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# Immunomodulatory role of omega-3 polyunsaturated fatty acid-derived pro-resolving lipid mediators on cell-mediated adaptive immunity

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**SUMMARY**

Inflammation represents a crucial physiological process mounted by immune system to react to tissue damage and pathogens. Acute inflammation, which is usually the quickest immune reaction to occur upon the insurgence of an infection, is a widely conserved mechanism that is triggered and regulated mostly by cells of the innate immunity (i.e. dendritic cells, monocyte/macrophages, neutrophils and NK cells) and is meant to be the first line of defense against microbes. Since innate immune cells only recognize a relatively limited number of pathogen-associated molecular patterns, higher efficiency requires the action of the specialized cells of the adaptive immune system (i.e. T and B lymphocytes), which potentiate innate functions. Acute inflammation can have two main outcomes: on one hand, prolonged immune reactions can lead to chronic inflammation, which involves the concerted action of macrophages and lymphocytes; on the other hand, inflammation can undergo resolution with complete return to tissue homeostasis. Resolution of inflammation is a pivotal process that is meant to actively quench phlogistic signals. Even though this mechanism has been thought for a long time to act in a passive way, in recent years it has been unequivocally established that it is instead a finely regulated and active process, governed by a new class of lipid mediators, which are synthesized by essential polyunsaturated fatty acids such as arachidonic acid (AA), eicosapentaenoic acid (EPA) and docosahexanenoic acid (DHA). These molecules, which have been termed pro-resolving lipid mediators (SPMs), orchestrate self-limitation of inflammation mostly by acting on innate immune cells; even though a growing number of works are starting to suggest that SPMs might also act on adaptive immunity, our current knowledge of the immunoregulatory actions exerted by SPMs on chronic inflammation and its main effectors, the lymphocytes, is still very scarce. Thus, we investigated the immunomodulatory effect of crucial DHA-derived bioactive lipids such as RvD1, RvD2 and Maresin 1 on pivotal populations of

cell-mediated adaptive immunity, i.e. $CD4^+$ and $CD8^+$ cells, as well as the most important T lymphocyte subsets ($T_H1$, $T_H2$, $T_H17$ and iTreg). In order to achieve this goal we analyzed immune functions by means of polychromatic flow cytometry alongside with well-established molecular biology and biochemistry techniques such as qRT-PCR, western blot and ELISA assays. We found that DHA-derived SPMs strongly inhibit the production of pro-inflammatory cytokines (i.e. IFN-γ, TNF-α and IL-17) in circulating $CD4^+$ and $CD8^+$ lymphocytes, and reduced the production of IL-2, a pivotal lymphocyte mitogen, without inducing apoptosis or necrosis. In order to further characterize the complete spectrum of immunoresolving actions elicited by DHA-derived SPMs, we assayed their ability to modulate *de novo* generation of T helper cells ($T_H1$, $T_H2$ and $T_H17$) and induced regulatory T cells (iTreg) from naïve T cell precursors. RvD1, RvD2 and MaR1 were able to reduce intracellular production and release of $T_H1$ and $T_H17$ signature cytokines (IFN-γ and IL-17 respectively), as well as inhibiting the expression of their pivotal transcription factors (T-bet and RORc), without affecting in any way $T_H2$ function. On the other hand they enhanced the immunoregulatory properties of iTreg cells by enhancing the expression of their key transcription factor Foxp3, while boosting the release of the anti-inflammatory cytokine IL-10 and the expression of their immunosuppressive receptor CTLA-4. These effects were mediated by the two well-known SPMs receptors, GPR32 and ALX/FPR2. The direct evidence of a role for SPMs in modulating cell-mediated immunity holds huge importance in that it unveils a new level of regulation that the immune system may use to balance inflammatory responses, and considerably improve our knowledge of the vast network of bioactive lipids that regulate inflammatory processes. This could be crucial in theorizing possible future therapeutic approaches for many T cell-dependent chronic inflammatory diseases

This thesis is based on the research work described in the following paper:

Chiurchiù V, **Leuti A**, Dalli J, Battistini L, Jacobsson A, Maccarrone M, Serhan CN. Pro-resolving lipid mediators D-series Resolvins and Maresin 1 are critical in modulating T cell responses: a novel role for regulation of cell-mediated adaptive immunity. Sci Trans Med. Under review

# I. INTRODUCTION

## 1. Inflammatory processes and their outcomes

Inflammation represents possibly one of the most anciently known biological processes, the oldest reports dating back to ancient Egyptian and Greek culture; as a matter of fact, the term edema, which describes an accumulation of liquid in inflamed tissues, is borrowed by modern sciences from Hippocrates, that coined the definition in 5$^{th}$ century BC, while the cardinal signs classically used to describe inflammation, namely *rubor* (redness), *calor* (warmth), *dolor* (pain) and *tumor* (swelling) were described by Celsus, in the first century; Galen, on the other hand is thought to have introduced the 5$^{th}$ sign, *functio laesa* (loss of function) which usually results from prolonged and/or detrimental inflammatory processes in tissues and organs (**Majno and Joris, 2004, Serhan et al., 2010**). Inflammation is a rather complex mechanism, or network of mechanisms by means of which the host reacts to tissue damage dealt by exogenous bodies or invading pathogens such as viruses, bacteria, fungi and helmints; it is characterized by intense vascular responses, local recruitment of immune cells in the site of infection and the production of a plethora of different chemical mediators such as cytokines, chemokines, molecules of the complement system (**Cotran et al., 2014**) eikosanoids (e.g. prostaglandins, leukotrienes and thromboxanes) (**Samuelsson, 2012**) and pro-resolving lipids (i.e. lipoxins, resolvins, maresins and protectins) (**Serhan et al., 2008**).

