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Cell-mediated peripheral tolerance in lymph nodes

Buněčně zprostředkovaná periferní tolerance v lymfatických uzlinách

Bakalářská práce

Vedoucí závěrečné práce/Školitel:

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Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracoval samostatně a že jsem uvedl všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

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Podpis

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Abstract

Tolerance of immune system to body-self constituents is a crucial issue for immunologists to solve. While the mechanisms of central tolerance are now described to well extent, antigen-specific tolerance mechanisms on immunological periphery are just beginning to be revealed, characterized and appreciated. Recently, novel models of peripheral tolerance emerged. Particularly, a model based mostly on lymph node stromal cells could be of profound importance, since it provides answers to some fundamental questions in tolerance immunology. So far, no review paper highlighting these newly discovered roles of lymph node stromal cells was published. Therefore, in this study we summarize data covering this topic, published up-to-date. Further, this text provides a basic overview of lymph node functional anatomy. To better illustrate the topic, we also show some experimental evidence demonstrating lymph node architecture and the localization of extrathymic *Aire*-expressing cells, one of the lymph node-resident populations, recently implicated in peripheral tolerance maintenance.

Keywords

Peripheral tolerance, lymph node stromal cells, fibroblastic reticular cells, lymphatic endothelial cells, blood endothelial cells, extrathymic *Aire*-expressing cells

Abstrakt

Tolerance imunitního systému vůči vlastnímu tělu je zásadní otázka, kterou musí imunologie vyřešit. Přestože mechanismy centrální tolerance jsou nyní už detailně popsány, mechanismy antigenně specifické tolerance na imunologické periferii teprve začínají být objevovány, popisovány a doceňovány. V nedávné době byly objeveny nové modely periferní tolerance. Zvláště model, který je založený na stromálních buňkách lymfatické uzliny, by mohl mít zásadní význam, jelikož přináší odpovědi na některé základní otázky v imunologii tolerance. Dodnes nebyl publikován žádný přehledový článek zaměřený na tyto nově objevené role stromálních buněk lymfatické uzliny. Proto v této práci shrnujeme doposud publikovaná data k tomuto tématu. Dále tento text poskytuje základní přehled funkční anatomie lymfatické uzliny. Pro lepší ilustraci tématu jsme zahrnuli taktéž jistá experimentální data demonstrující architekturu lymfatické uzliny a lokalizaci buněk exprimujících *Aire* mimo thymus, jedné z buněčných populací sídlících v lymfatických uzlinách, u nichž byla nedávno navržena účast na ustavení a udržování periferní tolerance.

Klíčová slova

Periferní tolerance, stromální buňky lymfatické uzliny, fibroblastické retikulátrní buňky, lymfatické endoteliální buňky, endoteliální buňky stěny cév, buňky exprimující *Aire* mimo thymus

Contents

List of abbreviations

A33	glycoprotein A33
AFP	α fetoprotein
Aire	autoimmune regulator
APCs	antigen presenting cells
BCR	B cell receptor
BECs	blood endothelial cells
BM	bone marrow
BSA	bovine serum albumin
DCs	dendritic cells
DN	double negative
Eif4g3	eukaryotic translation initiation factor 4 gamma 3
eTACs	extrathymic *Aire*-expressing cells
FCS	fetal calf serum
FRCs	fibroblastic reticular cells
Gad67	glutamate decarboxylase 67kDa isoform
HEVs	high endothelial venules
iFABP	intestinal fatty acid binding protein
Igrp	islet-specific glucose-6-phosphatase-related protein
KO	knockout
LECs	lymphatic endothelial cells
LN	lymph node
LNSC	lymph node stromal cells
Mart1	Melanoma antigen recognized by T cells 1
MHCI	major histocompatibility complex class I
MHCII	major histocompatibility complex class II
Mlana	Melanoma antigen recognized by T cells 1
MRCs	marginal reticular cells
mTECs	medullary thymic epithelial cells
NOD	non-obese diabetic
p	peptide
PBS	phosphate base saline
PFA	paraformaldehyde
pLNs	peripheral lymph nodes
PLP	proteolipid protein
Ret S	retinal s antigen
Rrad	Ras-related associated with diabetes
RT	room temperature
SA-PE	streptavidin coupled phycoerytrin

SCS	sub-capsular sinus
SLO	secondary lymphoid organs
TCR	T cell receptor
tOVA	truncated cytosolic form of chicken ovalbumin
TRA	tissue restricted antigen
Tregs	regulatory T cells
WT	wild type
αSMA	α smooth muscle actin

Introduction

Adaptive immunity provides body with extremely efficient system to specifically target and subsequently remove invading pathogens. This specificity is achieved via two types of cellular effectors of adaptive immunity, T and B cells and mediated via their antigen-specific receptors, T cell receptor (TCR) and B cell receptor (BCR), respectively. However, during the lifespan of an organism, its immune system can be challenged by vast diversity of pathogens, each of which can display a completely different set of immunologically-relevant antigens. In order to anticipate this diversity, variable parts of TCRs and BCRs are somatically rearranged by a stochastic process resulting in a tremendously diversified receptor repertoire able to recognize nearly every conceivable antigen. On the other hand, this remarkable receptor variability poses for immune system a problem of profound importance. Since TCRs and BCRs specific for body-self antigens are inevitably generated in this process, how is it achieved, that T cells and B cells do not recognize body-own tissues? Because recognition of body-own antigens may lead to fatal autoimmunity (Oliver et al., 2003), mentioned distinction becomes really a matter of life and death. To ensure that immunity is not directed against endogenous self-specific antigens, mammalian immune system is endowed with a set of efficient mechanisms that are collectively referred to as immunological tolerance. Since not much is known about mechanisms of tolerance enforcement to B cells, only T cell tolerance will be discussed further.

Majority of T cell mediated immune responses are initiated upon the recognition of the complex composed of a short peptide (p) presented in the context of the major histocompatibility complex class I or II (pMHCI or II) by TCR of cognate $CD8^+$ or $CD4^+$ T cells, respectively. Thymic education ensures, that T cells pass several checkpoints leading to T cell tolerization towards self-antigens as well as selection of only those T cells efficient enough in pMHC recognition. Paradoxically, the entire peripheral T cell repertoire is in the thymus selected on self-peptides. It means that while all T cells are self-reactive, the strength of their selfreactivity is crucial for their survivor or elimination during the thymic selection. This selection is performed in two consecutive steps, ensuring firstly T cell functionality and secondly T cell harmlessness. In the first step, T cells are tested to recognize pMHCI or II (with body-self peptide), in a process called positive selection. Those T cells which have too low affinity for any pMHCI or II are destined to die by neglect (Klein

et al., 2014). In a second step, T cells possessing too high affinity for pMHCI or II complexes and thus representing potentially self-reactive and harmful T cells, are deleted in a process called negative selection (Liston et al., 2003). Alternatively, those high-affinity self-reactive T cells can be phenotypically altered and functionally diverted to become regulatory T cells (Tregs) (Malchow et al., 2013). These cells can serve important immunosuppressory functions in the periphery. Collectively, these mechanisms of thymic positive and negative selection are termed central tolerance. Above described processes were summarized into the affinity model of thymic selection in which the fate of each particular T cell is decided by its affinity/avidity for thymic pMHC complexes, as described above. However, recently it became clear that the overall mechanism might not be that simple, since the processes of positive and negative selection are spatially and temporally separated, taking place in thymic cortex and medulla, respectively, and mediated by distinct populations of thymic epithelial cells (TECs) (Klein et al., 2014).

Since of presence of each particular antigen in thymus is needed in order to establish a central tolerance to it, the mechanism of central tolerance faces a logistic challenge. Specifically, how to ensure tolerance to antigens, whose expression is anatomically restricted to one or few extrathymic tissues? One mechanism ensuring tolerance to these so called tissue restricted antigens (TRAs; they are defined as self-constituents expressed in less than 5 of 60 tissues as assessed from available expression catalogues (Klein et al., 2014)) emerged with the identification of autoimmune regulator (AIRE) protein and its mouse homologue Aire.

