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Ο ρόλος της ενδογενούς έκφρασης της Οστεοποντίνης στην ανάπτυξη και την λειτουργία των Foxp3^{+} Τ ρυθμιστικών λεμφοκυττάρων

Σκόρδος Ιωάννης

Υποβολή μεταπτυχιακής διατριβής για την απόδοση Μεταπτυχιακού Τίτλου στον τομέα Της Μοριακής Βάσης των Ανθρωπίνων Ασθενειών του τμήματος Ιατρικής του Πανεπιστημίου Κρήτης

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Cellular Immunology Laboratory,
Center for Basic Research,
Biomedical Research Foundation Academy of Athens

University of Crete School
of Medicine

The role of endogenous Osteopontin expression in the development and function of Foxp3^{+} regulatory T cells

Skordos Ioannis

A thesis submitted in fulfilment of the requirements for the degree of Masters of Science in The Molecular Basis of Human Disease under the aegis of Medical Department, University of Crete

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Η παρούσα μεταπτυχιακή διατριβή πραγματοποιήθηκε στο εργαστήριο Κυτταρικής Ανοσολογίας, της ερευνήτριας Β. Πανουτσακοπούλου, το οποίο στεγάζεται στο Ίδρυμα Ιατροβιολογικών Ερευνών της Ακαδημίας Αθηνών (ΙΙΒΕΑΑ), στα πλαίσια του μεταπτυχιακού προγράμματος «Μοριακής Βάσης των Ανθρωπίνων Ασθενειών» του τμήματος Ιατρικής του Πανεπιστημίου Κρήτης.

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The following Master thesis carried out in the Cellular Immunology Laboratory which belongs to V. Panoutsakopoulou, PhD. The Lab is located at Biomedical Research Foundation Academy of Athens (BRFAA). This thesis was undertaken for fulfilment of the requirements for the degree of Master of Science in "The Molecular Basis of Human Disease", Medical Department of University of Crete.

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ΕΥΧΑΡΙΣΤΙΕΣ....

Κατ' αρχήν οφείλω να πω ένα τεράστιο ευχαριστώ στην καθηγήτρια-ερευνήτρια Βίλη Πανουτσακοπούλου, η οποία μου έδωσε την ευκαιρία να εργαστώ στο εργαστήριο της και να γνωρίσω τι πραγματικά σημαίνει ανοσολογία καθώς επίσης και ανοσολογική έρευνα. Σας ευχαριστώ μέσα από τα βάθη της καρδιάς μου για όλες τις επιστημονικές συμβουλές, κατευθύνσεις και ευκαιρίες που μου δώσατε και ελπίζω από την πλευρά μου να μην σας απογοήτευσα. Θα σας είμαι για πάντα ευγνώμων και υπόχρεος για την δυνατότητα που μου δώσατε να συμμετάσχω σε ένα από τα μεγαλύτερα και διεθνούς φήμης ανοσολογικά συνέδρια (WIRM conference), μέσα από το οποίο κατάφερα να προβάλω την εργαστηριακή μας δουλειά, επέκτεινα τις ήδη υπάρχουσες αναοσολογικές μου γνώσεις, καθώς επίσης άκουσα και γνώρισα, σε πρώτο πρόσωπο, πολλούς από τους <πατέρες> της ανοσολογίας.

Επίσης θα ήθελα να εκφράσω τις τεράστιες ευχαριστίες ευχαριστίας μου στον ερευνητή Νίκο Πασχαλίδη. Πραγματικά, ήταν ο άνθρωπος που πίστεψε σε μένα εξ' αρχής, με μύησε στον χώρο της ερευνητικής ανοσολογίας και ήταν πάντα δίπλα μου πρόθυμος να με βοηθήσει και να με συμβουλέψει για οποιαδήποτε μου απορία. Πρόκειται για τον πιο ολοκληρωμένο μέντορα που έχω γνωρίσει και έχει περάσει από την ερευνητική-φοιτητική μου ζωή μέχρι τώρα και πιστεύω ότι έχει συμβάλει τα μέγιστα στη διαμόρφωση της ερευνητικής μου ταυτότητας και της κρητικής μου σκέψης. Θεωρώ τον εαυτό μου πολύ τυχερό που συνεργάστηκα μαζί του και έχω να δηλώσω ότι μακάρι όλοι οι supervisors να έμοιαζαν στον Νίκο. Ένα μόνο μειονέκτημα έχει, είναι παοκτζής, αλλά δεν πειράζει γιατί κανείς δεν είναι τέλειος.

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ΥΓ. Δεν πρέπει να παραλείψω να ευχαριστήσω και τη Φατσούλα, το σκυλάκι μου, που μου κρατούσε συντροφιά όλα τα βράδια της συγγραφής της μεταπτυχιακής μου διατριβής.

Σε όλα τα μέλη της οικογένειάς μου…

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ABBREVIATIONS

Alum	Aluminum hydroxide
APC	Antigen presenting cells
BAL	Bronchoalveolar lavage
BSA	Bovine serum albumin
CD	Cluster of differentiation
cDCs	Conventional/classical dendritic cells
CTV	Cell Trace Violet
DI	Division Index
DC	Dendritic cells
ECM	Extracellular matrix
EOS	Eosinophils
FACS	Fluorescence activated cell sorter
FCS	Foetal calf serum
Foxp3	Forkhead box P3
FSC	Forward side scatter
GFP	Green fluorescent protein
GM-CSF	Granulocyte macrophage colony stimulating factor
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IL	Interleukin
iOpn	Intracellular Osteopontin
IRF	Interferon regulatory factor
iTregs	Induced regulatory T cell
KO	Knock-out
LMs	Lymphocytes
LN	Lymph node
MACs	Macrophages
MLN	Mediastinal lymph nodes
mLNs	Mesenteric lymph nodes
MMP	Matrix metalloproteinases
Neus	Neutrophils
NK	Natural killer cell
nTregs	Natural/thymic regulatory T cell
Opn	Osteopontin
OVA	Ovalbumin
PAS	Periodic acid-Sciff staining

PBS	Phosphate-buffered saline
pDCs	Plasmacytoid dendritic cell
PMA	Phorbol 12-myristate 13-acetate
PMAs	Post translational modifications
RGD	Arginine-glycine-aspartate domain
sOpn	Secreted Osteopontin
Spp1	Secreted phosphoprotein
TCR	T-cell receptor
TGF-β	Transforming growth factor-betta
Th cell	T helper cell
TNF	Tumor necrosis factor
WT	Wild type

ΠΕΡΙΛΗΨΗ

Η Οστεοποντίνη (Opn) είναι μία ισχυρά γλυκοζυλιωμένη φωσφο–πρωτεΐνη η οποία κάτω από φυσιολογικές συνθήκες εκφράζεται από μια μεγάλη ποικιλία κυττάρων, συμπεριλαμβανομένων των ενεργοποιημένων Τ-λεμφοκυττάρων. Η έκφραση της Opn σχετίζεται με διάφορες φυσιολογικές διαδικασίες όπως την κυτταρική κίνηση, προσκόλληση και απόπτωση. Πολλές μελέτες έχουν αποδείξει τον σημαντικό ρόλο της Opn στις ασθένειες που προκαλούνται από την απορύθμιση του ανοσοποιητικού συστήματος καθώς επίσης και τη συμβολή της στην καρκινική μετάσταση. Προγενέστερες ερευνητικές μελέτες του εργαστηρίου μας, πάνω σε πειραματικά ζωικά μοντέλα άσθματος σε ποντίκια, απέδειξαν την αντί-αλλεργική δράση της Opn κατά τη διάρκεια της δευτερογενούς απόκρισης στο αντιγόνο (Xanthou et al., 2007). Μη δημοσιευμένα ερευνητικά μας αποτελέσματα έδειξαν ότι τα Foxp3^{+} ρυθμιστικά Τ κύτταρα ήταν εν μέρει υπεύθυνα για τις προστατευτικές ιδιότητες της Opn. Προκαταρκτικές μελέτες έχουν δείξει ότι το γονίδιο της Opn (*Spp1*) είναι ένα από τα λίγα γονίδια που υπερεκφράζονται κατά την ενεργοποίηση των Foxp3^{+} Τ ρυθμιστικών κυττάρων. Ωστόσο ο ρόλος της ενδογενούς έκφρασης της Opn στην παραγωγή και στη λειτουργία των Foxp3^{+} Τ ρυθμιστικών κυττάρων, παραμένει άγνωστος. Έτσι ο πρωταρχικός στόχος μας ήταν να ερευνήσουμε τις συνέπειες της ενδογενούς έκφρασης της Opn στην ανάπτυξη και τη λειτουργία Foxp3^{+} Τ ρυθμιστικών κυττάρων.Τα αποτελέσματα της έρευνάς μας έδειξαν ότι η ενδογενής έκφραση της Opn είναι προαπαιτούμενη για τη βέλτιστη δημιουργία TGF-β-επαγόμενων Τ ρυθμιστικών κυττάρων, *in vitro*. Επίσης, μέσα από *in vivo* πειράματα μεταφοράς μη ενεργοποιημένων CD4^{+} Τ σε ανοσοανεπαρκή Rag$^{-/-}$ ποντίκια και της επακόλουθης μελέτης της ομοιοστατικής τους ανάπτυξης, παρατηρήσαμε μειωμένη επαγωγή του μεταγραφικού παράγοντα Foxp3 μόνο στα Τ λεμφοκύτταρα στα οποία έλειπε η ενδογενής Opn. Έπειτα εξετάσαμε τις συνέπειες της έλειψης έκφρασης του γονιδίου της Opn στη ανοσοκατασταλτική λειτουργία των επαγόμενων-Τ ρυθμιστικών κυττάρων *in vitro,* και βρήκαμε ότι η Opn είναι αναγκαία για τη βέλτιστη ανοσοκατασταλτική λειτουργία τους. Τέλος, χρησιμοποιώντας μοντέλα αλλεργικού άσθματος, μελετήσαμε την *in vivo* ανοσοκατασταλτική ικανότητα των

επαγόμενων Τ ρυθμιστικών κυττάρων. Συγκεκριμένα η μεταφορά ενεργοποιημένων επαγόμενων-Opn KO Τ ρυθμιστικών κυττάρων δεν κατέστειλε την ανάπτυξη του αλλεργικού άσθματος στα πειραματικά μοντέλα, υποδεικνύοντας ότι η ενδογενής έκφραση της Opn είναι απαραίτητη για τη βέλτιστη ανοσοκατασταλτική ικανότητα των επαγομένων Τ ρυθμιστικών κυττάρων και *in vivo*.

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ABSTRACT

Osteopontin (Opn) is a glycophosphoprotein that is normally expressed by a wide variety of cells types, including activated T cells. Opn is involved in many physiological processes such as chemotaxis, cell adhesion and apoptosis. Many studies have described a crucial role of Opn in immune mediated diseases and tumor metastasis. Previous work from our group has demonstrated that Opn has anti-allergic effects during secondary antigenic challenge in a mouse model of allergic airway inflammation (Xanthou et al., 2007). Further studies in our laboratory suggested that these protective effects were partially mediated by $Foxp3^+$ Tregs. Interestingly, Opn gene (*Spp1*) is one of the few significantly upregulated genes in stimulated $Foxp3^+$ Treg cells (Do et al., 2015; Marson et al., 2007); however its role in the generation and function of these cells remains elusive. The aim of our project was to investigate the effects of endogenous Opn on development and function of $Foxp3^+$ Treg cells. Our data showed that Opn expression is required for optimal Foxp3 induction in transforming growth factor-beta (TGF-β)-generated Tregs *in vitro*. We have also observed diminished Foxp3 induction in Opn knockout T cells in an *in vivo* model of homeostatic expansion of naïve T cells in $Rag^{-/-}$ mice. Furthermore, the effects of genetic deletion of Opn in the function of induced-Treg cells *in vitro* were investigated and results indicated that Opn is required for their optimal suppressive function. Finally, adoptive transfer experiments of iTreg cells, as a means of protection from the development of allergic airway inflammation, showed that endogenous Opn expression is indispensable for the optimal *in vivo* suppressive capacity of iTreg cells.

1. INTRODUCTION

1.1. Osteopontin

Osteopontin (Opn, or early T lymphocyte activation-1; Eta-1 or secreted phosphoprotein-1; Spp1) is a glyco-phosphoprotein (Christensen et al., 2005) that mediates diverse biological functions and belongs to the SIBLING (Small Integrin-Binding Ligand, N-linked Glycoprotein) family of proteins (Fisher and Fedarko, 2009). It is normally expressed by a wide variety of cells, it has two isoforms (in immune cells; the secreted, sOpn and the intracellular Opn, iOpn) (Inoue and Shinohara, 2011), interacts with integrin receptors and is very crucial for the cell adhesion and chemotaxis functions.

Opn was originally isolated as a protein secreted by transformed mammalian cells and human malignant tumors (Senger et al., 1979). In 1985 it was termed "Osteopontin" because it was found as sialoprotein derived from bovine bone matrix with a critical role in bone remodeling (Oldberg et al., 1986) and in 1989, due to the finding that Opn could act as a cytokine (Th1)-expressed by activated T cells (Patarca et al., 1989), it was the first time that Opn was directly connected to immunity (**Figure 1**).

Figure 1 Timeline of milestone research findings regarding the function of Opn.

1.1.1. Opn gene & protein

Opn is encoded by a single gene (*Spp1* locus*)* which is located on the mouse chromosome 5 and on the long arm of the human chromosome 4q13 (Fet et al., 1989; Young et al., 1990). *Spp1* locus contains 7 exons of which exon 1 is noncoding (Hijiya et al., 1994) and is directly related to four similar genes encoding for dentin sialophosphoprotein (DSPP), dentin matrix protein 1 (DMP1), bone sialoprotein (BSP) and matricellular proteins (including tenascin-c, galectins, periostin, syndecan-4 and others) (Crosby et al., 1995; Fedarko et al., 2004). Opn is expressed by a wide variety of cell types, including osteoblasts, endothelial cells, vascular smooth muscle cells, epithelial cells, neural cells, macrophages, dendritic cells and activated lymphocytes (T and B lymphocytes).

Three different splice variant forms of Opn cDNA have been identified in the human system: Opn-a, Opn-b and Opn-c, are made from alternative splicing in the N-terminal region of OPN and has been described to be implicated differently to the tumor survival and invasion (Anborgh et al., 2011). Opn-a encodes the entire Opn, Opn-b lacks the exon 5 (deletion of 14amino acids AA58-71) whereas Opn-c lacks the exon 4 (deletion of 27 amino acids AA31-57). Among the deleted regions are included O-glycosylated and phosphorylated sites, whose functional significance remains unknown.

Opn is composed of approximately 300-314 amino acids residues (Anborgh et al., 2011) and is highly conserved among many species. The molecular weight of sOpn is between 44–75 kDa due to high amount of post translational modifications (PMAs) such as phosphorylation, glycosylation and sulphation (Sodek et al., 2000). These modifications may impact both on Opn structure and function. As far as the dimensional structure of Opn , the use of MRI technique revealed that Opn exists as an open, flexible molecule in solutions completely devoid of secondary structures (Fisher et al., 2001). However this question is still controversial, due to current data which demonstrates that Opn's functional interactions with integrins or other binding partners can be modulated by intramolecular interactions (Yamaguchi et al., 2010).

The protein is located both as a non-collagenous immobilized molecule of the extracellular matrix (ECM) in mineralized tissues (brain , kidney and bones) and as a secreted protein (sOpn)-cytokine in body fluids such as milk, urine, saliva, blood,

seminal fluid and bile whereas elevated levels are observed in both physiological and pathophysiological conditions (Lund et al., 2009).

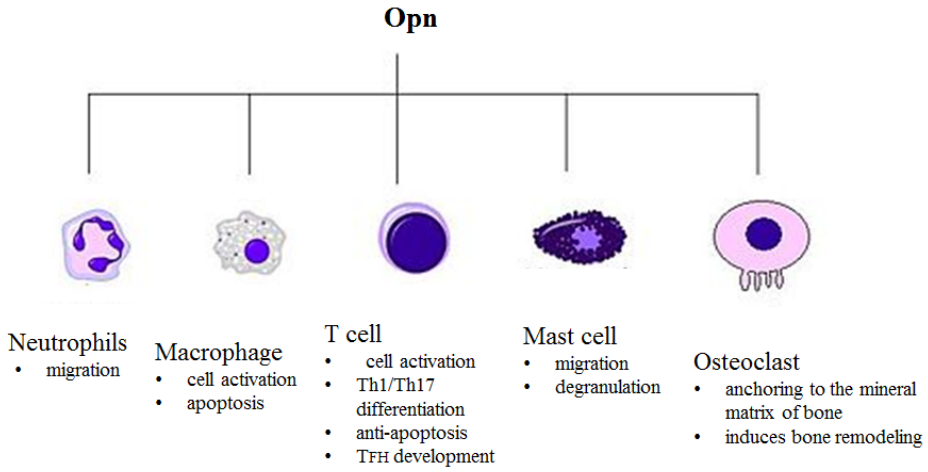

Figure 2 Known immunologic functions of Opn. Adapted from Biological functions of OPN, in Wikipedia, n.d. Retrieved June 22, 2015, from https://commons.wikimedia.org/wiki/File:Biological_functions_of_OPN.jpg.

