Příloha č. 3

Charles University in Prague

First Faculty of Medicine

Author's abstract

Mucosal immunity in upper respiratory tract diseases and autoimmunity diseases

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2016

Příloha č. 3

Doctoral study programmes in Biomedicine

Charles University in Prague and Czech Academy of Science

Study programme: Immunology

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Příloha č. 3

Table of contents

Abstract

Mucosal immune system comprises not only the major compartment of the immune system but also important interface with the outer environment. It is responsible in maintaining an intricate balance with the danger and non-danger stimuli of the outer world by employing specific anatomical features and unique functional mechanisms. Mucosal immune system has been long understudied, perhaps due to the limited accessibility, and its biological importance is thus still underevaluated. However, it has become evident that it is important to study mucosal immune system not only in local mucosal affections but also when uncovering pathogenic mechanisms and novel prevention strategies of organ specific autoimmune diseases such as type 1 diabetes.

Thus, the first, more clinically oriented part of this thesis is focused on mucosal immune system of the upper respiratory tract in disease conditions - in nasal polyposis (NP).

Because there is a substantial accumulation of eosinophils and neutrophils in the most frequent type of NP, we investigated and described increased expression of chemokine receptors CCR1 and CCR3 in NP versus nasal mucosa. Both innate immune mechanisms as well as homeostasis of epithelial cells may participate in NP. We have documented increased numbers of iNOS-positive and insulin-like growth factor-1 receptor (IGF-1R)-positive cells in both stroma and epithelium of NP.

The second part of the thesis deals with the interplay of the mucosal immune system, dietary gluten or gliadin in pathogenesis and a novel prevention strategy of autoimmune type 1 diabetes (T1D).

Because gluten-free (GF) diets highly prevent T1D in animal models we investigated the effect of dietary gluten on the mouse mucosal immune system. In the first two papers, we compared the effects of the standard, diabetes permissive vs. the diabetes preventive GF diet and found changes in proportion of mucosal γδ T cells, Th17 cells as well as a more proinflammatory cytokine signature associated with the standard, diabetes-permissive diet. Since not many studies addressed in detail the innate immune mechanisms of gliadin we next investigated innate signaling pathways utilized by gliadin fragments. We documented that apart from IL-1β induction, TLR2/4/MyD88/TRIF/MAPK/NF-κB innate signaling pathway and NLRP3 inflammasome activation are involved in wheat proteins signaling. Finally, we tested intranasal (i.n.) administration of gliadin as a novel vaccination strategy - using an environmental compound that may have etiological role in T1D. We showed that i.n. gliadin is able to substantially reduce diabetes incidence in NOD mice and also reduce diabetes incidence when applied much later to already prediabetic mice. The effect was associated with increased proportion of potentially regulatory γδ T cells, and to a lesser extent also $CD4^{+}Foxp^{+}$ Tregs, in mucosal compartments as well as with changes in their cytokine signatures.

In conclusion, this thesis deals with the mucosal immune system. We addressed some pathogenic processes in a local mucosal affection - NP as well as studied interactions of environmental factors and mucosal immune system in pathogenesis and also as possible novel prevention strategy in T1D.

Abstrakt

Slizniční imunitní systém představuje nejen hlavní složku imunitního systému, ale také důležité rozhraní s vnějším prostředím. Je zodpovědný za udržování jemné rovnováhy mezi bezpečnými a nebezpečnými podněty z okolí za využití specifických anatomických struktur a funkčních mechanismů. Slizniční imunitní systém nebyl dlouho dostatečně studován, také kvůli své omezené dostupnosti, a jeho biologický význam je podceňován. Nicméně je zřejmé, že jeho studium je důležité nejen v místních interakcích na sliznicích, ale také při odhalování patogenetických mechanizmů a nových strategií prevence orgánově specifických autoimunitních onemocnění jako například T1D.

První, více klinicky orientovaná část této disertace je zaměřena na slizniční imunitní systém horního respiračního traktu postižený chorobou – nosní polypózou (NP). Vzhledem k tomu, že nejčastěji se vyskytují polypy se značnou akumulací eosinofilů a neutrofilů, vyšetřovali jsme a popsali zvýšenou expresi chemokinových receptorů CCR1 a CCR3 v nosních polypech oproti nosní sliznici. K nosní polypoze mohou přispívat jak mechanismy přirozené imunity, tak homeostáza epitelových buněk. Zdokumentovali jsme zvýšený počet iNOS pozitivních a insulin-like growth factor-1 receptor (IGF-1R) pozitivních buněk jak ve stromatu, tak v epitelu NP.