AIRE was initially identified as a gene, mutations in which lead to a rare genetic human autoimmune disorder known as autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) (Consortium, 1997; Nagamine et al., 1997). Later it was determined that Aire is responsible for promiscuous gene expression of TRAs in the medullary thymic epithelial cells (mTECs) (Anderson et al., 2002). Furthermore it was shown, that Aire is essential for central tolerance enforcement to TRAs (Liston et al., 2003). Although a precise mechanism by which Aire drives expression of those TRA is still not completely clear, it was proposed, that Aire preferentially binds to promotors of transcriptionally silenced genes, which it recognizes by low levels of trimethylation of Histone 3 at lysin 4 and/or sequence-specific DNA binding. To these regulatory regions then Aire recruits transcription

elongation factor b complex and other factors, which together promote target gene expression. Presumably, those genes, which are transcriptionally silenced should, at least in part, represent genes encoding TRAs. Aire TRA specificity could be further ensured by sequence-specific DNA binding ability. Conclusively, Aire provides central tolerance with antigens, essentially needed for negative selection, which would otherwise be absent in the thymus (Peterson et al., 2008).

Recently however, it has been shown that although central tolerance is highly efficient, it is also incomplete, since some self-reactive T cells, particularly those with a low affinity for self-antigens, continuously escape to the immune periphery (Bouneaud et al., 2000). Furthermore, when Tregs, which normally suppress self-reactive T cells in the periphery, are conditionally depleted, dramatic autoimmunity develops in both newborn and adult mice (Kim et al., 2007). Together these data provide evidence that central tolerance alone is not sufficient to maintain complete T cell tolerance. These findings led to a newly formulated concept of mechanisms underlining antigen-specific peripheral tolerance, which would presumably keep those peripheral autoreactive T cells in check. Since the main secondary lymphoid organs (SLO), i.e. lymph nodes (LNs), spleen, Payer's Patches, and gut-associated lymphoid tissue, represent sites that are continuously percolated by naïve T cells, they logically became organs most suitable to study peripheral tolerance. Because LNs are arguably the best characterized member of SLO in terms of the identity and function of cell subpopulations which play a role in peripheral tolerance, these immune organs are the main focus of this work. However, to fully appreciate their roles in peripheral tolerance, the following sections, will first shortly review basic facts on LN morphology and functional anatomy.

Theoretical part

Lymph node morphology and functional anatomy

Lymph node integration into lymphatic and circulatory systems

The primary function of LN is to bring together naïve lymphocytes with their cognate antigens. In order to accomplish this goal, it is vitally needed to present a collection of antigens from surrounding tissues and allow lymphocytes to access it. As a result, LN creates one of few crossroads between lymph and blood circulation. The lymph mainly serves as the source of antigens and antigen presenting cells (APCs) while the blood brings lymphocytes into the LNs. This system greatly increases the efficiency of antigen scanning by lymphocytes, in comparison with patrolling all tissues one by one (Gretz et al., 1997). Each LN provides lymphocytes with representative set of antigens from tissue, which it drains, presented on APCs. Drained lymph can also bring the information about any potential inflammatory events. This information may in turn increases homing of leukocytes into LN (Swartz et al., 2008; von Andrian and Mempel, 2003). Thus, this system enables lymphocytes not only to monitor solely LNs but, coupled with inflammatory signals, it also ensures that lymphocytes enter preferably those LNs where they are most needed and provides them with effective APCs.

Basic morphological features of lymph node

Whole LN can be viewed as an extension of lymphatic vessel. It is enwrapped in the outer wall termed the capsule. Under the capsule spans sub-capsular sinus (SCS). Into the SCS enters one or more afferent lymphatic vessels on one side. On the other side of LN, referred to as the hilar, SCS divides into medullary sinuses. From this place exits usually only single efferent lymphatic vessel (Willard-Mack, 2006) (Fig. 1).

Basic structural unit of LN parenchyma is the lobule, which comprises the majority of stromal cells. The lobule was alternatively termed the compartment (Sainte-Marie, 2010) and is conventionally divided into three functionally and anatomically distinct regions: I) Outer or peripheral cortex, also referred simply as a cortex. It can be further divided into B cell follicles, comprising B cell zones, and spaces between them, known as interfolicular space; II) Paracortex, also termed inner or deep cortex, comprising T cell zone and finally III) Medulla, composed of medullary chords and sinuses (Gretz et al., 1997; Willard-Mack, 2006) (Fig. 1, also see *Experimental part*).

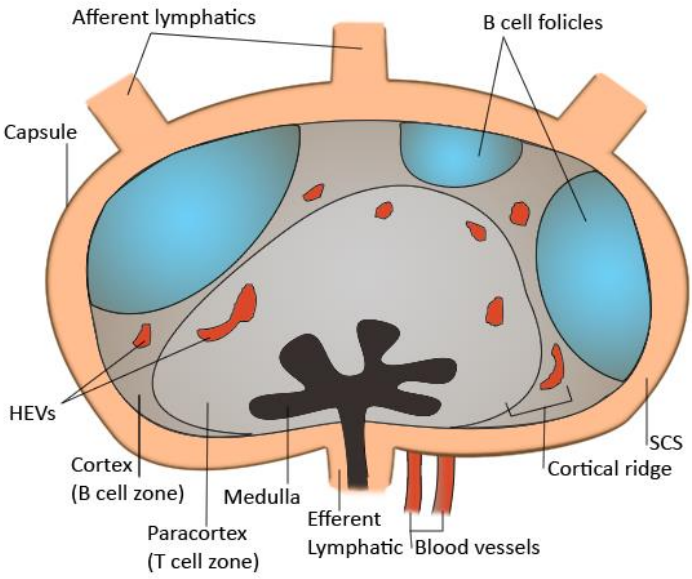

Fig. 1: Schematic representation of LN section. SCS: Sub-capsular sinus; HEVs: High endothelial venules. Inspired by: Malhotra et al., 2013; Mueller and Germain, 2009

LN can contain one or more lobules according to the size of an animal. In a mono-lobular LN, the single lobule is surrounded only by SCS, which is the case of most mouse LNs (Gretz et al., 1997). In a multi-lobular LN, lobules are separated by a transversal sinuses, which is the case of larger animals (Willard-Mack, 2006). Transversal sinuses were also referred to as cortical gaps (Sainte-Marie, 2010). From the SCS or transversal sinuses continue cortical sinuses, which are abundant within interfolicular regions and paracortex periphery (Willard-Mack, 2006).

The system of blood vessels passes through the lobule, both entering and exiting on the hilar side of LN through LN artery and vein respectively. Within paracortex and on the junction with peripheral cortex, blood venules form specific structures termed high endothelial venules (HEVs). Specific region, with high abundance of HEVs, was identified on the junction of peripheral cortex and paracortex and named cortical ridge (Katakai et al., 2004) (Fig. 1, also see *Experimental part*).

Lymph node stromal cells populations

Additionally to providing lymphocytes with their cognate antigens, LN serves as an incubator for lymphocytes undergoing activation and subsequent proliferation. Considering these facts, it becomes clear, that virtually every T cell undergoing activation has to pass through some LN at some point. Regarding this, the role of lymph node stromal cells (LNSCs) emerges as they form the environment, where this activation occurs, or at least, where those activated cells proliferate. In fact, stromal cells were initially thought only to serve as a scaffold for migrating lymphocytes and APCs. As research continued, it became

clear that LNSCs play crucial roles not only in processes such as lymphocyte homeostasis and migration, but recently also in immune tolerance.

Current approaches to identify distinct cell subsets depend mostly on identification of their cell surface markers. While these markers can provide a very limited but somewhat cell specific view on their gene expression profile, they also provide hints about their origin and potential physiological functions. LNSCs are identified as panhematopoietic marker CD45 negative cell subset. On the basis of glycoprotein gp38 and adhesion molecule CD31 surface expression, they can be further divided into four groups, distinct in both function and localization within LNs: fibroblastic reticular cells (FRCs; $gp38^{+}CD31^{neg}$), lymphatic endothelial cells (LECs; $gp38^{+}CD31^{+}$), blood endothelial cells (BECs; $gp38^{neg}CD31^{+}$) and double negative cells (DN; $gp38^{neg}CD31^{neg}$) (Fletcher et al., 2010; Link et al., 2007).