1.1.2. Intracellular Opn

As mentioned previously apart from the secreted Opn there is another form of Opn which can be found in the cytoplasm and the nucleus (Gomaa et al., 2013; Junaid et al., 2007). This form of Opn has been termed as intracellular Opn (iOpn). Both sOPN and iOpn are produced from *Spp1* gene by translation of different initiation sites and they have different and distinct biological function (**Figure 3**). Specifically, iOpn, in contrast to sOpn, exerts its effect by binding to MyD88, which is a downstream molecule of the Toll-like receptor (TLR). It has been shown, while viral infection, in pDCs (plasmocytoids dendritic cells) that iOpn is responsible for the regulation of the MyD88-IRF7 (interferon regulatory factor 7)-mediated production of type 1 interferons (IFNs) (Inoue and Shinohara, 2011), while in murine macrophages iOpn is suggested to be a negative regulator of both TLR4/MyD88 signaling and TLR-induced IFN-β production, thus playing an important role in inflammation-related

hepatocarcinogenesis (Fan et al., 2015; Zhao et al., 2010). Furthermore, in cDCs (conventional dendritic cells), it is demonstrated that the expression of iOpn negatively regulates the IL-27 expression and drives the Th17 differentiation (Shinohara et al., 2008). In addition, it was recently found, through experiments with iOpn knock–in and *Spp1*$^{-/-}$mice, that iOpn is important for Bcl-6-dependent functional differentiation of follicular regulatory T (T_{FR}) cells (Leavenworth et al., 2015a). Finally, it was also described the importance of iOpn (as an intermediate within the IL-15 signaling pathway) in survival and function of natural killer (NK) cells (Leavenworth et al., 2015b). However, it is still debatable and under investigation whether iOpn is expressed naturally in naïve or activated T cells and how this expression affects T cell differentiation and function.

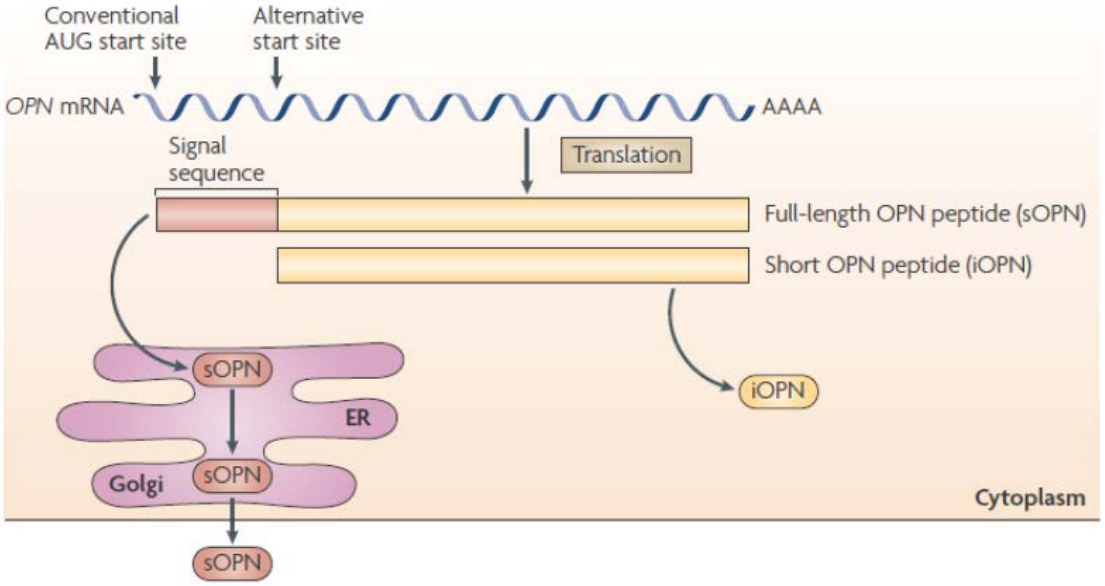

Figure 3 Generation of iOpn and sOpn by alternative translational mechanism. Following transcription and processing, mature osteopontin (Opn) mRNA has a conventional AUG translation start site and a downstream alternative translation start site. Translation from the conventional start site results in the generation of the full-length Opn peptide, which includes a signal sequence that targets the nascent peptide to secretory vesicles, resulting in its secretion from the cell (sOpn). Translation from the alternative translation start site results in a short Opn peptide product that lacks a signal sequence and remains inside the cell (iOpn). ER, endoplasmic reticulum (Cantor and Shinohara, 2009).

1.1.3. sOpn structure and receptors

The sOpn protein has a variety of highly conserved motifs, of different functions, among mammalian species within the total sequence (**Figure 4**). Importantly, an arginine-glycine-aspartate (RGD) domain is located at the center of the molecule. This is a classical cell binding motif that is recognized by different cell surface RGD-recognizing integrins (αvβ1, αvβ3, αvβ5, αvβ6 and α5β1) (Yokosaki et al., 2005). These integrin receptors are expressed by variety of cells, including immune system cells. Next to the RGD domain of Opn, it is located a thrombin-plasmin cleavage site (Christensen et al., 2010). After the cleaving of sOpn by proteases, a cryptic binding site is exposed (SLAYGLR in mice; SVVYGLR in humans) which is recognized by non-RGD-recognizing integrins such as α4β1, α9β1, α4β7 integrins (Grassinger et al., 2009; Smith et al., 1996) and has been implicated in inflammation (Hasegawa et al., 2009). Immune cells such as macrophages and T cells express in their surface α4β1 integrins which can recognize both the intact and the thrombin cleaved forms of sOpn. Furtherly, sOpn can be cleaved by matrix metalloproteinases (MMP-3/ MMP-7) (Agnihotri et al., 2001) at a position between G and L within the SVVYGLR sequence and this creates a sOpn form which is recognizable only by the α4β1 integrin receptor (Ito et al., 2009). However, the biological significance of protease modifications of sOpn, *in vivo* is still under investigation.

Another important group of receptors for sOpn are the spliced variant forms of CD44 (CD44v), but not the standard form of CD44 (CD44s). Until now, it has not been identified the precise binding site within OPN which is recognized by the CD44v receptors, although it has been suggested that CD44v interacts with both the C- and the N-termini regions of sOpn(Weber et al., 1996). The binding of OPN to CD44v mediates adhesion, chemotaxis and promotes cell proliferation of bone marrow cells and fibroblasts. During inflammatory responses, the CD44v expression is upregulated in antigen stimulated lymphocytes, so that the interaction between sOpn and CD44v by lymphocytes to induce the chemotaxis and the subsequent migration of activated lymphocytes into the site of inflammation (Denhardt et al., 2001; Senger et al., 1996; Weiss et al., 2001; Zohar et al., 2000). Also, the sOpn-CD44v ligation on macrophages is known to downmodulates the IL-10 expression, resulting in the inhibition of Th2 immune responses, while on the other hand the sOpn-αvβ3 ligation

on macrophages enhances the IL-12 expression, favoring the generation of Th1 cells (Ashkar et al., 2000).Recently, it was found that another matricellular protein like sOpn, the galectin 9 was shown to interact with the CD44 receptor and enforce iTreg cell differentiation and maintenance(Wu et al., 2013). Besides that, in a tumor model with a pancreatic carcinoma cell, line β1 integrin expression was shown to be essential for OPN binding to CD44 variants (Katagiri et al., 1999).

Finally, sOpn contains another two well conserved motifs; an aspartate-rich region and two putative heparin binding domains (HBD). Although, immunological functions have not been attributed to these regions, syndecan-4 is known to bind one of the two HBD on sOpn and protects against OPN-mediated injury (hepatotoxicity) by masking functional domains on the sOpn molecule (Kon et al., 2008).

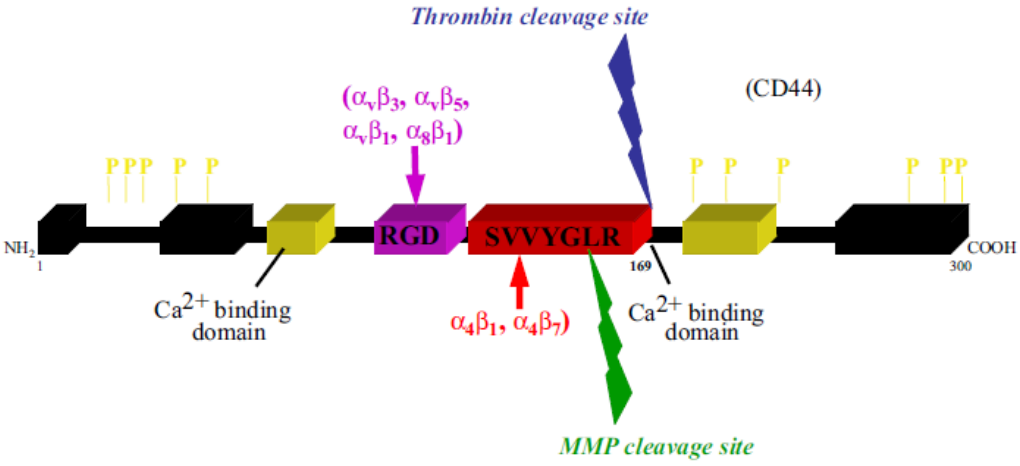

Figure 4 Opn structure and receptors for sOPN. Opn contains a classical cell-binding motif, RGD and non-classical binding motif of SVVYGLR, which is exposed after protease digestion. Osteopontin is also cleaved by other proteases. SVVYGLR is recognized by a9b1 and a4b1 integrins. Matrix metalloproteinase (MMP)-cleaved SVVYG fragment can be recognized by α4β1, but not α9β1 integrin (Lund et al., 2009).

1.2. Opn regulates T cell immune responses

It has been shown that sOpn is a key cytokine for the polarization of T helper (Th) cells and the inhibition of apoptosis of stimulated T cells. It modulates the T cell

phenotype by direct effects either on APCs or T cells. Specifically, the binding of sOpn to $\alpha_v\beta_3$ integrin on murine macrophages stimulates the pro-inflammatory interleukin (IL)-12 cytokine release and at the same time through the interaction with the CD44 receptor downmodulates the anti-inflammatory IL-10 expression. As a consequence sOpn induces Th1 immune responses which are STAT4-mediated (Afkarian et al., 2002; Ashkar et al., 2000; Mullen et al., 2001).

In turn, it has been demonstrated that activated T cells secrete high amounts of sOpn which is controlled by T-bet, a member of the T box family of transcription factors. T-bet is the key transcription factor for induction of Th1 immune responses. *Opn mRNA* expression during T cell activation is positively regulated by T-bet and this T-bet-mediated sOpn expression in T cells is critical for skewing of naïve $CD4^+$ T cells toward Th1 cell-lineage commitment phenotype (Shinohara et al., 2005). In human T cells the expression of sOpn induces CD3-mediated IFN-γ production as well as CD40L (ligand) expression. So on, it creates a positive feedback loop which in turn augments the production of IL-12 by macrophages (T cell-mediated) (O'Regan et al., 2000). It is still debatable, at least in mice, if IFN-γ expression induces or not sOpn expression (Croitoru-Lamoury et al., 2011; Hu et al., 2012; Li et al., 2003; Murugaiyan et al., 2010); the most prevalent version is that high concentrations of IFN-γ suppresses *Opn* expression (Murugaiyan et al., 2010). Inoue and Shinohara *at* 2011 (Inoue and Shinohara, 2011) suggested that increase of *Opn* expression is an early event during Th1 polarization and in progress of Th1 immune response while increased levels of IFN-γ inside the cytokine milieu create a feedback loop mechanism which in turn downregulate *Opn* expression (**Figure 5**).

Studies have also demonstrated the important role of sOpn in polarization of Th17cells, during inflammation. It has been described that sOpn is capable of inducing expression of IL-17, both in murine and human activated $CD4^+$ T cells (Murugaiyan et al., 2008) and directly to influence Th17 differentiation (Chen et al., 2010). The mechanism of sOpn-mediated-Th17 cell differentiation is suggested to be independent of IL-6/STAT3 pathway, at least in human $CD4^+$T cells, and to be mediated by the upregulation of RORγ/RORα transcription factors and their increased accessibility to *il17A* gene locus (Chen et al., 2010). In addition, the usage of $Opn^{-/-}$ mouse models for the study of Th17-mediated inflammatory diseases has also indicated that sOpn may support skewing towards Th17 phenotype (Frenzel et al.,

2015). However, it is debatable on which integrin (β3,β1) or CD44 receptor, on cell surface of activated $CD4^+$ T cells, sOpn binds in order to induce Th17 differentiation (Chen et al., 2010; Murugaiyan et al., 2008). It has to be emphasized that, an indirect role in development of Th17 cells has also the expression of iOpn by cDCs. This iOpn expression inhibits the production and secretion of IL-27, a Th17-cell inhibitory cytokine, by cDCs and so on enhances Th17 polarization. The expression of iOpn in cDCs is negatively regulated by IFN-γ (type 1 IFNs) which has been found to be highly expressed during sOpn-induced $CD4^+$ T cell (Th17/Th1) polarization (Murugaiyan et al., 2010; Shinohara et al., 2008).

It has been shown that Opn is also implicated in Th2 cytokine polarization of conventional T cells. During Th2-mediated allergic airway inflammation in mice, Opn expression was found to exert opposing effects on mouse Th2 effector cells. Thus, Opn expression could be either a pro-or an anti-inflammatory cytokine and as consequence to promote or to abrogate Th2 immune responses (Xanthou et al., 2007). Furthermore in humans, It was also found that the administration of recombinant OPN on nickel-specific T cell clones (skin allergic inflammatory disease) dampens the Th2 cell phenotype by down-modulating the IL-4 expression, exerting an anti-inflammatory role (Seier et al., 2010). However, Opn expression was found to be negatively regulated by the presence of two characteristic Th2-specific cytokines, IL-4 and IL-13 (Konno et al., 2006), which indicates the implication of Opn expression in Th2 polarization and vice versa. Although, Opn expression has been shown to modulates Th2 effector responses it has not been identified yet the exact mechanism of Opn action to skewing of conventional T cells into a Th2 lineage commitment phenotype during Th2-mediated inflammation.

Finally, it has been shown that in stimulated $Foxp3^+$ Treg cells the *Spp1* gene is one of the few significantly upregulated genes (Marson et al., 2007). Furthermore, it has been recently described that iOpn expression is important for Bcl-6 stability in follicular regulatory T cells (another subtype of $Foxp3^+$ Treg cells) and that Bcl-$6^{-/-}$ Tregs have defects in their suppressive ability *in vivo* (Leavenworth et al., 2015a; Sawant et al., 2012). However, it has not been investigated yet the importance of Opn expression in $Foxp3^+$Treg cell differentiation and function.

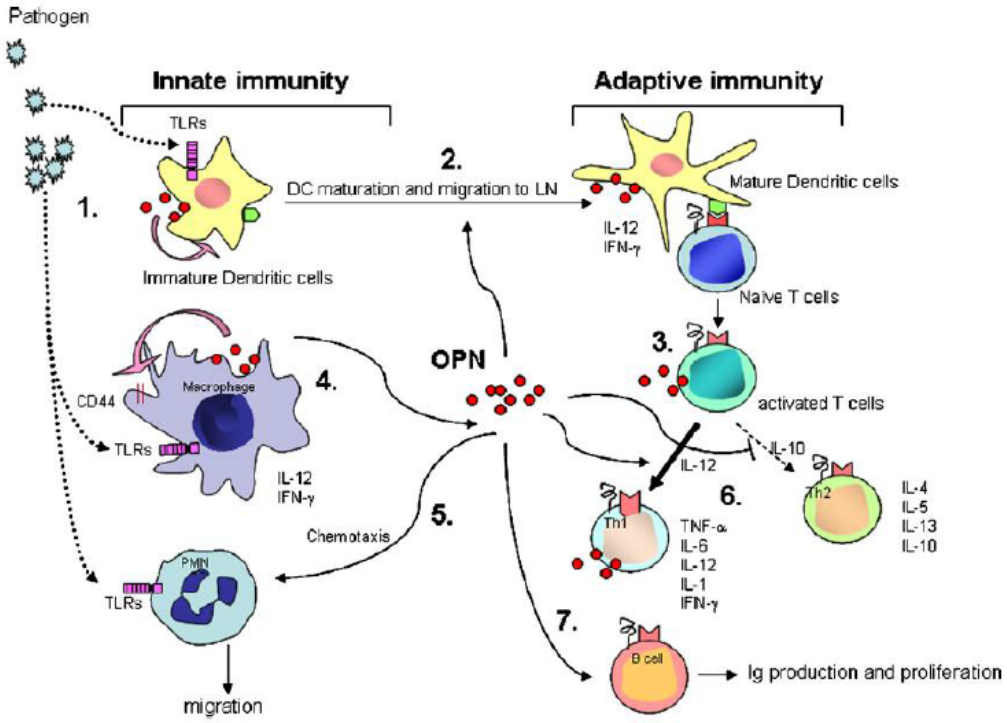

Figure 5 Opn's role in the regulation of innate and adaptive immunity. (1) Exogenous pathogens activate TLRs on the surface of cells, including macrophages, neutrophils and immature dendritic cells. OPN is secreted by macrophages and dendritic cells when challenged by foreign antigens and enhances the activation and functions of these cells. OPN promotes neutrophil migration towards the site of injury. (2) OPN promotes immature dendritic cells to mature and migrate to draining lymph nodes, where they present processed antigens through the MHC to naive T cells and initiate a cell-mediated immune response. (3) Signals from DCs activate naive T cells and determine the polarization of T cells to Th1 or Th2 type cytokine responses. (4) Macrophages produce large amounts of OPN, which in an autocrine/paracrine manner contributes to the migration of macrophages and the expression of the pro-inflammatory cytokine IL-12. (5) OPN produced by various immune cells at inflammatory sites promotes infiltration of neutrophils. (6) Activated T cells are promoted by IL-12 to differentiate towards the Th1 type, producing Th1 cytokines (IL-12, IFN-g). OPN inhibits production of the Th2 cytokine IL-10, which leads to an enhanced Th1 response. (7) OPN promotes B lymphocyte proliferation and immunoglobulin production. Retrieved by STUDY OF THE ROLE OF THE CYTOKINE OSTEOPONTIN IN TYPE-2 ALLERGIC RESPONSES ,Greek National Archive of PhD Thesis page 44, Themis Alissafi. Retrieved June 22, 2015, from http://thesis.ekt.gr/thesisBookReader/id/28839#page/1/mode/2up

1.3. Opn and allergic airway inflammation (asthma)

Due to the fact that sOpn interacts with receptors on immune cells and regulates a lot of lymphocyte's functions, it is well established that an excessive or deregulated expression of sOpn is correlated with the development of inflammatory diseases,

autoimmunity disorders and tumor metastasis (Coppola et al., 2004). Specifically, elevated levels of sOpn have been observed to participate in the pathophysiology of many Th1/Th17-mediated chronic inflammatory diseases including multiple sclerosis (MS), rheumatoid arthritis (RA), Crohn's disease, obesity, diabetes, and many other disorders. The molecular mechanism of OPN in the development of these diseases has been extensively described in many excellent review articles (Cantor and Shinohara, 2009; Chen et al., 2010; Kahles et al., 2014; Lund et al., 2009). However, apart from the significant role of sOpn in the development of Th1 and Th17-associated diseases, sOpn is also involved in the development ofTh2-associated diseases, including allergic airway inflammation (allergic/asthma) (Konno et al., 2011).