### *1.1. Acute inflammation*

Acute inflammatory response (or angiophlogosis), depending on the kind of pathological phenomenon, occurs within the first few hours or days after the injury and represents the earlier event of a chain of protective mechanisms that the immune system mounts upon the recognition of noxious stimuli; it is characterized by a profound modification in the permeability of local blood and lymphatic vessels, formation of edema and massive recruitment of innate immune cells from the bloodstream (**Levy and Serhan, 2014**). Indeed, the increasing of neutrophils (polymorphonuclear leukocytes, PMNs) in the injured site during the first 6-24 hours, their replacement by monocytes in the following 24 to 48 hours and the production of vast amounts of reactive oxygen and nitrogen species (ROS and RNS), autacoids, complement system proteins, vasoactive peptides (e.g. histamine) and inflammatory cytokines (e.g.: IL1-β and TNF-α) represent typical hallmarks of the most common forms of acute inflammatory reactions and are part of the machinery that eventually lead to pathogen elimination and, hopefully, restoration of tissue homeostasis (**Cotran et al., 2014**). As summarized in **Fig. 1.1** acute inflammation can have different outcomes:

i) If the injury is limited or short-lived and the cells are able to regenerate, dead cells and debris are removed, the edema is resorbed through the lymphatic stream and the tissues heal completely (**Kumar et al., 2014; Serhan et al., 2007**). This process, which is characterized by complete return to functionality, is called resolution and will be discussed in detail later.

ii) When the damage is too extended, it involves cells that cannot regenerate or the inflammatory response has led to the production of vast quantities of connective tissue proteins that cannot

be adequately cleared, the healing process is characterized by the replacement of the functional tissue with a fibrous scar (**Ueha et al., 2012**).

iii) If inflammation cannot be cleared and the stimulus persists, or if the other healing processes are hindered, acute inflammation evolves into chronic inflammation (**Murakami and Hirano, 2012**)

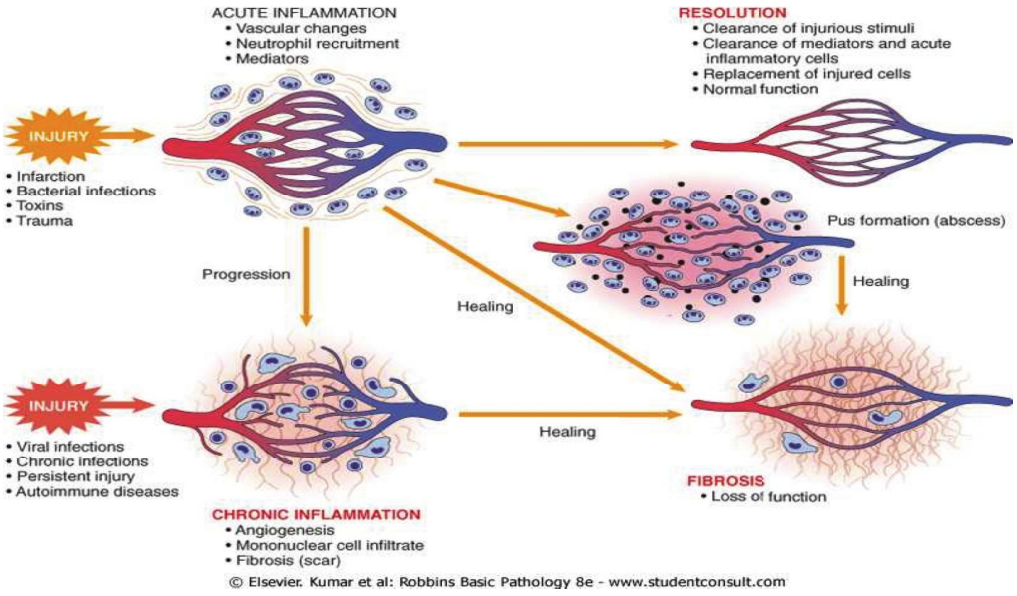



**Fig. 1.1** *Outcomes of acute inflammation.* Depending on the inflammatory condition and the nature and persistency of the noxious stimuli, acute inflammation can evolve into chronic inflammation, undergo organization with the formation of a fibrous scar or resolve completely, restoring tissue functions.

### *1.2. Chronic inflammation*

The immune mechanisms that are meant to get rid of pathogens, should ideally cease upon elimination of the stimulus without further consequences. However the actual result of acute inflammation depends on many factors, including the nature of the pathogen, the tissue involved and its ability to regenerate damaged cells and the ability of the host to react to such pathogens. Indeed, certain microbes that induce delayed host defenses (e.g.: fungi, helmynthes and parasites, mycobacteria and certain viruses) (**Hotez et al., 2008; Saunders and Britton, 2007; Di Rosa and Barnaba, 1998; Matsuzaki et al., 2007**), the presence of exogenous or endogenous toxic agents that continuously feed the immune response due to constant tissue damage (e.g.: asbestos or atheromas during atherosclerosis) (**Pascolo et al., 2015; Rocha and Libby, 2009**) or aberrant immune reactions to exogenous or endogenous molecules, can lead to chronic inflammatory responses, (termed also hystophlogosis). Chronic inflammation can either evolve from acute conditions or can generate from certain immune responses characterized by chronic features from the onset.