Druhá část práce se zabývá souhrou slizničního imunitního systému, glutenu či gliadinu z diety v patogenezi a v nové strategii prvence autoimunitního diabetu 1. typu (T1D). Protože bezlepková (GF) dieta u zvířecích modelů vysoce zabraňuje T1D, studovali jsme efekt glutenu z diety na myší slizniční imunitní systém. V prvních dvou publikacích jsme srovnávali efekt standartní, diabetes-permisivní s diabetes preventivní GF dietou a našli jsme rozdíly v proporci slizničních γδ T buněk, Th17 buněk stejně jako zvýšenou produkci prozánětlivých cytokinů spojených se standartní, diabetes permisivní dietou. Protože pouze málo studií se zabývalo detailně mechanismy přirozené imunity u gliadinu, jako další jsme vyšetřovali signalizační dráhy přirozené imunity užívané fragmenty gliadinu. Zdokumentovali jsme, že kromě indukce IL-1β, jsou v signalizaci pšeničných proteinů zahrnuty i TLR2/4/MyD88/TRIF/MAPK/NF-κB signalizační dráha a aktivace inflamazomu NLRP3. Nakonec jsme testovali intranasální (i.n.) administraci gliadinu jako novou vakcinační strategii – užití složky prostředí, která může hrát roli v etiologii T1D. Ukázali jsme, že i.n.gliadin je schopen značně redukovat incidenci diabetu u NOD myší a také redukuje incidenci diabetu, když je podán mnohem později již prediabetickým myším. Tento efekt byl spojován se zvýšeným podílem potenciálně regulačních γδ T buněk, a v menší míře také CD4+Foxp+Tregs ve slizničních kompartmentech, stejně jako se změnou cytokinových profilů.

Závěrem, tato práce se zabývá slizničním imunitním systémem. Zaměřili jsme se na určité patogenetické procesy v místních slizničních vztazích - NP a současně jsme studovali interakce mezi faktory vnějšího prostředí a slizničního imunitního systému v patogenezi a také v možné novátorské prevenční strategii u T1D.

1. Introduction

Mucosal immune system represents the major compartment of the immune system that is also responsible for its interactions with the outside world. Mucosal surfaces serve as barrier against but also as an interface to the outer environment. Thus, mucosal immune system is instrumental as the first line of defense against danger signals form the outer environment as well as for maintaining immune homeostasis with non-danger events such as many inhaled agents, dietary components and commensal microbes inhabiting mucosal surfaces (nose, oral cavity, gut etc.). Mucosal immune system has been long understudied, perhaps also due to the limited accessibility, however, it is now for many years established that local immune mechanisms and tissue specific immune responses are important in pathogenesis of organ specific diseases. The topic of this thesis is mucosal immune system in disease - in a local affection of nasal mucosa, i.e. nasal polyposis as well as its role (in an accord with environmental factors) in an autoimmune disease – type 1 diabetes.

Nasal polyposis (and chronic rhinosinusitis, CRS) is a recurrent and most likely multifactorial chronic disease of the upper respiratory tract with still unclear etiology and pathogenesis (Fokkens et al., 2005; Fokkens et al., 2012). This is perhaps also due to a non-existence of a good animal model for this rather frequent nasal affection within ENT practice. Nevertheless, the massive eosinophil, and to a lesser extend neutrophil infiltration typical for most common type of nasal polyposis represent together with various proinflammatory and allergy related cytokines and chemokines an important phenomenon for experimental studies (Pawankar, 2003; Stoop et al., 1993). Several studies in pathogenesis of NP and CRS focused on the inflammatory infiltrate, proinflammatory cytokines and chemokines and/or adaptive immune responses (Pawankar and Nonaka, 2007; Bachert et al., 2003). However, both epithelial cells as well as innate immune mechanisms - as the first line of defense against pathogens and environmental agents, may play important role in pathogenesis of NP (Kato, 2015; Hamilos, 2014).