FRCs constitute T cell zone and partially B cell zone stroma where they form reticular network. They produce extra cellular matrix which they then ensheathe forming tube like structures, termed conduits. This reticulum spans through whole T cell zone, some parts of B cell zone and connects to sleeves around HEVs which are also partially formed by FRCs (Kaldjian et al., 2001). LECs generally cover sinus walls starting in afferent lymphatics and SCS and ending in medullary sinuses and efferent lymphatic (Cohen et al., 2014). BECs form blood-vessel walls, while their subpopulation forming HEVs is defined by altered phenotype optimized for lymphocyte homing (Katakai et al., 2004).

Recent research discovered few novel cell subsets within LNSCs subpopulations described above, based mainly on additional surface markers (Chang and Turley, 2015; Katakai et al., 2008; Malhotra et al., 2012; Togoo et al., 2014). However, roles of most of these populations in peripheral tolerance have not yet been evaluated. For further description see respective subsections.

T cell trafficking

A main road of T cell entry to LN is through the HEVs. T cell recirculating through blood stream first engages loose tethering to HEV wall followed by slow rolling on the surface. When T cell encounters chemotactic signal it undergoes firm arrest on HEV wall. Finally T cell migrates through the wall to LN parenchyma (von Andrian and Mempel, 2003). Alternatively, T cell can enter LN via afferent lymphatics, however in most cases this T cell

already undergone HEV mediated entry in upstream LN (Masopust and Schenkel, 2013). After transmigration through HEV, T cell finds itself in a sleeve usually constituted by FRCs (Kaldjian et al., 2001). T cell then connects to FRCs and migrates along the reticulum which FRCs create (Bajénoff et al., 2006). It is noteworthy that similar mode of migration was observed in spleen (Bajénoff et al., 2008). After some time, T cell crosses medullary sinus wall, comprised of LECs, and exits LN via efferent lymphatics (Cohen et al., 2014). It should be noted that this is only a brief review and much more is known about T cell trafficking in LNs, however it is out of scope of this study to discuss this data and it is well reviewed elsewhere (Masopust and Schenkel, 2013; von Andrian and Mempel, 2003).

Peripheral tolerance in lymph nodes

Classic models of peripheral tolerance

Perhaps the oldest known mechanism of peripheral tolerance is a physical separation of naïve T cells from non-lymphatic parenchymal tissues. However, this mechanism is not operational for T cells which already encountered their cognate antigen and in an ongoing inflammation even immunologically privileged organs, which are normally protected by elaborate system of barriers, are endangered (see below). Another mechanism, supporting tolerance, depends on Tregs generated in thymus (see *Introduction* section), which suppress functions of effector T cells with the same antigen specificity. It has been shown that antigen-specific interactions occurring in the periphery may also lead to the establishment of T cell regulatory phenotype which could consequently maintain the same function as Tregs derived from thymus. Alternatively, tolerogenic antigen-specific interactions may lead to anergy or deletion of T cells, with the latter as the most reliable outcome in tolerance enforcement (Mueller, 2010).

First concepts of antigen-specific tolerance in the periphery were formulated on the basis of experiments showing that bone marrow (BM)-derived APCs are able to promote both $CD8^+$ (Kurts et al., 1997) and $CD4^+$ T cell tolerance (Adler et al., 1998), via T cell deletion or anergy in antigen-specific manner. This tolerization leads to the prevention of response after further re-stimulation (Adler et al., 1998) and was even able to ensure prolonged protection from self-reactive T cells constitutively produced in the thymus (Kurts et al., 1997). It was ascertained, that those BM derived APCs are not required to endogenously produce antigens to which they enforce tolerance and that they rather acquire those antigens from parenchymal tissues and present them to cognate T cells which they subsequently tolerize (Adler et al., 1998; Kurts et al., 1997). The model became more precise with findings, that BM-derived dendritic cells (DCs) are able to produce very similar effects if either provided with exogenous antigen (Hawiger et al., 2001) or apoptotic cells carrying this antigen (Ferguson et al., 2002). These studies have also shown that DCs are able to promote both $CD8^+$ (Ferguson et al., 2002) and $CD4^+$ (Hawiger et al., 2001) T cell tolerance. However, the identification of DCs as a main population responsible for this kind of tolerance carries with it another problem. DCs under inflammatory conditions undergo maturation and mature DCs are one of the most efficient APCs for the induction of T cell

immune responses (Mueller, 2010). Indeed, the outcome of both antigen delivery pathways and their presentation was altered from tolerance to immunity when provided with maturation stimuli mediated by CD40 ligation (Ferguson et al., 2002; Hawiger et al., 2001).

Taken together, the model of DC-mediated tolerance can be summarized as follows: I) Under steady state conditions, immature DC captures antigens from peripheral tissues; II) the antigens are then presented to naïve T cells; III) cognate T cells are thereafter deleted, anergized, or converted to Tregs, ultimately leading to tolerance to those antigen(s) (Mueller, 2010; Steinman et al., 2003). From the data presented it is clear that main problem with such scenario is its inefficiency under inflammatory conditions. Therefore, one could hypothesize that when body goes through inflammation from other source, such as viral infection, reduced capacity of DCs to toleraze self-reactive T cells will lead to the breakdown of peripheral tolerance and progression of autoimmunity. Indeed, it has been experimentally shown that when immunized with particular self-antigen, autoimmunity can develop even inside immunologically privileged organs, showing how fragile this 'privilege' is (Oliver et al., 2003). Mechanistically, such antigen immunization can mimic the escape of self-antigen from sites to which naïve T cells normally do not have access, which could otherwise result from tissue disruption during infection. Together with inflammatory environment, the antigen escape can lead to fatal autoimmunity.

Recently another mode of peripheral tolerance has emerged. This mode relies mostly on LNSCs. Therefore, in the following chapters, various functional and structural aspects of each LNSC subset (as described in the subsection *LNSCs populations*) in peripheral tolerance will be evaluated to our knowledge up-to-date.

Fibroblastic reticular cells

Probably the most studied subpopulation of LNSCs are FRCs. They were proposed to play crucial roles in multiple immunological processes in LNs, including T cell migration (Bajénoff et al., 2006; Bajénoff et al., 2008; Gretz et al., 2000) (see also subsection *T cell trafficing*), T cell (Link et al., 2007) and B cell homeostasis (Cremasco et al., 2014) and important structural function in SLO (Kaldjian et al., 2001). However, data regarding their involvement in peripheral tolerance are still a little bit scarce. Questions about FRC mediated tolerance arose during studies using the mouse model expressing neo-self antigen, which was initially used to determine spatial aspects of $CD8^+$ T cell-mediated autoimmunity

(Lee et al., 2007). In such a model, a neo-self antigen, here truncated cytosolic form of chicken ovalbumin (tOVA), is expressed under a selected endogenous promotor, here intestinal fatty acid binding protein (iFABP). It was expected that this neo-self antigen would be expressed only in the small intestine. However, finding that tOVA was expressed also in LNs, but not spleen, led to further study which resulted in establishing of LNSCs role in peripheral tolerance (Lee et al., 2007). In fact, studies by Lee et al. and that of Nichols et al. were first to reveal such phenomenon and opened a new field in tolerance immunology (Lee et al., 2007; Nichols et al., 2007). It was later identified, that cells responsible for tOVA expression in LNs of iFABP-tOVA mice are FRCs defined as $gp38^{+}CD31^{neg}$ (Fletcher et al., 2010). It is also worth noting, that using sorting strategy described in subsection *LNSCs populations* (Fletcher et al., 2011), newly described population termed $MAdCAM^{+}$ marginal reticular cells (MRCs) (Katakai et al., 2008), forms a subset of FRCs. These MRCs are found mainly on the border of B cell follicles, underneath the SCS (Katakai et al., 2008). However, no effort has been made so far as to their potential role in peripheral tolerance or to determine if some of observed functions of FRC are performed by this subpopulation.

