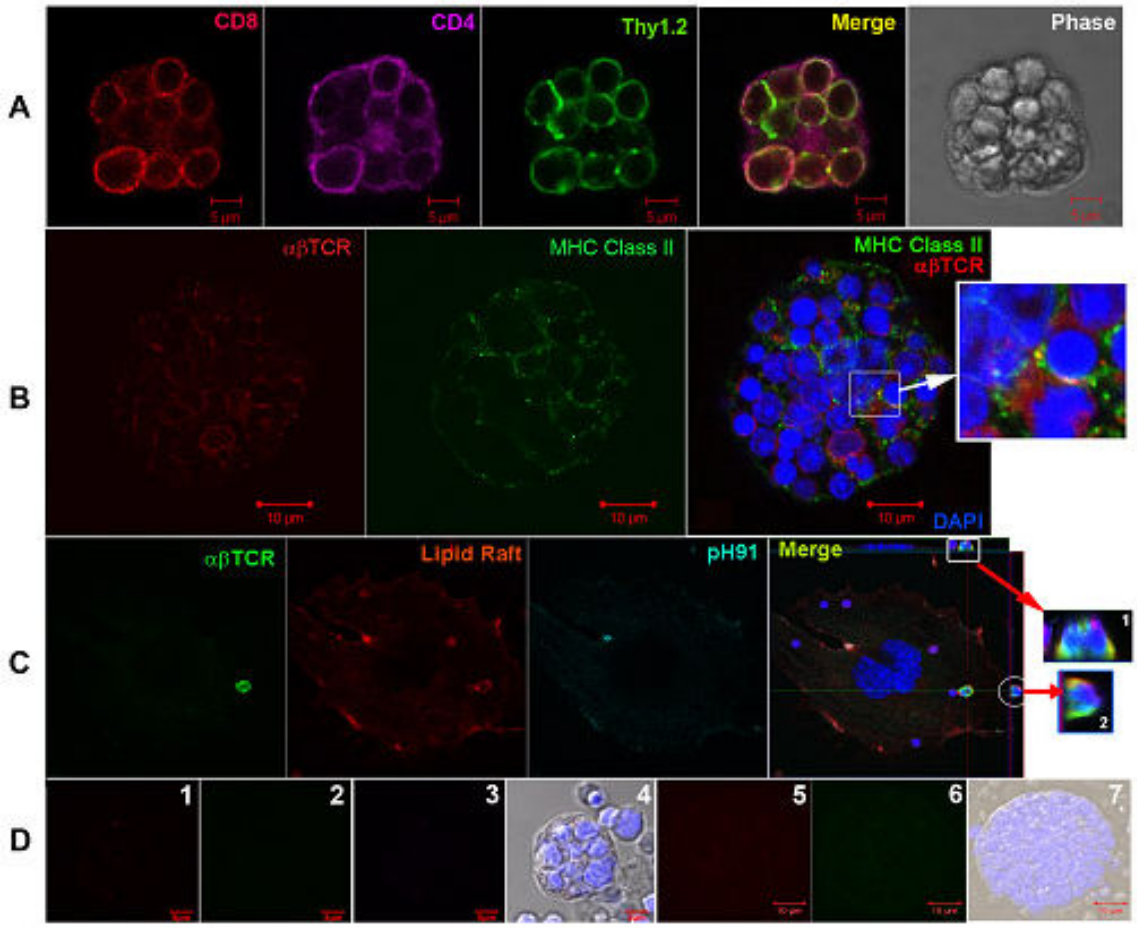

A
CD8
CD4
Thy1.2
Merge
Phase
B
αβTCR
MHC Class II
MHC Class II
αβTCR
DAPI
C
αβTCR
Lipid Raft
pH91
Merge
1
2
D
1
2
3
4
5
6
7

Figure. 11: K5 and K8 cytokeratin profiles of TNCs *ex vivo*: Freshly isolated TNCs stained with K5 (red) and K8 (green). The double positive TNC appears as yellow in the merge (Panel A). Original magnification: 40X. (B) Frequency of $K8^+$ and $K8^+K5^+$ populations in freshly isolated TNCs determined by manual counting of 1000 cyto-spun TNCs after immunostaining with anti-K5 and anti-K8 antibodies. TNCs stained with pH91 were also stained with K5 and K8 antibodies, cells were counted manually to determine the percentage of $pH91^+$ TNCs that were double positive for the keratins. Data for (A) and (B) is representative of three independent experiments .

Figure 11:

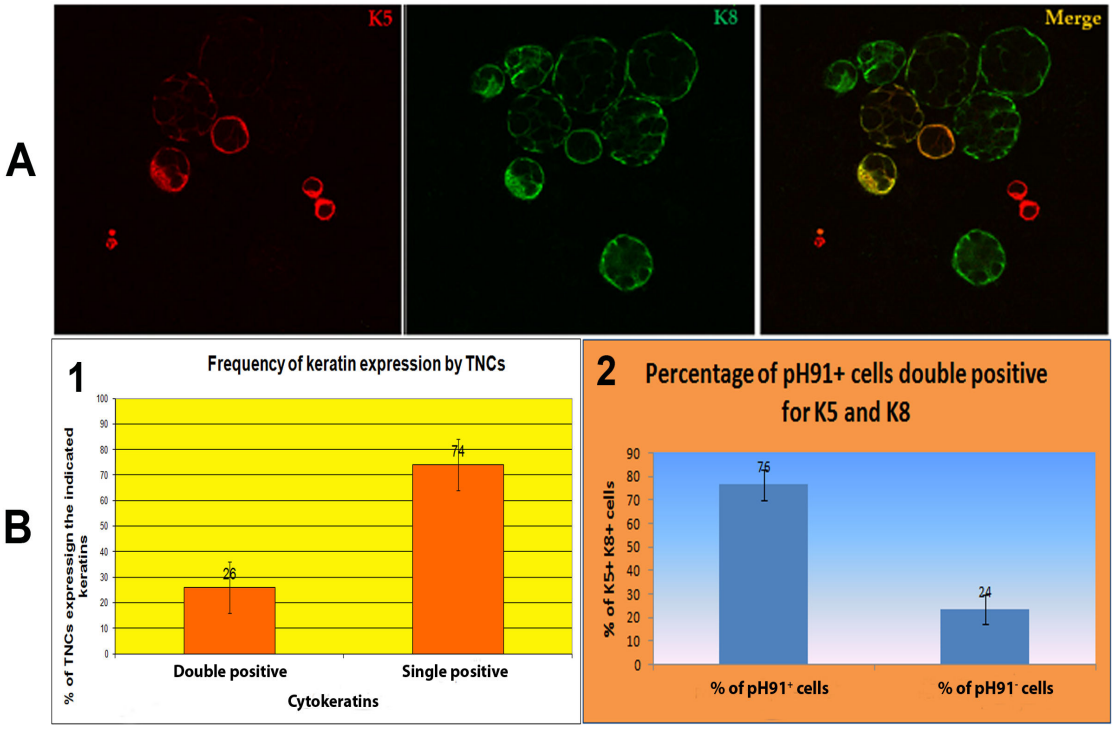

A
K5
K8
Merge
B
1
Frequency of keratin expression by TNCs
% of TNCs expressign the indicated keratins
26
74
Double positive
Single positive
Cytokeratins
2
Percentage of pH91+ cells double positive for K5 and K8
% of K5+ K8+ cells
75
24
% of pH91+ cells
% of pH91- cells

Figure. 12: **Expression of K8 and K5 by pH91-labeled TNCs:** (A) Thymic sections stained with anti-K8 (magenta), anti-K5 (red), and pH91 (green) Abs. The cortex and medulla are indicated by "C" and "M", respectively, and the cortico-medullary junction by a dotted line. The merge shows areas of co-localization. Original magnification: 40X. (B) Magnified area of section in panel A. The triple stained TNC exhibits a fenestrated structure. (C) Freshly isolated TNCs stained with anti-K8 (magenta), anti-K5 (red), and pH91 (green) Abs. Both $K8^{+}K5^{+}$ and $K8^{+}$ only TNCs are visible. (D) Shows secondary Ab controls for K8, K5, and pH91 as well as a phase image. Data is representative of three independent experiments.