Environmental factors play a major role in the relatively recent increase of type 1 diabetes (T1D) in developed countries (Bach, 1994; Graves and Eisenbarth, 1999). As during a relatively short period of a few decades the incidence substantially increased, especially in the developed countries. Several studies in both NOD mice as well as BB rats have documented that diets influence diabetes incidence (Scot, 1996). It has been shown that gluten-free diet highly decreases diabetes incidence in NOD mice (Funda et al., 1999; Schmid et al., 2004). Gliadin, the component of wheat gluten that triggers celiac diseases in susceptible individuals,

was shown to activate innate immune (Jelinkova et al., 2004). Thus, gliadin and mucosal immune mechanisms have been also considered in pathogenesis of type 1 diabetes (Visser et al., 2009). As patients diagnosed with both celiac disease and T1D usually develop diabetes first and not vice versa (Cosnes et al., 2008), it suggests a beneficial effect of gluten-free diet in humans. Several human trials with intranasal or oral administration T1D autoantigens such as insulin or GAD65 have so far failed to show protective effects (Hanninan and Harrison, 2004). However, since environmental factors are clearly behind the recent rapid increase of T1D incidence in developed countries, environmental factors and their (mucosal) mechanisms should be researched for the disease prevention and/or cure.

2. Rationale and Aims

In this thesis I studied the mucosal immune system in a local mucosal affection - in nasal polyposis, but also in an organ specific autoimmune disease - type 1 diabetes, in which mucosal delivery of not only autoantigens, but also environmental entities (dietary components, probiotics) should be "re-searched" for their use in secondary prevention of the disease. Mucosal immune system in diseases – especially mucosal immune system of the upper respiratory tract and of the gut is subject of this thesis.

This thesis consists of two parts that also reflect my involvement in both experimental immunology research as well my clinical ENT practice. They are interconnected not only by my interests but also by the common mucosal immune system phenomenon, that e.g. allows for intranasal vaccination in type 1 diabetes prevention.

Thus, the first, more clinically focused, part deals with the role of mucosal immunity of the upper respiratory tract in disease conditions - more specifically in nasal polyposis, a recurrent, chronic inflammatory condition, which pathogenesis and etiology are not well understood. We focused :

- on mechanism of the massive eosinophilic as well as neutrophilic infiltration in pathogenesis of NP by studying pattern of expression of CCR1 and CCR3 chemokine receptors in nasal polyps versus nasal mucosa.
- on the possible role of epithelial cell homeostasis and innate immune mechanisms in pathogenesis of NP by evaluating expression of insulin-like growth factor-1 receptor (IGF-1R) and iNOS in stroma and epithelium of NP compared to nasal mucosa.

The second part of this thesis deals with the role of mucosal immunity and environmental factors in autoimmune diseases - more specifically, in the spontaneous animal model (the NOD mouse) of type 1 diabetes. We investigated effects of environmental factors such as gluten-free diet and gliadin on the mucosal immune system and prevention of type 1 diabetes, and also addressed some innate mechanisms of gliadin signaling by:

- studying possible mechanisms by which gluten-free diet highly prevents type 1 diabetes in NOD mice; in particular its influence or regulatory T cells and Th17 cells in mucosal compared to systemic lymphoid compartments.
- further investigating the diabetes-protective effect of gluten-free diet by assessing the balance of pro-inflammatory and anti-inflammatory cytokines in T cells in the mucosal versus systemic lymphoid compartments.
- mapping innate signaling pathways of pepsin-digest of gliadin (such as TLR2/4/MyD88/TRIF/MAPK/NF-κB and a NLRP3 inflammasome activation), also in relation to celiac disease.
- testing intranasal vaccination strategy with gliadin, an environmental agents, that may have etiological role in type1 diabetes, for both prevention or even early cure of type 1 diabetes. Describing induction of Tregs and γδ T cells and their cytokine profiles in relation to this immunointervention strategy.

In addition, two reviews that cover the two parts described above are presented in this thesis.

3. Materials and Methods

A wide array of methods and materials have been used in presented six original papers. They are in detail described in respective papers. Herewith a list of methods is provided.

