Novel **I**nter**l**eukins for immuno**ther**apy of **A**sthma

"NILTHERA"

Final report

Summary

Allergies are increasing at an alarming rate, being particularly widespread in Western populations. Asthma, the chronic inflammation of the airways is becoming more and more prevalent, with one out of 10 children being currently asthmatic. Lambda interferons or interleukins-28/29 (IFNλ/IL-28s) or type III IFNs are the latest addition to the class II cytokine family. They have potent antiviral and anti-tumor functions but their full spectrum of activities remains poorly characterized. In the present study, we suggest a new role for IL-28, one of the members of type III IFNs, as a key endogenous regulator of allergic airway disease. By the use of genetically engineered mice deficient in IL-28R signalling, we show that endogenous IL-28 is critical for driving Th1 cell differentiation while limiting Th2 cell generation in a mouse model of allergic airway disease. In accordance, IL-28Rα$^{-/-}$ mice developed exacerbated allergic airway inflammation and hyper-responsiveness associated with augmented Th2 responses. IL-28-induced immune shifting to a Th1 cytokine profile was mediated through the modulation of DC function rather than direct effect on T cells. Moreover, transcriptome profile analysis of IL-28-treated DCs revealed molecular pathways modulated by IL-28 and leading to Th1 polarization. Finally, the generation of a novel IL-28a/eGFP knock-in reporter mouse allowed for the screening of potential therapeutic regimes that induce IL-28s and can regulate the allergic immune response. The observations made have the potential to lead to novel immunotherapeutic approaches for the treatment of allergic asthma.

Table 1: Timelines and activities in NILTHERA

ACTIVITY	YEAR 1		YEAR 2		YEAR 3	
WP1: Investigation of the role of endogenous IFNλ/IL-28s in allergic airway disease.	→	→				
WP2: Dissection of the cellular events involved in IFNλ/IL-28-mediated immunomodulation.		→	→			
WP3: Identification of the molecular events that promote IFNλ/IL-28-mediated immunomodulation.		→	→	→		
WP4: Design of novel immunotherapeutic agents for the treatment of allergic airway disease.			→	→	→	→
Reports, publications.				★		★

Progress towards objectives and details for each task:

WP1: Investigation of the role of endogenous IFNλ/IL-28s in allergic airway disease.

The host laboratory has previously identified an important role of exogenous IFNλ/IL-28s administration in suppressing allergic airway disease. More specifically, intratracheal (*i.t.*) treatment of C57BL/6 wild type (wt) mice with an IL-28-expressing adenovirus (AdIL-28) one day before challenge with the allergen ovalbumin (OVA), led to reduced production of the Th2 cytokines IL-5 and IL-13 in the bronchoalveolar lavage fluid (BALF), as well as in the lung-draining mediastinal lymph nodes (MLNs). In parallel, expression of the Th1 cytokine IFN-γ was induced. This observed shift in the Th-cytokine profile reduced allergic airway disease, as documented by the decreased lung resistance after metacholine challenge, the significantly reduced leukocyte infiltration in the lung tissue, and the significantly decreased goblet cell metaplasia and hypersecretion of airway epithelial mucus in lung tissue from AdIL-28- versus Ad0- or PBS-treated mice. Thus, IL-28 *in vivo* treatment had a protective role in the allergic airway disease model.

To further investigate whether endogenous levels of IFNλ/IL-28 affect allergic airway disease, we established an *in vivo* model of allergic disease in mice lacking the alpha chain of the IL-28 receptor (IL-28Rα$^{-/-}$ mice). These mice are not responsive to any of the type III IFNs (Ank *et al*. *J Immunol* (2008) 180:2474-2485). Wt or IL-28Rα$^{-/-}$ C57BL/6 mice were sensitized and subsequently challenged with the allergen (OVA/OVA mice) according to the protocol described in Figure 1A. Control mice were treated with PBS alone (PBS/PBS mice). All mice were sacrificed 24h after the last challenge.