Hystophlogosis is rather different from angiophlogosis, in that it is characterized by massive recruitment of mononuclear cells (monocytes/macrophages and lymphocytes) on the inflamed site (**Shi and Pamer, 2011; Pène et al., 2008**). Within 48 hours from the arising of the inflammatory stimulus, monocytes invade the tissue, following the same cytokine and chemochine gradient that drove the influx of neutrophils in the first acute reaction. Monocytes that reach the target site come in contact with a torrent of different factors that drive their differentiation into macrophages, including Toll-like receptor ligands coming from the pathogen and damaged cells, as well as cytokines (e.g. IFN-γ) mainly produced

by the T lymphocytes and NK cells (**Martinez and Gordon, 2014**). Once activated by these mediators, macrophages produce ROS, RNS, cytokines, proteases and growth factors that, if on one hand represent the arsenal required for pathogen destruction, are also the main mediators of the tissue damage occurring during the chronic inflammatory response (**Murray and Wynn, 2011**). During this process, injured cells feed the recruitment of other monocytes and thus, the persistence of the phlogistic condition. Alongside monocytes/macrophages, lymphocytes represent the other prominent population of immune cells invading tissues during chronic inflammation. These two cells work in a reciprocal way: macrophages release cytokines and chemokines that recruit leukocytes and drive the differentiation of T cells, while T cells produce several macrophage-activating cytokines which are pivotal in the induction of inflammation clearance of debris and tissue reparation (**Martinez and Gordon, 2014**). Pathogen removal is also achieved by plasma cells, the activated and mature form of B cells, that produce antibodies in order to facilitate the inactivation of microbes as well as their uptake from phagocytes.

### *1.3. Resolution phase of inflammation*

For a long time, resolution of inflammation has been thought to be a passive process, simply consisting in the dilution and clearance of the inflammatory factors occurring after the elimination of the pathogenetic source. However in the past two decades it has become increasingly evident that resolution is a rather active and well-orchestrated process that, similarly to inflammation, possesses its own cellular and molecular events and its own lipidomic fingerprint (**Serhan, 2014**). Indeed, the studies conducted on animal models of self-resolving inflammation has led to the

discovery of a novel class of molecules with pro-resolving properties that act as master regulator of the resolution phase of inflammation. These lipids, that altogether have been termed as pro-resolving lipid mediators (SPMs), are synthesized from ω-3 and ω-6 essential polyunsaturated fatty acids that the body takes from dietary intake and, consist of four main families of molecules: lipoxins, resolvins, protectins and maresins (**Serhan and Chiang, 2008**). Their metabolism and role in immune responses will be discussed in detail later. Upon induction of resolution, the same immune cells that triggered inflammation in the first place (i.e. PMNs and macrophages, but also platelets) undergo a biochemical paradigm shift, commonly referred to as "lipid mediator class switch", during which they stop producing prostaglandins and leukotriene $B_4$ and start synthesizing high levels of lipoxins (**Basil and Levy, 2016**). The large amounts of prostaglandins that the inflammatory milieu is enriched of during the phases of inflammation preceding resolution represent quite likely the signal that triggers the biosynthesis of new resolution mediators, inasmuch as pharmacologic inhibition of cyclooxigenase 2 (COX2) significantly delays resolution (**Schwab et al., 2007; Chan and Moore, 2010**). After the synthesis of lipoxins, the inflammatory exudate rapidly enriches in EPA- and DHA-derived SPMs (i.e. E-series and D-series resolvins, protectins and maresins); as these lipid mediators orchestrate resolution, the first noticeable cellular event occurring as inflammation start fading, is the cessation of neutrophil influx (**Maderna and Godson, 2009**); SPMs are also able to modulate macrophage functions in order to skew the M1/M2 balance towards M2 phenotype, thus enhancing macrophage-mediated clean up of debris and apoptotic cells, a process often referred to as efferocytosis (**Serhan, 2014**). The complete elimination of the phlogistic sources driven by SPMs clears the way to the processes that lead to functional recovery and tissue healing; nonetheless, to date, a number of studies

have also suggested that these lipids can also directly promote tissue regeneration and wound healing (**Wang et al., 2012; Tantg et al., 2013**). The main events that occur during resolution are summarized in **Fig. 1.2.**