Preparation of frozen histological specimens; immunoflourescence staining, fluorescence microscopy and computer image analysis, preparation of cell suspension from lymphoid organs, fluorescence staining for flowcytometry, in vitro polyclonal restimulation, intracellular staining, FACS data analysis, preparation of pepsin digest on agarose, E-toxate test, cell cultures of PBMCs, preparation and cultures of bone marrow-derived dendritic cells, ELISA, FLICA staining, Western blotting, DNA extraction, PCR, intranasal administration, isolation of NALT, insulitis scoring, glycaemia measurements, and statistical analysis.

4. Results

Clinically focused studies: mucosal immunity of the upper respiratory tract in nasal polyposis and CRC.

4.1. **Fundová P**, Funda DP, Kovář D, Holý R, Navara M, Tlaskalová-Hogenová H. Increased expression of chemokine receptors CCR1 and CCR3 in nasal polyps: molecular basis for recruitment of the granulocyte infiltrate. ***Folia Microbiol*** **(Praha), 2013, 58(3):219-24**. IF (2015) = 1.335

The aim of this study was to investigate the expression of chemokine receptors CCR1 and CCR3 in nasal polyps compared to nasal mucosa. Both computer image analysis and fluorescence microscopy with calibrated eyepiece graticule were used to asses the differences in expression. All samples were collected form patients with no sign of asthma and allergies. We found increased number of cells expressing CCR1 and CCR3 in the stroma of nasal polyposis. Next, we also examined the pattern of CCR1 and CCR3 expression and documented increased positivity of CCR3 within the epithelial compartment of NP. In conclusion, our data document molecular basis for recruitment of the neutrophil and eosinophil infiltrate in NP. Increased or dysbalanced production of chemokines and cytokines may facilitate accumulation of immune cells expressing corresponding receptors CCR1 and CCR3. Because eosinophils and neutrophils form the predominant cellular component in NP, we think they may also represent important targets for novel therapies. Their active role in pathogenesis of NP should be further investigated as novel mechanisms such us extracellular DNA traps were described with respect to these granulocytes (Goldmann and Medinna, 2013).

4.2. **Fundová P,** Filipovský T, Funda DP, Hovorka O, Holý R, Navara M, Tlaskalová-Hogenová H. Expression of IGF-1R and iNOS in nasal polyps; epithelial cell homeostasis and innate immune mechanisms in pathogenesis of nasal polyposis. ***Folia Microbiol*** **(Praha), 2008; 53(6):558-62.** IF (2015) = 1.335

In this study we examined expression of insulin-like growth factor-1 receptor (IGF-1R) in nasal polyps and showed statistically significantly increased numbers of IGF-1R-positive cells in both stroma and epithelium of NP compared to nasal mucosa. We think the increased expression of the receptor for insulin-like growth factor-1 in NP may be connected with prolonged survival or increased proliferation of epithelial cells that both have been described in NP (Mendelsohn et al., 2001). One of the innate immune mechanisms that control various

infections entities is production of nitric oxide by cytokine inducible nitric-oxide synthase (Nathan and Shiloh, 2000). Thus, we investigated the expression of inducible nitric-oxide synthase (iNOS) in biopsies of nasal polyps and nasal mucosa and found substantially increased number of iNOS-positive cells in both stroma and within epithelium of NP. We think our results support recent opinion that both epithelial cells as well as innate immune mechanisms may play important role in pathogenesis of NP.

4.3. **Fundová P**, Navara M, Tlaskalová-Hogenová H., Pathogenetic mechanisms in nasal polyposis (in Czech), ***Alergie,* 2009; 1: 68-71.** Review. IF (2015) = none

This review covers the topic of the first, more clinically focused part of my thesis and is intended as an overview on history and pathogenesis of nasal polyposis for Czech ENT (and other) clinicians dealing with NP in their routine outpatient clinics. It consists of chapters covering basic characteristics and history of the disease, prevalence of NP, also in relation to asthma, the contribution of genetic factors and possible environmental factors that were considered but not confirmed in etiology of NP. The next three paragraphs then deal with possible immune mechanisms involved in pathogenesis of NP - role of mucosal immunity and epithelial cells, innate immune mechanisms, and chemokines and cytokines. One recent topic not covered by this review is the relation of nasal microbiom to development of NP and CRS.