Th2 cell immunity is something of a two-edged sword. These cells evolved to fight off helminthes and other parasites through the induction of Th2–cytokine expression (IL-4, IL-13, IL-5, IL-9), but they are also responsible for allergic diseases. Allergic asthma is a Th2-immune mediated disease, which affects approximately 20% of the population in industrialized countries and it is life-threating due to the causality of anaphylaxis. It is characterized by airway eosinophilia, increased mucus production by goblet cells, and structural remodeling of the airway walls. This leads to variable airway obstruction and to bronchial hyperresponsiveness to nonspecific stimuli (Ngoc et al., 2005). It is an IgE-mediated allergic disease due to the high levels of allergen-specific IgE in patients which are a reflection of an aberrant Th2 cell immune response to common inhaled environmental allergens (Platts-Mills, 2001).

In both human cases and murine model of allergen-induced airway inflammation, it was reported that the levels of sOpn in both sputum and bronchial lavage (BAL) fluid from asthmatic patients and mice were elevated and much higher than that of healthy controls (Kohan et al., 2007; Samitas et al., 2011). The increased levels of sOpn expression, during allergic airway disease, were demonstrated to have a dual role which was attributed to its influence on the recruitment of different DC subsets to the site of the antigen response. Specifically, in a mouse model of OVA-induced allergic airway inflammation, Opn had a pro-inflammatory role during the sensitization phase but an anti-inflammatory and protective effect during antigenic challenge. These sOpn-mediated effects were attributed to the fact that during the primary antigen sensitization, sOpn expression blocked the migration of pDCs to the draining lymph nodes (DLN) and induced airway hyperresponsiveness , whereas during the secondary

antigenic challenge sOpn expression inhibited the migration of cDCs to the DLN, and attenuated the established Th2-inflammatory response by decreasing IgE production (Xanthou et al., 2007) (**Figure 6**). In regard to this, it was found that administration of anti-OPN antibody significantly decreased the number of eosinophils in BAL fluid obtained from an asthmatic mouse model (Takahashi et al., 2009) and that there was the significant correlation between sOpn protein levels and the number of eosinophils in the sputum and BAL fluid obtained from asthmatic patients (Puxeddu et al., 2010; Takahashi et al., 2009). It was also observed that sOpn could augment both IgE-mediated mast cell degranulation and mast cell chemotaxis *in vitro* (Nagasaka et al., 2008) and that sOpn was able to suppress antigen specific production of IL-13 when $CD4^+$ T cells were treated with recombinant OPN *in vitro* (Kurokawa et al., 2009). Moreover, it was demonstrated that the upregulated levels of sOpn in lung biopsies and BAL fluid in both asthmatic humans and mice were directly correlated with markers of bronchial tissue remodeling, such as increased collagen deposition, reticular basement membrane thickening and subepithelial fibrosis (Kohan et al., 2007; Simoes et al., 2009).

A critical T cell population for controlling immune responses and the development of asthma is regulatory T (Treg) cells. Naturally occurring and induced Treg cells downmodulate the exaggerated Th2 cytokine-mediated responses and create a mechanism for balancing between pro- and anti-inflammatory cytokines at mucosal interfaces which in subsequence prevents asthma. Furthermore, $Foxp3^+$ Treg cells numbers were observed to increase in patients undergoing allergen immunotherapy (O'Hehir et al., 2009; Pereira-Santos et al., 2008; Radulovic et al., 2008). Thus, by considering the significant role of Treg cells in dampening Th2-immune mediated responses, the excessive Opn levels in the inflammatory milieu during asthma, as well as the anti-inflammatory effects of Opn during antigenic challenge phase, it would be possible that these Opn-regulated protective effects are partially mediated by the induction of regulatory T (Treg) cells.

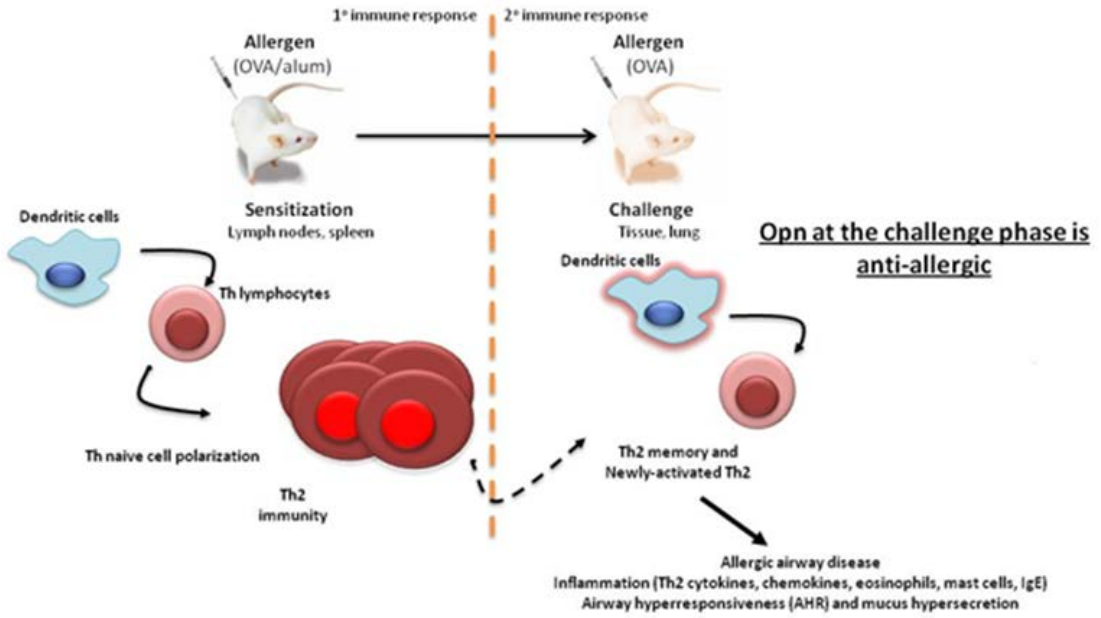

Figure 6 Dual role of sOpn expression in Th2-mediated allergic airway inflammation mouse models. Opn expression during sensitization phase exerts pro-inflammatory role by blocking the migration of tolerogenic pDCs to lung MLNs whereas during challenge phase exerts anti-allergic effects by blocking the migration of pathogenic cDCs to lung MLNs.

1.4. Foxp3+Regulatory T cells

$CD4^+$ regulatory T cells (Tregs) comprise a specialized subpopulation of T cells which suppresses immune effector activation and function and maintains immune homeostasis and self-tolerance (Sakaguchi, 2004). Normally, Tregs suppress and keep in check peripheral autoreactive T cells that escape thymic negative selection (Sakaguchi, 2004). There are many regulatory populations which contribute to immune homeostasis, but Foxp3+ Treg cells play the most critical role in the maintenance of the immunological balancing. Forkhead box P3 (Foxp3) is a master regulator in the development and function of a certain subset of Treg cells. It is the signature transcription factor of Tregs that promotes the Treg gene suppressive program while repressing key genes characteristic of effector T (T_{eff}) cells, such as *IL-2*, *IL-4* and *IFN-γ* (Fontenot et al., 2003; Williams and Rudensky, 2007). Spontaneous mutation of *Foxp3* leads to widespread lymphocytosis and autoimmunity development in the scurfy mouse and in humans with immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) (Lahl et al., 2007; Lin et

al., 2007). Further studies have demonstrated that Treg cells are also important in regulating many other immune responses, including environmental allergens, pathogenic agents, tumors and transplanted tissues.

Three major mechanisms of Foxp3^{+} Treg cells generation have been demonstrated until now. Foxp3^{+} Treg cells could be either generated naturally in thymus (tTreg or nTreg) or can be induced peripherally (pTreg) or *in vitro* (iTreg cells) (**Figure 7**). The proportion of peripheral Tregs is approximately ***5-10%*** of the total CD4^{+}T cells compartment and their suppressive functions could be performed either by the secretion of immunoregulatory cytokines, such a IL-10 or TGF-β, or by the consumption of IL-2 required for survival and proliferation of effector T cells, or directly by cell to cell contact.

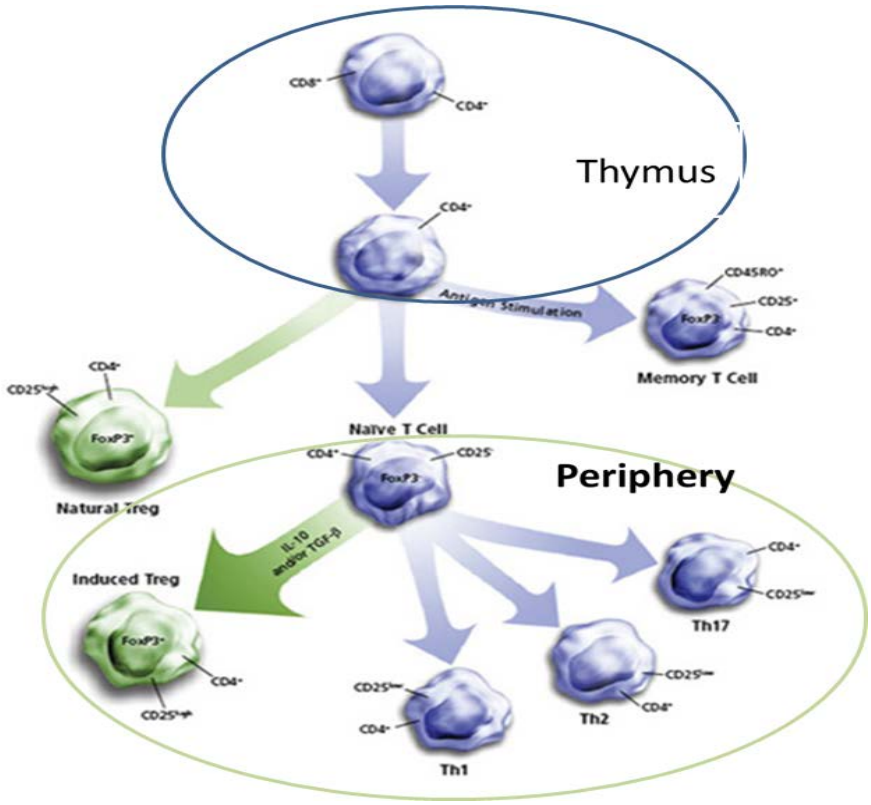

Figure 7 Generation of Foxp3^{+} T reg cells. Foxp3 expressing Treg cells can be either generate in thymus tTreg or nTreg cells or in the periphery and *ex-vivo* under the appropriate cytokine stimulation (TGF-β and IL-10) from conventional T cells and termed peripheral or induced Treg cells (pTreg or iTreg cells). Adapted from Regulatory T cells, in BDbiosciences, n.d. Retrieved April 22, 2015, from https://www.bdbiosciences.com/us/applications/research/t-cell-immunology/regulatory-t-cells/m/745680/workflow/abouttregs.

1.4.1. Thymically derived Foxp3$^+$nTreg cells

The majority of circulating Tregs has been assumed to be of thymic origin (Shevach and Thornton, 2014). Studies carried out in transgenic mice demonstrated that generation of nTreg cells is driven by the specificity of TCR to self-peptide. Specifically, following positive selection and development of single positive CD4$^+$ thymocytes, nTreg lineage commitment was shown to be occurred in thymic medulla through selection events on medullary epithelia cells and DCs (Aschenbrenner et al., 2007; Fontenot et al., 2005). These selection events result in formation of a cell-pool comprised by nTreg cells which express a broad TCR-repertoire with a relatively high affinity for self-antigens, which only partially overlap with that found on conventional CD4$^+$ T cells (Apostolou et al., 2002; Aschenbrenner et al., 2007; Fontenot et al., 2005; Hsieh et al., 2006). Induction of Foxp3 expression during nTreg cell generation has been well described in many excellent reviews (Sakaguchi et al., 2010; Yuan and Malek, 2012). Briefly, during nTreg cell generation, TCR activation in conjunction with costimulatory signaling (CD28) results in activation of transcription factors including AP1 (activator protein-1), NFAT (nuclear factor of activated T cells) and NF-kB (Nuclear Factor-kappa Beta). These transcription factors bind to promoter of *Foxp3* gene and induce Foxp3 expression (Maruyama et al., 2011). Another critical transcription factor for promoting sustained Foxp3 expression *in vivo* is Runx-1. Runx-1 interacts with CBFb and forms Runx-CBFb complex which binds in both to promoter of *Foxp3* gene and its enhancer regions as well (on conserved non-coding sequence 1 and 3;CNS1,CNS3) (Rudra et al., 2009) in order to enhance Foxp3 expression. It creates a positive auto-regulatory feedback loop mechanism with Foxp3 itself and enhances its action (Zheng et al., 2010). Studies have shown that complete maturation and maintenance of nTreg cells are mediated by activation of (TGF-β-dependent) Smad3 and (IL-2-dependent) Jak3/STAT5 transcription factors (Burchill et al., 2007; Horwitz et al., 2008). These transcription factors provide growth survival signals and act on *Foxp3* gene to induce heightened levels of Foxp3expression which is required for Treg cell function program (Cheng et al., 2013; Ouyang et al., 2010; Yao et al., 2007). Mature nTreg cells seed peripheral immune tissues and maintaining immune homeostasis by suppressing peripheral autoreactive T cells that were not deleted during thymic negative selection.

1.4.2. Peripherally induced Foxp3+ Treg cells

Many studies demonstrated that under appropriate environmental cues, conventional $CD4^+$ $Foxp3^-$T cells can differentiate into $Foxp3^+$ Treg (pTreg) cells in periphery with similar suppressive function to nTreg cells (Chen et al., 2003). This differentiation of naïve $CD4^+$ $Foxp3^-$T cells into $Foxp3^+$ Treg cells is mediated by inhibition of $CD4^+$ T cell lineage-specific transcription factors, including RORγτ/STAT-3, IRF-4 /GATA-3, T-bet and Bcl6 (induce Th17, Th2, Th1, follicular regulatory T (T_{FR}) cells ,respectively) and the subsequence transactivation of *Foxp3* gene expression. pTreg cell regulate antigen-specific immune responses and play an pivotal role for maintaining immune tolerance to mucosal tissues and pregnancy conferring to maternal tolerance to fetus (Aluvihare et al., 2004; Josefowicz et al., 2012; Samstein et al., 2012). TCR-activation and CD28-co-stimulation mediate pTreg cell development, which is highly dependent upon TGF-β signaling (Josefowicz et al., 2009; Tone et al., 2008). Specifically, the strength of both TCR and co-stimulation signaling (CD28) regulate pTreg cells generation. Low level of TCR signaling is required for induction of pTreg Foxp3 expression (Kretschmer et al., 2005) whereas pTreg induction is inhibited by strong CD28 ligation (Benson et al., 2007; Kim and Rudensky, 2006). As mentioned above, TGF-β phosphorylates and activates Smad3 which in turn binds to CNS1 (enhancer region of Foxp3) and directly induces Foxp3 expression. It also needs to be mentioned that TGF-β expression is also indispensable for IL-6–dependent Th17 and IL-4-dependent Th9 cell differentiation from naïve T cells and mediated by RORγτ and IRF-4 transcription factors, respectively, (Bettelli et al., 2006; Schmitt et al., 1994; Staudt et al., 2010; Veldhoen et al., 2006; Zheng, 2013). The majority of pTreg development *in vivo* is found at mucosal sites (lamina propria), where epithelial cells, macrophages and $CD103^+$ DCs produce highly amounts of TGF-β and retinoid acid. Both TGF-β and retinoid acid induce pTreg cell differentiation at mucosal sites and maintain mucosal tolerance (Coombes et al., 2007; Soroosh et al., 2013; Sun et al., 2007). Other factors which contribute to pTreg cell differentiation *in vivo* are the plurality of commensal bacteria and food antigens inside gut and the presence of aerosolized harmless antigens in lung airways (Atarashi et al., 2013; Cording et al., 2013; Soroosh et al., 2013). Studies at mucosal sited have

demonstrated the unique specificities of pTreg cell TCR for recognizing environmental antigens which are only restricted to pTreg cell populations and not to nTreg cells (Kuhn and Stappenbeck, 2013). These specificities are likely derived from conventional T cells because when individual TCR specificities from the gut mucosa were expressed in bone marrow chimeras, they were not selected in the pool of nTreg cells (Kuhn and Stappenbeck, 2013).