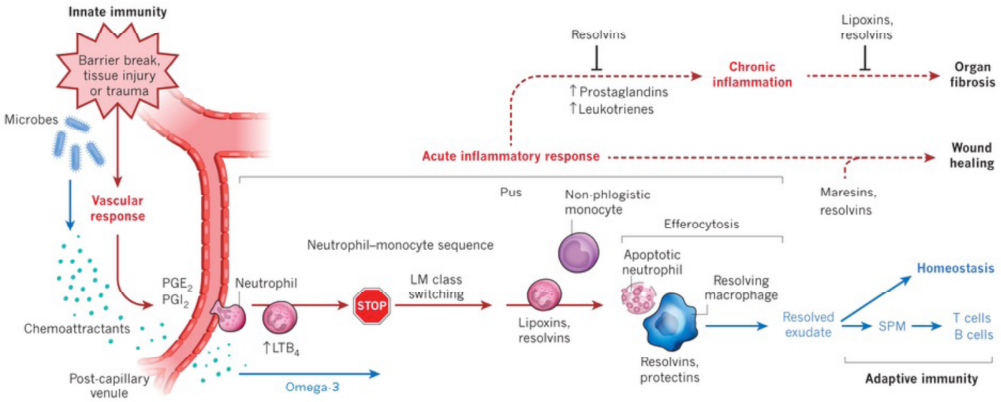



**Fig. 1.2** *Resolution phases of inflammation.* Innate immune cells and epithelium stop producing prostanoids that fuel acute inflammation and start producing SPMs that promote the resolution of the inflammatory processes and the return to tissue homeostasis. Furthermore, by acting on innate immunity, they promote wound healing while inducing debris clearance, thus hindering fibrotic processes. PG= prostaglandins; LTB4= Leukotriene B4; SPMs= Specialized pro-resolving lipid mediator.

# 2. Immune system

## *2.1. Innate immunity*

Innate immune system represents the first line of defense against infections and injuries and one of the most evolutionary conserved biological functions in all eukaryota, its molecular components sharing many functional and structural features even between plants and animals (**Ronald and Beutler, 2010**). Innate immunity serves critical functions in host defense such as the initial recognition, control and elimination of invading microbes and plays a crucial role in continuously searching for damage or infection signals in order to trigger the elimination of pathogens, dead cells and debris and initiating repair processes. Furthermore, after its first encounter with noxious microorganisms, the innate immune system alerts and instructs adaptive immunity, which, in turn works by enhancing the natural ability of innate immune system to eliminate microbes (**Abbas, et al., 2014**).

The innate immune cells constantly sense the tissue they inhabit, and can detect specific molecular structures that are associated with infection (pathogen-associated molecular patterns, PAMPs), cell damage (damage-associated molecular patterns, DAMPs) that are released by damaged (but non apoptotic) cells, and resolution of inflammation (resolution-associated molecular patterns, RAMPs) (**Akira et al., 2006; Schaefer, 2014; Shield et al., 2011**). Most common PAMPs are represented by microbial products that are vital for the invading microorganism such as viral double-stranded DNA, CpG DNA sequences that are typical of many bacteria, glycoprotein components of bacteria cell wall (e.g. lipopolysaccharide or lipoteichoic acid) or mannose rich oligosaccharides. In order to

efficiently recognize these signals, innate immune cells employ a vast array of pattern-recognition receptors (PRRs) that endow them with the ability to recognize PAMPs and DAMPs; upon binding a particular molecular pattern, PRRs transduce signals that promote the production of pro-inflammatory mediators or trigger anti-microbial reaction that, in turn, initiate and modulate effector responses (**Akira et al., 2006**). The arsenal of PRRs that macrophages and dendritic cells (DC) can count on, include membrane toll-like receptors (TLR), that recognize extracellular and intracellular PAMPs and DAMPs and induce APC activation, intracellular NOD-like receptors (NLR) that modulate the activation and release of pro-inflammatory cytokines, such as IL-1β and IL-18, and RIG-like receptors (RLR) that work as cytoplasmic viral RNA sensors that trigger the release of type-I interferons (**O'Neill et al., 2013; Hornung and Latz, 2010; Loo and Gale, 2011**). The two main processes by which innate immune system operate in the defense of the host are the inflammation and the anti-viral responses which are carried out by its cellular components, represented by the epithelial barriers, by the phagocyte cells system, and the natural killer cells (NK). Epithelial barriers perform their task at multiple levels: on one hand, the stratified layers of cells they are made of, physically isolate tissues from the external environment and the microbes that swarm in it; on the other hand, epithelial cells are also able to produce bactericide peptides (e.g. defensins and cathelicidins) (**Selsted and Ouellette, 2005**). Furthermore, epithelia are also inhabited by many immune cells that participate to maintain tissue homeostasis, such as macrophage-like Langerhans cells and γδ T-cells, the latter being intraepithelial lymphocytes which are able to recognize a limited number of microbial antigens (**Hayday and Tigelaar; 2003**). The phagocytic system, especially neutrophils and macrophages, represent the vanguard of the immune system against invading microorganisms that have succeed in violating

the epithelial barriers (**Dale et al., 2008**). These phagocytic cells are able to internalize microbes and kill them, a function in which neutrophils are rather efficient; on the other hand, macrophages represent highly plastic cells that are entrusted with a rather various list of tasks, such as fire-starting acute inflammation upon internalization of pathogens by releasing pro-inflammatory cytokines and regulating tissue repair after infections or insults (**Kolaczkowska and Kubes, 2013; Martinez and Gordon, 2014**). Dendritic cells (DCs), another kind of phagocyte, represent arguably the most versatile and efficient sensors of PAMPs and DAMPS in the entire body; upon internalizing microbes and digesting them, they migrate to the lymph nodes where they load pathogen-associated protein antigens on their membrane and present them to T cells, thus triggering adaptive immune responses (**Merad et al., 2013**). This process is called antigen presentation and will be discussed later. Cells such as macrophages and DCs that can process antigens and present them to the adaptive immune cells are commonly referred to as professional antigen-presenting cells (APCs). Finally, NK cells are lymphocytes that exert important roles such as the elimination of transformed cells or cells infected by viruses and enhance the ability of macrophages to eliminate phagocytized microbes (**Vivier et al., 2008**).