Experimentally focused studies: mucosal immunity and environmental factors in autoimmune type 1 diabetes mellitus.

4.4. Antvorskov JC, **Fundová P,** Buschard K, Funda DP. Impact of dietary gluten on regulatory T cells and Th17 cells in BALB/c mice. ***PLoS One,* 2012;7(3):e33315.** IF (2015) = 3.057

In this study we compared the effect of standard, gluten-containing diet and the diabetes preventive, gluten-free diet on various regulatory T cell subsets and Th17 cells in immunocompetent BALB/c mice. The diet was fed to the experimental animals prenatally and cell subsets were assessed by flowcytometry in mucosal (MLN, PLN, PP) compared to non-mucosal (spleen, ILN) lymphoid organs. The standard, gluten-containing diet led to general decrease of γδ T cells in all lymphoid compartments studied. The standard, gluten-containing diet also influenced proportion of $CD4^+CD45RBlow^+$ and $CD4^+CD45RBhigh^+$ T cells. Fewer $CD4^+$ T cells expressed CD62L in PP and increased proportion of $CD103^+$ mucosa-homing T cells was noted in spleen and PLN. There was no effect dietary effect on $CD4^+Foxp3^+$ Tregs.

Finally, significantly increased proportion of Th17 cells was detected in PLN of mice fed the standard diet. In conclusion, we showed that the diabetes-permissive, standard diet versus gluten-free, diabetes-protective diets influence various T cell subsets e.g. γδ T cells and Th17 cells, preferentially in mucosal compartments.

4.5. Antvorskov JC, **Fundová P**, Buschard K, Funda DP. Dietary gluten alters the balance of pro-inflammatory and anti-inflammatory cytokines in T cells of BALB/c mice. ***Immunology,* 2013; 138(1):23-33.** IF (2015) = 4.078

This study is a follow-up of previous paper. In animal models of T1D, the dietary prevention of the disease is well documented, but the mechanisms behind the diet-mediated modification of diabetes incidence are not well understood. In this study we investigated cytokine profiles of polyclonally (PMA + ionomycin) stimulated $Foxp3^+$ Tregs as well as $Foxp3^-$ T cells from mucosal (MLN, PLN, PP) and non-mucosal (spleen, ILN) lymphoid organs from immunocompetent BALB/c mice that were fed neonatelly a standard, gluten-containing, diabetes permissive diet compared to litters fed gluten-free, diabetes preventive diet. Within $CD4^+$ T cells the gluten-containing, standard diet increased numbers of IFN-γ, IL-17 and IL-2 producing cells in all organs studied. In Foxp3+ Tregs, there was a shift towards more IL-2, and IL-17 (in mucosal organs) as well as IL-4 and IFN- γ positive cells (in both compartments). On the other hand, the gluten-free diet was associated with increased proportion of TGF-β positive $CD4^+$ T cells. Collectively, our results document that the gluten-containing, diabetes permissive diet is causing a more proinflammatory cytokine signature in both $CD4^+$ T cells as well as $Foxp3^+$ Tregs. We think the proinflammatory properties of the diabetes permissive, gluten-based diet points towards a hypothesis, that a higher gluten intake could be associated with an increased diabetes incidence.

4.6. Palová-Jelínková L, Dáňová K, Drašarová H, Dvořák M, Funda DP, **Fundová P,** Kotrbová-Kozak A, Cerná M, Kamanová J, Martin SF, Freudenberg M, Tučková L. Pepsin Digest of Wheat Gliadin Fraction Increases Production of IL-1β via TLR4/MyD88/TRIF/MAPK/NF-κB Signaling Pathway and an NLRP3 Inflammasome Activation. ***PLoS One,* 2013; 8(4):e62426**. IF (2015) = 3.057