1.4.3. *In vitro* induced Foxp3^{+} Treg (iTreg) cells

In vitro induction of iTreg cells, at least in mouse models, is mediated by TCR-stimulation (anti-CD3/anti-CD28 or antigen-specific) of naïve conventional CD4^{+} T cells (CD25$^{-\ or\ lo}$ CD62L^{+}) in the presence of TGF-β and IL-2 (Chen et al., 2003; Zheng et al., 2007). However, it still remains unclear whether iTreg and pTreg cells are similar populations that use the same suppressor mechanisms. Apart from the significant role of IL-2 in the *in vitro* induction of Foxp3 expression on iTreg cells, studies revealed that IL-2 also plays a critical role in iTreg stability *in vivo*, by maintaining Foxp3 expression and iTreg–suppressive function *in vivo* (reviewed by Shevach and Thornton, 2014). Also another critical molecule which confers generation and stability of Foxp3^{+} iTreg cells during inflammation is the glycan-binding protein galectin 9, which increases Smad3-driven *Foxp3* transactivation (Wu et al., 2013). In addition, the induction of Foxp3 is also dependable from the TCR signaling. It has been demonstrated that iTreg induction is inhibited by increased concentration of anti-CD3 (Kim and Rudensky, 2006), as well as that the premature termination of TCR signaling or inhibition of any of the TCR-signaling downstream enzymes (PI3K/AKT/mTOR) augments iTreg induction (Battaglia et al., 2005; Delgoffe et al., 2009; Kopf et al., 2007; Powell et al., 2012). Notably, as it was mentioned in a recent review article, many studies have provided evidence that adaptive Treg cells (iTreg or pTreg) are not so stable population comparing to nTreg cells and that can suppress in vivo immune responses by different mechanisms than that are used by nTreg cells (Shevach and Thornton, 2014).

To date, no single marker has been identified to perfectly differentiate nTreg from adaptive pTreg or iTreg cells. The transcription factor Helios as well as the cell surface antigen 28europilin-1 (Nrp1) are both used for discrimination between nTreg (Helios^{+}, Nrp1^{hi}) and pTreg (Helios^{-}, Nrp1^{-}) cells however there are data which show

that both molecules under specific circumstances can also be expressed on pTreg cells, as it is indicated in a current review (Shevach and Thornton, 2014).

1.4.4. Function of Foxp3+ Treg cells

Foxp3$^+$ Treg cells TCR activation and IL-2 presence are indispensable for their suppressive function (de la Rosa et al., 2004; Takahashi et al., 1998; Thornton et al., 2004a, 2004b) while costimulation via CD28 is dispensable (Takahashi et al., 2000). There are numerous inhibitory mechanisms on how CD4$^+$ Foxp3$^+$ Treg cells suppress the activation, proliferation and function of effector T (Teff or Tconv) cells and maintain immune homeostasis. That variability on methods of suppression is attributed to the fact that Foxp3$^+$ Treg cells express a huge number of distinct mediators that downmodulate immune responses, including IL-10, TGF-β, IL-35, granzyme, Galectin 1 and 9, cytotoxic T-lymphocyte antigen-4 (CTLA-4), CD39, CD73, lymphocyte activation gene-3 (LAG-3), programmed cell death-1(PD-1), TIGIT and fibrinogen-like protein-2 (FGL-2) (Joller et al., 2014; Schmidt et al., 2012; Shevach, 2009; Wu et al., 2014; Yamaguchi et al., 2011; Yu et al., 2009). Each mediator molecule belongs to a specific category of *in vivo* inhibitory mechanisms. Those mechanisms of Treg–mediated cell suppression of Teff cells include cell-cell contact dependent inhibition, modulation of APC function, cytolysis of target cells, metabolic disruption, secretion of immunosuppressive cytokines and competition for environmental IL-2 (Povoleri et al., 2013) (**Figure 8**).

In a brief description, it has been suggested that the direct interaction of Tregs/Teff and the following release of perforin and granzyme-A or -B induce cell apoptosis in Teff cells (Bluestone, 2011). Furthermore, another way of cell to cell dependent inhibition is the abrogation of IL-2 transcription and proliferation of Teff cells by the Treg-mediated release of the immunosuppressive factor cAMP into Teff cells through the gap junctions (Bopp et al., 2007). Treg cells induce indirectly inhibition of activation and proliferation of autoreactive Teff cells, by modulating the antigen presentation of APCs. CTLA-4 and PD-1 are very crucial surface molecules for this mechanism as well. These molecules are constitutively expressed in both human and murine Treg cells and exposed on the surface after Treg cell activation. Both of these

molecules interact with B7.1 (CD80) and B7.2 (CD86) on surface of APCs and compete the costimulatory molecule CD28 binding to CD80/CD86. Thus, through that interaction, Treg cells downregulate CD80/CD86 expression on APCs (transendocytosis) and indirectly inhibit the activation of Teff cells by limiting the Teff-APC contact and by preventing the pro-survival IL-2 expression (Bluestone, 2011). Furthermore, CTLA-4 also induces IDO (indoleamine 2,3-dioxygenase) expression in APCs, thereby limiting T cell proliferation (Curti et al., 2007; Fallarino et al., 2003). Additionally, activated Treg cells produce immunosuppressive cytokines, such as IL-10, IL-35 and TGF-β that either indirectly or directly inhibit Teff cell proliferation (Collison et al., 2010). Finally, immunosuppression is further enhanced by the overexpression of IL-2 receptor (CD25) on Tregs cells, which increase the consumption of the crucial for T effectors survival IL-2 (Pandiyan et al., 2007).

Recent reports has led to the concept that Foxp3$^+$ Treg cells are not all identical, but rather comprised of multiple, functionally diverse subtypes with distinct phenotypes and specialized functions (reviewed by Shevach and Thornton, 2014). The specialization of Foxp3$^+$ Treg subtypes to selectively regulate specific effector T cell responses and control inflammation at defined anatomical tissues is mediated by the expression of the hallmark transcription factor of each specific Teff subtype. It has been demonstrated that a Foxp3$^+$ Treg cell subset upregulate IRF-4 and GATA-3 expression in response to Th2 responses, as well as other Foxp3$^+$ Treg cells express T-bet and STAT3 or RORγτ to regulate Th1 and Th17 immune responses, respectively (Chaudhry et al., 2011; Hall et al., 2012; Koch et al., 2012). Correspondingly, Treg cells with a deficiency in IRF-4, T-bet, STAT-3 and Bcl6 expression showed selective inability to control Th2, Th1, Th17 and B–cell immune responses respectively (Chaudhry et al., 2009; Koch et al., 2009; Zheng et al., 2009). However, the molecules that mediate these selective effector functions remain largely unknown. It is suggested that this ectopic expression of lineage-specific transcription factors could upregulate the expression of specific Th homing receptors on surface of Foxp3$^+$ Treg cells, such as CXCr3, CCR8 and CCR6, in order to migrate to specific inflamed tissue sites and to regulate Th1, Th2 and Th17 responses, respectively. Further studies have demonstrated that the specific expression of each lineage transcription factor by Foxp3$^+$ Treg cells induces different immunosuppressive

molecules and cytokine expression patterns which are distinct and specialized for the prevention of Th1, Th17 and Th2-inflammatory responses (Chaudhry et al., 2011; Hall et al., 2012; Koch et al., 2012). However, there are still concerns about the stability of those Foxp3$^+$ Treg cells due to the fact that it has been observed that under inflammation many Foxp3$^+$ Treg cells lose their Foxp3 expression (termed exFoxp3) and secrete pro-inflammatory cytokines (Oldenhove et al., 2009). For instance, if this dual-expressing Foxp3$^+$ Treg cells loses Foxp3 but retains the other lineage-specific transcription factor, such as IRF4, it may cause expression of pro-inflammatory cytokine, as was shown for exFoxp3 cells recovered from different tissues of diabetic mouse models (Bettelli et al., 2006).

Until now it was believed that there were not any functional differences between adaptive iTreg and nTreg cells to maintain immune homeostasis. However, recent studies revealed that iTreg and nTreg function differently *in vivo*. Studies with EAE and AIG (autoimmune gastritis) mouse model demonstrated that *ex vivo* polyclonal stimulated Foxp3$^+$ nTreg cells suppressed antigen–specific effector T cells inflammatory responses by inhibiting their trafficking from the draining lymph nodes to the site of inflammation (Thangada et al., 2010) and not by limiting their proliferation. That was mediated by the downregulation of specific chemokine and integrin receptors expression, including CXCR5, syndecan and sphingosine phosphate receptor 1 (S1P1) on the surface of Teff cells, following the entry of T cells into the lymph node (Tang et al., 2004). Normally, during the priming of T cells the S1P1 expression is upregulated and through the interaction with the S1P ligand in the circulation, the antigen-specific Teff cells exit lymph nodes. On the other hand, in the same experimental mouse models, Foxp3$^+$ iTreg cells mediated immune suppression by inhibiting the priming of conventional T cells from specialized antigen-priming DCs, rather than by preventing the trafficking of Teff cells. That was partially mediated by the induction of immunosuppressive IL-10 secretion from antigen-specific-Foxp3$^+$ iTreg cells. IL-10 impaired the antigen presentation function of DCs, by downregulating the availability of MHC-II class molecules and their accessibility to Tconv cells, and as a consequence prevented the priming of Tconv cells, limited their proliferation and inhibited their secretion of pro-inflammatory cytokines (Chattopadhyay and Shevach, 2013; DiPaolo et al., 2007; Fantini et al., 2004). In addition, it has also been described that Foxp3$^+$ nTreg cells co-operates with CD4$^+$ T

cells and induce their Foxp3 expression and as a consequence their differentiation into Foxp3^{+} iTreg and pTreg *in vivo*, in order to promote infection tolerance. This mechanism of Foxp3 induction is LAP-TG-β/GARP-mediated (Edwards et al., 2013; Li et al., 2007; Xu et al., 2007).

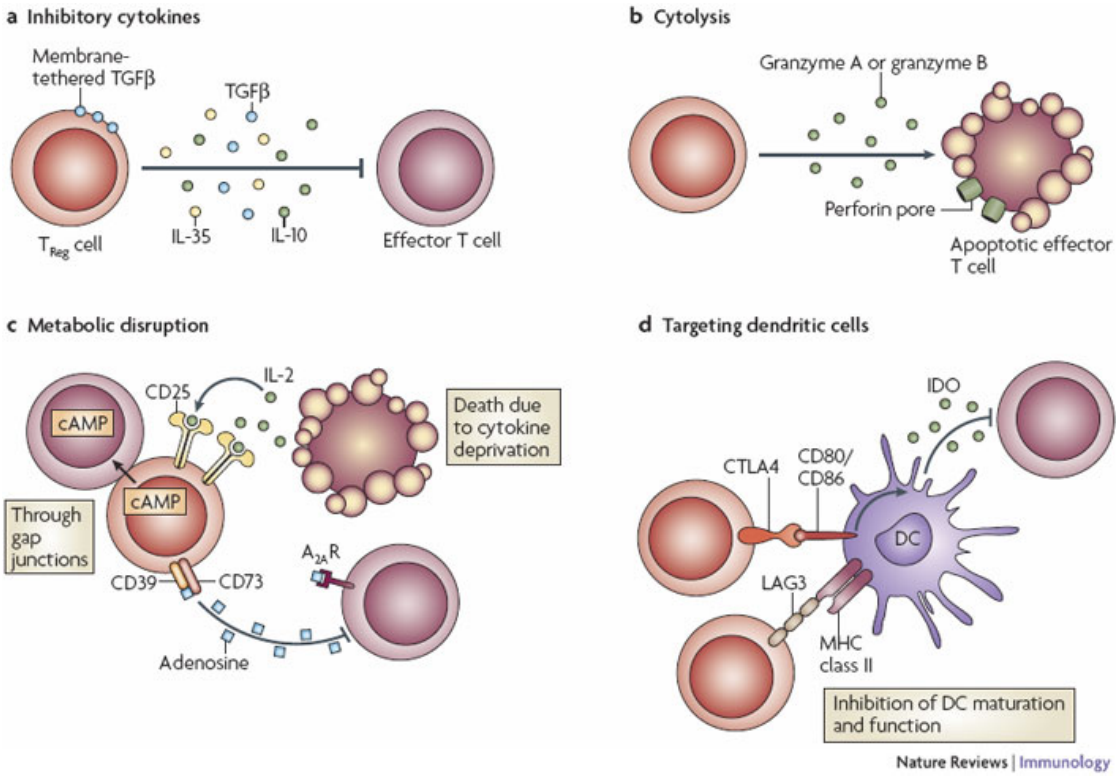

Figure 8 Basic suppressive mechanisms of Treg cells. a) secretion of suppressive cytokines, including IL-10, IL-35 and TGF-β. b) inhibition of Teff proliferation by elevated secretion of cytolysis proteins, including perforin a and b. c) another mechanism is the metabolic disruption by the overconsumption of the survival for Teff cells IL-2, or by increased expression of cAMP. d) Indirect mechanism of suppressive function by targeting dendritic cells (DCs). It includes mechanisms that modulate DC maturation and/or function such as lymphocyte-activation gene 3 (LAG3; also known as CD223)–MHC-class-II-mediated suppression of DC maturation, and cytotoxic T-lymphocyte antigen-4 (CTLA4)–CD80/CD86-mediated induction of indoleamine 2,3-dioxygenase (IDO), which is an immunosuppressive molecule made by DCs (Vignali et al., 2008) .

1.4.5. Foxp3+ Treg cell integrin receptors expression

As far as the role of integrin/integrin receptors interaction into the trafficking of $CD4^+$ $Foxp3^+$ regulatory T cells to lymphoid and non-lymphoid tissues the field is still debatable and under investigation. It seems that integrin CD103 ($\alpha_E\beta7$) receptor plays a crusial role for the recruitment of $Foxp3^+$ Tregs into the draining lymph nodes of gut mucosa (Lehmann et al., 2002; Wagner et al., 1996). CD103 has been found to interact with CCR2 and E-cadherin (Wei et al., 2006). However, the absence of integrin β7 largely reduced Treg trafficking to the gut mucosa but Tregs still suppressed colitis, suggesting that suppression at least in part may occur in draining lymph nodes (Denning et al., 2005). In addition, $CD103^+$ $Foxp3^+$ Tregs, comparing to $CD103^-$ favoring migration into tissue sites and expressing enhanced suppressive activity due to high levels of Treg suppressor molecules (Banz et al., 2003; Feuerer et al., 2010; Lehmann et al., 2002; McHugh et al., 2002). Further studies depicted in humans the existence of two different $CD4^+$ $CD25^+$ $Foxp3^+$ T regulatory cell subpopulations, based on different integrin receptor expression on their surface. Although, both subpopulations display strong suppressive activity for conventional $CD4^+$ T cells and show an activation-dependent expression of Foxp3, they differ in their ability to induce secondary suppressor cell populations (Tr1, Th3). The one population expresses the $\alpha_4\beta7^+$ and the other one the $\alpha4\beta1^+$ integrin receptor. The first subset is specialized to migrate to mucosal tissues to counteract autoreactive T cells and generate Th3 cells, thereby preventing chronic mucosal inflammation, whereas the second one migrate to inflamed tissues and inhibits Teff cells by inducing Tr1 cells (Stassen et al., 2004). In addition, it has been described that many Tregs express different isoforms of the CD44v receptor (Darrasse-Jèze et al., 2009). Also, the interaction of galectin 9, a chemoattractant glycoprotein with a lot of similarities with Opn, with the CD44 receptor was found to regulate the stability and function of iTreg cells (Wu et al., 2013).

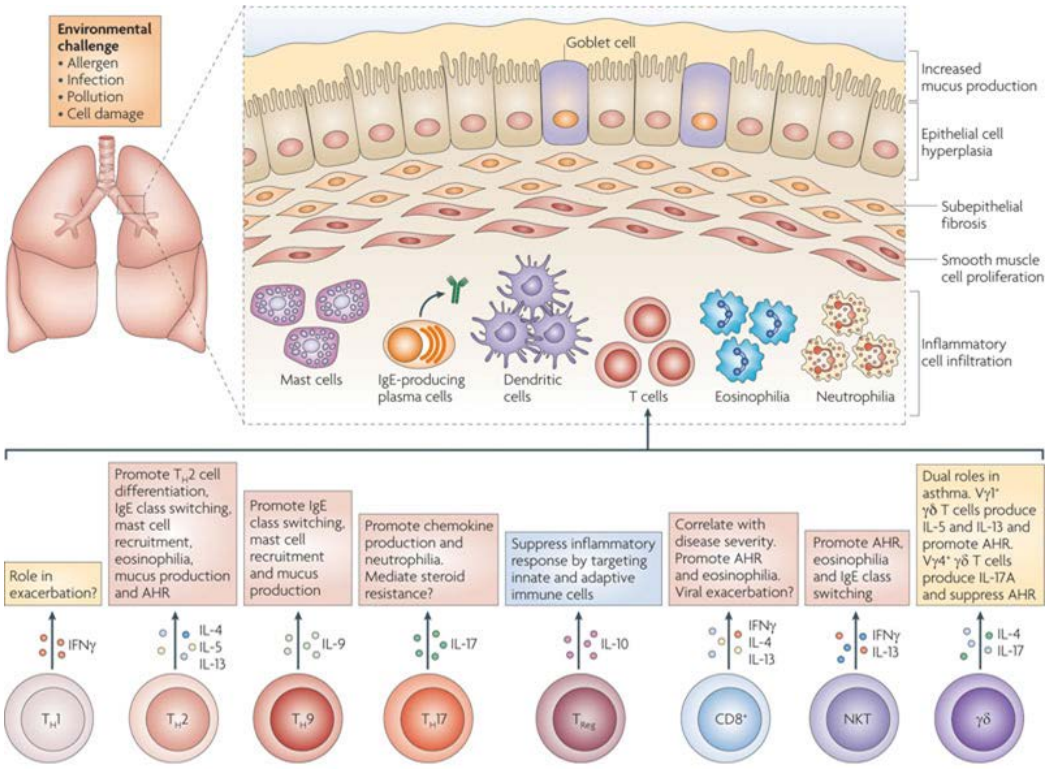

Figure 9 T cells involved in the induction of the allergic phenotype. Asthma is a heterogeneous disease that is characterized by AHR, recruitment of inflammatory leukocytes to the lung and tissue remodelling, including mucus production and airway smooth muscle changes. A number of different T cell subsets are thought to influence the nature and magnitude of the allergic immune response by the cytokines that they secrete. T_H2 cells promote eosinophil recruitment, in conjunction with NKT cells and $CD8^+$ T cells. By contrast, T_H1 cells and T_H17 cells are thought to be associated with severe, steroid-resistant asthma, which is often marked by neutrophilic infiltrates. Treg cells and subtypes of γδ T cells are able to downregulate pulmonary immune responses and are thought to be important for maintenance of immune homeostasis in the lungs. The nature and magnitude of allergic inflammation in the lung is influenced by external environmental stimuli, such as exposure to allergens and pollution as well as infection with pathogens. IFNγ, interferon-γ; IL, interleukin (Lloyd and Hessel, 2010).