### *2.2. Adaptive immunity*

Adaptive immunity represents the highly specialized branch of immune system that works by either improving the natural ability of innate immune system of getting rid of invading microbes or by directly eliminating infected cells that can't be wiped out by means of other mecha-

nisms. Adaptive immune system makes use of three strategies to fight pathogens:

- T cell-enhanced elimination of microbes that have been ingested by phagocytes.
- Secretion of antibodies that recognize and bind to specific determinants on the surface of microbes, thus hindering their ability to infect cells and flagging them to be ingested by phagocytes.
- Direct elimination of cells that have been infected by pathogens that are inaccessible to antibody or phagocytosis, operated by cytotoxic T lymphocytes (CTL).

Adaptive immunity acts through discrete steps, each one corresponding to a different event associated with lymphocytes activation. The starting event is represented by the first contact in lymph nodes between APCs, mostly DCs, and T lymphocytes. After ingesting microbes, DCs digest them and process the resulting peptide antigens, exposing them on their own cell membrane bound to MHC molecules. Naïve T lymphocytes express a specific surface T cell receptor (TCR) that is able to bind a particular MHC-peptide complex (**Merad et al., 2013**). On the other hand, intact microbes, or free microbial antigens that enter lymph nodes without being processed by APCs are recognized by B lymphocytes that, instead, express B cell receptors (BCR) (**Cooper, 2015**). Prior to this event, each and every naïve T or B cell that circulates between the bloodstream and lymph nodes can recognize and bind a specific antigen by means of its unique TCR or BCR. These antigen-specific lymphocyte clones exist before and independently of the antigen exposure and develop during cell maturation in a process meant to maximize the amounts of antigens that adaptive immune system can recognize (**Jenkins et al., 2010**). Upon recognition, the lymphocyte specific clone that was able to bind a certain MHC-antigen

complex, activate and undergo clonal expansion, with naïve T cells turning into effector T cells, that enhance innate immune cells ability of getting rid of pathogens, and naïve B cells converting into plasma cells that release antibodies (**Jenkins et al., 2010**). Activated lymphocytes drive the adaptive immune responses and, after the elimination of microbes and antigens they are deleted through apoptosis in order to restore homeostasis. The initial lymphocyte activation also generates a pool of long-living cells that survive the mass death of activated T and B cells that occurs in the aftermath of infections. These memory lymphocytes represent the components of the immunological memory that allows a prompt reaction upon reinfection and are also the basis of immunization after vaccines.

### *2.2.1. T cells and cell-mediated immunity*

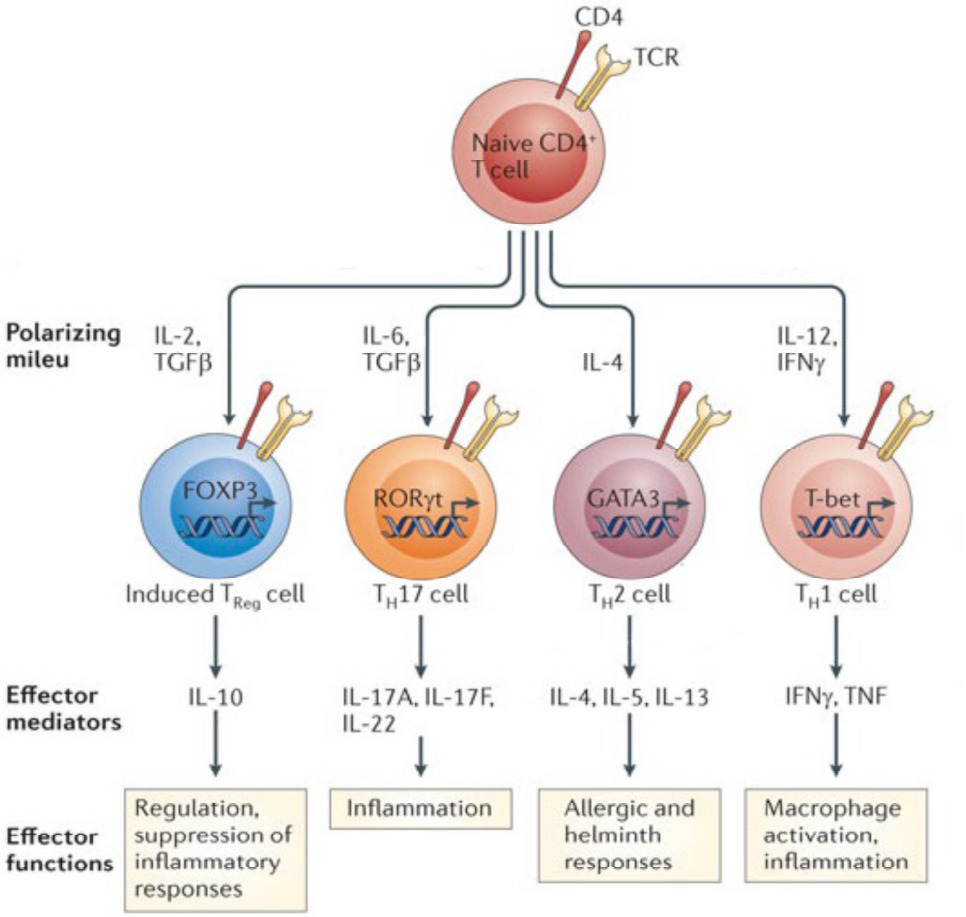
T lymphocytes historically take their name from the thymus, which is their final destination of their maturation process (**Takahama, 2006**). They drive the cell-mediate branch of adaptive immunity in that they boost the native ability of innate immune system of eliminating microbes, viruses, helminths and fungi; they are also able to drive regulative mechanisms in order to self-limit inflammatory processes and avoid aberrant phlogistic states that would lead to severe pathologies (**Patsoukis et al., 2015; Vignali et al., 2008**). Nonetheless, T cells are also believed to be responsible for a number of pathophysiological inflammatory conditions such as autoimmunity, asthma, allergy and tumor immunity (**Zhu et al., 2010**). During adaptive immunity-driven responses, T lymphocytes streaming through lymph nodes interacti with the DCs that migrated there after microbial phagocytosis: T cells with the right specificity, make contact with APCs expressing MHC-peptide complexes that can be bound