Figure 12:

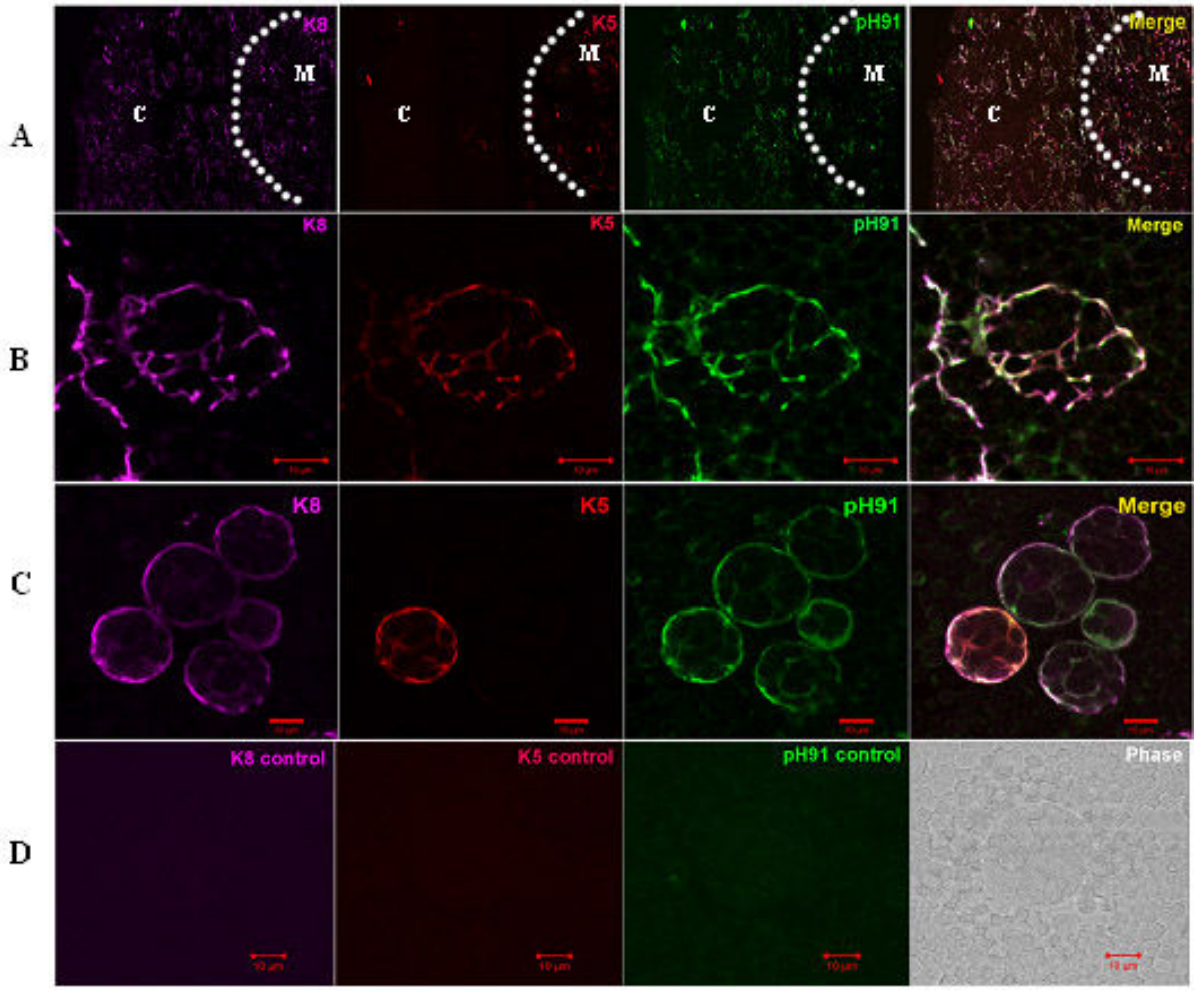

A
K8
K5
pH91
Merge
C
M
B
K8
K5
pH91
Merge
C
K8
K5
pH91
Merge
D
K8 control
K5 control
pH91 control
Phase

Figure. 13: Expression of p63 by pH91^{+} TECs: Panel A shows embryonic thymic anlagen at E12.5 saggital section. Sections were stained for p63 (Red), pH91 (Green), DAPI (Blue) and all the three together (Merge). Expression of p63 is seen in the nucleus of pH91 positive epithelial cells. Panels B-E shows labeling for p63 (Red), pH91 (Green), Merge at stages E13.5, E16.5, E18.5 and 2 weeks after birth respectively. Inset shows TECs that are positive for pH91 that express p63 (Arrow) and also TECs that are pH91^{+} but do not express p63 (Arrow head). Panle F shows the secondary and isotype control for p63 and pH91 respectively.

Figure 13:

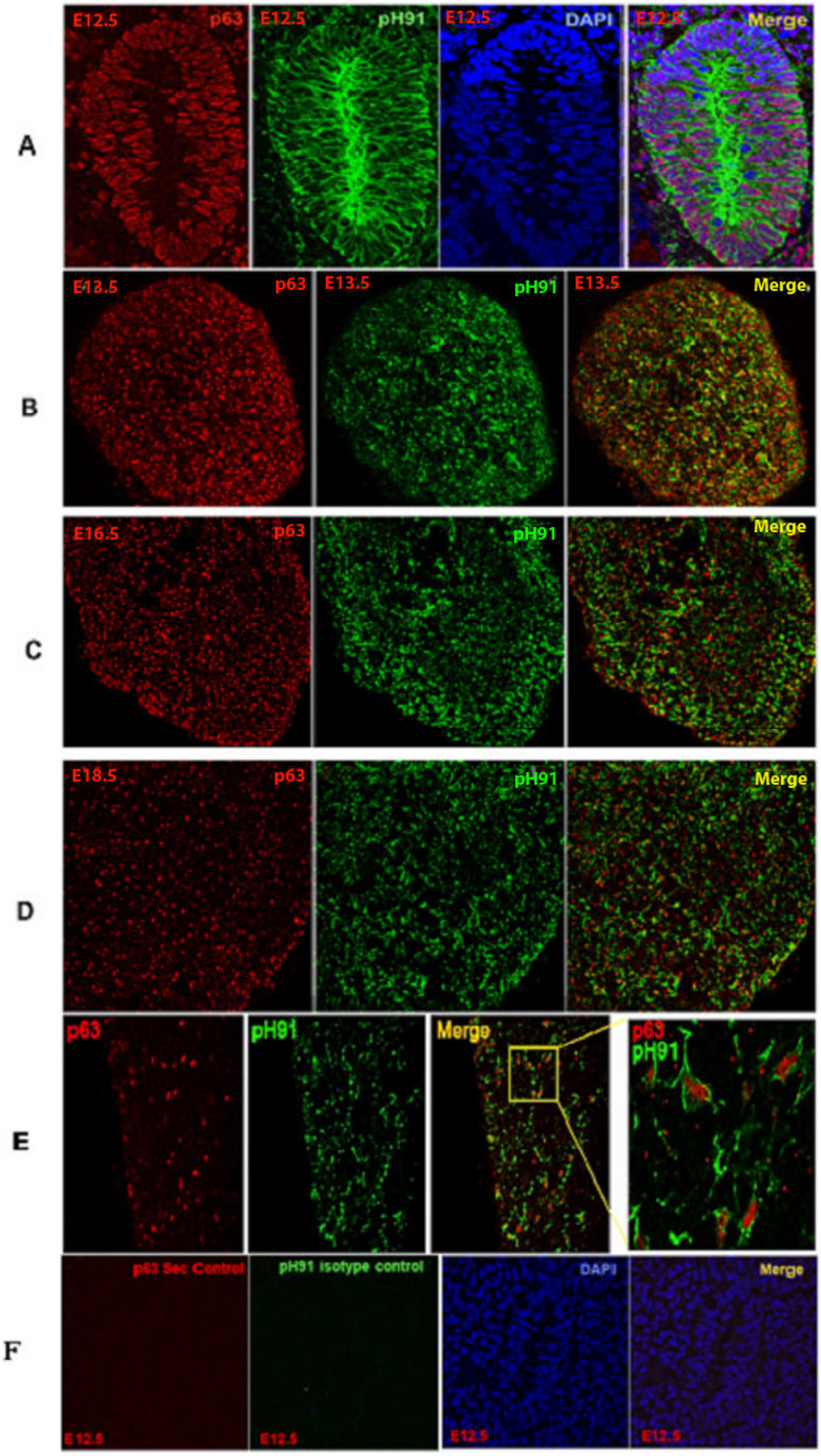

A
E12.5
p63
E12.5
pH91
E12.5
DAPI
E12.5
Merge
B
E13.5
p63
E13.5
pH91
E13.5
Merge
C
E16.5
p63
pH91
Merge
D
E18.5
p63
pH91
Merge
E
p63
pH91
Merge
p63
pH91
F
p63 Sec Control
pH91 isotype control
DAPI
Merge
E12.5
E12.5
E12.5
E12.5

Figure 14. Trp63 expression of freshly isolated TNCs. (A1-5) Confocal micrograph of a TNC labeled with Ab against p63 (red) and pH91 (green). Merge shows cell surface staining with pH91, nuclear localization of p63, and DAPI stained nuclei. Panel A5 shows that not all TNCs (white arrows) express p63. (B) Confocal micrograph of a TNC labeled with Ab against p63 (red), K5 (magenta), and K8 (green) with DAPI stained (nuclei). Merge shows cell surface staining with pH91, nuclear localization of p63, and DAPI stained nuclei. (C) TNCs stained with secondary controls for p63 (IgG) and pH91 (IgG_{2a}) with phase image. (D) TNCs stained with secondary controls for K5 and K8 showing phase image and DAPI stained nuclei

Figure 14:

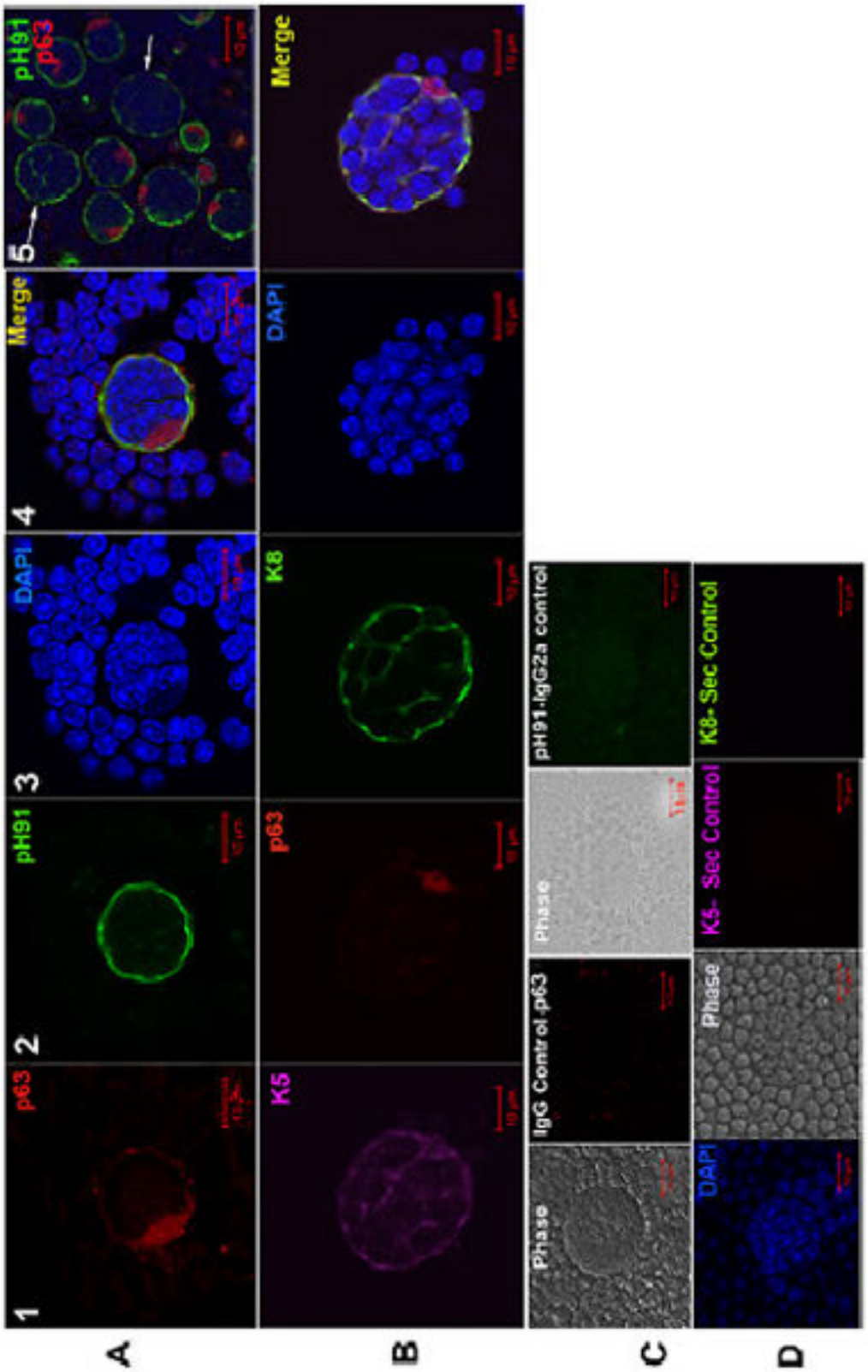

A
B
C
D
1
p63
2
pH91
3
DAPI
4
Merge
5
pH91
p63
K5
p63
K8
DAPI
Merge
Phase
IgG Control-p63
Phase
pH91-IgG2a control
DAPI
Phase
K5- Sec Control
K8- Sec Control