1.4.6. The role of Foxp3+ Treg cells in Th2-allergic airway disease

Immunity against pathogen is mediated through the induction of antigen-specific T helper (Th) type 1 and type 2 lymphocytes. Aberrant Th2 cell activation against environmental antigens is possible to induce allergy and asthma. Asthma is a chronic airway disease characterized by Airway hyperresponsiveness (AHR) and airway inflammation. AHR is mediated by Th2 cells and the release of Th2 specific cytokines, including IL-4, IL-5, IL-13, and IL-9 (McGee and Agrawal, 2006). This cytokine milieu which is created by the aberrant activation of Th2 cells causes chronic

inflammation, and pulmonary eosinophilia, leading to mucus cell hyperplasia, destructive airway tissue remodeling and contraction of smooth muscle cells (Pueringer and Hunninghake, 1992) (**Figure 9**).

As previously mentioned $Foxp3^+$ Treg cells are responsible for regulating Th2 immune responses and maintaining immune homeostasis. $Foxp3^+$ Treg cells have demonstrated to maintain airway balance in mouse models of allergic airway disease and increased numbers of Treg cells or their activity have been associated with current treatments that reduce allergic and asthmatic symptoms. $CD4^+$ $CD25^+$ $Foxp3^+$ Treg cells numbers and frequency have been observed to increase in asthmatic patients undergoing allergic immunotherapy, for instance after exposure to escalating doses of HDM , venom and grass pollen (O'Hehir et al., 2009; Pereira-Santos et al., 2008; Radulovic et al., 2008). On the other hand lower frequencies $Foxp3^+$ Treg cells with decreased suppressive functions have been found in the circulation and in the BAL of asthmatic patients, indicating the significant role of Treg cells in regulating Th2-mediated allergic airway inflammation (Hartl et al., 2007; Nguyen et al., 2009). As far as mouse models of allergic airway inflammation, it has been shown that nTreg cells are present in the lung tissue of sensitized mice and increase upon inhaled allergen challenges (Langier et al., 2012). Furthermore, inhibition of nTreg cells augments respiratory allergen-induced AHR and IgE production, as well as Th2 cytokine levels in BAL fluid (van Oosterhout and Bloksma, 2005). Additionally,, it has been also observed mouse models of asthma that both nTreg and iTreg cells can reverse allergic airway inflammation, after the adaptive transfer of $Foxp3^+$ Treg cells in pre-immunized mice (Chen et al., 2003; McGee and Agrawal, 2009; Xu et al., 2012).

To date, no single mechanism of $Foxp3^+$ Treg cells-mediated suppression of allergic airway disease has been emerged from multiple studies on experimental asthmatic models. It has been proposed that $Foxp3^+$ Treg cells either upregulate their expression of immunosuppressive IL-10 and control effector T cell responses by inducing anergy (inhibition of costimulation signaling; ICOS:ICOSL; inhibition of IgE) (Akdis et al., 1996; Becker et al., 1994) or express membrane bound-TGF-β which mediates inhibition of allergic inflammation; Hes-1-mediated inhibition of T cell activation (Akbari et al., 2002; Eagar et al., 2004; Joetham et al., 2007). Specifically, the inhibition of IL-10 secretion of $Foxp3^+$ Treg cells promotes allergic airway disease, in

asthmatic mouse models (Rubtsov et al., 2008). In addition, a recent study depicted that Treg suppress the progression of allergic airway inflammation by expressing OX40L and as a consequence inhibiting the degranulation of pathogenic mast cells (Gri et al., 2008). Also, the expression of CTLA-4 and the secretion of IL-35 cytokine by Foxp3$^+$ Treg cells are also limiting factors for the development of asthma disease (Collison et al., 2007; Strickland et al., 2006).

1.5. Aim of the study

Opn has been widely studied under normal and pathological conditions in various organs (Lund et al., 2009). It has been well demonstrated the role of Opn expression in regulating the cross talk between the innate and the adaptive immune system and the subsequent polarization of Th1, Th2 and Th17 cells as well (Inoue and Shinohara, 2011). Clinically, increased Opn levels in plasma have been shown to correlate with many autoimmune diseases, including lupus multiple sclerosis and rheumatoid arthritis (Comabella et al., 2005; Kariuki et al., 2009; Sennels et al.; Vogt et al., 2010). Previous work from our group has demonstrated that Opn has also anti-allergic effects during secondary antigenic challenge in a mouse model of allergic airway inflammation (Xanthou et al., 2007). Our work suggested that these protective effects were mediated partly by Foxp3$^+$ Treg cells. Work from other groups showed that *Spp1* gene is one of the few significantly upregulated genes in stimulated Foxp3$^+$ Treg cells (Do et al., 2015; Marson et al., 2007), however its role in the generation and function of these cells remains elusive. Regarding all published and unpublished data, our aim was to investigate the effects of endogenous Opn on development of Foxp3 expressing Treg cells. We also tested the effects of genetic deletion of Opn in the function of induced-Tregs *in vitro* and our final goal was to assess the effect of endogenous Opn expression in the function of Foxp3$^+$ Treg cells *in vivo*, in a model of allergic airway inflammation.

2. MATERIALS AND METHODS

2.1. Mice

The strains C57BL/6, C57BL/6.CD45.1, and C57BL/6 Foxp3/GFP-reporter were used. For some experiments C57BL/6 Foxp3/GFP mice backcrossed to the Spp1-/- background were also used as these mice were previously generated in the lab. For the majority of the experiments (unless otherwise stated) all mice were females 6 to 8.Foxp3/GFP-reporter mice were used as a source of $Foxp3^+$ regulatory T cells also C57BL/6.$Rag^{-/-}$ were used for *in vivo* homeostatic expansion experiments. All experiments were conducted following the guidelines of the Biomedical Research Foundation Academy of Athens (BRFAA) department of Animal Care and use Committee.

2.2. CD4+ T cell isolation and culture *in vitro*

Mouse cells were harvested from spleen and lymph nodes and single cell suspensions were prepared. Briefly, tissues were gently dissociated by mechanical force; lymphocytes passed through a Falcon Cell Strainer (BD, 100µm pore) and diluted in PBS-1X supplemented with 1% BSA and 2mM EDTA to avoid clumps formation. Cells were counted with a Neubauer hemocytometer and naïve $CD4^+$ $CD62L^+$ $CD25^-$ T cells were isolated by using magnetic beads ($CD4^+$ $CD62L^+$ T cell Isolation Kit II, MACS Miltenyi Biotec, CA) following the manufacturer's instructions For some experiments, alternatively, naïve T cells were also isolated by using fluorescence cell sorting cytometry (Appendices Figure 4) (FACS Aria III, BD). In some experiments a CD4 enrichment step preceded FACS sorting isolation of T cell population to reduce experimental time and cellular stress. To this aim CD4 cells from spleen and lymph node cell suspensions were isolated using the CD4+ T cell isolation kit MACS following the manufacturer's instructions The $CD4^+$ T cell purity was >99%. Isolated $CD4^+$ T cells were labeled with the appropriate fluorescence dye labeled-antibody cocktail (anti-CD3, anti-CD4, anti-CD25 and anti-CD62L). The isolated naïve $CD4^+$ T cells were cultured on 48 or 96-well plates at a density of 10^6 cells/well in complete

RPMI 1640 GlutaMAX (Gibco, UK) medium supplemented with 10% heat-inactivated Fetal Calf Serum (FCS), 100 U/ml penicillin-streptomycin, 2 mM L-glutamine (all from Gibco, UK) and 50 μM β-mercaptoethanol (Life Technologies) under the appropriate stimulation and cytokines.

2.3. Flowcytometry analysis of Foxp3^{gfp} Treg cell populations in Opn WT and KO mice

Single cell suspension of lymphocytes from WT and Opn KO Foxp3/GFP-reporter mice from spleen, axillary/inguinal lymph nodes, thymus and mesenteric lymph nodes were prepared as described above (2.2). Single cell suspension were also collected from the *lamina propria (LP)* For isolation of LP cells, mesenteric fat tissue was carefully removed and colons were flushed of their laminal content with cold PBS-1X, opened longitudinally and were cut into 1 cm pieces. Epithelial cells were removed by 2 x 20minutes incubation with predigesting solution containing Ca/Mg free Hank's balanced salt solution (HBSS) supplemented with 5 mM EDTA and 1 mM dithiothreitol (DTT) (Sigma) with slow rotation at 40 g in room temperature. Colon pieces were then incubated with digestion solution containing 0.05 g of collagenase D (Roche), 0.05 g of DNase I (Sigma) and 0.3 g of dispase II (Roche) in 100 ml of PBS-1X for 2 x 20 minutes rotating at 40 g. Subsequently mononuclear cells (MCs) were enriched with Percoll density gradient. Following centrifugation, the LPMCs were visible in a white ring at the interphase of the two different Percoll solutions (40/80). All cells were stained with a specific fluorescence dye-labeled antibody cocktail (anti-CD4, anti-CD25) and analyzed via flowcytometry analysis.

. 2.4. *In vitro* generation of iTreg cells

Naïve $CD4^+$ $CD25^-$ $CD62L^+$ $Foxp3^-$ T cells from Opn WT and Opn KO Foxp3/GFP-reporter mice isolated from spleen and lymph nodes with FACS sorting as previously described **(section 2.2)**. Isolated cells were stimulated with polyclonal anti-CD3/CD-28 dynabeads (Life Technologies) at a 1:1 ratio (25μL of beads per 10^6 cells) and differentiated in the presence of rhTGF-β (10 ng/ml;Peprotech) and rhIL-2 (100 U/ml) for 5 days. In some experiments different concentrations of rhTGF-β (0.001, 0.001,

0.01, 0.1, 1, 10 ng/ml) were used. Foxp3^{gfp} expression was assessed by flow cytometry (as described in section 2.3).

2.4.1. Assessment of iTreg cell proliferation and cell death

In some experiments the Cell Trace Violet (CTV) Cell Proliferation kit (Invitrogen, US) was used in order to assess the proliferation rate of WT and Opn KO naïve T cells during their differentiation to iTreg cells. After sorting, naïve T cells were counted and labeled with 5 μM CellTrace Violet (diluted in DMSO) at a ratio of 1μl 5 mM CTV per 10^6 naïve T cells in 1 ml of PBS-1X. Then cells were incubated for 20 minutes at 37^0C and after that, complete medium added to the sample and cultured for 5min at room temperature. Finally, cells washed and resuspended in fresh complete medium. CTV labeled naïve T cells cultured *in vitro* under Foxp3 polarizing conditions as previously described (**2.2**) and the proliferation rate of iTreg cells was analyzed by Flowcytometry. Opn KO and WT iTreg cell death was determined by using PE Annexin V Apoptosis Detection Kit with 7-AAD (Biolegend, CA). After 5 days of culture cells were harvested and washed twice with PBS-1x, 0.5% BSA, 2mM EDTA and finally resuspended in Annexin V Binding Buffer at a concentration of 0.25-1.0 x 10^7 cells/ml. After that, 100 μL of cell suspension were transferred in a new tube and 5 μL of PE Annexin V and 5 μL of 7-AAD Viability Staining solution were added. Cells were incubated at room temperature for 15 minutes in dark. iTreg cells were analyzed for early (Annexin V^+ / 7-AAD $^-$ -or late-stage Annexin V^+ / 7-AAD apoptosis or by FACS cytometry.

2.4.2. Restimulation of iTreg cells

Following TGF-β-driven differentiation, Opn KO and WT iTreg cells were harvested counted and their dynabeads were removed by washes with MACS buffer in a magnet holder. WT and Opn KO iTreg cells were isolated by resorting cells based on their Foxp3^{gfp} expression. Live differentiated Foxp3^{gfp} iTreg cells were isolated and cultured in 48-well plate (5x10^5 cells/well) with fresh complete RPMI medium under the presence of anti-CD3/CD28 dynabeads at a ratio of 25 μL/10^6 iTreg cells and rhIL-2 (100 U/ml). After 5 days the maintenance of Foxp3 expression was assessed by flow cytometry.

2.4.3. iTreg cells in vitro suppression assay (ISA)

iTreg cells were generated *in vitro* as described previously (section 2.2). iTregs were resorted based on their Foxp3^{gfp} expression and co- cultured at different ratios, with naïve CD4^{+} CD25^{-} CD62L $^{+}$ CD45.1 T cells and anti-CD3/CD28 dynabeads. naïve T cells were isolated as described above and stained with Cell Trace Violet (CTV). After 72 hours in culture, cell division of naïve T cells was assessed by dilution of CTV dye in the gated CD4^{+} CD45.1^{+} populations via FACs indicating the *in vitro* suppressive capacity of Opn KO and WT iTreg cells. Division indices of proliferating cells were assessed with FACS and level of Treg-mediated suppression of proliferation was analyzed

2.5. *In vivo* homeostatic expansion of naïve T cells following adoptive transfer to Rag-/- recipients

Naïve CD4^{+} CD25^{-} CD62L $^{+}$ Foxp3^{-} T cells purified from WT and Opn KO Foxp3/GFP-reporter mice as described above were labeled with CTV and adoptively transferred intravenously into B6.Rag$^{-/-}$ recipient mice. Cells were transferred at a density of 10^{6} /mouse. After 10 days, spleens of recipient mice were analyzed for Foxp3^{gfp} expression, as well as proliferation capacity with Flow cytometry as described above.

2.6. Induction of experimental allergic airway inflammation and iTreg adoptive transfer

Mice were sensitized by intraperitoneal (i.p.) injections of 0.01 mg OVA (Sigma-Aldrich) mixed with 0.2 ml aluminum hydroxide,, (Serva) at days 0 and 12. Alum is known to initiate strong Th2-immune responses. Mice were challenged with aerosolized OVA (5% in PBS-1X) for three consecutive days (days 17, 18, and 19). Two days before the first allergen challenge (day 15) with aerosolized OVA, 2.5 x 10^{6} resorted WT and Opn KO Foxp3^{gfp} iTreg cells were intravenously injected into mice via retro-orbital vein. A group that did not receive iTreg was also included. Mice were euthanized 48 h after the final challenge.

2.6.1. Lung histopathology of asthmatic mice

Paraffin embedded sections of experimental mice were stained with Periodic acid-Sciff (PAS) staining for determine airway mucus secretion. 20-30 consecutive airways from all group of mice were categorized according to the abundance of PAS-positive goblet cells and assigned numerical scores (0, <5% goblet cells; 2, 5-25% 2, 25-50% 3, 50-75% 4, >75%). The sum of airway scores from each lung was divided by the number of airways examined for the histological goblet cell score (expressed as mucus score in arbitrary units; U).

2.6.2. Bronchoalveolar lavage fluid (BAL) cellularity

Following mouse's euthanasia, the trachea was cannulated, and lungs were lavaged three times with 400 µl PBS-1X supplemented with 0.07% NaCl, to collect BAL fluid and then stored on ice. BAL fluid was centrifuged at 1500 rpm for 5 minutes. Cell pellet was resuspended in 1 ml of complete medium, whereas BAL fluid was used for cytokine expression analysis. Cells recovered from BAL fluid were counted with a Neubauer hemocytometer and 5 x 10^4 cells loaded in cytoslides and centrifuges in a cytospin funnel (3 minutes at 600 rpm). Cytoslides allowed to dry overnight and stained with May-Grünwald-Giemsa stain solution in order to discriminate through microscopy the different cell populations inside lungs of asthmatic mice.

2.6.3. Differential Counting- May-Grünwald-Giemsa staining

In order to count the numbers of eosinophils, neutrophils, monocytes and leucocytes in lung of asthmatic mice, cytospins should be stained with May-Grünwald-Giemsa protocol. Firstly, cytoslides were fixed with MetOH for 10 minutes and then stained for 15 minutes in May-Grünwald (BDH) 1:1 Sorense's buffer (BDH) following with a 15 minutes staining in Giemsa (1:16 diluted in Sorense's buffer). After that, cytoslides were transferred in Sorense's buffer for 5 minutes following by one deep in water to wash dyes. Slides leaved to dry in air for 30 minutes and put on xylene (one deep). Finally, a cover slip is glued on cytoslides with one drop of Enthelan (Merk) To calculate frequencies of lymphocytes,400 cells should be counted.