Tolerance induction by fibroblastic reticular cells. It was reported that OT-I $CD8^{+}$ T cells, specific and with high affinity for MHCI ($H\text{-}2K^{b}$):tOVA (amino acids 257–264) complex, were activated and subsequently deleted in all LNs of iFABP-tOVA mice (Lee et al., 2007). Taken this data together with tOVA expression in FRCs, one can hypothesize, that FRCs directly present TRAs to cognate T cells. This hypothesis was proven to be true, at least in *in vitro* conditions. Specifically, FRCs from peripheral lymph nodes (pLNs) of iFABP-tOVA mice promote OT-I T cell activation and proliferation, as assessed by *in vitro* co-culture experiments (Fletcher et al., 2010). Although, these *in vitro* experiments prove involvement of FRC in tolerance to tOVA, they do not particularly disapprove involvement of other subpopulations in this process. Therefore, another experiment was conducted using $ß2m^{neg/neg}$ mouse model. These mice have no MHCI expression. Using BM from these mice it was proved, that tolerance to OT-I cells was not dependent on radio sensitive cells in iFABP-tOVA, ruling out most of the hematopoietic populations (Lee et al., 2007). However, other radioresistant hematopoietic populations, such as Langerhans cells, could still be involved. In contrast, direct involvement of hematopoietic DCs was suggested in another

study, which reported that DCs are able to transfer pMHCII complexes to FRCs, which are then used to anergize cognate T cells (Dubrot et al., 2014).

Mechanisms of fibroblastic reticular cells mediated tolerance. Regarding molecular mechanisms underlining FRC antigen presentation and tolerance, not much data is yet available. It was reported that upon antibody-mediated blocking of PD-1:PD-L1 pathway, which is known to inhibit T cell proliferation (Carter et al., 2002), in iFABP-tOVA mice, injected with OT-I T cells dramatic intestinal autoimmunity occurs. Furthermore very similar symptoms appear when iFABP-tOVA mice are injected with OT-I T cells deficient in PD-1 molecule (Reynoso et al., 2009). This evidence strongly suggests the involvement of PD-1:PD-L1 axis in tolerance to tOVA in these mice. However, it is not clear if this tolerance disruption can be solely appointed to FRC-mediated tolerance, or if it impairs other mechanisms, such as DC-mediated tolerance to tOVA, since both FRCs and DCs were shown to express PD-L1 (Fletcher et al., 2010; Reynoso et al., 2009). In conclusion, presented evidence would suggest that PD-1:PD-L1 axis could be involved in FRCs mediated tolerance.

Spatial aspects of fibroblastic reticular cells mediated tolerance. For each subpopulation involved in tolerance induction is important to clearly state its anatomical location and position and establish the extend to which it comes in contact with naïve T cells *in vivo*. This are largely overlooked functional aspects when describing the particular mechanism of peripheral tolerance, yet, this knowledge would have a profound importance in any clinical application. Contact of FRCs, residing in T cell zone, with T cells is well established. FRCs are known to direct T cell migration within LNs *in vivo* (see subsection *T cell trafficking*). Importantly FRCs do so by direct contacts with T cells, since migrating T cell follows a reticulum composed of FRCs (Bajénoff et al., 2006). On the other hand it was shown that FRC-mediated T cell homeostasis is independent of cell-cell contacts (Link et al., 2007). Nonetheless, when migrating along FRC-formed reticulum, T cells have plenty of opportunities to scan it for pMHC complexes. Moreover, in this way, T cells are able to sample substantial portion of T zone FRCs for their presented antigens and therefore, potentially self-reactive T cell has higher probability to encounter its cognate antigen. Such a large scale scanning would become even more beneficial if FRCs were not uniform in their TRA expression but rather form a combinatorial mosaic of particular TRAs, as was shown

for mTECs (Cloosen et al., 2007) . On the other hand the above mentioned MRCs were not shown to be able to interact with T cells, so far.

Tissue restricted antigens expression by fibroblastic reticular cells. In spite of experimental indications concerning tolerogenic properties of FRCs discussed above, it would not be possible for them to induce tolerance *in vivo* if FRCs did not express at least some TRAs. To this end, the expression of number of different TRAs was studied among LNSCs. These studies revealed that FRCs express following TRAs on the level of mRNA: *Melanoma antigen recognized by T cells 1* (*Mart-1*, melanocytes); *Retinal S antigen* (*Ret S,* retina; low transcript levels); *Ras-related associated with diabetes* (*Rrad,* pancreas); *a fetoprotein* (*Afp,* liver); *proteolipid protein* (*Plp,* central nervous system); and *glutamate decarboxylase* 67kDa isoform (*Gad67,* pancreas and neuronal tissue) (Cohen et al., 2010; Fletcher et al., 2010).

Another matter of interest is, how is this TRAs expression regulated in FRCs. The above mentioned Aire is known to play a crucial role in TRA expression in the thymus (see *Introduction* section). However, its expression on the periphery remains largely elusive (see *DN cells* subsection). In one study, *Aire* mRNA was detected in all LNSCs subsets although at very low levels and major *Aire* transcript levels were detected only in DN cells subpopulation (Fletcher et al., 2010). In other study, Aire was found to be present only in DN and $CD45^+$ hematopoietic populations (Cohen et al., 2010). Aire involvement was ruled out in FRCs at least for *Mart1* and *Gad67*, since their expression shown no difference in Aire knockout (KO) and wild type (WT) mice. Surprisingly, *glycoprotein A33* (*A33*, intestinal epithelium TRA) mRNA expression was upregulated in Aire KO animals in comparison to WT. Taken together, there is no evidence that the expression of TRAs in FRC is Aire-dependent as it is comparable between the wild-type and Aire KO animals (Cohen et al., 2010).

Interestingly mRNA of another transcription regulator, *Deaf1*, which was also proposed to control TRA expression (Yip et al., 2009), was detected in all LNSCs and was proposed be involved in the regulation of some TRAs expression (Fletcher et al., 2010). This notion was supported by a decrease in LN expression of TRAs associated with diabetes in non-obese diabetic (NOD) mice, correlating with a decrease in levels of conventional Deaf1. Furthermore, Deaf1 function seemed to be inhibited by alternative isoform of Deaf1 itself

(Yip et al., 2009). Later, Deaf1 was shown to possess even much broader role in the regulation of gene expression since it seems to control expression of eukaryotic translation initiation factor 4 gamma 3 (*Eif4g3*). Strikingly, the expression of both *Deaf1* and *Eif4g3* was reduced in FRCs of 12 weeks old NOD mice, a time correlating with diabetes progression. Therefore Deaf1 could control TRA expression on the level of translation, even though this mode of action can be very generic and not specific to TRAs (Yip et al., 2013). In other concerns none of TRAs mentioned above were shown to be expressed at protein level, which would be needed for tolerance induction. Thus, FRCs express specific set of TRAs, however mechanism controlling their expression remains unclear.

Lymphatic endothelial cells

Perhaps the second most studied population of LNSCs are LECs. The study of their role in peripheral tolerance was largely influenced by the discovery that they express tyrosinase gene (pubmed ID: 22173), the expression of which is conventionally restricted to skin melanocytes and retinal pigment epithelium cells (Giménez et al., 2003). The main advantage of using this model TRA is the existence of well-studied tyrosinase gene KO mouse mutant, termed the albino mouse (Nichols et al., 2007). Very first hints about the expression of tyrosinase in LN emerged in study by Nichols et al. (Nichols et al., 2007). Tyrosinase mRNA has been detected in the thymus, suggesting that intrathymic clonal deletion may ensure tolerance to tyrosinase (Derbinski et al., 2001). However, tyrosinase mRNA was also detected in both pLN and mesenteric LNs, but not in spleen (Nichols et al., 2007). Nichols et al. devised a system combining a novel mouse model and transgenic T cells: I) AAD mice: In these mice tyrosinase epitope 369 (Tyr_{369}; comprising residues 369–377 of tyrosinase protein) is presented on chimeric MHCI molecule, modified by peptide binding domain of human HLA-A*201 and the CD8-binding domain from $H2\text{-}D^d$. II) FH cells: T cells specific for Tyr_{369}/AAD complex. One of advantages of such system is that model TRA (here tyrosinase) is expressed at physiological level and it is not modified in any way, which could alter its folding and localization and therefore expression regulation on multiple levels. On the other hand, antigen presentation is rather artificial. Using this system, the authors were able to provide evidence that tolerance to the particular epitope Tyr_{369} is independent of central tolerance in the thymus, as confirmed with thymus transplantation experiments (Nichols et al., 2007). However it should be noted, that central tolerance still can be involved in tolerance to other tyrosinase epitopes. Cells expressing

tyrosinase in LNs were later identified as $CD45^{neg}gp38^{+}CD31^{+}$, in other words LECs (Cohen et al., 2010; Fletcher et al., 2010).