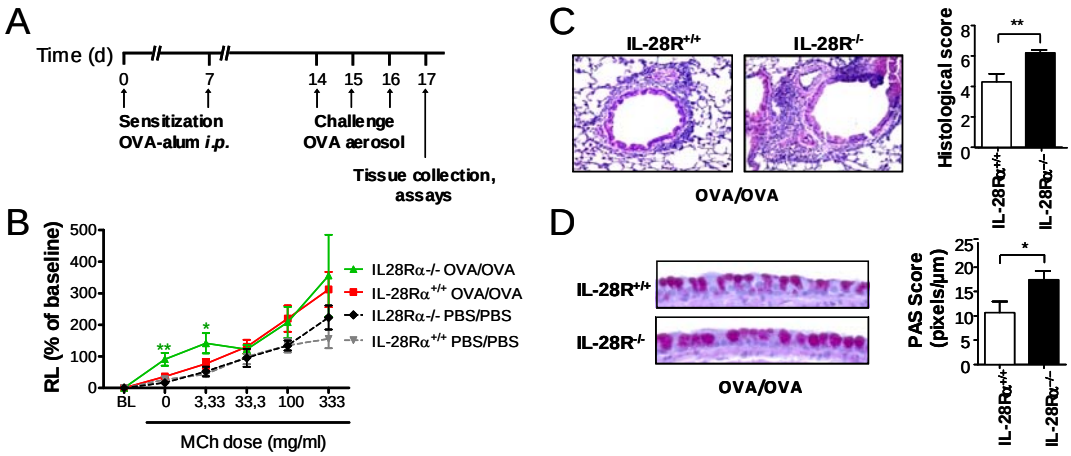


Figure 1: IL-28Rα-deficient mice develop increased airway inflammation and hyper-responsiveness in a mouse model of allergic airway disease. (A) Experimental protocol: wt and IL-28Rα$^{-/-}$ C57BL/6 mice were sensitized twice with OVA/alum and challenged for three consecutive days with inhaled OVA (OVA/OVA). Control mice were challenged with PBS (PBS/PBS). (B) Airway hyper-responsiveness measured as metacholine-induced increases of total lung resistance (RL) in mechanically ventilated wt and IL-28Rα$^{-/-}$ mice. Data are expressed as mean values of percentage increase from baseline of the total RL ± SEM from 10 mice per group pooled from two independent experiments. (C) Histological assessment of lung inflammation in wt and IL-28Rα$^{-/-}$ mice. H&E stained lung sections and histological scoring expressed as mean values ± SEM from 5-7 mice/group are shown. (D) Histological assessment of mucus secretion in wt and IL-28Rα$^{-/-}$ mice. PAS stained sections and morphometric analysis expressed as mean values ± SEM from 5-7 mice/group are shown.

When pulmonary resistance (RL) in mechanically ventilated mice was assessed, we found that OVA-sensitized and challenged IL-28Rα$^{-/-}$ mice exhibited significantly increased airway hyper-responsiveness in response to low metacholine doses as compared to wt controls (Figure 1B). No difference in lung resistance between PBS sensitized and challenged IL-28Rα$^{-/-}$ and wt mice was observed (Figure 1B). The increased airway hyper-responsiveness was accompanied by significantly enhanced

inflammatory infiltrates in the lung, as documented by haematoxylin and eosin (H&E) staining (Figure 1C), and increased goblet cell metaplasia, as documented by periodic acid-Schiff (PAS) staining (Figure 1D), in the airways of IL-28Rα$^{-/-}$ mice compared to wt controls. These data indicate that endogenously produced IFNλ/IL-28s are involved in dampening allergic airway inflammation and hyper-responsiveness in mice.

WP2: Dissection of the cellular events involved in IFNλ/IL-28-mediated immunomodulation.

The host laboratory has found that administration of IFN-λ2/IL-28A either in the initial allergen sensitization or the subsequent allergen challenge phase potently suppressed allergen-specific Th2 cytokines in the bronchoalveolar lavage fluid (BALF) and subsequent disease development. We thus analyzed T cell responses after IL-28 treatment in the allergic airway disease model.

Mice were sensitized and challenged with the allergen as described in Figure 2A. Some animals were treated intratracheally (*i.t.*) with an IL-28-expressing adenovirus (AdIL-28) one day before the first challenge or with a mock adenovirus (Ad0) or with PBS as controls (Figure 2A). All mice were sacrificed 24h after the last challenge. T cell responses were determined in the lung-draining mediastinal lymph nodes (MLNs) after in vitro re-stimulation with OVA. Figure 2B shows that IL-28 suppressed the production of the Th2 cytokines IL-5, IL-10 and IL-13, and induced the expression of the Th1 cytokine IFN-γ. Of interest, increased percentages of the naturally occurring regulatory T cells, defined by the phenotype CD4$^+$CD25$^+$Foxp3$^+$, among CD4$^+$ T cells were detected in the MLNs of AdIL-28- versus Ad0- or PBS-treated animals (Figure 2C). In summary, IL-28 had an impact on the Th1-Th2 cytokine balance and enhanced regulatory T cells.