2.6.4. Analysis of CD4+ T cells in mediastinal lymph nodes of immunized mice

Following mouse's euthanasia, lung mediastinal lymph nodes (MLN) of asthmatic mice were pooled and live $CD4^+$ T cells were purified and analyzed via FACS sorter (anti-CD3, anti-CD4, Foxp3gfp) fluorescence staining, as described in section 2.2. Sorted $CD3^+$ $CD4^+$ T cells (5 x 10^4 cells/well) were cocultered with mitomycin C-treated antigen presenting cells (APCs) (2 x 10^5 cells/well) and OVA to assess their recall responses to OVA. APCs were purified from spleen of naïve WT mice. Spleen pooled and cells were isolated (see section 2.2), counted and treated with 50 μg/ml mitomycin C (for 5 x 10^7 whole splenic cells) for 20 minutes at 37°C in dark. Three washes with complete RPMI 1640 were followed and then mitomycin C-treated APCs were counted and placed in cultures. After 72 hours of antigen-OVA stimulation, supernatants of culture were collected pro-inflammatory cytokine production (IL-4, IL-13 and IFN-γ) were measured with via ELISA kits (R&D systems, MN USA), Optical density was determined at 450nm by spectrophotometry (ELISA reader).

2.6.5. Intracellular cytokine analysis of CD3+ CD4+ T cells from MLNs of asthmatic mice

Sorted $CD3^+$ $CD4^+$ T cells (10^5 cells/well) were stimulated *in vitro* with 10 ng/ml phorbol 12-myristate 13-acetate (PMA) and 250 ng/ml ionomycin cultured in 96-well plates for 9 hours. Following this incubation intracellular staining was performed using the Cytofix/Cytoperm plus Fixation/Permeabilization KIT (BD). Cells were harvested, and treated with 1 μl/ml Golgi Plug (BD). After 4 hours, cells were washed with MACS buffer and stained for extracellular-surface markers (a-CD4) with the appropriate cocktail of antibodies. Cells were then washed two times with MACS buffer and resuspended in Fixation/Permeabilization solution for 20 min. at 4°C. In subsequence, cells were washed two times with BD Perm/Wash buffer and resuspended in Perm/Wash buffer containing the appropriate amount of fluorescence dye labeled-antibody cocktail for intracellular cytokines. Two washes with BD Perm/Wash were followed and cells were resuspended again in MACS buffer. Their intracellular cytokine profile was assessed by flowcytometry analysis.

2.7. Statistical analyses

FlowJo V10 software was used for all statistical analyses of flowcytometry data. Statistical analyses were performed with a two-tailed, unpaired Student's t-test, with GraphPad Prism V6 software. A P value <0.05 was considered statistically significant. No exclusion of data points was used.

3. RESULTS

3.1. Endogenous Opn expression is required for optimal Treg differentiation and suppressive function *in vitro*

3.1.1. Opn deficiency impairs the proper induction of Foxp3 during TGF-β driven differentiation of iTreg cells, *in vitro*

Previous unpublished work from our group showed that Opn is also expressed by $Foxp3^+$ Treg cells during their *in vitro* stimulation and differentiation and that is a direct target of Foxp3 transcription factor (**Appendix figure 1**). We examined whether endogenous Opn could affect Foxp3 induction. To address this, we isolated naïve $CD4^+$ $CD25^-$ $CD62L^+$ $Foxp3^-$T cells from Opn KO and Opn WT$Foxp3^{gfp}$ mice and induced Foxp3 expression *in vitro*. We found that during *in vitro* differentiation of iTreg cells Opn KO T cells displayed impaired Foxp3 expression compared to their wild-type controls. The mean values of the % of Foxp3 expressing cells were 70 for the WT compared to 56 for the KO (**Figure 10A**)

We further tested whether impairment of Foxp3 induction in Opn KO cells was attributed to an increased apoptosis or differences in proliferative capacity (rate of death vs rate of conversion vs rate of proliferation) We found that this reduction of Foxp3 expression in Opn KO $Foxp3^{gfp}$ iTreg cells was not due to increased early ($AnnexinV^+$/$7\text{-}AAD^-$) or late ($AnnexinV^+$/$7\text{-}AAD^+$) apoptosis in comparison to Opn WT $Foxp3^+$ iTreg cells (**Figure 10B**). Finally we did not observe any significant differences in the division indices between Opn WT and KO $Foxp3^+$ iTregs or the whole population examined (**Figure 10C**)

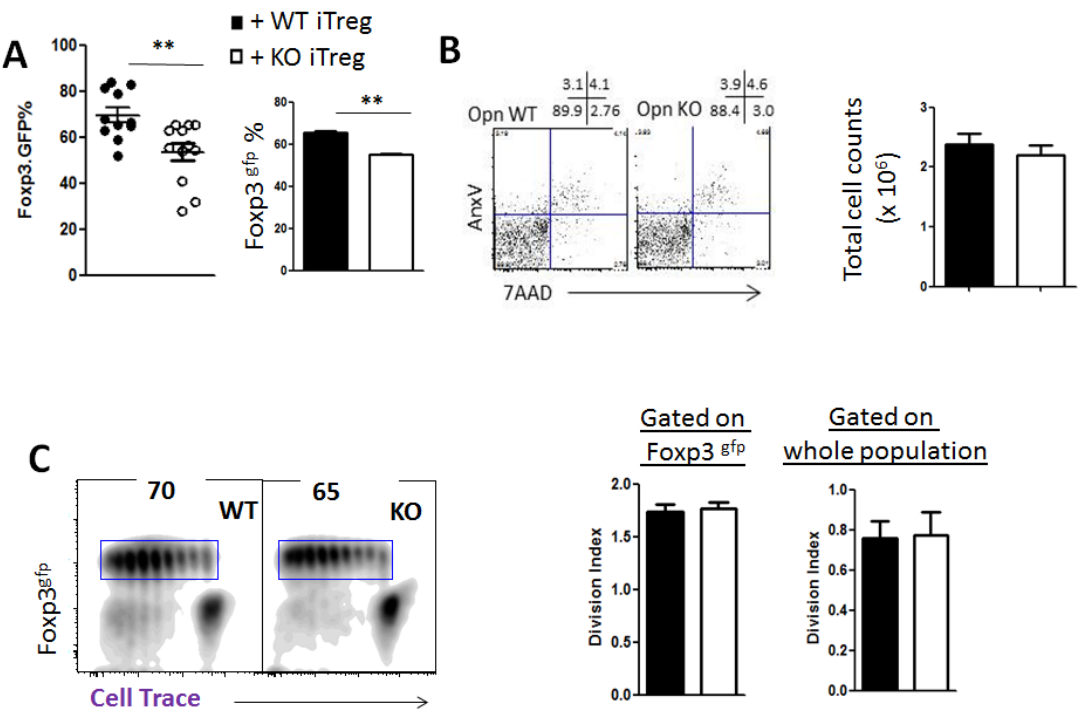

Figure 10 Endogenous Opn expression in naive T cells is required for optimal Foxp3 induction. **(A)** Naïve $CD4^+$ $CD62L^+$ $CD25^-$ T cells from WT and Opn KO Foxp3/GFP–reporter mice were differentiated in vitro. Quantification of frequency of WT and Opn KO $Foxp3^{gfp}$ iTreg cells was determined by flow cytometry: Percentages of WT and Opn KO $Foxp3^{gfp}$ iTreg cells on $CD4^+$ T cells gated for their $Foxp3^{gfp}$ expression. **(B)** Quantification of apoptosis of WT and Opn KO $Foxp3^{gfp}$ iTreg cells labeled with AnnexinV/7-AAD apoptotic markers was determined by flowcytometry. Total number of apoptotic cells (right panel). **(C)** In vitro proliferation capacity of CTV-labeled Opn KO and WT naive $CD4^+$ $CD62L^+$ $CD25^-$ T cells was monitored by CTV dye dilution and quantified using flow cytometry analysis. Data are representative of three independent experiments, $n \geq 4$ mice.NS, non-significant, *P < 0.05; **P <0.01 (two-tailed unpaired student's t-test, error bars, S.E.M).

3.1.2. Opn deficiency impairs Foxp3 induction in $CD4^+$ T cells *during in vivo* homeostatic expansion

The previous results prompted us to test whether this defect of Foxp3 induction would also be apparent *in vivo*. It is already known that the transfer of naïve $CD4^+$ T cells into $Rag^{-/-}$ host mice leads to an increased proliferation of donor T cells (Le Campion et al., 2002). That homeostatic proliferation has been shown to regulated by the development of $Foxp3^+$ Treg cells (Shen et al., 2005). To this aim, naïve $CD4^+$ $CD25^-$ $CD62L^+$ T cells were isolated from Opn KO and WT Foxp3/GFP-reporter mice, cells were labeled with CTV and they were adoptively transferred into immunodeficient $Rag^{-/-}$ recipient mice. After 10 days, spleens from mice were pooled and analyzed for

the presence of iTreg cells (**Figure 11A**). We found an impairment of Foxp3 induction *in vivo* in Opn deficient $CD4^+$ donor T cells compared to WT $CD4^+$ donor T cells. The mean values of the % of Foxp3 expressing cells were 11.5 for the WT compared to 5.8 for the KO significantly decreased (**Figure 11B**). However, homeostatic proliferation rate of Opn KO $Foxp3^+$ Treg cells was the same as their WT control Treg cells.(Figure 11B) suggesting that the lack of endogenous Opn expression does not affect the *in vivo* proliferation capacity of Treg cells.

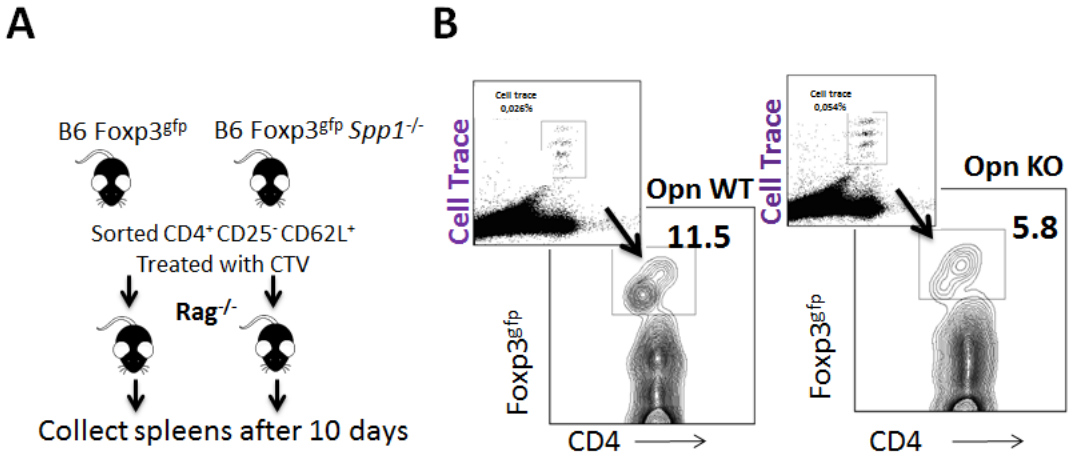

Figure 11 Endogenous Opn expression is required for the optimal induction of Foxp3 expression during homeostatic expansion of T cells. (A) Schematic representation of the homeostatic expansion experiment. WT and Opn KO $CD4^+$ $CD62L^+$ $CD25^-$ T cells were isolated from Foxp3/GFP-reporter mice. Cells were labeled with CTV and adoptively transferred into $RAG^{-/-}$ host mice. **(B)** After, 10 days T cells were pooled from spleens of host mice and the *in vivo* generation of WT and Opn KO $Foxp3^{gfp}$ Treg cells percentages were analyzed using flow cytometry. iTreg cells division and survival rate were monitored by CTV dye dilution. Dot plots are from a representative experiments, $n \geq 4$ mice.

3.1.3. The lack of endogenous Opn expression affects the suppressive capacity of Treg cells, *in vitro*

Multiple studies have shown the indispensable role of iTreg cells in the maintenance of immune homeostasis (Chen et al., 2003). To test whether the lack of endogenous Opn expression affects the suppressive function of Treg cells *in vitro*, we resorted Foxp3 expressing Opn KO and WT iTreg cells and used them in *in vitro* suppression

assay. We found that Opn-deficient iTreg cells exhibited lower suppressive capacity which was indicated by the increased % of Teff proliferation (82.4) compared to control WT iTreg cells (67.6) (**Figure 12A**). That significant difference is observed at lower concentrations of iTreg cells in the coculture (iTreg: Teff cells ratio, 1:4 and 1:8), as indicated by the increased division index of CTV-Teff cells and by the dilution of cell trace violet (**Figure 12B**).

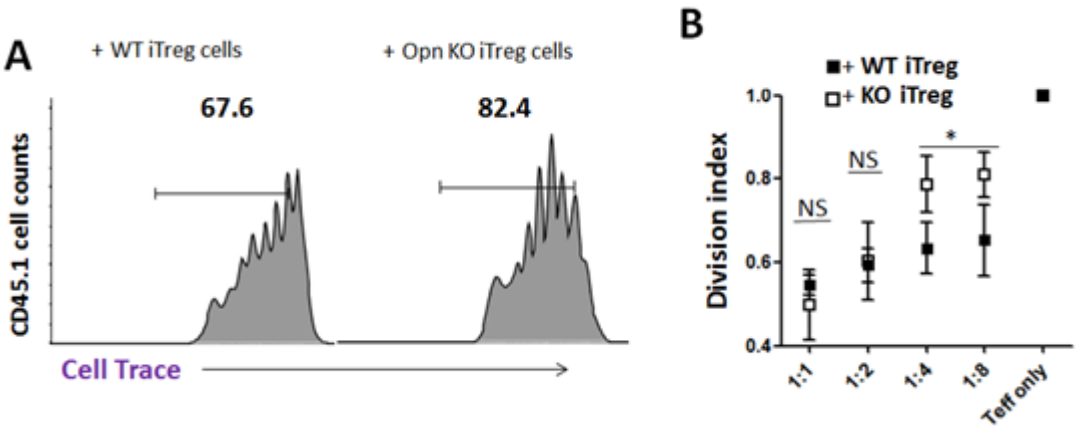

Figure 12 Endogenous Opn expression is indispensable for the optimal suppressive capacity of iTreg cells, in vitro. *In vitro* suppression assay with resorted WT and Opn KO Foxp3^{gfp} iTreg cells cocultered with CTV-labeled WT CD45.1^{+} CD4^{+} CD62L^{+} CD25^{-} naïve T cells for 3 days under anti-CD3/CD28 dynabeads polyclonal stimulation. Cells were cocultured at different iTreg: Teff ratios. After 3 days the rate of proliferation of CD45.1^{+} CD4^{+} Teff cells was analyzed by using flowcytometry. Proliferation of CD45.1 Teff cells at the ratio of iTreg cells Teff 1:8 **(A)** and their division index (DI) was calculated based on the dilution pattern of CTV dye (DI, the average number of divisions undergone by a cell in the starting population) **(B).** Representative data of 3 independent experiments, n ≥ 4 mice. NS, non-significant, *P < 0.05; **P <0.01 (two-tailed unpaired student's t-test, error bars, S.E.M).

3.2. Endogenous Opn expression is required for normal Treg development *in vivo*

We further questioned whether the lack of endogenous Opn expression affects the development of Treg cells *in vivo.*

To investigate our hypothesis, we compared the frequencies of $CD4^+$ $Foxp3^{gfp}$ Treg cells in primary and secondary lymphoid organ (thymus; Thy, axillary/ inguinal lymph nodes; LN, spleen; SP and mesenteric lymph nodes; mLNs) from naive Opn KO and WT Foxp3/GFP-reporter mice. We found significantly decreased percentages % of $CD4^+$ $Foxp3^+$ Treg cells frequencies in thymus (0.17), spleen (8.93) , and mLNs (3.41) of Opn KO mice compared to WT controls (**Figure 13A**). However, significant differences in the number of Foxp3 expressing $CD4^+$ Treg cells were only observed in the SP and the mLNs of Opn deficient mice odes (**Figure 13B**). The differences in the mLNs were particularly interesting since it has been previously reported that under steady state (or inflammatory conditions) iTreg are generated and accumulated in the gut (Coombes et al., 2007; Siddiqui and Powrie, 2008). In addition, it is known that inside gut there is a constant accumulation of $Foxp3^+$ Treg cells which every day maintain the immune gastrointestinal homeostasis by inhibiting the activation of Tconv cells against oral-food antigens (Sun et al., 2007,). For those reasons, We also compared the percentages of $CD4^+$ $Foxp3^{gfp}$ Treg cells frequency in *lamina propria* (LP) of Opn KO and WT Foxp3/GFP-reporter mice. We interestingly observed similar to mLNs reduced % levels of Foxp3 expressing CD4+ T cells in Opn KO mice (9.87) compared to WT mice (10.7) with a subsequent reduction in their $CD4^+$ $Foxp3^{gfp}$ Treg cells numbers (**Figure 13B**).These data suggested that the endogenous Opn expression is required for Treg cells differentiation *in vivo* and it seems more crucial for the induction of adaptive (iTreg and pTregs) Treg cells. We are currently testing whether these cells express nTreg markers (Neuropilin1, Helios) to distinguish nTreg/iTreg population in these mice.