In this study we investigated the innate signaling pathways of pepsin digest of wheat gliadin fraction (PDWGF). Using PBMCs and monocytes from active celiac disease patients and healthy controls, we documented significantly increased secretion of IL-1β and IL-1α and

slightly increased production of IL-18 in response to PDWGF in CD patients. We then showed that PDWGF leads to de novo pro IL-1β synthesis and that the follow up processing is caspase-1 dependent. IL-1β secretion was promoted by K^+ efflux and independent of P2X7. Using set of MAPK inhibitors we showed that the pro-IL-1β synthesis is induced via the MAPK-NF-κB pathway. Experiments on KO mice then documented that the caspase-1 dependent induction of IL-1β also requires ASC and NLRP3. Following the documentation of NF-κB pathway in IL-β we then explored the upstream TLR signaling pathway using TLR2, 4, 2/4 as well as MyD88 and TRIF KO mice and found the IL-1β secretion is dependent on TLR4, MyD88, and TRIF and influenced by TLR2. In conclusion, we documented that pepsin digest of wheat gliadin activates innate immune signaling via TLR2/4/MyD88/TRIF/MAPK/NF-κB and the NLRP3 inflammasome pathways. This study on the role of innate immune mechanisms of gliadin actions is also relevant to type 1 diabetes (Visser et al., 2009), in which more likely its innate than adaptive immune mechanisms influence the development (or prevention - see next paper) of type 1 diabetes.

4.7. Funda DP, **Fundová P**, Hansen AK, Buschard K. Prevention or Early Cure of Type 1 Diabetes by Intranasal Administration of Gliadin in NOD Mice. ***PLoS One*, 2014; 9(4):e94530.** IF (2015) = 3.057

In this study we explored whether, mucosal, intranasal administration of gliadin or gluten may prevent development of diabetes in NOD mice. We showed that i.n. administration of gliadin, but not gluten, at age of 4 weeks led to significant decrease of diabetes incidence in NOD mice fed a standard gluten-containing diet. Interestingly, this intervention was less but still statistically significantly preventing diabetes when applied to 13-week-old NOD mice, just before clinical manifestation of the disease. Following the i.n. administration of gliadin or OVA we then assessed proportions of Tregs and their cytokine signatures in both mucosal (NALT, MLN, PLN) and control, systemic lymphoid organs (spleen, inguinal lymph nodes). There was a clear mucosal induction - at the site of the application, i.e. in the NALT, and in the MLNs and PLNs, of $CD4^+Foxp3^+$ T cells and even more significant increase of γδ T cells after i.n. gliadin administration. Increased induction of IL-10 and decreased proportion of IL-2, IL-4 and IFN-γ in $CD4^+Foxp3^+$ Tregs, as well as decreased proportion of IFN- γ in γδ T cells was observed after i.n. gliadin in preferentially mucosal lymphoid organs.

Interestingly, there is evidence for regulatory role of mucosal γδ T cells in T1D as intranasal aerosol application of the whole insulin molecule also led to induction of CD8+ γδ T

cells that were capable of preventing T1D in the adoptive co-transfer model (Harrison et al., 1996). We suggest that perhaps innate immune properties of gliadin may be responsible for the diabetes preventive effect - similar to prolonged or no exposure to LPS. In conclusion, intranasal application of gliadin, an environmental antigen that may have an etilogocal influence in T1D, represent a novel and safe approach for prevention or even early cure of T1D.

4.8 Tlaskalová-Hogenová H, Stěpánková R, Kozáková H, Hudcovic T, Vannucci L, Tučková L, Rossmann P, Hrnčíř T, Kverka M, Zákostelská Z, Klimešová K, Přibylová J, Bártová J, Sanchez D, **Fundová P**, Borovská D, Srůtková D, Zídek Z, Schwarzer M, Drastich P, Funda DP. The role of gut microbiota (commensal bacteria) and the mucosal barrier in the pathogenesis of inflammatory and autoimmune diseases and cancer: contribution of germ-free and gnotobiotic animal models of human diseases. **Cell Mol Immunol., 2011; 8(2):110-20.** Review. IF (2015) = 5.193

This review addresses the importance of interplay of microbioms and mucosal barriers in shaping mucosal immune responses in postnatal development, and in development of inflammatory, autoimmune as well as neoplastic diseases. Use of germ-free and environmentally (microflora) defined, gnotobiotic animal models is accented in studying pathogenesis of these human diseases. More specifically, this review focuses on inflammatory bowel disease, celiac disease, type 1 diabetes, neurological and psychiatric diseases, rheumatic diseases, cardiovascular diseases and obesity, allergies and cancers.