by their TCR, and undergo activation, thus acquiring powerful functional capabilities. However, in order for this process to induce a stimulation, TCR-MHC binding must be coupled to the concomitant presence of specific co-stimulatory signals on the APC surface, represented by B7-1 (CD80) and B7-2 (CD86) (**Kapsenberg, 2003; Chen, 2004**): these proteins are highly expressed by activated macrophages and DCs and, during T cell activation, bind to a stimulatory receptor expressed on T cells surface, which is CD28. After the formation of TCR-MHC synapsis, lymphocyte functions are regulated by the time-dependent differential expression of several proteins (**Von Adrian and Mackay, 2000**): lymphocytes upregulate CD69 in order to be retained in the lymph nodes. Subsequently, the expression of CD25 (IL-2R) drives clonal expansion, while the expression of CD40L mediates T cell effector functions on innate immune cells. The immune regulation required after the ceasing of the phlogistic stimulus is later achieved through the expression and activetion of inhibitory receptors such as CTLA-4 (cytotoxic t lymphocyte antigen 4) and PD-1 (programmed death 1), two other members of the CD28 family that, contrarily to CD28, transduce pro-apoptotic and suppressive signals upon binding APC-expressed B-7 proteins and play, thus, a crucial role in immune regulation and tolerogenic processes. Of course, even though both CTLA-4 and PD-1, along with CD28, are grouped in the same class of receptors, their role in the adaptive immune response varies dramatically, and the outcome of the contact between T cells and APCs strictly depends on the balance between the activatory and inhibitory ligands of B7 proteins on lymphocyte surface (**Chen, 2004; Greenwald et al., 2005**). For this process to work with the proper flexibility that the immune system demands in order to cope with such a huge variety of pathogens and situations, a heterogeneous group of role-specific lymphocyte phenotypes must be generated from a common precursor. This T cell precursor is represented

by naïve T cells, which are a plastic population of lymphoid cells that, after thymic selection, recirculate the bloodstream and lymphatic torrent, waiting to make contact for the first time with macrophage- or DC-processed antigens, their destiny bound to the signals they will receive upon engaging an APC. They are commonly grouped in two main classes, depending on the exclusive expression of the surface glycoproteins $CD4^+$ and $CD8^+$ T cells; the recognition of APC-presented antigens along with the presence of polarizing cytokines, mostly produced by innate immune cells, lead to the differentiation of $CD4^+$ and $CD8^+$ naïve T lymphocytes into effector T helper cells and cytotoxic T cells (CTL) respectively.

#### *2.2.1.1. $CD4^+$ T cells*

T lymphocytes are defined by the expression of high amounts of surface CD4 glycoprotein, which works as a TCR co-receptor during antigen recognition. Depending on the type of polarizing cytokines they receive by innate immune cells during the early stages of immune response, $CD4^+$naïve T cells can differentiate into several classes of T-helper ($T_H$) cells, the most studied of which are $T_H1$, $T_H2$ and $T_H17$, or T regulatory cells (Treg) (**Swain et al., 2012**). Each of these cell subset is specifically induced by cytokines produced by innate immunity in response to those microbes or noxious stimuli that these CD4 cells are programmed to respond to; furthermore polarized T cells often produces cytokines that further self-promote polarization, hindering other commitment pathways. Interestingly, $T_H$ cells exhibit remarkable plasticity, being able to cross-differentiate between different subsets (**Zhou and Paul, 2010; Gagliani et al., 2015**), although this feature seems to depend on the differentiation state of the lymphocytes (**Murphy et al., 1996**). **Fig. 2.1** illustrates the




pathways undertaken by naive T cells in order to differentiate into effector T cells.

**Fig. 2.1** *Variability of T cell response.* Activated APCs release a set of specific cytokines according to the pathogen they encountered in the first place, and this drives naïve T cells differentiation towards several types of effector phenotypes. APC= Antigen-presenting cell; TCR= T-cell receptor; $T_H$= T helper; Treg; T regulatory cell; IL=Interleukin; TGF-β= Transforming growth factor-β; IFN-γ= Interferon-γ; TNF= Tumour necrosis factor; Foxp3= Forkhead box p3; T-bet= T-box expressed in T cells; RORγt= RAR-related orphan receptor γt; GATA3= Trans-acting T-cell-specific transcription factor

#### *2.2.1.1.1. $T_H1$ lymphocytes*

$T_H1$ lymphocytes are effector cells that play a pivotal role in the host response against intracellular microbes. They were firstly identified alongside with $T_H2$ cells and until the discovery of the other $T_H$ populations, they represented the only two known sides of the T cell heterogeneity paradigm. Their development is driven by IL-12 and IFN-γ produced by APCs and NK cells respectively, the former representing the most potent $T_H1$-polarizing agent. Upon engaging their receptors on $CD4^+$ naïve T cells surface, these cytokines promote the activation of early transcription factors such as the signal transducer and activator of transcription 1 and 3 (STAT1 and STAT3), which, in turn, activates the signature transcription factor of $T_H1$ cells, i.e. T-bet. This last transcription factor operates on the nuclear transcriptional machinery and triggers the production of IFN-γ, the $T_H1$ signature cytokine, which is responsible for macrophage activation and elimination of intracellular microbes. $T_H1$ cells are also good IL-2 producers and can also release other pro-inflammatory cytokines such as TNF-α and lymphotoxin (formerly known as TNF-β).