Figure. 15: Embryonic staining of pH91 with Foxn1: Panel A shows embryonic staining of Foxn1 (Magenta) along with pH91 (Green) in E11.5 mouse thymic primordium. Expression of Foxn1 is localized to the ventral region. pH91 expression is seen along the entire thymic and para-thyroid primordium. Panel B shows staining of E12.5 thymic anlagen with Foxn1 (Magenta) and pH91 (Green). Merge images show overlay of Foxn1 and $pH91^{+}$ cells. Both the sections are saggital in orientation. Panel C shows secondary antibody control for Foxn1 antibody (Magenta) and isotype control for pH91 (Green). Magnification 40X

Figure 15:

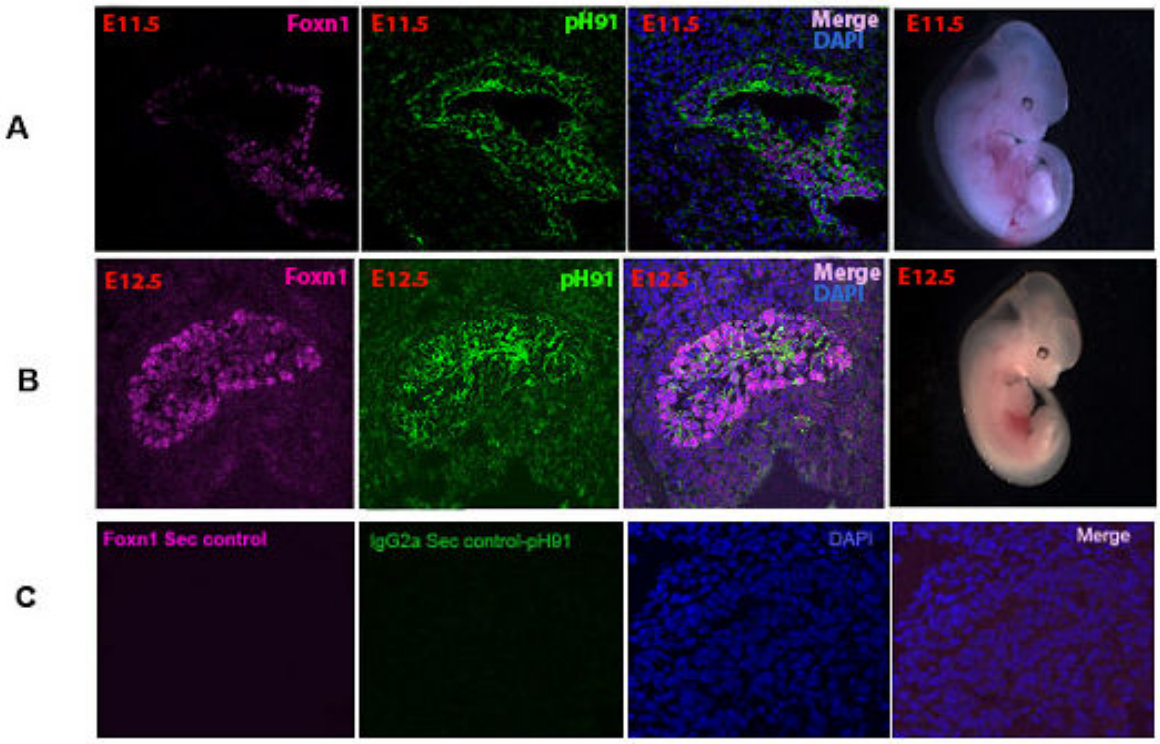

A
E11.5
Foxn1
E11.5
pH91
E11.5
Merge
DAPI
E11.5
B
E12.5
Foxn1
E12.5
pH91
E12.5
Merge
DAPI
E12.5
C
Foxn1 Sec control
IgG2a Sec control-pH91
DAPI
Merge

Figure. 16: Developmental expression of Foxn1 and pH91: Thymic section at embryonic stage E13.5 (Panel A), E16.5 (Panel B) and E18.5 (Panel C) were stained anti-Foxn1 antibody (magenta) and pH91mAb (green). Magnification 20X

Figure 16:

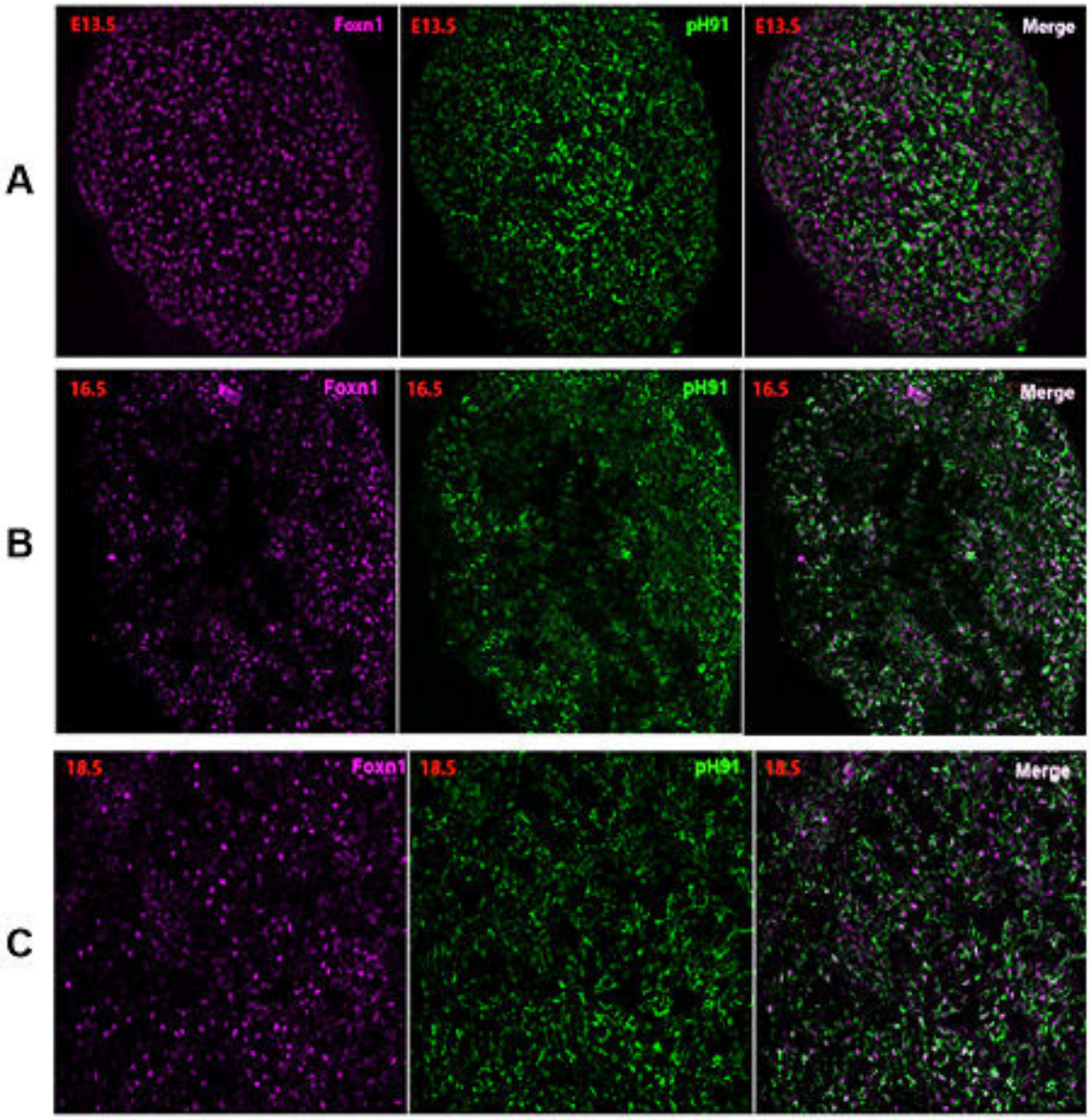

A
E13.5
Foxn1
E13.5
pH91
E13.5
Merge
B
16.5
Foxn1
16.5
pH91
16.5
Merge
C
18.5
Foxn1
18.5
pH91
18.5
Merge

Figure.17: Expression of Foxn1 along with pH91 in thymic tissue section two weeks after birth. Sections were stained with anti-Foxn1 (Panel A-Magenta) along with pH91 (Panel B-Green). Some of the pH91 cells can be seen not expressing any Foxn1 (Panel C, Arrows). A larger number of mTECs can be seen to express Foxn1 devoid of pH91 expression (Panel C-Arrow heads).