5. Discussion

In the first, more clinically oriented part of this theses we have investigated increased expression of chemokine receptors CCR1 and CCR3 in NP versus nasal mucosa. With respect to the hypothesis on the role of innate immune mechanisms and homeostasis of epithelial cells in NP we documented increased number of iNOS-positive and insulin-like growth factor-1 receptor (IGF-1R)-positive cells in both stroma and epithelium of NP.

Both pathogenesis and etiology of NP is not fully understood, what results in a lack of causative treatments. Experiments to uncover pathogenetic mechanisms and etiological agents are not easy to design, as there is no good experimental model of NP. It is therefore difficult to distinguish what it the causative mechanism and what is just an outcome of the pathological processes. Thus, no single agent or list of etiological agents has been identified in nasal polyposis. NP is considered at present as a multifactorial disease, in which the interplay of

various environmental factors and genetic predisposition leads to its development. Innate immune mechanisms, epithelial cells and above all the granulocyte infiltrate are most likely the major key players in the development of the disease. It is a bit surprising, that although there is a massive, eosinophilic and neutrophilic infiltrate present in the most common type of NP, these cell types have been a bit avoided or not adequately studied. As regards the role of environmental factors, interaction of nasal mucosa with nasal microbiom deserves much more attentions (Chalermwatanachai et al., 2015). Last but not least, the definition of NP is rather old, based mainly on histology. With the rapidly advancing techniques of molecular immunology, there is a high need for a better definition of new (sub-) phenotypes of nasal polyposis..

The second part of this thesis deals with the role of mucosal immunity and environmental factors (gluten-free diet and gliadin) in pathogenesis and prevention of type 1 diabetes.

Environmental factors (diets, microflora) play an important role in the recent increase of type 1 diabetes (T1D) in developed countries. Because gluten-free (GF) diets highly prevent T1D in animal models we investigated the effect of dietary gluten on the mouse mucosal immune system. We also investigated innate signaling pathways of wheat proteins and tested intranasal gliadin as a vaccination strategy for prevention or even early cure of type 1 diabetes in NOD mice. The mechanisms of environmental factors e.g. gluten-free diet are difficult to study because of the complexity of the topic. Although type 1 diabetes is often reported as autoimmune disease it is good to keep in mind that it does not fulfill one of the important criteria of autoimmunity as defined by the Witebsky and Rose (Rone and Bona, 1993); the disease-induction with a beta cell specific autoantigen has never been achieved in T1D. Immunizations with neither beta cell autoantigens nor pancreatic extract together with adjuvants were able to induce T1D (Chatenoud and Bach, 2005). Several of the beta-cell autoantigens that have prevented T1D in animal models proceeded to human trials e.g. oral or intranasal insulin, s.c. and i.v. insulin or s.c. GAD65/alum. However none of these human trials has so far showed a protective effect. This is also case in other autoimmune diseases (Hanninen and Harrison, 2004). In addition, there is always a danger of induction of autoimmunity when using beta cell autoantigens for tolerance induction. Based on this evidence and also on the fact that environmental factors play important roles in the recent increase of T1D, we think environmental antigens related to T1D, such as gluten-free diet or intranasal gliadin and their mechanisms of action should be re-searched as novel prevention strategies.

6. Conclusions

In the clinically oriented part of this thesis, we have focused on the role of mucosal immunity of the upper respiratory tract in disease conditions by studying possible pathogenic mechanisms of nasal polyposis - a very frequent diagnosis in ENT clinical practice, which pathogenesis as well as etiology is not resolved.

(A) We documented increased expression of chemokine receptors CCR1 and CCR3 in nasal polyps versus nasal mucosa. Increased number of CCR1 and CCR3 positive cells within nasal stroma and increased epithelial expression of CCR3 was showed in NP. These data document molecular basis for the increased migration and also accumulation of neutrophils and eosinophils in NP. Both CCR1 and CCR3 molecules may represent promising therapeutical targets in a local chronic inflammatory process such as NP.

(B) We studied the expression of insulin-like growth factor-1 receptor (IGF-1R) and inducible nitric-oxide synthase (iNOS) in nasal polyps and showed statistically significantly increased numbers of IGF-1R-positive and iNOS-positive cells in both stroma and epithelium of NP compared to nasal mucosa. These findings may have implications for the role of epithelial cell homeastasis and innate immune mechanism in pathogenesis of NP.