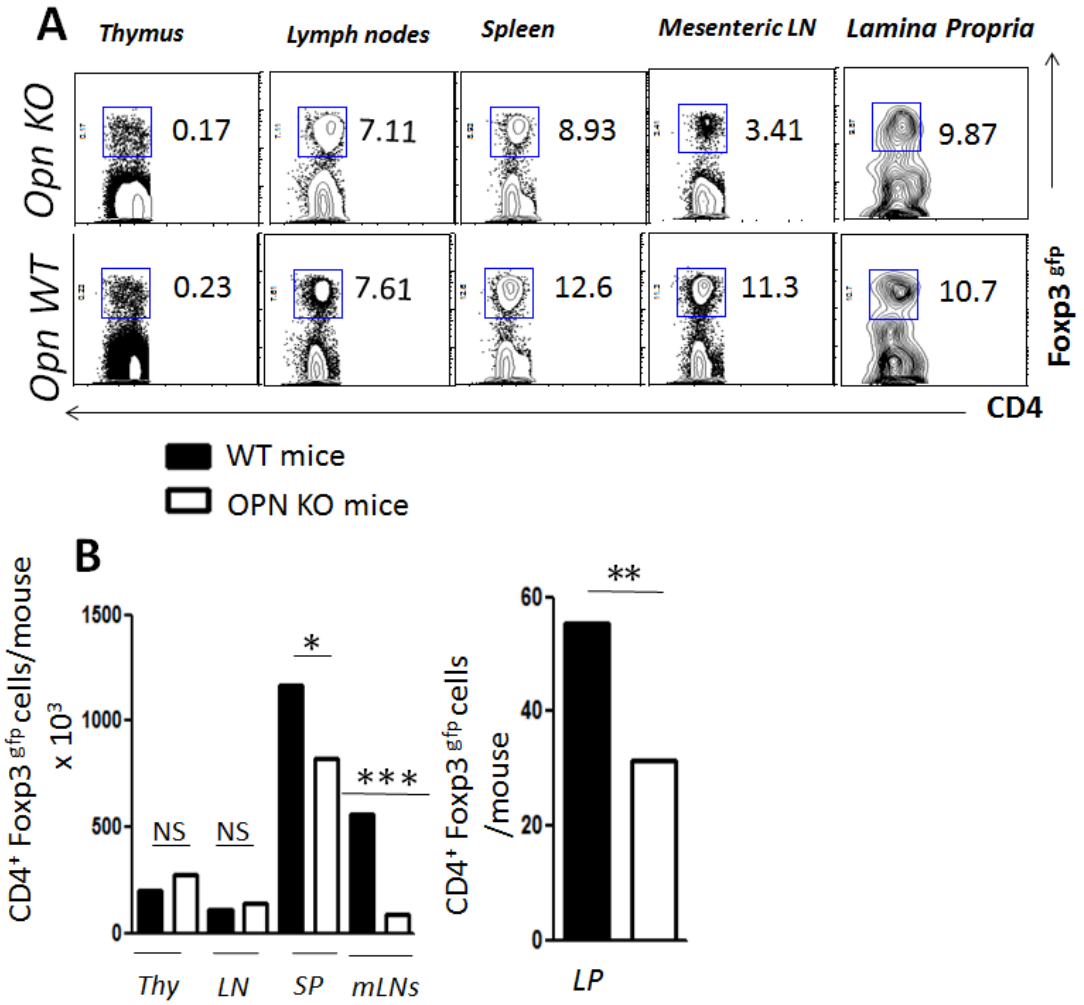

Figure 13 Endogenous Opn expression is indispensable for development of Foxp3$^+$ Treg cells, *in vivo*. CD4$^+$ T cells were pooled from lymphoid tissues and the lamina Propria of WT and Opn KO Foxp3^{gfp} –reporter mice and analyzed for the presence of Foxp3$^+$ Treg cells. **(A)** Percentages of Foxp3^{gfp} Treg cells in Thy, LN, SP, mLNs and LP were assessed with the use of flowcytometry. **(B)** Total CD4$^+$ Foxp3^{gfp} T cell counts in Thy, LN, SP, MLN and LP of naïve WT and Opn KO Foxp3/GFP–reporter mice. software and data are representative of 1 experiment, n=2 mice/group. Non-significant (NS);*P < 0.05; **P <0.01***P<0.001 (two-tailed unpaired student's t-test, error bars, S.E.M).

3.3. Endogenous Opn expression is required for optimal suppressive function of Treg *in vivo*

The differences in Foxp3 induction and Treg cell development in vivo prompt us to investigate possible differences in the genetic profile of Opn WT and KO Tregs. We performed microarray gene analysis in 16 hours anti-CD3/CD28-in vitro-activated

$CD4^+$ $Foxp3^{gfp}$ Treg cells which were sorted from Opn WT and KO Foxp3/GFP-reporter mice. We found that Opn KO $Foxp3^{gfp}$ Treg cells had elevated expression of Th2 lineage-specific genes, such as *irf4*, *il4 and il13* in comparison to control WT Treg cells (**Appendix figure 2**). For this reason, we hypothesized that this altered gene expression would affect the suppressive function of Opn KO Treg cells *in vivo*. In order to test our hypothesis, we assessed the suppressive ability of Opn KO and WT Tregs in a Th2- mediated allergic airway inflammation mouse model, It has been previously described that the adoptive transfer of iTreg cells effectively attenuates murine airway allergic inflammation (Xu et al., 2012). So on, isolated naïve $CD4^+$ $CD25^-CD62L^+$ T cells from WT and Opn KO Foxp3/GFP-reporter mice were differentiated into $Foxp3^+$ iTreg cells *in vitro* under polyclonal stimulation, and were resorted for their $Foxp3^{gfp}$ expression. Purified iTreg cells were adoptively transferred into OVA-pre-immunized mice, 2 days before intranasal challenges with OVA (**Figure 14A**).

One pathologic characteristic of mice and patients suffering from asthma is an excess of airway mucus that can contribute to airway obstruction, and increased risk of death. We observed a reduction of lungs PAS positive cells in lungs of asthmatic mice only when we transfer WT $CD4^+$ $Foxp3^{gfp}$ iTregs (PAS score: 1.41) (**Figure 14B upper panel**) but not while Opn KO iTreg cells were transferred (PAS score: 1.68).

In addition, Th2-mediated airway inflammatory responses are characterized by an increased infiltration of cells of the innate immune system in the lungs contributing to asthma exacerbation. Analysis of BAL fluid from immunized mice revealed that the transfer of iTreg cells did not affect eosinophils intensity within lungs however a significant increased accumulation of macrophages was observed in mice, to whom Opn KO iTreg cell were transferred (4.18×10^5) in comparison to WT iTreg cells group (2.18×10^5) (**Figure 14C**).

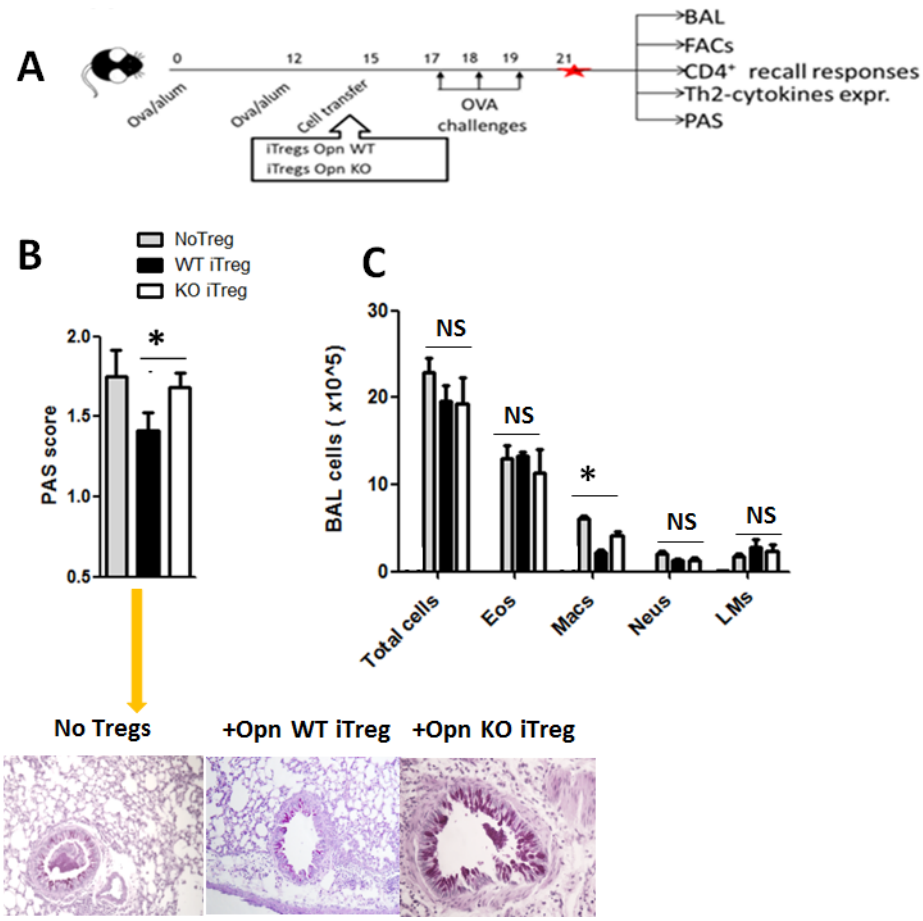

Figure 14 Adoptive transfer of Opn KO iTreg cells does not prevent histopathologic features of allergic airway inflammation. (A) Schematic representation of experimental protocol used to prevent development of Th2-mediated allergic airway inflammation by adoptively transferred intravenously Opn WT and KO Foxp3^{gfp} iTreg cells (2.5x10^6 cells/mouse, n=4/group). **(B)** Excessive mucin expression in small airway epithelial cells was detected by PAS-staining (red color), scale bar, 40 μM. Lung mucus secretion in upper panel, indicated by PAS score *P < 0.05; **P <0.01 (two-tailed unpaired student's t-test, error bars, S.E.M). **(C)** BAL differentials (x10^5) are expressed as means +/- S.E.M Data are representative of 2 independent experiments.

Furthermore, lung mediastinal lymph nodes of asthmatic mice were pooled and single cell suspensions were made. The recovery of adoptively transferred Foxp3gfp expressing CD4$^+$ T cells was the same as between the two groups as it was indicated with the use of flow cytometry (approximately 10 x 10^3 cells) (**Figure 15A**). In addition, we resorted CD4$^+$ T cells from MLNs of immunized mice and we cultured them *in vitro* for downstream applications. We either stimulated sorted CD4$^+$ T cells with PMA/Ionomycin, a strong cytokine inducer, in order to analyze the discrepancy of pro-inflammatory CD4$^+$ IL-4$^+$ T cells within MLNs of two groups or used them for recall responses to OVA and study their cytokine expression pattern.. It was found,

with the use of intracellular staining and flowcytometry, , that the numbers of $CD4^+$ $IL\text{-}4^+$ T cell producers within MLNs of Opn KO iTreg group were doubled (103 x 10^3 cells) compared to WT iTreg group. of mice (49 x 10^3 cells) (**Figure 15B**) Also, recall responses to OVA experiments revealed that the $CD4^+$ T cells, which were isolated from the MLNs of Opn KO iTreg cells group, had higher expression of Th2 pro-inflammatory IL-4 and IL-13 cytokine (8.4 pg/ml and 995.5 pg/ml respectively) than the WT iTreg cell control group (0.6 pg/ml and 713 pg/ml respectively). Also, that cell express higher amounts of IFN-γ (71.75 pg/ml) in comparison to the control group (26 pg/ml) (**Figure 15C**). Collectively, our data suggest that the WT $Foxp3^+$ iTreg cells display a slightly stronger inhibitory ability in suppressing the activity of Th2-effector cells and inhibit the progression of the lung allergic inflammation compared to Opn KO iTreg cells and suggest that the lack of endogenous Opn renders iTreg cells unable to inhibit Th2-immune responses, *in vivo*.

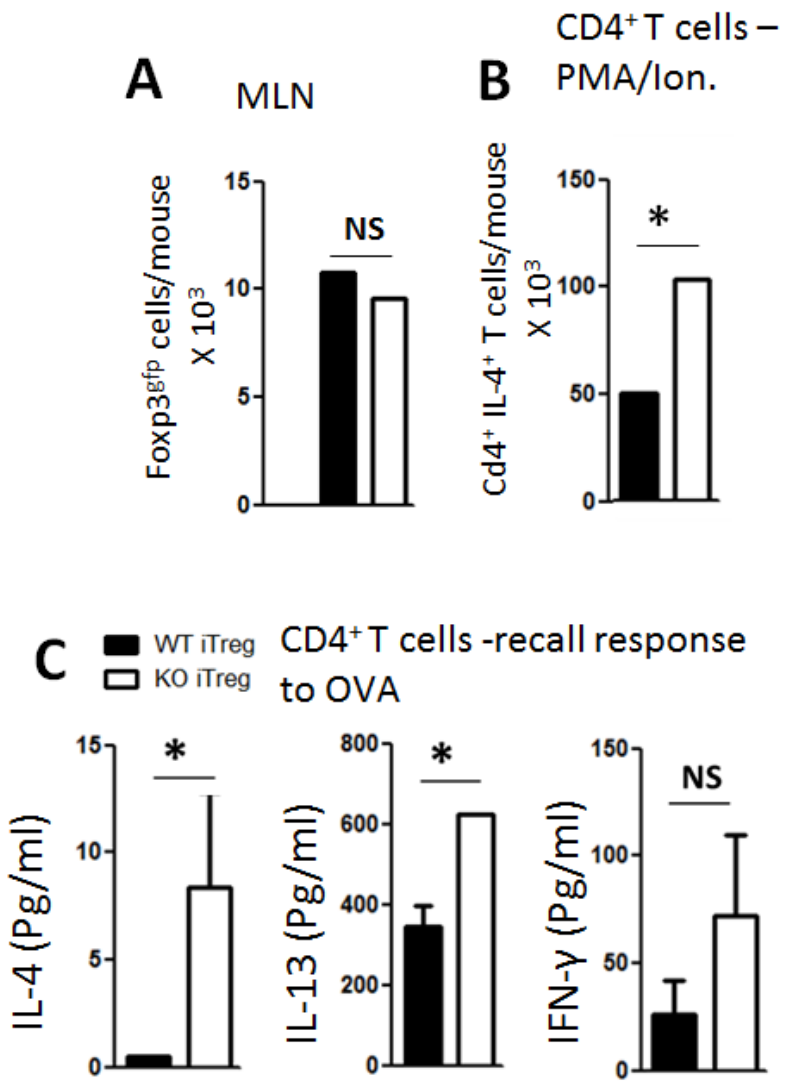

Figure 15 The endogenous Opn expression in iTreg cells is required for their in vivo suppressive function. **(A)** Total Foxp3^{gfp} iTreg cells counts (x10^3) isolated from MLNs of OVA-challenged mice. **(B)** Total CD4$^+$ T cell counts (x10^3) which purified from MLNs of asthmatic-mice and express IL-4 cytokine upon restimulation with PMA/Ionomycin (PMA/Ion) were determined by intracellular flow cytometry analysis. **(C)** Levels of pro-inflammatory IL-4, IL-13, IFN-γ cytokines in supernatants of OVA-stimulated CD4$^+$ T cells isolated from the MLNs of OVA-Th-2 mediated allergic airway diseased mice were determined by the use of ELISA kits. Data are representative of 2 independent experiments, n=4 mice/group. Non-significant (NS);*P < 0.05; **P <0.01***P<0.001 (two-tailed unpaired student's t-test, error bars, S.E.M).

4. DISCUSSION

Opn is a highly phosphorylated glycoprotein that mediates diverse biological functions. Excessive or deregulated expression of Opn has been correlated with chronic inflammatory disorders. It has been demonstrated that Opn is expressed by activated $CD4^+$ T cells, is regulated by the expression of T-bet, and many studies have revealed the involvement of Opn in T cell mediated immunity (Th1, Th2 and Th17) (Cantor and Shinohara, 2009; Xanthou et al., 2007). Further studies from our group have revealed that Opn is highly expressed in the lungs of asthmatic patients and in the lungs of mice with allergic airway inflammation (Th2-mediated) exerting both pro-and anti-inflammatory effects (Xanthou et al., 2007). Previous unpublished data from our group suggested that these anti-inflammatory effects could be partially mediated by Treg cells. In addition, other groups have shown that on stimulated $Foxp3^+$ Treg cells the *Spp1* gene is significantly upregulated (Do et al., 2015; Marson et al., 2007). Furthermore, a recent study demonstrated that iOpn exerts a crucial role in the generation and stability of follicular regulatory T cells (another $Foxp3^+$ Treg cell subtype) (Leavenworth et al., 2015a). However, until now the level of Opn expression (iOpn and sOpn) in Treg cells and the biological importance of this has not been investigated.

Of great importance to our study previous experiments from our lab showed that Opn is also expressed by $Foxp3^+$ $CD4^+$ Treg cells, during their *in vitro* differentiation and activation, and also that the promoter of *Spp1* gene is a direct target of Foxp3 transcription factor (**Appendix figure 1**). We investigated whether endogenous Opn expression is also critical for Foxp3 induction. It is well known that TGF-β in combination to TCR-stimulation and IL-2, induces Foxp3 expression in naïve $CD4^+$ T cells and differentiates them into iTreg cells (Chen and Konkel, 2010; Fu et al., 2004). We observed that the absence of endogenous Opn expression impairs Foxp3 induction. We also observed a similar defect in Opn KO T cells under homeostatic expansion *in vivo*. It has been demonstrated that naïve $CD4^+$ T cells when adoptively transferred into immunodeficient host mice ($Rag^{-/-}$) under homeostatic conditions, they proliferate (Le Campion et al., 2002). This has been shown to be mediated by the strength of the interaction of their TCR with MHC-molecules of recipient mice (Smith et al., 2001). In addition, it has been shown that Foxp3 Treg cells are also

developed in order to control the homeostatic proliferation after the transfer of naïve $CD4^+$ T cells into $Rag^{-/-}$ mice (Shen et al., 2005). We found that the lack of endogenous Opn expression in T cells impairs the conversion into $Foxp3^+$ Treg cells under homeostatic expansion without affecting their proliferative capacity. It is important to emphasize here that we did not observe any increase of apoptosis or changes in the division indices of these cells. This is crucial since Opn has been described to promote T cell survival, possibly in an autocrine way, and also that stimulated Opn KO $CD4^+$ T cells are predisposed to early or late-stage cell death, due to increased abundance of Bim and Bak pro-apoptotic proteins (Hur et al., 2007). Collectively, this part of the study suggests that the endogenous Opn expression is indispensable for Foxp3 induction and it is required for optimal $Foxp3^+$ Treg cells differentiation both *in vitro* and *in vivo*.