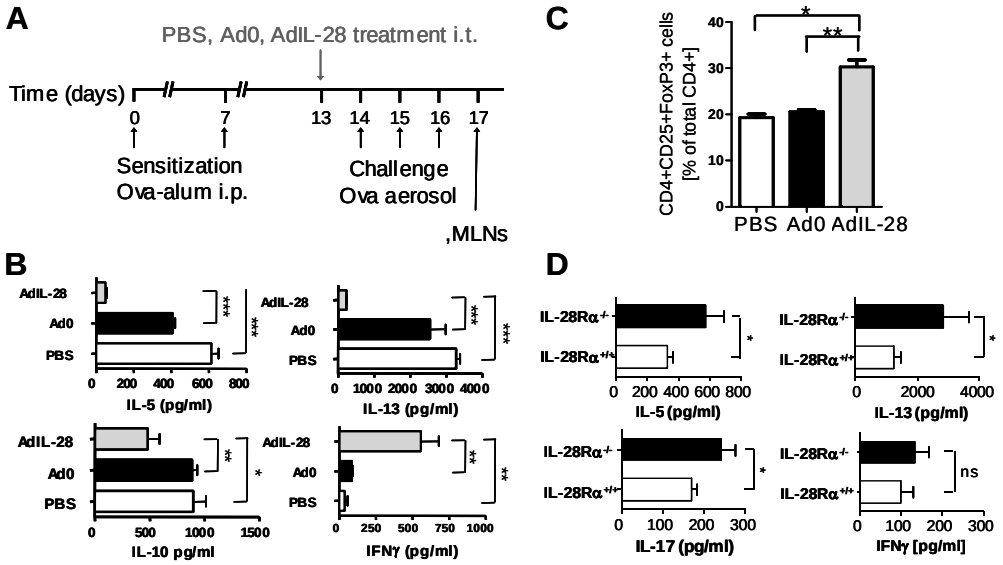


Figure 2: IL-28 mediates Th1 immune skewing and suppresses allergic airway disease. (A) Experimental protocol: C57BL/6 wt mice were subjected to sensitization and challenge with OVA, and in addition treated with PBS, mock adenovirus (Ad0) or IL-28-expressing adenovirus (AdIL-28) as designated. (B) Mice were sacrificed on day 17, and cytokine levels were determined by ELISA in the S/N of MLN cultures after *in vitro* restimulation with OVA. (C) CD4$^+$CD25$^+$Foxp3$^+$ regulatory T cell % among total CD4$^+$ T cells were determined in the MLNs of PBS-, Ad0- and AdIL-28-treated mice. (D) Secondary T cell responses in the lung-draining MLNs of OVA sensitized and challenged wt and IL-28Rα$^{-/-}$ C57BL/6 mice. Cytokine levels are expressed as mean values ± SEM of 7 mice per group from two independent experiments. *, $p< 0.05$; **, $p< 0.01$; ***, $p< 0.001$.

Further, when the ability of wt and IL-28Rα$^{-/-}$ mice to produce cytokines after a secondary response to the allergen was compared, we found that Th2 cell responses were profoundly augmented in IL-28Rα$^{-/-}$ mice. MLN cells from IL-28Rα$^{-/-}$ mice produced significantly higher levels of IL-5, IL-13 and IL-17 in response to OVA than their wt counterparts, whereas IFN-γ levels were not affected (Figure 2D). Taken together, these findings suggest that endogenously produced type III IFNs act as an inhibitor of Th2 and Th17 cell differentiation *in vivo* by favouring Th1 responses, thus limiting the development of allergic airway inflammation and hyper-responsiveness.

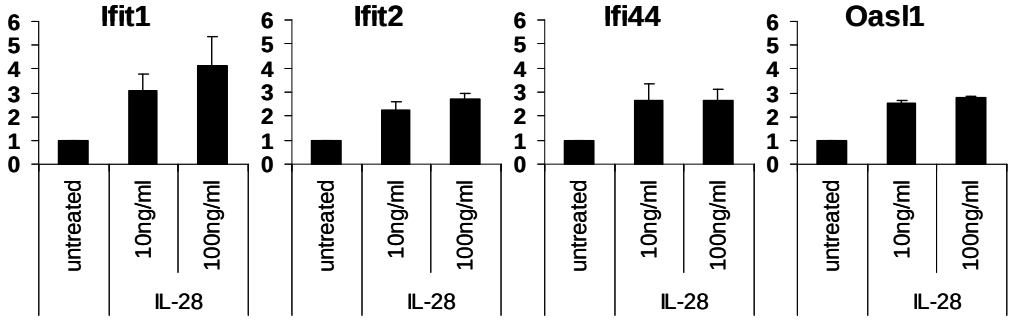
WP3: Identification of the molecular events that promote IFNλ/IL-28-mediated immunomodulation.