Tolerance induction by lymphatic endothelial cells. Significance of tyrosinase expression in LECs was evaluated and it was established that transferred FH cells are deleted in tyrosinase^{+} and not in albino mice. This deletion is preceded by activation and abortive proliferation based on CD69 (T cell activation marker) expression. Also FH cells activated in tyrosinase^{+} mice displayed pro-apoptotic phenotype, as assessed by annexin V staining (Nichols et al., 2007). Based on co-culture experiments, cells responsible for this deletion were identified as $CD45^{neg}gp38^{+}CD31^{+}$ LECs. This finding is consistent with tyrosinase expression in this subpopulation. Moreover, other subpopulations ($CD45^{+}$; FRCs; BECs; DN) were unable to efficiently activate FH cells in these *in vitro* experiments, what suggests their inability to do so *in vivo* as well (Cohen et al., 2010). It should be noted, that $gp38^{+}CD31^{neg}$ (BECs) subset retained some potential to activate FH cells, however the level of activation was comparable to albino control and therefore could be due to unspecific mechanisms. Furthermore, the involvement of DCs or other hematopoietic populations ($CD45^{+}$) in FH cells abortive activation and deletion was ruled out by BM transfer experiments. AAD^{+} BM reconstitution of AAD^{neg} mice did not rescue FH cells activation and reciprocal transfer did not impair it (Nichols et al., 2007). While these experiments rule out the involvement of the hematopoietic populations, some radioresistant subsets can still activate FH cells. Therefore, Langerhans cells, main DC subset known to be partially radioresistant (Cole, 1986), were depleted by prolonged exposure to diphtheria toxin in double transgenic AAD mice crossed with mice expressing diphtheria toxin receptor under Langerin promoter. Even under such conditions FH cells activation was not impaired as assessed by CD69 presence (Nichols et al., 2007). Thus, tolerance to Tyr^{369} occurs via abortive activation and subsequential deletion of Tyr^{369} specific T cells and moreover this deletion is independent of hematopoietic populations as well as LNSC subpopulations other than LECs. Also it was shown, that LECs are able to enforce tolerance to $CD4^{+}$ T cells, using pMHCII complexes acquired form DCs (Dubrot et al., 2014).

Mechanisms of lymphatic endothelial cells mediated tolerance. Research on LECs continued in identification of molecular mechanisms by which deletional tolerance occurs. Phenotypic screen revealed that LECs fail to express most of the major co-

stimulatory molecules (CD80; CD86; CD137L; CD252; CD70). On the other hand LECs expressed multiple co-inhibitory molecules (PD-L1; HVEM; CD48) (Tewalt et al., 2012). However, when studied under inflammatory conditions, i.e. provided by PolyI:C, LECs upregulated CD80 and also further increased PD-L1 expression (Fletcher et al., 2010). FH cells phenotype was also assessed and receptors for indicated inhibitory molecules (PD-1; CD160; BLTA; LAG-3) were present, with exception of 2B4 (receptor for CD48). Such phenotypes would allow two possible mechanisms of T cells deletional tolerance: I) Deletion via TCR activation and concurrent absence of co-stimulation, which would suffice abortive proliferation observed. II) Employment of inhibitory molecules, which would predispose T cells for deletion, regardless of preceding proliferation. It was shown that these two mechanism are both employed in FH cells deletion and that they intertwine to mediate this deletion. Furthermore, PD-L1:PD-1 axis was identified as a single inhibitory pathway involved in this deletion (at least among several pathways studied) and CD137L as at least one co-stimulatory molecule able to rescue FH cells from this deletion (involvement of CD134, the receptor for CD252, was ruled out of this process). The model of LEC-induced FH cells deletional tolerance can be divided into 3 steps: I) TCR specific recognition and signaling and simultaneous lack of CD137L signaling leads to II) the upregulation of PD-1 on FH cells, and III) High levels of PD-L1 on LECs mediate PD-1 signaling in FH cells, which later leads to their deletion. This model was supported by direct demonstration that PD-1 levels on FH cells are substantially lower when CD137 agonist was provided to tyrosinase^{+} mice. This ultimately led to rescue of FH cells from deletion, as did blocking of PD-L1, which compromises PD-L1:PD-1 signaling. Also this model was supported by vitiligo occurrence in PD-L1$^{neg/neg}$tyrosinase^{+} mice and in tyrosinase^{+} mice treated with CD137+CD134 agonist, suggesting an autoimmune destruction of melanocytes in the absence of PD-L1:PD-1 signaling or presence of exogenous co-stimulation (Tewalt et al., 2012).

In conclusion, FH cell deletion occurs by combination of lack of co-stimulatory signals and the presence of inhibitory signals. While multiple inhibitory pathways were suggested to be involved in FH cells deletion, only PD-L1:PD-1 pathway turn out to be essential in this process. This conclusion was confirmed by either perturbation of this pathway or administration of exogenous co-stimulation in which both led to vitiligo autoimmunity.

Spatial aspects of lymphatic endothelial cells mediated tolerance. Regarding direct contacts between LECs and T cells, a model where only medullary subpopulation of LECs mediates tolerance was recently proposed (Cohen et al., 2014). This is in compliance with current model of T cell migration through LN, since this composition would ensure contact with nearly every recirculating T cell since all T cells exit LN parenchyma via medullary sinuses (see subsection *T cell trafficking*). To propose such model, one needs to discriminate between various LEC subpopulations. This was done on the basis of four different markers: PD-L1, ICAM-1, MAdCAM-1 and LtβR. Using this method, three subsets of LECs were identified within LN (Cohen et al., 2014) (Tab. 1). Exclusive role of medullary subpopulation in deletional tolerance was demonstrated by co-culture experiments. In these experiments, LN LECs subpopulations were either pulsed with Tyr_{369} (as a positive control) or left untreated and co-cultured with FH cells. Strikingly, only medullary LECs were able to induce FH cells proliferation when not pulsed with Tyr_{369}. Furthermore only medullary LECs expressed tyrosinase mRNA at substantial level. Additionally, peripheral LECs (represented by diaphragm and colon LECs) were ruled out of tolerance induction (Cohen et al., 2014). Thus, LEC subset able to induce tolerance was identified as LN medullary subpopulation. This data suggests that tolerance induction occurs when T cells attempt to leave LN and necessarily cross medullary sinus wall comprised by LECs.

LECs subpopulation	Marker expression			
	PD-L1	**ICAM-1**	**MAdCAM-1**	**LtβR**
Subcapsular	High	High	+	Low
Cortical	Intermediate	Intermediate	-	+
Medullary	High	High	-	+

Tab. 1: LECs subpopulations within LN

Tissue restricted antigens expression by lymphatic endothelial cells. LECs (defined as $CD45^{neg}gp38^{+}CD31^{+}$) expression of other TRAs then tyrosinase was further analyzed and mRNA of following TRAs was detected using RT-PCR: *A33* (intestinal

epithelium); *pancreatic polypeptide* (*Ppy,* pancreas); *Rrad* (pancreas); *Afp* (liver); *Plp* (nervous system) (Cohen et al., 2010; Fletcher et al., 2010). However, it should be noted that while the *Afp* expression was predominant in FRCs, its levels in LECs were much lower and therefore could be due to cell sorting-related contamination (Fletcher et al., 2010). Similarly to FRCs, no evidence was provided that TRAs, other than tyrosinase, are expressed at protein level and that they are presented to cognate T cells by LECs. As discussed above for FRCs another issue is the mechanism that controls TRA expression in LECs. Mainly, no transcription modulator was identified as responsible for TRA expression in LECs. As described above (see *FRCs* subsection), Aire expression in LECs is questionable, but they were found to express mRNA of *Deaf1* (Fletcher et al., 2010). Since the expression of *A33* and *Tyrosinase* by LECs remained unchanged in Aire KO mice, it is probable that TRA expression in LECs is independent of Aire. An involvement of Deaf1 in TRA expression in LECs has not been studied yet. Thus LECs, as well as FRCs, express specific set of TRA, however their expression control remains unknown.