Seminal studies have shown that Foxp3 gene expression requires TCR activation, TGF-β receptor and IL-2 receptor signaling (Chen et al., 2011; Horwitz et al., 2008; Shevach et al., 2008). We doubt the possibility that endogenous Opn is affecting positively Foxp3 induction through integration with TCR signals since recent studies have shown in $CD4^+$ T cells, PI3K (which has been shown to negatively correlate with Foxp3 expression) is activated by iOpn (Leavenworth et al., 2015a). However, forced expression of iOpn in Opn KO T cells saves their differentiation into $Foxp3^+$ $Bcl\text{-}6^+$ T_{FR} cells (Leavenworth et al., 2015a). Possible interactions of Opn with the TGF-β or IL-2 signaling in this context have not been investigated and future experiments could shed more light into this.

An alternative explanation to the deficiency in Foxp3 induction in Opn KO T cells could be the presence of cytokines in these cultures. It has been previously described that iTreg cells differentiation is very sensitive to pro-inflammatory cytokines, such as IL-4 (Beal et al., 2011; Mantel et al., 2007; Wei et al., 2007). IL-4 was found to be expressed in increased levels in the supernatants of Opn KO iTreg cells during their TGF-β-driven differentiation (**Appendix figure 3**). This could probably lead to a trans-differentiation of the Opn KO T cells into Th2 and Th9 cells (Dardalhon et al., 2008).

The above data came in agreement with our Foxp3 T reg cell characterization in the Opn KO Foxp3/GFP reporter mice *in vivo*. By comparing the frequencies of $Foxp3^+$

Treg cells in lymphoid and non-lymphoid tissues from Opn KO and WT Foxp3/GFP-reporter mice, we observed significantly decreased in the numbers of $Foxp3^+$ Treg cells especially in spleen, mesenteric lymph nodes and *lamina propria* of Opn KO mice, indicating the requirement of endogenous Opn expression for the normal Treg cells generation *in vivo*. Significantly, the decreased numbers of Treg cells on mesenteric lymph nodes and *lamina propria* were particularly interesting since it has been previously described that under steady state (and inflammatory conditions) iTreg cells are generated and accumulated at mucosal surfaces of gut in order to maintain gut immune homeostasis. Thus our data suggest that in the absence of endogenous Opn expression, there is a defect in iTreg cell differentiation *in vivo*. Although, we found diminished numbers of $Foxp3^+$ Treg cells in the gut of Opn deficient mice (indicating an *in vivo* functional problem of Opn KO $Foxp3^+$ Treg cells), we did not observe any spontaneous gut inflammation, as it has been previously described (Josefowicz et al., 2012), which is probably attributed to the fact that Opn is also absent from $CD103^-$ cDCs, which has been previously described to express Opn and become highly pathogenic for induction of murine intestinal inflammation (Kourepini et al., 2014).

Of great importance to our study, investigation of the suppressive function of these cells also revealed that Opn deficiency impairs the function of Tregs both *in vitro* and *in vivo*. Opn KO iTreg cells were not as effective as their WT controls in suppressing the proliferation of naïve $CD4^+$ T cells upon polyclonal stimulation *in vitro*. As mentioned above we haven't observed any abnormalities with the Opn KO iTregs in terms of their apoptotic predisposition or proliferation rates. Previous seminal studies have shown that in this context Tregs can suppress T cell proliferation by either production of cytokines (IL-10 and TGF-β) or through cell-contact inhibition with the latter to be considered as the dominant (Povoleri et al., 2013). In addition other mechanisms have also been suggested such as production of granzymes, "IL-2 sink" (though their high expression of high affinity receptor CD25) and modulation of APC function (Joller et al., 2014; Schmidt et al., 2012; Shevach, 2009; Wu et al., 2014; Yamaguchi et al., 2011; Yu et al., 2009). Due to the absence of APCs in our *in vitro* suppression assays we aim to believe that the lack of endogenous Opn in iTreg cells could be affecting their cytokine expression or their cell-contact inhibitory abilities. None of these mechanisms have been yet investigated so far. In future experiments,

the use of the transwell system, which can discriminate between cell-contact dependent and cytokine secretion suppression, could be used to expand our understanding of these results.

The breakdown of tolerance in the airways leads to an abnormal response to harmless airborne antigens, characterized by Th2 type inflammation and airway hyperresponsiveness. Among several possibilities for maintaining the balance between airway tolerance and airway inflammation, regulatory T cells have been proposed to be an essential protective mechanism mouse model (Lloyd and Hawrylowicz, 2009). Interestingly, the adoptive transfer of polyclonal stimulated iTreg cells before airway antigenic challenges were able to suppress effector T cell activation during the peak of inflammatory response and attenuate mouse asthmatic lung-inflammation (Duan et al., 2011; Xu et al., 2012). These reasons prompted us to test whether the lack of endogenous Opn expression affected the suppressive function of $Foxp3^+$ iTreg cells *in vivo* in a well-established model of allergic inflammation. We generated polyclonal-stimulated WT and Opn KO $Foxp3^+$ iTreg cells and we adoptively transferred them into OVA-pre-immunized mice before the first allergen challenge. Both Opn KO and WT iTreg cells were tracked and recovered in the same frequencies from the MLNs of immunized mice indicating that the lack of endogenous Opn in iTreg cells did not affect their *in vivo* migratory capacity to lymphoid tissues and the inflammatory site. However, in the recipient group of asthmatic mice to which Opn KO iTreg cells were adoptively transferred, we didn't observe a significant suppression of allergic disease in comparison to the control group which received WT iTreg cells. Analysis of recall responses from these mice revealed that mice that received Opn KO iTregs had increased amounts of Th2-cytokine expressing T cells in their lung mediastinal lymph nodes. In addition, these mice had increased accumulation of macrophages in their lungs. It has been shown that these cells can secrete IL-13 as well as IL-33 and contribute to increased mucus expression and fibrosis underlying the exacerbation of IgE-mediated airway inflammation (Mizutani et al., 2013). It has to be noted here that the typical eosinophilia that is observed in this experimental allergic inflammatory model was not affected by either groups. This could be due to number of iTreg cells transferred. Additional studies could be performed using increased amounts of iTreg cells to test whether eosinophilia is affected on this context.

These data could be partially explained by previous unpublished studies of our group indicating that Opn KO Tregs have increased Th2 cytokine expressing genes (*Il4*, *Il13*, *Irf4*) (**Appendix figure 2**). Interestingly, a recent study has showed Bcl-6 KO Treg cells present defect in their Th2-suppressive ability *in vivo* (Sawant et al., 2012). These cells present similar phenotypic alterations with the Opn KO iTregs. In support to our study another recent study has shown that endogenous Opn is also indispensable for Bcl-6 stability in follicular regulatory T cells (Leavenworth et al., 2015a). Collectively these data imply that the Opn/Bcl-6 axis is vital to Treg stability and *in vivo* function.

This study suggests that Opn expression is important for the stability of $Foxp3^+$ Treg cells and indispensable for optimal Treg cell differentiation and suppressive function, both *in vivo* and *in vitro*. Additional studies are required to elucidate the mechanisms behind the biological role that Opn has in Treg cells. Future experiments have to confirm a possible role for candidate genes *Il4*, *Irf4* and *Il13* which we found to be overexpressed into stimulated Opn KO $Foxp3^+$ Treg cells compared to WT $Foxp3^+$ Treg cells as well as cytokines IL-4 and IL-9 production from differentiating Opn KO T cells. Finally, the *in vivo* function of Opn KO iTreg cells and nTregs in a more clinically relevant mouse model of allergic inflammation, such as the house dust mite (HDM)-induced should be investigated.

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6. APPENDICES

6.1. Opn is expressed by Foxp3 +Treg cells in vitro during their differentiation and activation

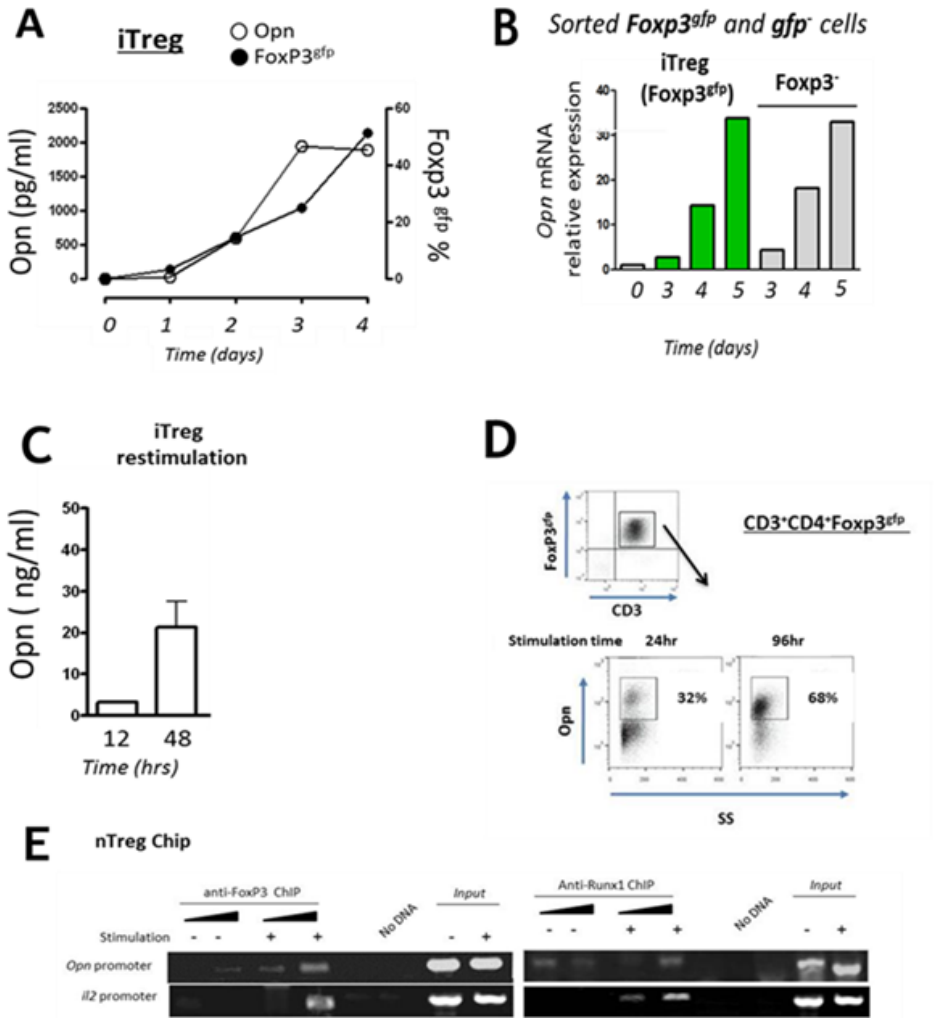

Figure 1. **Foxp3^{+} Treg cells express Opn. (A)** Naïve CD4^{+}CD62L^{+}CD25^{-} T cells from WT Foxp3^{gfp}-reporter mice were differentiated *in vitro* into iTreg cells (see methods). Time course of secreted Opn protein expression in iTreg cells was determined by ELISA in their supernatants and the Foxp3 expression was determined by flowcytometry analysis. **(B)** Quantitative real-time PCR analysis of the time course of the expression of *Opn* mRNA in WT iTreg cells and Teff cells. **(C)** Opn expression in re-sorted WT Foxp3^{gfp} iTreg cells was determined by ELISA. **(D)** CD4^{+} CD62L^{+} CD25^{+} Foxp3^{gfp} nTreg cells were isolated from WT Foxp3^{gfp}-reporter mice and cultured under the presence of anti-CD3/CD28 dynabeads and IL-2 for 96hrs (see methods). Their expression of secreted Opn was determined by flowcytometry analysis. **(E)** The binding of Foxp3 and Runx1 transcription factors to *Opn* promoter in *in vitro* polyclonal activated naïve T cells for 16hrs was assayed by ChIP-PCR (see methods). The *IL-2* promoter was used as a positive control for Foxp3 and Runx1 binding and samples without DNA were used as negative controls. Another positive control, the PCR was done directly on input DNA purified from chromatin before immunoprecipitation, right panels. Data are representative of three independent experiments with n ≥ 4 mice.

6.2. Microarray analysis of Opn KO iTreg cells

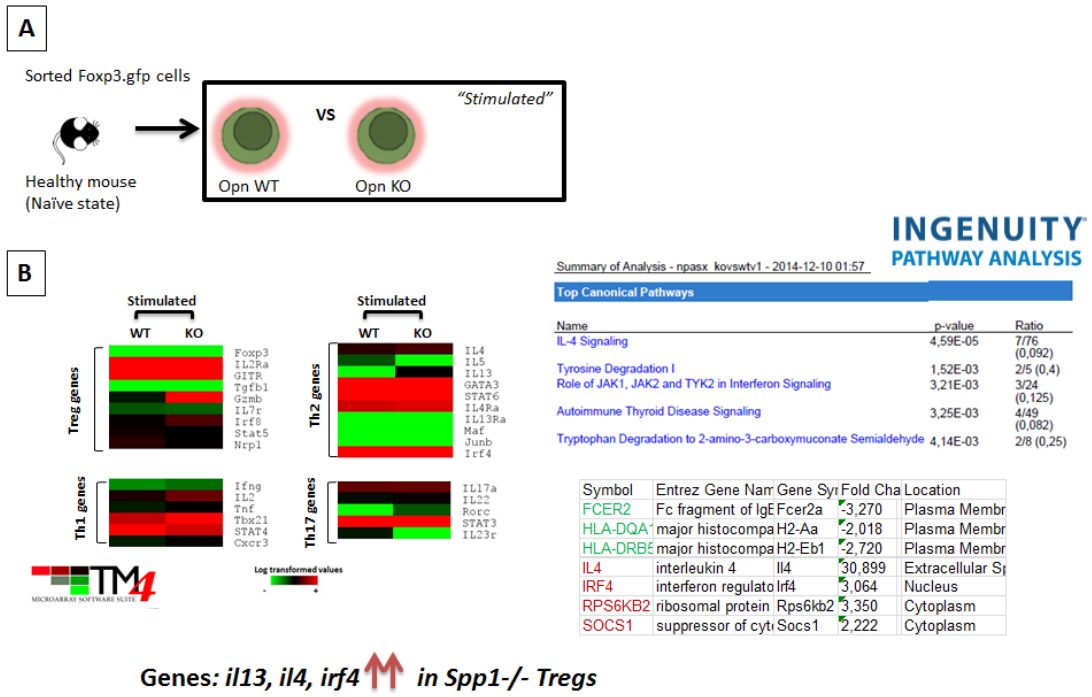

Figure 2. **Opn expression is required for the stability of activated Treg cells**. Naïve Foxp3^{gfp} Treg cells were isolated from naïve WT and Opn KO Foxp3/GFP-reporter mice. Isolated cells were cultured for 16 hours under anti-CD3/CD28 stimulation and analyzed for their gene expression pattern with the use of microarray analysis. Schematic representation **(A)**. Microarray data revealed increased expression of *il13*, *il4* and *irf4* genes in stimulated Opn KO Treg cells compared to stimulated WT Treg cells **(B)**.

6.3. Opn KO CD4+ T cells cytokine secretion during TGF-β-driven iTreg cell *in vitro* differentiation

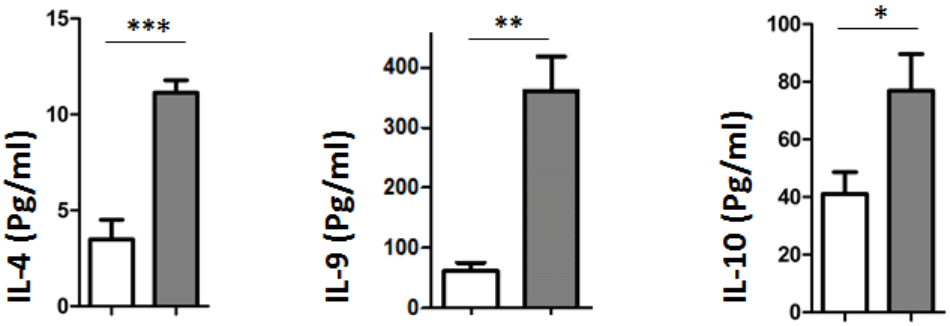

Figure 3. Increased expression of Th9-cytokine during the *in vitro* differentiation of Opn KO iTreg into iTreg cells. Naïve CD4+ T cells were isolated from WT and Opn KO Foxp3/GFP-reporter mice. Cells were cultured in *vitro* under TGF-β (10 ng/ml), IL-2 (100 ng/ml) and anti-CD3/CD28 dynabeads at a ratio 1:1. After 5 days of culturing we measured the IL-4, IL-9 and IL-10 cytokines in culture supernatants with the use of ELISA kits. Data are representative of two independent experiments, n ≥ 4 mice. GraphPad software was used to analyze our data. Non-significant (NS);*P < 0.05; **P <0.01***P<0.001 (two-tailed unpaired student's t-test, error bars, S.E.M).

6.4. Antibodies list used for extracellular and intracellular staining of mouse T cells

Antibody	Clone	Company
Anti-CD4	GK1.5	Biolegend
Anti-CD3	145-2C11	Biolegend
Anti-CD25	PC61.5	ebioscience
Anti-CD62L	MEL 14	Biolegend
Anti-IL4	11B11	Biolegend
Anti-CD45.1	A20	Biolegend

Figure 4 Antibodies used for Flowcytometry.