Myeloid DCs have been shown by the host laboratory to express the IFNλ/IL-28 receptor chains and to mediate the Th2-suppressive effect upon IFNλ/IL-28 stimulation. The aim of this WP is to identify the molecular events altered by IFNλ/IL-28 signalling that confer Th2-polarizing function to DCs. The transcriptome profile of IFNλ/IL-28-treated versus control DCs is under investigation. *In vitro* generated bone marrow DCs (BMDC) were cultured with titrating amounts of IFNλ2/IL-28A or vehicle control for varying time points and their transcriptome profile was determined with the Affymetrix GeneChip Mouse Exon 1.0 ST Array in collaboration with Dr. Vassili Soumelis, Institut Curie, Paris, France. A total of 23.332 genes were analyzed in biological duplicate experiments. Figure 3A shows a summary of the total number of genes that were regulated upon IL-28 treatment. Of note, signature-genes, which are known targets of IL-28 signalling, were validated to be significantly upregulated. Among them, the anti-viral genes interferon-induced protein with tetratricopeptide repeats 1 (Ifit1), interferon-induced protein with tetratricopeptide repeats 2 (Ifit2), interferon-induced protein 44 (Ifi44) and 2'-5' oligoadenylate synthetase-like 1 (Oasl1) were readily induced at 3h after IL-28 stimulation (Figure 3B). Additional gene ontology-based clustering and pathway analyses was performed in collaboration with the Bioinformatics Department of the BRFAA, while interesting candidates were validated using real-time PCR, as shown for the gene NOS2 or iNOS in Figure 3C, which is a type I immune mediator that we found to be upregulated by IL-28 treatment.

A

	3h				24h			
	10ng/ml IL-28		100ng/ml IL-28		10ng/ml IL-28		100ng/ml IL-28	
	Exp.1	Exp.2	Exp.1	Exp.2	Exp.1	Exp.2	Exp.1	Exp.2
↑ genes	113	161	96	183	158	274	57	165
↓ genes	69	120	57	192	18	176	30	160

B



C

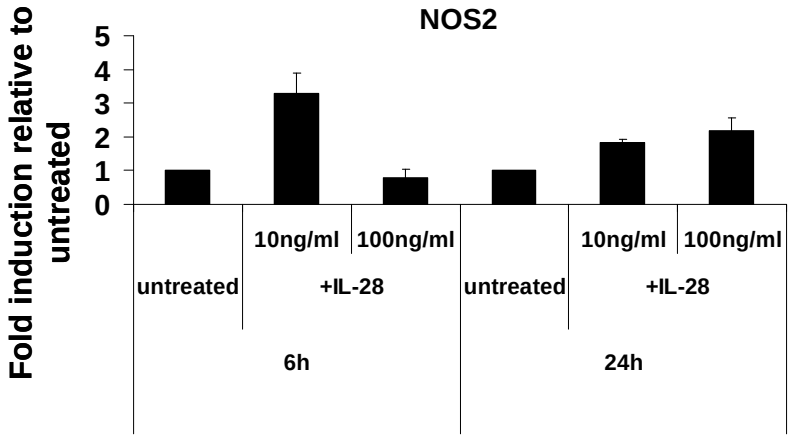


Figure 3: Transcriptome profile analysis of IL-28-stimulated BMDCs. (A) Summary of total genes regulated after IL-28 treatment, as analyzed with the GeneChip Mouse Exon 1.0 ST Array. (B) mRNA expression of the anti-viral genes Ifit1, Ifit2, Ifi44 and Oasl1 in BMDCs treated with either 10 or 100ng/ml IL-28 for 3h, as determined with the GeneChip Mouse Exon 1.0 ST Array. Results are expressed as fold induction (mean levels ± SEM of duplicate experiments) relative to expression levels of untreated BMDCs. (C) Expression levels of the NOS2 gene as validated by qPCR analysis. Results are expressed as fold induction (mean levels ± SEM of duplicate experiments) relative to expression levels of untreated BMDCs.