Blood endothelial cells

So far, there is no study focused directly on the role of BECs in peripheral tolerance. However, some hints can be taken from the work on other LNSCs subsets and from related studies. Firstly, BECs have an excellent access to naïve T cells, both from the apical and basolateral side. While T cells roll on, they are arrested by BECs and finally they travel across BECs in HEV endothelium, while entering LN (see *T cell trafficking subsection*). Secondly, the expression of a specific set of TRAs was found in BECs: *Ret S* (retina), *Rrad* (pancreas), *Afp* (low expression, liver), *Plp* (nervous system), *A33* (intestinal epithelium) (Cohen et al., 2010; Fletcher et al., 2010). The control mechanism of expression of these TRAs remains unknown, with the exception of *A33* which seems to be somehow under the control of Aire, since *A33* transcript was not found in BECs from Aire KO mice (Cohen et al., 2010). Despite this observation, the expression of Aire by BECs is still questionable. *Deaf1* mRNA is also expressed in BECs, although its predominant expression was found in FRCs (Fletcher et al., 2010). In spite of these characteristics, BECs were not yet observed to enforce T cell tolerance. Only one study suggested that they may do so, although indirectly, via presenting pMHCII complexes acquired from DCs. However, this tolerance mechanism does not delete T cells, but rather anergize them (Dubrot et al., 2014). In conclusion BECs

possess many features needed for cells to mediate peripheral tolerance, but very little is known about their actual ability to do so.

Double negative cells

The major issue regarding studies of DN cell subset is, that this is indeed a heterogeneous population of cells. DN cells consist of multiple populations, which are negative for gp38 and CD31 markers. Only recently these populations are being described but goal to definitively sort out LNSCs populations will require a lot of work in the future. Despite this pre-maturity of our knowledge about DN cells, some of their features has been described and will be discussed now.

Double negative cells populations. First population identified among DN cells and consequently the best characterized today is the population of FDCs. FDCs are known to play important roles in B cell zone maintenance, B cell homeostasis and particularly in the presentation of antigens bound in immunocomplexes to B cells residing in LNs (Chang and Turley, 2015), although recently it has been shown that some of these functions can be accounted for by FRCs (Cremasco et al., 2014). From those populations described recently, ITGA7^{+} pericytes were the first. This population should be of some importance, since it represents more than 50 % of DN cells in LN. Cells of this population seem to resemble FRCs, since they form sleeves around blood vessels and they are contractile (Malhotra et al., 2012), which are two typical features of FRCs (Kaldjian et al., 2001; Link et al., 2007). Another approach, using alpha-smooth muscle actin (αSMA) promoter driven GFP, identified new subsets in both gp38^{+}CD31^{neg} (FRCs) and DN cells subsets. Although the relationship between these subsets and those previously characterized is not clear, it seems likely that FRCs can be further divided into αSMA^{+} and αSMA^{neg} subpopulations. Also it seems probable, that αSMA^{+} DN cells strongly overlap with IAPs, if they are not outright identical, since αSMA is a marker of pericytes (Togoo et al., 2014). Although the identification of these novel subpopulations brings new insights into LNSCs diversity, their role in peripheral tolerance remains so far unknown. One recently identified DN subpopulation strongly implicated in this process is population of extra-Thymic *Aire*-expressing cells (eTACs). Therefore only those will be discussed further.

Extrathymic Aire expressing cells identification. As the name suggests, interest in eTACs arose from the finding that both *Aire* mRNA and Aire protein were found within LNs of both mice (Adamson et al., 2004; Anderson et al., 2002; Halonen et al., 2001; Heino et al., 2000; Lee et al., 2007) and humans (Consortium, 1997; Heino et al., 1999; Nagamine et al., 1997). However, while all studies agree on expression of Aire in the thymus, there are conflicting reports of Aire expression in the periphery (including LNs) (Hubert et al., 2008). This prompted the question, which, if any, subpopulation is responsible for the expression of *Aire* in LNs. To answer this question, novel mouse transgenic model was created and designated Adig mouse. In these mice, a fusion protein of islet-specific glucose-6-phosphatase-related protein (Igrp) and GFP is expressed under *Aire* promotor (Gardner et al., 2008) (see also *Experimental part*). However, even employment of such a model did not clear the issue entirely. At first, it was reported, that *Aire* mRNA was restricted to $CD45^{neg}$ subset (Lee et al., 2007) and on the basis of this finding population of $CD45^{neg}$ eTACs was described (Gardner et al., 2008).

Later however *Aire* mRNA was also found in $CD45^{+}$ subset (Cohen et al., 2010) and in humans even $AIRE^{+}$ nodal DCs were described. Strikingly, these $AIRE^{+}$ DCs displayed a tolerogenic phenotype (Poliani et al., 2010). Also the distribution of *Aire* mRNA among LNSC subsets became unclear, since different studies shown their different Aire expression profiles (Cohen et al., 2010; Fletcher et al., 2010). Regarding this new data, concept of eTACs was reevaluated and eTACs were described as $CD45^{low}$ hematopoietic cells. Authors explained these findings by inclusion of population of $CD45^{low}$ cells in $CD45^{neg}$ subset in previous experiments and argued that eTACs are in fact exclusively of hematopoietic origin. This claim was supported by observations that chimeras of WT mice with Adig BM possessed ability to activate Igrp specific $CD8^{+}$ T cells and that, in same mice, GFP signal was detectable in LNs, while in the LNs of Adig mice reconstituted with WT BM was not, however sorting strategy used in this experiment included only $DAPI^{neg}CD45^{low}$ cells. Further, the authors claimed that GFP signal co-localized with actual anti-Aire signals only in BM-derived cells, which they showed only demonstratively (Gardner et al., 2013).

While these experiment strongly established the existence of BM-derived eTACs, they did not necessarily disprove the existence of other eTAC subpopulations, which could be of stromal origin, particularly when *Aire* mRNA expression is concerned. Nonetheless,

BM derived eTACs were further characterized and were found to express low level of CD45 (marker of hematopoietic cells), CD11b (marker of macrophages) and CD11c (marker of DCs). Interestingly, BM eTACs were found to express Zbtb46 (another marker of DCs) mRNA at levels similar to those found in DCs, suggesting their common origin. Also, BM eTACs were universally positive for MHCII (Gardner et al., 2013). While these findings are in compliance with the data from humans (Poliani et al., 2010), they are in conflict with previous report that in mice, no DC population expresses Aire on protein level (Hubert et al., 2008). Regardless of uncertainties about origin of eTACs, their role in peripheral tolerance is well established and will be discussed below.

Extrathymic Aire expressing cells in peripheral tolerance. Using above described Adig mice it was shown, that eTACs are able to mediate tolerance to Igrp-specific $CD8^+$ 8.3 T cells. This tolerance occurs as abortive proliferation and subsequential deletion of $CD8^+$ 8.3 T cells, at day 3 and up to 14 days after T cell transfer, respectively. It is noteworthy, that both abortive proliferation and deletion of $CD8^+$ 8.3 T cells occurred even in BM chimeras of Adig mice reconstituted with $ß2m^{neg/neg}$ BM. B2M deficient cells lack MHCI expression and are therefore incapable of antigen presentation to $CD8^+$ T cells (Gardner et al., 2008). Taking into account proposed hematopoietic origin of eTACs this effect could be explained by radioresistence of eTACs, while this can also be explained by the presence of multiple eTAC population, some of which could be of stromal origin.

Later BM eTACs were shown to mediate even $CD4^+$ T cell tolerance, while utilizing another mouse model AdBDC mouse. In these mice, BDC mimotope peptide is inserted into MHCII-associated invariant chain and expression of this transgene is together with GFP driven by *Aire* promoter. BDC mimics peptide, derived from chromogranin A, which is expressed in pancreas aside from other tissues and was implicated in diabetes. This system ensures presentation of a part of BDC on MHCII molecules in these mice. MHCII in complex with peptide derived either form chromatogranin A or BDC is recognized by BDC2.5 CD4+ T cells. It was shown that BDC2.5 $CD4^+$ T cells proliferate in both spleen and inguinal and pancreatic LNs of AdBDC mice 3 day after transfer, while the same cells proliferate only in pancreatic LNs and to a lesser extent at same time point in WT mice. However the proliferation in AdBDC mice did not lead to the same outcome as in 8.3/Adig system, since 14 days after transfer there was still considerable population of BDC2.5 $CD4^+$

T cells in all studied SLO of AdBDC mice. On the other hand, these T cells did not cause diabetes, since AdBDC mice did not have increased glucose levels in blood, nor they display pancreatic islet disruption. Also, the involvement of conventional DCs was ruled out, since DCs provided with BDC derived peptide via αDEC1040 (endocytic receptor) were not able to protect WT SCID mice from diabetes caused by BDC2.5 $CD4^+$. Although phenotypical analyses of residual T cells from AbBDC mice injected with BDC2.5 $CD4^+$ T cells revealed the enrichment of $Foxp3^+$ Treg cells, tolerance enforcement was not Treg dependent, since it remained unchanged after Treg conditional depletion. Furthermore, those BDC2.5 $CD4^+$ Tregs induced in AbBDC mice were not able to suppress naïve BDC2.5 $CD4^+$ T cells, when co-transferred to WT SCID mice.