Figure 17:

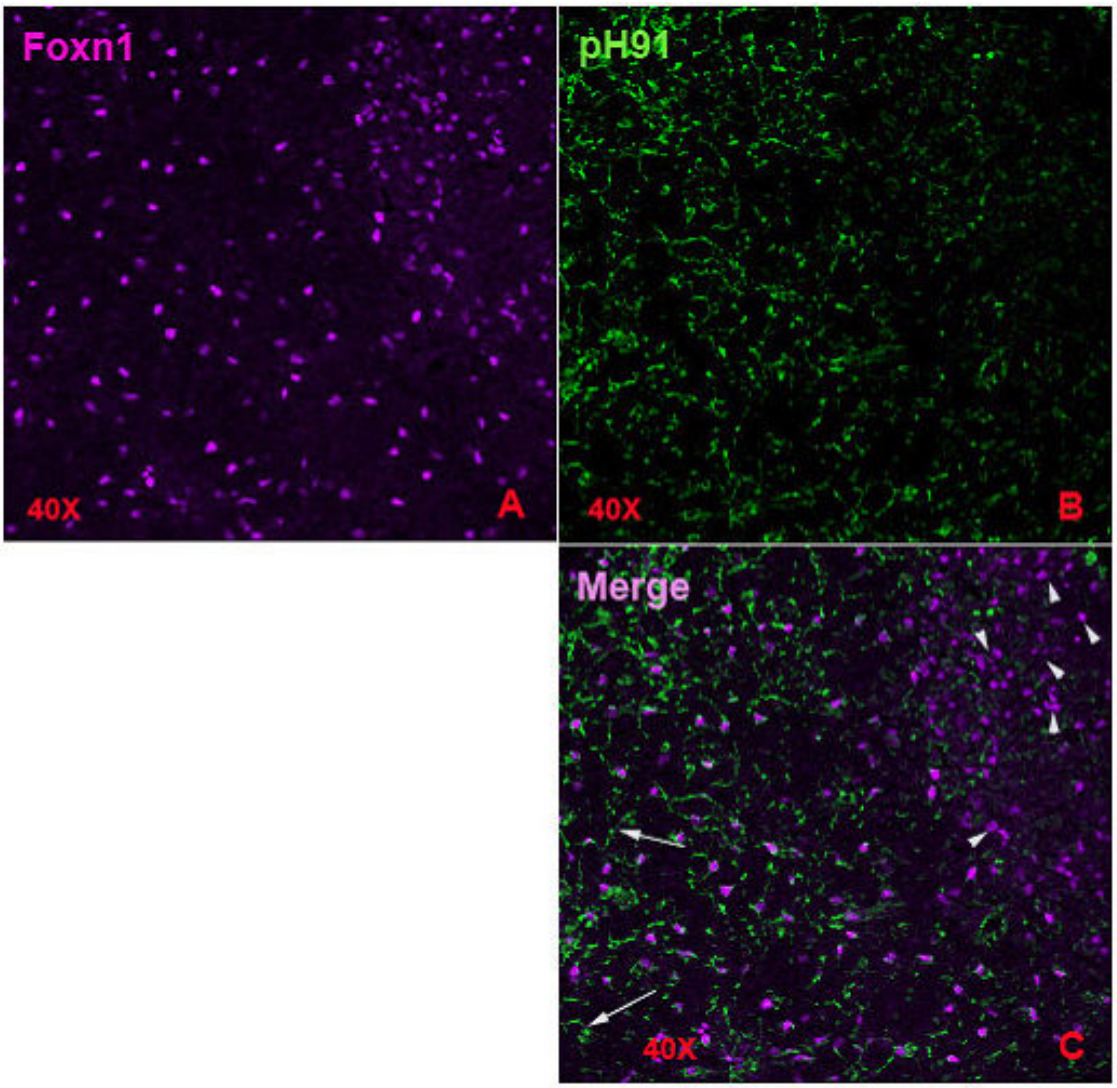


Figure.18: Expression of Foxn1 in TNCs *ex vivo*: Using antibodies against Foxn1, expression was observed *ex vivo* in the TNCs. Foxn1 nuclear expression is seen only in the TNC nucleus (Panel A). pH91 staining is seen in (Panel B), DAPI nuclear staining Panel C, Merge (Panel D). None of the thymocytes show Foxn1 expression. Magnification 40X.

Figure 18:

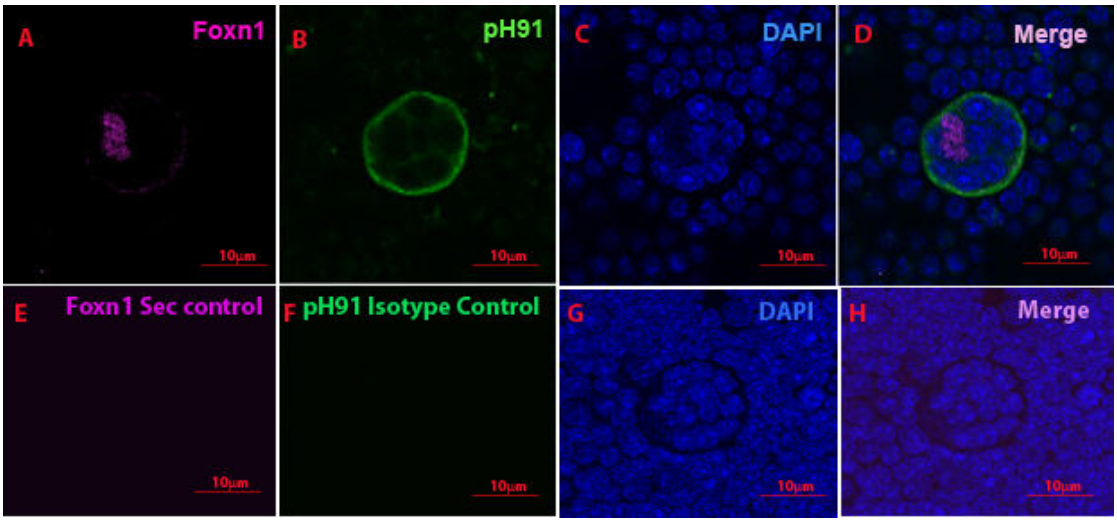

A
Foxn1
10µm
B
pH91
10µm
C
DAPI
10µm
D
Merge
10µm
E
Foxn1 Sec control
10µm
F
pH91 Isotype Control
10µm
G
DAPI
10µm
H
Merge
10µm

Figure. 19: Expression of Foxn1 and p63 in freshly isolated TNCs *ex vivo* and in thymic sections *in vivo*: Expression of Foxn1 and p63 was observed in freshly isolated TNCs by using antibodies against Foxn1, p63 along with pH91. Panel A-E show isolated TNC with Foxn1 expression (Panel A), p63 expression (Panel B), pH91 (Panel C), DAPI nuclear staining (Panel D) and Merge (Panel E). Expression of the transcription factors can be clearly seen in the TNC nucleus. Expression of these transcription factors was observed *in vivo in* adult mice. Differential expression was visible between these transcription factors in $pH91^{+}$ cells (boxes 1-3, read from left to right). Box 1 shows cells that express only Foxn1 and p63, box 2 shows two cells expressing Foxn1 and p63 and one cells expressing p63 only. Box 3 shows one cell expressing only Foxn1 without p63. Panels J-M represents controls. Magnifications 40X.