#### *2.2.1.1.2. $T_H2$ lymphocytes*

$T_H2$ cells are key regulators of a number of different immune processes: they develop from $CD4^+$ naïve T cells in response to TCR-MHC ligation and IL-4 produced by mast cells, macrophages and, possibly, eosinophils during helminth infections. Furthermore they are also involved in some allergic and asthmatic conditions (**Zhu, 2015**). $T_H2$ cells are known to produce a wide array of different cytokines such as IL-4, IL-5 and IL-13, which are usually considered their signature cytokines, but are also able to release other factors such as IL-9, thus being able to elicit a wide spec-

trum of effects: IL-4 triggers B cells class switching towards IgE which, in turn, drives eosinophils and mast cells degranulation, while IL-5 and IL-9 recruit eosinophils and mast cells in lungs and other tissues; furthermore, $T_H2$ cytokines can act in concert during airway hyper-responsiveness in that they drive mucus production and act on smooth muscle cells (**Paul and Zhu, 2010**). On the other hand, IL-4 can also exert anti-inflammatory effects by acting on macrophages, triggering M2 alternative activation, which, in turn, leads to the debris clearance, tissue regeneration and the release of other anti-inflammatory cytokines (**Zhu, 2015; Martinez and Gordon, 2014**). The master regulator of $T_H2$ differentiation upon IL-4 ligation is represented by the transcription factor GATA-3 that acts in concert with STAT6 to commit $CD4^+$ naïve T cells towards $T_H2$ phenotype (**Ho et al., 2009**). Of note, since $T_H2$ cells are good producers of IL-4, after initial stimulation they are able further drive their own development by sustaining a positive feedback.

*2.2.1.1.3. $T_H17$ lymphocytes*

The initial adaptive immunity paradigm, which was conceived 25 years ago as being characterized by the $T_H1/T_H2$ binomial (**Mossman and Coffman, 1989**), was later expanded by the discovery of a third subset of T cells that produce IL-17 (hence the name $T_H17$) and elicit an array of effector functions that are distinct from $T_H1$ and $T_H2$ (**Korn et al., 2009**). The main function of $T_H17$ cells appears to be the eradication of pathogens that are not efficiently eliminated by either $T_H1$ or $T_H2$ responses. Nonetheless, $T_H17$ effector function has been correlated with a number of human and experimental pathological autoimmune conditions (**Dong, 2014; Hernandez et al., 2015**). During antigen presentation, several

bacteria and fungi recognized by DCs, induce the production of factors such as IL-1β, IL-6 and IL-23 (**McGeachy and Cua, 2008**) which work in concert with TGF-β, a cytokine which is produced by many cell types, to induce $T_H17$ development; interesting enough, even though this last cytokine has been classically associated with anti-inflammatory functions, its presence seems to be crucial during $T_H17$ development, a T cell subset mostly endowed with pro-inflammatory properties (**Volpe et al, 2008; Manel et al., 2008**); TGF-β has also been suggested to work indirectly, by inhibiting $T_H1/T_H2$ lineage development, thus preparing $CD4^+$ naïve T cells to react to IL-1β and IL-6. The role of other cytokines such as IL-23 or IL-21 in the $T_H17$ development seems to be working alongside with TGF-β, IL-1β and IL-6 to unlock the expression of key transcription factors such as STAT3 and RORγt that in turn, trigger the synthesis of IL-17 (**Manel et al., 2008**). To date, $T_H17$ *in vivo* effector functions are still uncertain and since they are not the only source of the cytokines they produce, which also include IL-17F, IL-21 and IL-22 (**Yeste et al., 2014; Jin and Dong, 2013**), they might work in a tissue-dependent fashion, and act in concert with other $T_H$ subsets.

*2.2.1.1.4. Regulatory T lymphocytes*

Regulatory T cells (Treg) were firstly identified in 1995 as a new subset of $CD4^+$ T lymphocytes expressing high levels of IL-2 receptor (CD25) and highly endowed with suppressor as well as autoimmunity-preventing activity (**Sakaguhi et al., 1995; Fehervari and Sakaguchi, 2004**). This subset of T lymphocytes is also characterized by the expression of high levels of the forkhead box p3 (Foxp3) transcription factor, which appears to be crucial, although not sufficient, in the maintaining of Treg suppres-