WP4: Design of novel immunotherapeutic agents for the treatment of allergic airway disease.

Upon identification of IL-28s as main regulators of the allergic airway response, we focused our efforts on the screening of innovative therapeutic agents that govern IL-28 production and subsequently the regulation of the allergic immune response. We have thus generated a novel Il28a_T2A_eGFP constitutive knock-in reporter mouse, in which the T2A-eGFP coding sequence was inserted in between of the last codon and the STOP codon of the Il28a exon 5, respecting the Il28a translation frame (Figure 4A). This allele expresses a chimeric transcript harboring the Il28a exons 1 to

5 fused with the T2A and the eGFP sequences. The presence of the T2A sequence induces a co-translational cleavage between the Il28a-T2A and the eGFP proteins, resulting in co-expression of the Il28a-T2A and the EGFP proteins under the control of the endogenous Il28a promoter. This construct allows for the equimolar production of the IL-28a and eGFP proteins, consisting thus a reliable tool for the detection of IL-28 production. In order to screen for potential agents that induce IL-28 production, we have generated Flt3L-derived BMDC that give rise to the two major DC populations of conventional DC (cDC) and plasmacytoid DC (pDC), and sorted them to high purity defined as $CD11c^{+}B220^{-}$ and $CD11c^{+}B220^{+}$ respectively (Figure 4B). Sorted cells were placed in culture with several TLR ligands and induced IL-28a/eGFP expression was monitored by flow cytometry. Figure 4C shows cumulative data of eGFP expression induced in cDC by the TLR3 ligand poly(I:C) and to a lesser degree by the type A CpG-ODN1585 binding to TLR9. Moreover, ODN1585 is the best inducer of IL-28a/eGFP in pDCs that also respond to the TLR7 ligands R848 and CL097 to produce lower amounts of IL-28a/eGFP. The specificity of our reporter system was further validated by IL-28a mRNA expression which was restricted only in the GFP^{+} cells, and by the detection of IL-28 protein levels in cell culture supernatants which was respectant to the GFP expression (data not shown). Finally, the use of the Il28a_T2A _eGFP reporter mouse in our allergic airway mouse model allowed for the identification of IL-28a/eGFP-expressing cells in the inflamed airways of mice (Figure 4D). The Il28a_T2A _eGFP reporter mouse consists thus a valuable tool for the screening of IL-28-inducing agents *in vitro* and *in vivo*.