Figure 19:

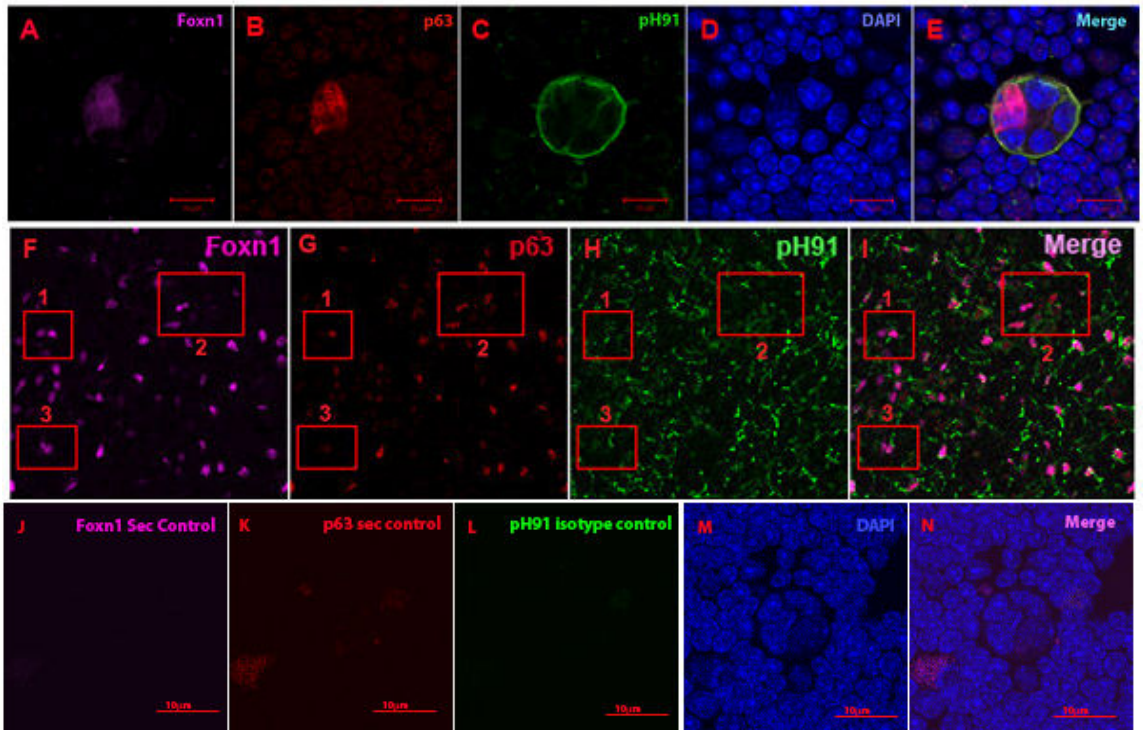
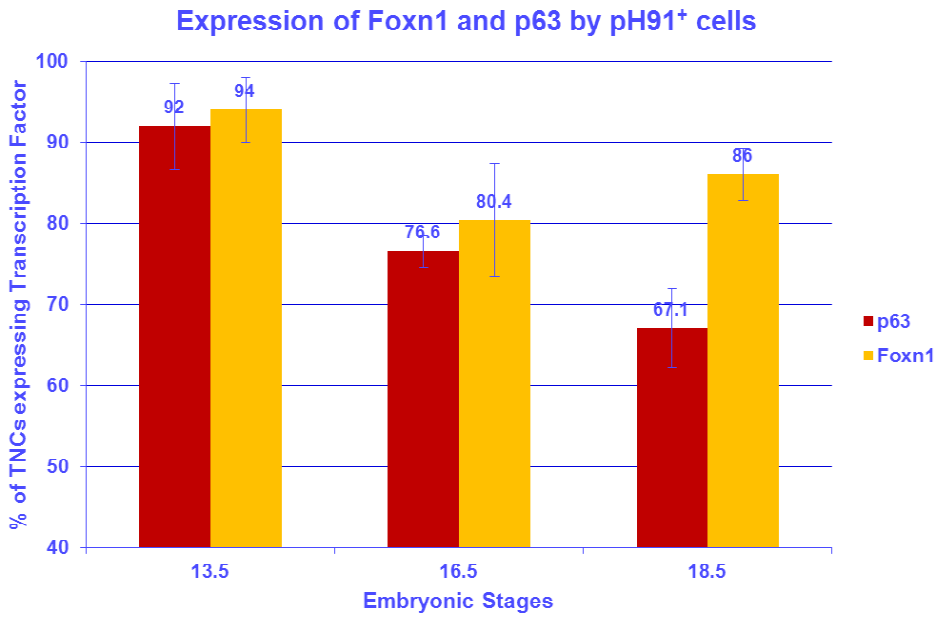

A
Foxn1
B
p63
C
pH91
D
DAPI
E
Merge
F
Foxn1
G
p63
H
pH91
I
Merge
1
2
3
J
Foxn1 Sec Control
K
p63 sec control
L
pH91 isotype control
M
DAPI
N
Merge

Figure. 20: Quantification of TNC/pH91^{+} cells expressing transcription factors Foxn1 and p63: TECs were isolated from thymi from C57BL/6 mice different embryonic stages of development E13.5, E16.5 and E18.5, cyto-spun onto slide and were quantified manually with respect to expression of Foxn1, p63 and pH91. Panel A shows the expression of p63 and Foxn1 by pH91^{+} cells embryonically. Panel B shows expression of p63 and Foxn1 by freshly isolated TNCs *ex vivo*

Figure 20:

A


Expression of Foxn1 and p63 by pH91+ cells
% of TNCs expressing Transcription Factor
100
90
80
70
60
50
40
92
94
76.6
80.4
67.1
86
13.5
16.5
18.5
Embryonic Stages
p63
Foxn1