Further analysis of eTAC-mediated $CD4^+$ T cell tolerance revealed that it is mainly achieved via the lack of co-stimulation by eTACs which express low amounts of CD80 and CD86. In accordance with this notion, anti-CD28 agonistic antibody partially impaired tolerance induction as measured by INF-γ expression by residual BDC2.5 T cells. A model similar to those shown for LECs and FRCs mediated tolerance. Moreover this lack of co-stimulation leads to impaired TCR signaling in residual T cells, as measured by impaired Erk phosphorylation, ultimately switching BDC2.5 T cells to anergic phenotype (Gardner et al., 2013).

In conclusion, few novel cell subsets were identified within DN cells population. However, only one of them, eTACs, has been studied with regards to its role in peripheral tolerance. eTACs were shown to mediate both $CD4^+$ and $CD8^+$ T cells tolerance via induction of anergy and clonal deletion, respectively, and can therefore be viewed as a population strongly contributing to peripheral tolerance. However, while the mechanism by which eTACs induce anergy in $CD4^+$ T cells was clarified at least to some extent, the mechanism of eTACs mediated $CD8^+$ T cells deletion remains unclear. Moreover, origin, phenotype and number of eTACs populations remain a matter of debate.

Discussion of theoretical part

In the recent years a novel concept of antigen-specific peripheral tolerance emerged in the form of tolerogenic DCs. Even more recently, those concepts undergone dramatic changes, with the discovery of unexpected abilities of LN resident cells, mainly of stromal origin, to enforce tolerance in an antigen-specific manner. In this discovery may lay an answer to one of most troubling questions: if DCs are the only cell subset which mediates tolerance, then how is tolerance imposed and autoimmunity prevented under inflammatory conditions? Here, it is important to realize that TRAs can escape from a tissue disrupted by inflammation and presence of TRAs in the pro-inflammatory environment was shown to have severe consequences (Oliver et al., 2003). This is due to the fact that DCs which mature in the presence of inflammatory stimuli are designed to support immune responses and therefore even enhance autoimmunity progression (Mueller and Germain, 2009). This conclusion was further supported by the observation that such mature DCs can promote self-reactive T cell expansion even when loaded with self-antigens, acquired from apoptotic cells (Ferguson et al., 2002; Hawiger et al., 2001). Thus, the main question is whether there exist other cell populations in the periphery which can counteract DC-mediated activation of selfreative T cells, and if yes, what's their nature.

Data discussed above have not only proved involvement of different LN-resident populations in the peripheral tolerance, they also provided evidence that these cells, in contrast to DCs, do not change their properties to immunogenic APCs under inflammation. If anything, LNSCs even increase their ability enforce tolerance in inflammatory conditions, since all LNSCs subsets were shown to upregulate their surface PD-L1 expression (Fletcher et al., 2010). Furthermore, all the LNSCs subsets, except DN cells, were shown to upregulate surface MHCII (Malhotra et al., 2012). While it has been recently shown that this may be due to transfer of MHCII from DCs, their endogenous upregulation is also possible, since MHCII expression in LNSCs is INFγ-dependent. In either case, MHCII upregulation seems to serve a tolerogenic role, since LECs, FRCs and BECs were able to induce activation unresponsiveness in $CD4^+$ T cells (Dubrot et al., 2014).

Regarding the involvement of PD-1:PD-L1 axis in LNSC-mediated tolerance, and especially its importance in both LEC- and FRC-mediated tolerance (see respective

subsections) it is tempting to speculate on broader significance of this pathway in the mechanism of peripheral tolerance, for example as an universal tolerogenic mechanism in LNSC-mediated tolerance.

Although, much work has been done, many questions still remain unanswered and many new questions arose. Particularly control mechanism(s) of TRA expression in different LNSCs subpopulations remains largely elusive. While it would be intuitive to presume employment of mechanisms similar to that operating in central tolerance, direct involvement of Aire was demonstrated only in eTACs. Another possibility is the potential involvement of the transcription regulator Deaf1, since *Deaf1* mRNA was found in all LNSCs. However, given the inhibition of Deaf1 function by its alternative isoforms, it will be important to determine if this alternative splice-variant is also expressed in other LNSCs then FRCs. If so, it would also be interesting to ascertain if changes in the expression of these variants correlate with changes in TRA expression, as was shown in diabetes progression (Yip et al., 2009). However, future work on Deaf1 might be impaired by a recent finding that it controls the expression of *Eif4g3* mRNA (Yip et al., 2013) and thus it could have other important functions related to cell transcription/translation. Therefore, additional studies are needed to clarify whether the effects of Deaf1 on TRA expression are specific or rather a consequence of its other regulatory activities in the mechanisms of gene expression.

Finally, most of the work showing tolerogenic properties of LNSCs was done using neo-self antigen expression. Now it will be important, although probably complicated, to translate this knowledge into more physiological animal models and show that LNSCs are able to enforce tolerance to endogenous self-antigens. Furthermore, until now, T cells with transgenic TCRs, which possess high affinity to their cognate pMHC complexes, were mostly used. Therefore, other possible line of work should focus on LNSC-mediated tolerance to T cells with lower affinities, to mirror physiological conditions, since such T cells continually escape central tolerance (Bouneaud et al., 2000) and thus represent major targets of peripheral tolerance.

Experimental part

Lymph node compartmentalization and localization of extrathymic *Aire* expressing cells

Objectives

For better illustration of LN anatomy discussed in *Theoretical part*, we decided to visualize basic anatomical structures of LNs. For this purpose we used immunofluorescence on frozen LN sections. Since eTACs possess important tolerogenic properties and yet represent so far ill-defined subpopulation of LNSCs, we decided to further study their localization in LN.

Materials and methods

Mice. Adig mice (a gift from prof. Ludger Klein, LMU Munich) were described previously (Gardner et al., 2008) (also see *DN cells* subsection). Both Adig and WT mice were bred on the Balb/c background. All mice were bred in the animal facility of the Institute of Molecular Genetics, ASCR, Prague under specific pathogen free conditions in individualy ventilated cages.

Reagents and chemicals. Following chemicals and other reagents were used in this study: paraformaldehyde (PFA, Polyscience), methanol (Lachner), bovine serum albumin (BSA, Serva), fetal calf serum (FCS, Gipco), Sucrose (Sigma-Aldrich), Phosphate buffer saline (PBS, in house), TritonX-100 (Bio-Rad).

Antibodies. Phycoerythrin coupled streptavidin (SA-PE) was obtained from eBioscience. Anti PNAD (clone Meca79) antibody was obtained from BioLegend. Anti-GFP and anti-B220 antibodies were produced in the lab. Anti rabbit-Ig antibody coupled with Alexa-488 was obtained from Life Sciences. All primary antibodies, except Anti-GFP antibody, were biotinylated and SA-PE was used as secondary reagent. For anti-GFP secondary staining, anti rabbit-Ig antibody coupled with Alexa-488 was used.