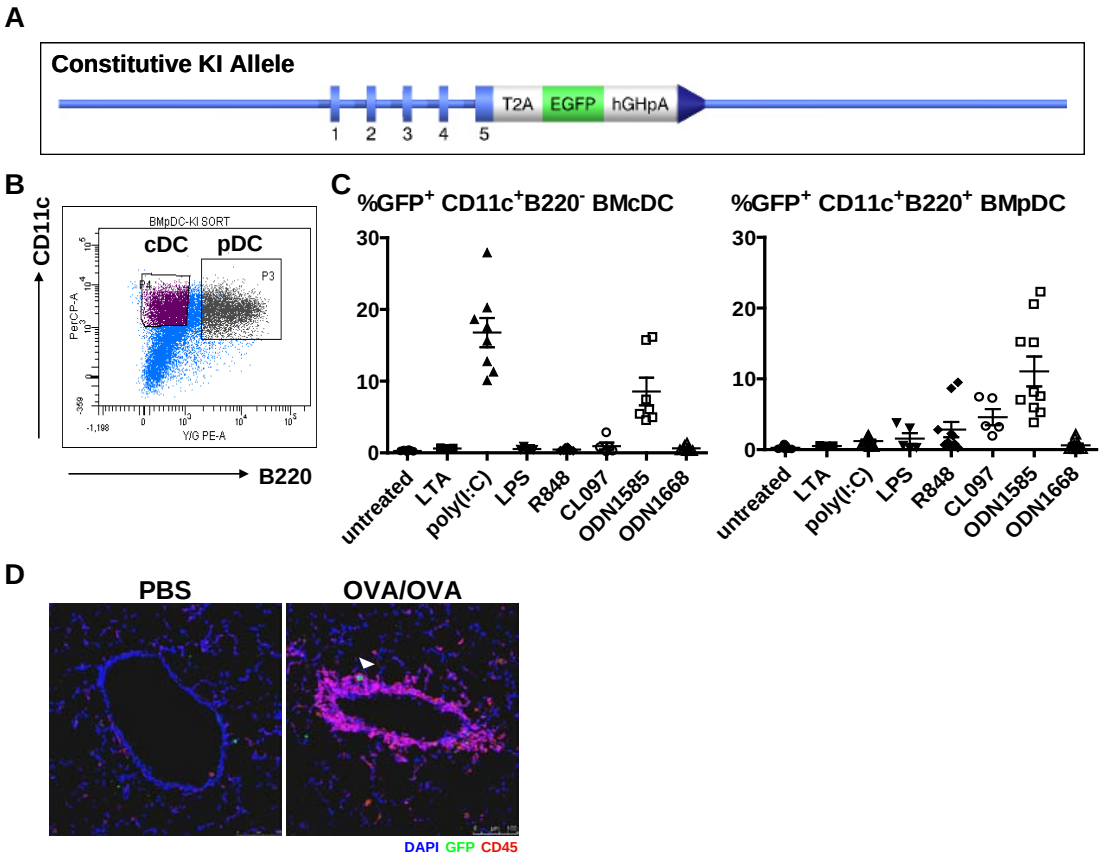


Figure 4: Generation of a constitutive Il28a_T2A _eGFP knock-in mouse. (A) Cassette used for the generation of the Il28a_T2A_eGFP knock-n mouse. (B) Gating strategy for the FACS sorting of the two distinct DC subpopulations, conventional DCs (cDC) and plasmacytoid DC (pDC), after 7 days of culture with 200ng/ml

rhFlt3L (C) GFP expression of Flt3L-derived DC subsets after treatment with TLR ligands for 12h. Results are expressed as percentages of GFP^+ cells among the total DC population. Every point corresponds to one individual experiment. Arrow bars represent mean levels ± SEM. (D) *In vivo* IL-28a/eGFP expression induced in the lungs of OVA-sensitized and challenged mice, localized in an inflamed airway. $eGFP^+$ cells are shown in green, $CD45^+$ infiltrating leucocytes in red, and nuclei in blue stained with DAPI.

Overall, we trust we have fully addressed the project's main goals according to plan. In addition, we have developed novel technology by the generation of the Il28a_T2A _eGFP mouse that will be a useful tool for the screening of potential therapeutic regimes that induce IL-28 production, which in turn shifts the T cell response to a Th1 phenotype and suppress allergic airway disease.

Acknowledgments
PhD students Ourania Koltsida, MD and Athanasios Stavropoulos have contributed to this work.

Significant results of NILTHERA

- Identification of the role of endogenous IFNλ/IL-28s in controlling allergic airway inflammation and hyper-responsiveness in mice by shifting Th cytokine balance.
- Identification of the role of IFNλ/IL-28s in the regulation of allergen-specific T cell responses and protection from allergic airway disease.
- In-depth analysis of the precise mechanism of action of IFNλ/IL-28s by defining the molecular pathways altered by IFNλ/IL-28 treatment that confer to DC regulatory function in allergic airway disease.
- Development of a novel tool (Il28a_T2A _eGFP mouse) for the screening of prospective immunotherapeutic agents.

Progress of the researcher training activities/transfer of knowledge activities/integration activities:

During the reporting period, the fellow has successfully incorporated in the host laboratory and her home country. She has expanded her laboratory skills, by being introduced in animal allergic airway disease models and experimental aspects for evaluation of disease severity. Further, she gained useful experience on novel technologies, such as mRNA expression profile analysis, by collaborating with in-house (Bioinformatics Department, BRFAA), as well as international collaborators (Dr. Vassili Soumelis, Institut Curie, Paris, France)..
Further, within the frames of the host laboratory and host institution, the fellow had the opportunity to participate in several seminars and meetings, including internationally-renowned scientists. Of importance, the fellow had the opportunity to expand her leadership qualities by training and supervising diploma and MSc students.
With regards to the scientific goals, the fellow has contributed in the investigation of the immunomodulatory role of IL-28s in allergic airway disease, by showing that endogenous production of IFNλ/IL-28s is crucial for the control of allergic airway inflammation and hyper-responsiveness in mice, through the modulation of T cell responses. As DCs were identified as the main responders to IL-28-treatment, the fellow took over the analysis of the molecular profiling of the events taking place

after IL-28 treatment, and has identified interesting candidates that have set the basis for further work in the host laboratory. Finally, she took over the task of analysing a novel tool introduced in the lab of Dr. Andreakos, the unique Il28a_T2A _eGFP reporter mouse, leading to innovative findings on IL-28 biology and consisting a useful screening approach for immunotherapeutic agents.