The second part of this thesis comprises experimental studies on the role of mucosal immunity and environmental factors in autoimmune diseases. More specifically, we studied the effect of environmental factors (diets, gliadin) on mucosal immune system of BALB/c mice and prevention of type 1 diabetes in the spontaneous NOD mouse model. We also addressed some innate mechanisms of gliadin actions.

(C) Using immunocompetent BALB/c mice, we compared effects of the diabetes-preventive gluten-free diet and the standard, gluten containing diet on multiple regulatory T cell subsets and Th17 cells. We found diet-related changes in γδ T cells, $CD4^{+}CD62L^{+}$ and $CD4^{+}CD45RB^{low}$ T cells, Th17 cells, but not in $CD4^{+}Foxp3^{+}$ Tregs, preferentially in mucosal lymphoid organs (MLN, PP, PLN). Our data thus show that the two tested diets - diabetes permissive and diabetes preventive (in NOD mice) influence multiple regulatory T cell subsets and Th17 cells, especially in the mucosal lymphoid compartment.

(D) We assessed the cytokine profiles of polyclonally stimulated T cells in BALB/c mice fed the diabetes-preventive gluten-free diet and the standard, gluten containing diet. We found an altered, more proinflammatory cytokine signature associated with the diabetes-permissive, gluten containing standard diet in both mucosal but also systemic lymphoid organs. We think the shift towards a more proinflammatory cytokine profile may be directly related to the diabetes-permissive character of the standard diet.

(E) We investigated the innate signaling pathways of pepsin digest of gliadin and found that it leads to robust induction of IL-1β and IL-1α as well as some induction of IL-18 in PBMCs and monocytes from celiac disease patients. We then documented the IL-1β is induced via the MAPK-NF-κB pathway, the process is caspase-1 dependent and requires NLRP3 and ASC. Using various strains of KO mice we documented the TLR2/4/MyD88/TRIF signaling pathways are involved in the innate signaling by the pepsin digest of gliadin. Collectively, these data describe that innate immune pathways, such as TLR2/4/MyD88/TRIF/MAPK/NF-κB and NLRP3 inflammasome activation are involved in wheat proteins signaling.

(F) We tested a novel vaccination strategy in T1D by intranasal administration of an external environmental substance to NOD mice. I.n. application of gliadin led to significant prevention of diabetes and development of insulitis in NOD mice. It was even able to reduce diabetes incidence in prediabetic mice with advanced insulitis. We described changes in mucosal T cells, especially γδ T cells and their cytokine profiles. In conclusion, intranasal application of gliadin, an environmental antigen that has possible etilogocal influence in T1D, may represent a novel and safe approach to prevention or even early cure of T1D.

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8. Publications

Publications *in extenso* related to the presented thesis:

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Příloha č. 3

Abbreviations

APC	Antigen-presenting cell
BB	Biobreeding
BMDC	Bone marrow derived dendritic cells
CD	Celiac disease
CRS	Chronic rhinosinusitis
ENT	Ear-nose-throat
GAD	glutamic acid decarboxylase
GF	Gluten-free
IGF-1R	Insulin-like growth factor-1 receptor
i.n.	Intranasal
LPS	Lipopolysaccharide
iNOS	Inducible nitric oxide synthase
MAPK	Mitogen-activated protein kinase
MLN	Mesenteric lymph nodes
MyD88	Myeloid differentiation primary response gene 88
NALT	Nasal-associated lymphoid tissue
NFκ-B	Nuclear factor κB
NLRP1,3	Nod-like receptor family containing pyrin domain 1,3
NO	Nitric oxide
NOD mice	Non-obese diabetic mice
NP	Nasal polyps
NM	Nasal mucosa
PBMC	Peripheral blood mononuclear cells
PDWGF	Pepsin digest of wheat gliadin fraction
PLN	Pancreatic lymph node
PMA	Phorbol myristate acetate
PP	Peyers Patches
PRR	pattern recognition receptor
Tregs	Regulatory T cells
T1D	Type 1 diabetes mellitus