Immunofluorescence. Mice were sacrificed by cervical dislocation and pLNs and thymi were extracted immediately. Tissues were washed in PBS and fixed in 4% solution of PFA for 1 hour at room temperature (RT). Tissues were then incubated in 30% sucrose solution over night at 4 °C and frozen in TissueTec Cryomold in freezing medium (JUNG) on dry ice and stored at -80 °C. Tissues were then sectioned to 10 µm thick sections using

cryomicrotome (Leica CM 1950) and stored at -20 °C or used immediately. Sections were fixed in 4% solution of PFA for 15 minutes at RT, washed in PBS for 5 minutes at RT and permeabilized, using 100% methanol for 10 minutes at -20 °C. Further, sections were washed three times in PBS for 5 minutes at RT and blocked for 1 hour at RT using 2.5% FCS/ 2.5% BSA diluted in PBS. Primary antibodies diluted in 4% TritonX solution (diluted in PBS) were added on sections immediately after blocking step and incubated over night at 4 °C. Sections were then washed three times with PBS for 5 minutes at RT and incubated with secondary antibodies and/or SA-PE diluted in 4% TritonX solution (diluted in PBS). Secondary staining was performed for 1 hour at RT. Sections were then washed three times with PBS for 5 minutes at RT and stained with DAPI (3,4 μg/ml) for 5 or 10 minutes. Sections were finally washed three times with PBS for 5 minutes at RT, immersed with Vectashield (Vector Laboratories, Inc.) and assembled for microscopy with cover slip. For co-staining experiments, all solutions and reagents were prepared as indicated and diluted accordingly.

Microscopy. All the images presented were obtained with Leica SP5 confocal microscope.

Results

Lymph node architecture. To visualize LN structures we used three color immunofluorescence, utilizing markers of specific LN structures. Firstly, we visualized B cell zones using B220 (used as marker of B cells). B cell zones formed oval shapes on the border of the LNs, presumably under SCS (Fig. 2A and C). Secondly, we visualized HEVs using PNAD (marker of HEV cells (Katakai et al., 2004)). HEVs were distributed more centrally, presumably in the paracortex or in the junction regions of cortex (B cell zone) and paracortex (T cell zone) (Fig. 2B and D). The localization of all identified structures was in agreement with previous reports (Katakai et al., 2004; Willard-Mack, 2006).

Extrathymic Aire expressing cells localization within lymph nodes. To visualize eTACs we used Adig mouse model (Gardner et al., 2008) (also see DN cells subsecion). In these mice GFP is expressed under Aire promoter. Therefore all cells with active Aire promoter should express GFP and should be visualized with anti-GFP staining on the fixed LN sections. Using this approach we were able to visualize eTACs in all the LNs from Adig mice examined, although they were relatively rare (Fig. 2 C, D and data not shown). As expected, GFP^+ eTACs were undetectable in LN sections from WT mice (Fig. 2A, B and data

not shown). Using the co-staining with above mentioned markers we were able to determine, that, at least in Adig mice, eTACs are not localized in B cell zones, nor are they immediately connected to HEVs.

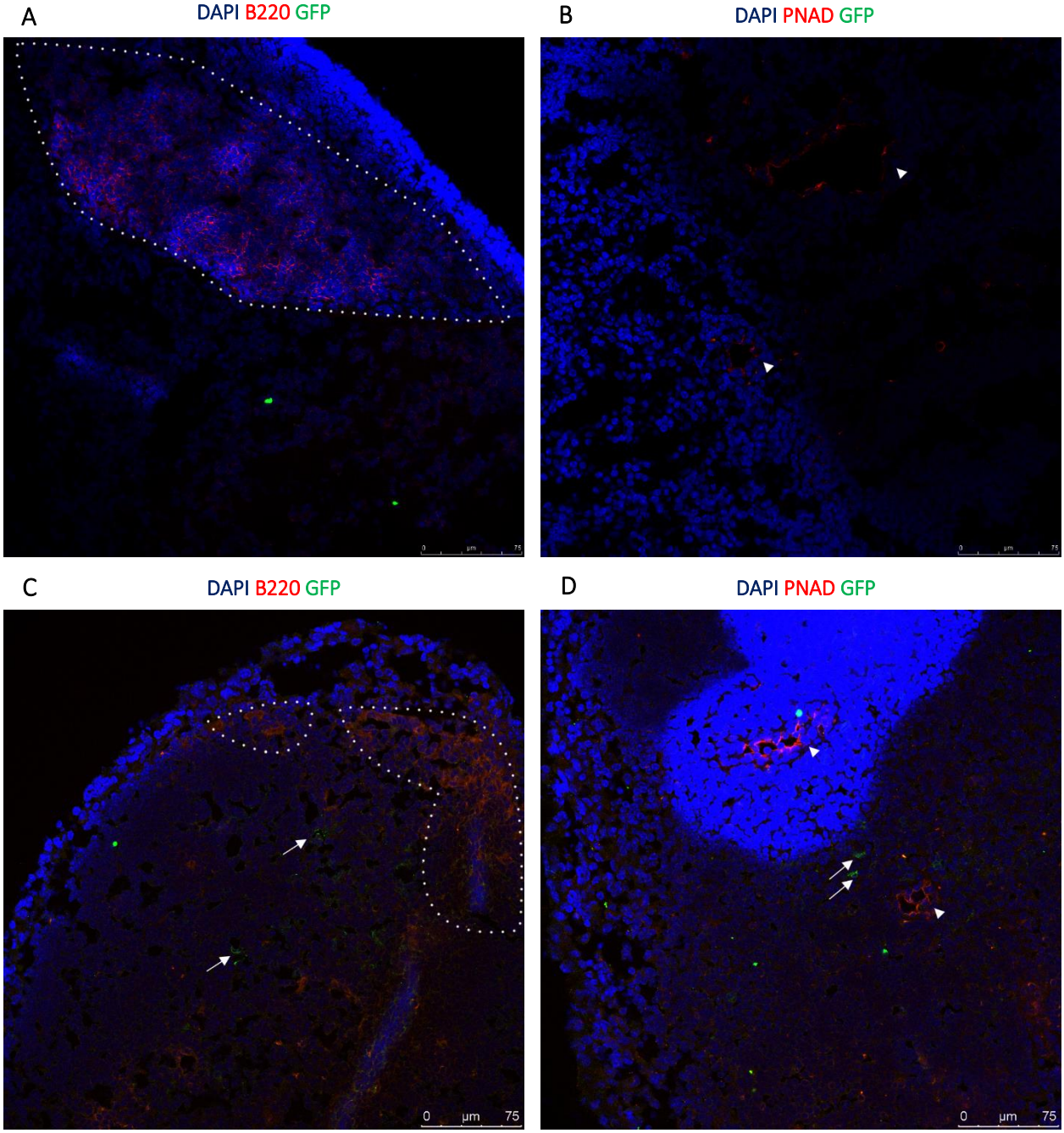

Fig. 2: LN architecture and eTACs localization. Sections of LNs from WT (A-B) and Adig (C-D) mice were stained using immunofluorescence and analyzed by confocal microscopy. ***A,C)*** Immunostaining of B cell zone marker B220 (red). Dotted lines indicate B cell zones. ***B,D)*** Immunostaining of HEV marker PNAD (red). Arrowheads indicate HEVs. ***A-D)*** All sections were stained with DAPI to monitor LNs cellularity and visualize nuclei (blue). All sections were also stained with anti-GFP (green), using **A** and **B** as negative control and in **C** and **D** to determine eTAC localization within LNs. ***C-D)*** Arrows indicate GFP^+ eTACs.

Discussion of experimental part

We demonstrated at least some basic anatomical structures of LNs. This data should be viewed as illustrative supplement for *Theoretical part*. Regarding eTACs localization, we were able to determine position of GFP^+ eTACs in Adig reporter mouse LNs. From data presented, we conclude that those GFP^+ eTACs are not localized to B cell zones nor are identical or immediately adjacent to HEVs. Furthermore, from eTACs localization in the center of LNs and from the relative proximity to HEVs we propose, that they might be localized in the T cell zone or at the junction of T and B cell zones, in compliance with previous reports (Gardner et al., 2008; Gardner et al., 2013).

Although Adig mouse model proved useful for eTACs identification, promotor activity does not necessarily lead to protein production. Therefore we attempted to visualize protein directly, using anti-Aire staining. However, while we were able to detect thymic Aire protein expression using this approach (data not shown), we failed to reproducibly detect Aire within LNs using the very same two-step staining protocol. This could be due to rarity of eTACs as well as their lower expression levels of Aire protein. Therefore, further work is required to more accurately visualize eTACs localization within LNs. Towards this end, a straightforward approach would be to generate a new mouse knock-in transgenic strain where Aire is fused to a red flourescent protein. This would allow a direct visualization of eTACs in LNs and assessment and their origin, migration pattern and localization in respect to other LN populations. The generation of this strain is currently underway in our laboratory.

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