B

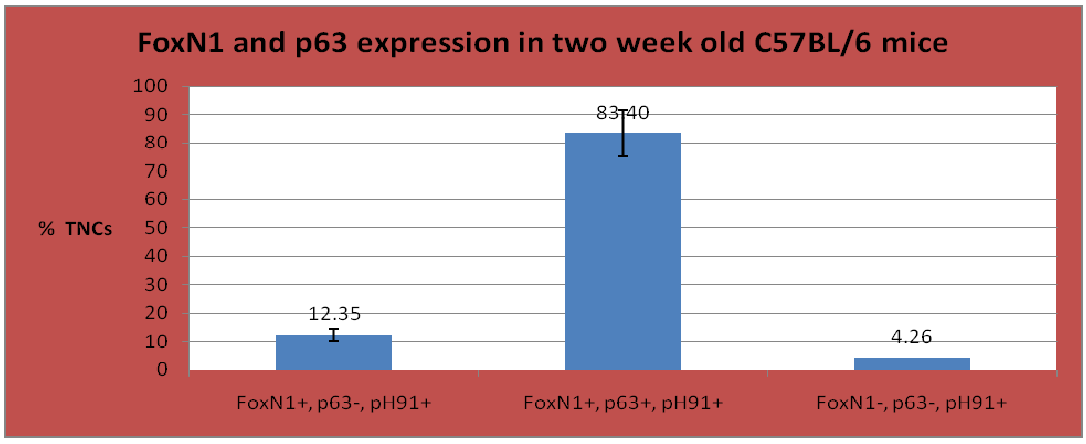

FoxN1 and p63 expression in two week old C57BL/6 mice
% TNCs
100
90
80
70
60
50
40
30
20
10
0
12.35
83.40
4.26
FoxN1+, p63-, pH91+
FoxN1+, p63+, pH91+
FoxN1-, p63-, pH91+

Figure. 21: Expression of pH91 and Oct4 in the embryonic tissue: Embryonic sections at stage E7.5 were stained for the expression of pH91 and Octamer binding transcription factor (Oct3/4). Panels A and D show expression of Oct4 (red). Panels B and E show expression of pH91 (green). pH91 expression can be seen at varying intensity on the surface of the cells (green) also expressing Oct4 (Arrows, Panel F). Some of the $pH91^{+}$ cells seen on the rim do not express Oct4 (Arrow Heads, Panel C and Panel F). Panels G and H represents the secondary control for Oct4 and isotype control for pH91 respectively. Results were representative of three independent repeats.

Figure 21:

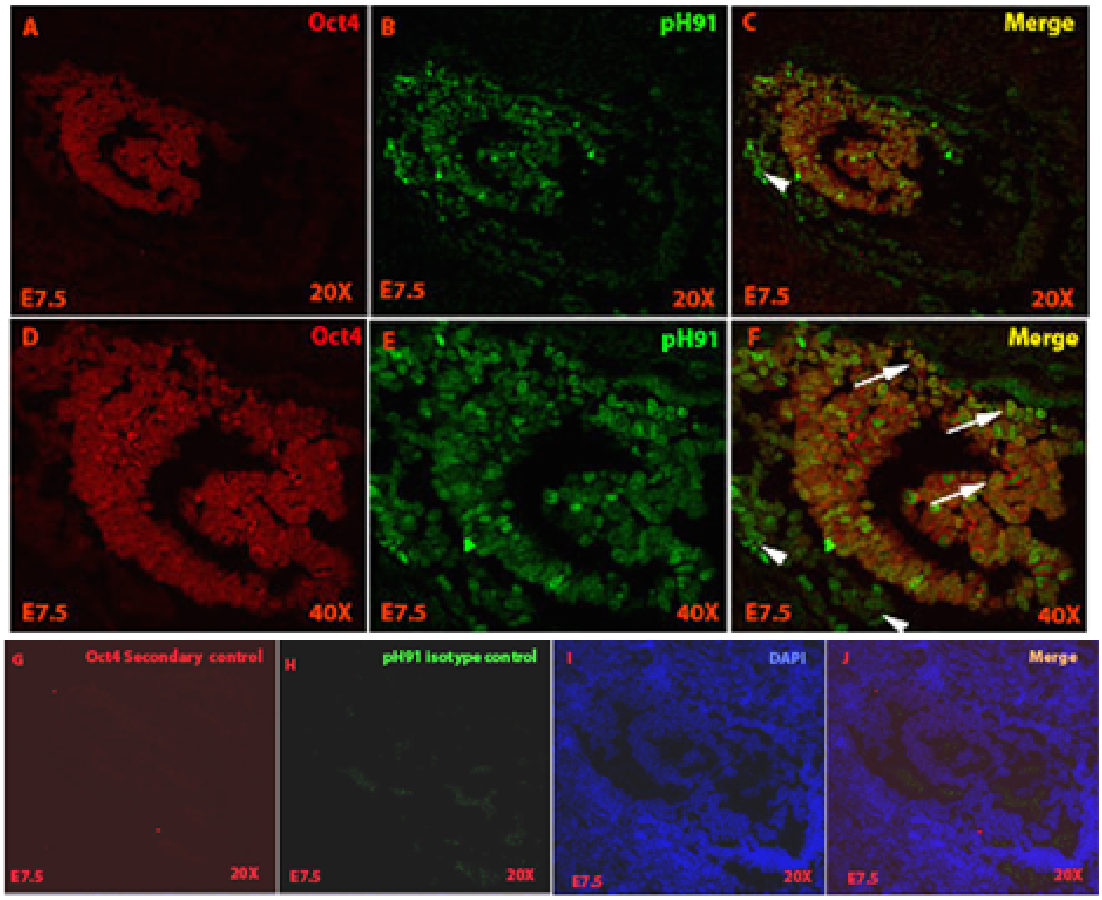

A
Oct4
E7.5
20X
B
pH91
E7.5
20X
C
Merge
E7.5
20X
D
Oct4
E7.5
40X
E
pH91
E7.5
40X
F
Merge
E7.5
40X
G
Oct4 Secondary control
E7.5
20X
H
pH91 Isotype control
E7.5
20X
I
DAPI
E7.5
20X
J
Merge
E7.5
20X

Figure. 22: TNC ontogeny and MHC Class II expression on their cell surfaces: Development of TNCs was followed embryonically beginning at E13.5. TECs were isolated from stages E13.5, E14.5 and E17.5. Isolated cells were cyto-spun onto slides and stained with pH91 antibody (Green) and DAPI nuclear stain (Blue). $PH91^{+}$ cells were seen at different stages of cell cycle, cells were seen to be bi-nucleated (Panel A, arrow), some of the pH91 positive cells were seen to have fibrous extensions (Panel B). Cells positive for pH91 were also seen to be undergoing cell division (Panel C). Panel D shows a small TNC complex isolated form E17.5 embryonic thymi, TNC seen with internalized $CD4^{+}CD8^{+}Thy1.2^{+}$ thymocytes. Panel E shows FACS data for the expression of pH91 and MHC class II by isolated TECs at embryonic stages E13.5, E16.5 and E18.5. Data is representation of three repeats for the given time points. Panels A-C magnification 40X, Panel D magnification 63X

Figure 22:

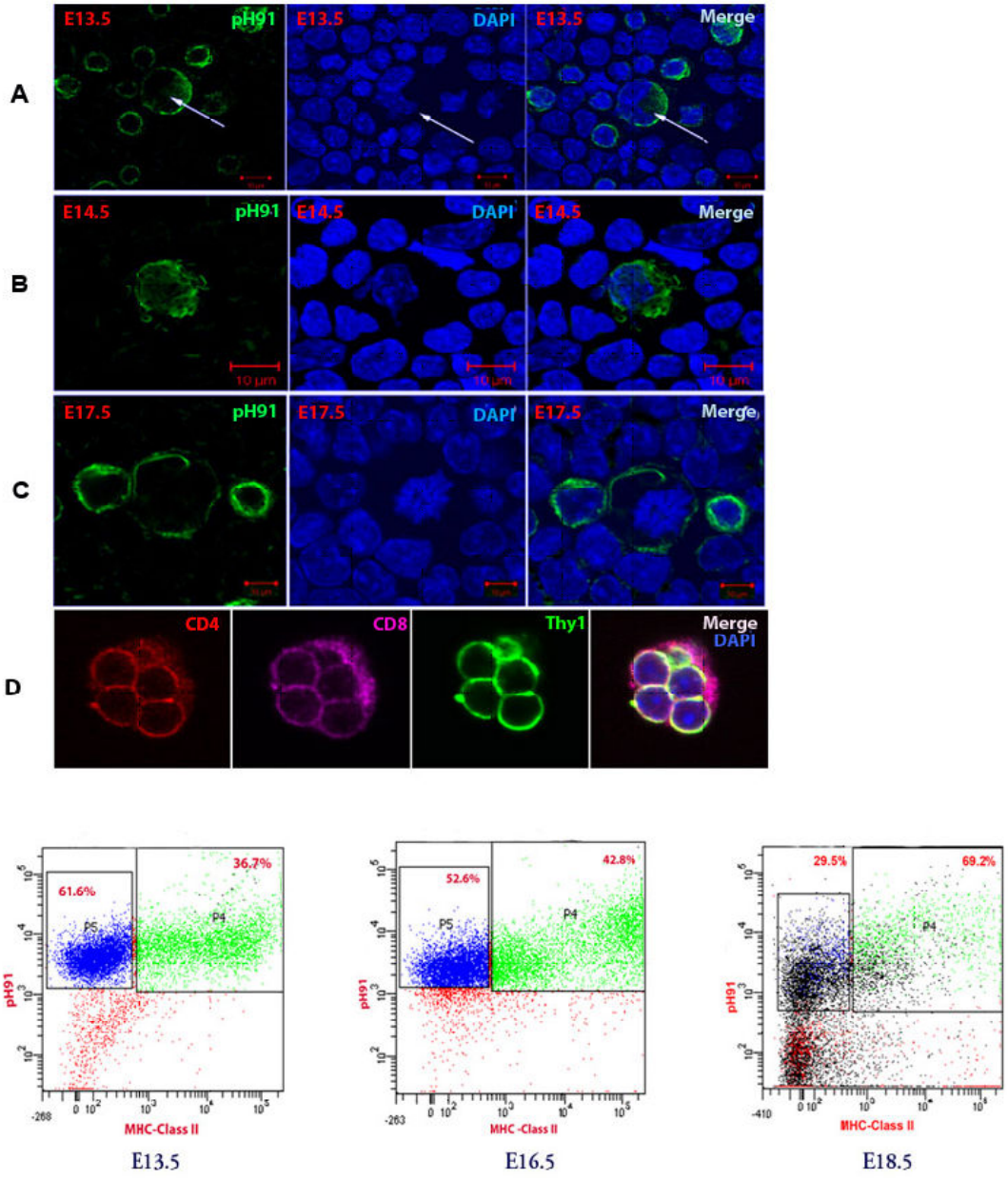
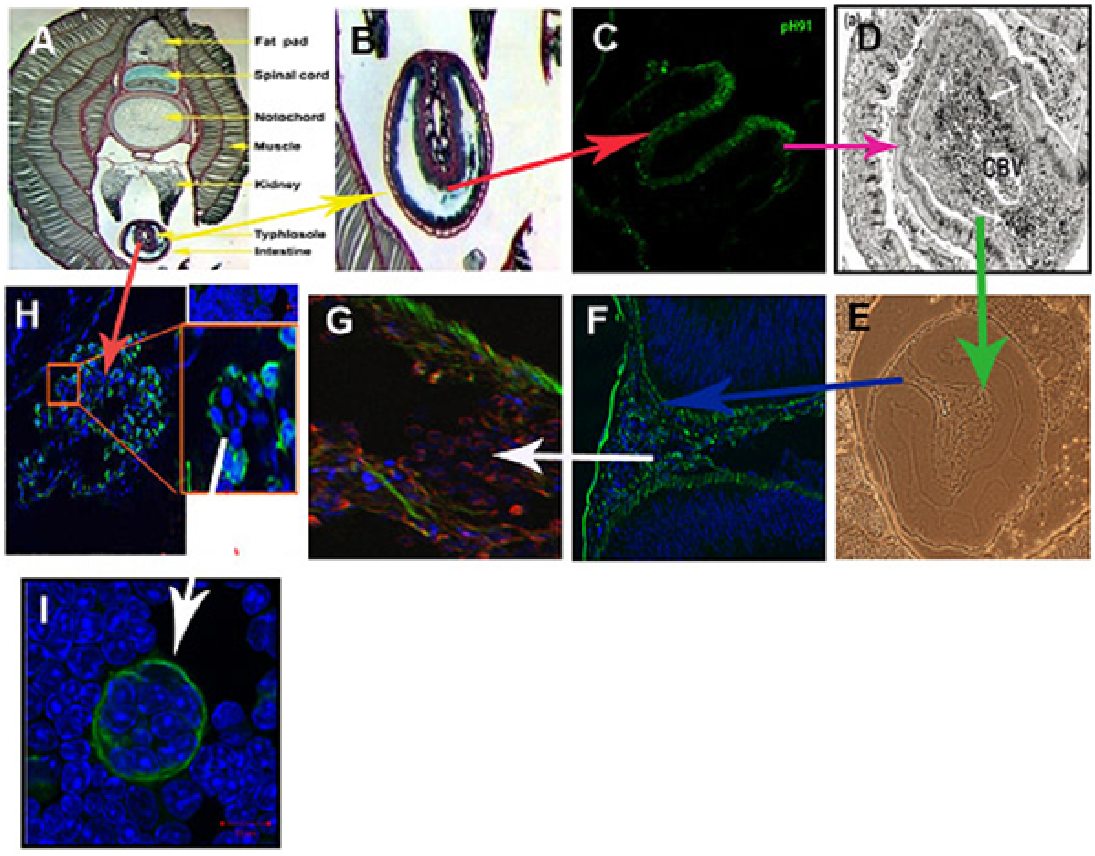

A
E13.5
pH91
E13.5
DAPI
E13.5
Merge
B
E14.5
pH91
E14.5
DAPI
E14.5
Merge
C
E17.5
pH91
E17.5
DAPI
E17.5
Merge
D
CD4
CD8
Thy1
Merge
DAPI
E
pH91
MHC-Class II
61.6%
36.7%
P5
P4
52.6%
42.8%
29.5%
69.2%
E13.5
E16.5
E18.5

Figure. 23: Detection of pH91+ cells in the typhlosole of *Lamprey:* Typhlosole is one of the immune structures emanating from a lamina of mesenchymal cells located between the dorsal aorta and the upper intestine in Lampreys (Panel A-H). Panel C shows typhlosole stained with pH91 antibody (green). Panel D shows TEM micrograph of the typhlosole with blood cells (white arrow), central blood vessel (CBV). Panel E shows the phase image of the typhlosole as seen in Panel D. Panel F shows the typhlosole loop stained with pH91(green). Panels G shows the region interior to the loop of typhlosole stained with pH91 (green), lymphocytes were stained with anti-rat Thy1 antibody (Panel G, Red). Structures inside the typhlosole seen in panel D, arrow are stained with pH91(Panel H). Inset shows complex-like structures with cells enclosed in it. These structures appear similar to the TNC complexes isolated from mice (Panel I). *(Panel A, courtesy Nil Rata Saha. Panel D Ref: Curr Opin Immunol. 2007 October; 19(5): 535–541)*

Figure 23:


A
Fat pad
Spinal cord
Notochord
Muscle
Kidney
Typhlosole
Intestine
B
C
pH91
D
CBV
E
F
G
H
I

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