sor activity (**Wan and Flavell, 2007; Campbell and Koch, 2011**). Two subsets of Treg, which might work in slightly different ways and contexts, have been identified thus far: naturally occurring Treg cells (nTreg), originate in the thymus and require high affinity TCR-MHC binding, whereas induced Treg cells (iTreg) differentiate in the periphery, possibly after exposition to non self antigens and specific cytokines such as TGF-β (**Hsieh et al., 2006**). Both nTreg and iTreg seem to rely, on the secretion of inhibitory cytokines such as IL-10 and TGF-β for their suppressive abilities (**Vignali et al., 2008; Hawrylowicz and O'Garra, 2005; Joetham et al., 2007**) although a number of other mechanisms have been identified. Indeed, Treg can induce cytolysis in target cells by producing granzymes and perforins (**Grossman et al, 2004**). Interestingly, many authors theorized that they can induce metabolic disruption, by leeching IL-2 from contiguous cells using the high amounts of CD25 they express (**Pandiyan et al, 2007**). Moreover Treg cells can also work through the concerted action of CD39 and CD73, two membrane ectonucleotidases that interfere with triphosphate nucleoside metabolism, the former catalyzing the hydrolysis of their β phosphates, thus depleting the milieu of ATP while producing AMP, and the latter further dephosphorylating AMP to adenosine which exerts inhibitory action on T cells (**Borsellino et al., 2007**; **Deaglio et al., 2007; Zarek et al., 2008**; **Bopp et al., 2007**). They can also act by upregulating CTLA-4, which signals immunosuppressive actions on activated or autoreactive T cells (**Read et al., 2007**)

#### *2.2.1.2. Cytotoxic CD8 T cells*

Cytotoxic T cells (CTLs) have been historically defined by the expression of high surface levels of CD8 glycoprotein protein on their surface, which acts as a TCR-associated co-receptor. CTLs activation, as all T cells, requires the contact between their TCR and the peptide-MHC complex expressed on APCs but, depending on the kind of infection these cells are called to face, they may or may not require the intervention of $CD4^+$ helper T cells. If antigen presentation process is efficient, APC can induce alone $CD8^+$ T lymphocytes activation and differentiation; when this doesn't happen, $CD4^+$ lymphocytes may help in this process either enhancing APC-dependent CTL action in a CD40/CD40L-dependent manner, or by secreting $CD8^+$-boosting cytokines (**Bevan, 2004; Wong and Pamer, 2003**). Upon activation, $CD8^+$ T lymphocytes undergo clonal expansion and acquire the ability to express special proteins such as perforins and granzymes, in the form of cytoplasmic granules, which endow CTLs with the ability to kill target cells. In order to achieve this goal, the TCR on CTLs and its CD8 co-receptor, alongside with other secondary signals (e.g. LFA-1 integrin), must make contact with the target cell's peptide-MHC complex. The list of effector action elicited by CD8 also includes the secretion of a wide variety of pro-inflammatory cytokines and chemoattractants such as IFN-γ, TNF-α, RANTES and MIP-1α (**Kristensen et al., 2004**) After activation, CTLs also express Fas ligand (FasL) that induces programmed cell death upon binding is Fas counterpart in the cells that must be eliminated (**Russel and Ley, 2002**).

### *2.2.2. B cells and humoral immune response*

Humoral immunity functions are elicited through the antibodies that are produced by cells of the B lymphocytes lineage. These cells can work in two different ways: on one hand they can directly engage microbial proteins by means of their BCR, a membrane-tethered immunoglobulin that can be endowed with the ability to recognize a great deal of antigens; on the other hand, B cells are APCs and, not differently from macrophages or DCs, can present antigens to T cells that, in turn, activate the B cells themselves by releasing cytokines. When the unique BCR expressed on a B cell surface has sufficient affinity to bind a free antigen, that lymphocyte clone activates and undergo clonal expansion, thus maturating into antibody-producing plasma cells.

## 3. Specialized pro-resolving lipid mediators

Specialized pro-resolving lipid mediators (SPMs) are a class of recently elucidated bioactive lipid mediators that have received a huge deal of attention due to their remarkable list of immunomodulatory effects (**Serhan CN, 2014**). The discovery of molecules with pro-resolving activity, which are biosynthesized from ω-3 and ω-6 essential polyunsaturated fatty acids (PUFA), has subverted the over simplistic theory that saw in resolution of inflammation a passive process, only consisting of the cessation of the inflammatory stimuli and the dilution of chemoattractants, and has demonstrated that it is instead a finely orchestrated mechanism which, just like inflammation, possessed his own specific mechanisms and lipidomic fingerprint.

### *3.1. Essential polyunsaturated fatty acids*

Polyunsaturated fatty acids (PUFAs) represent a rather vast class of lipids, containing one or more double bond in their carbon skeleton, that play important roles in a huge number of key biological processes, such as energy supply, inflammatory signaling and structure and function of cell membranes (**Castro et al., 2016**). These molecules are synthesized through the action of two classes of enzymes, namely fatty acyl desaturases (Fads) and elongation of very-long fatty acid proteins (Elovl) (**Schmitz and Ecker, 2008**) that work in concert to extend the aliphatic chain of lipid precursors while sequentially adding up double bonds. Among PUFAs, lipids such as arachidonic acid (AA, 20:4n-6) eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoid acid (DHA, 22:6n-3) are three essential lipids that play pivotal roles in inflammation and resolution. Indeed, AA is arguably the most important ω-6 PUFA known by far,

as it is the precursor of all the autacoids that trigger and govern inflammatory processes, while EPA and DHA are ω-3 polyunsaturated lipids are responsible for the generation of SPMs. Both these two classes of PUFA are substrates to cyclooxygenases (COX) and lypooxygenases (LOX), which are thus involved in the formation of either pro- or anti-inflammatory mediators. Since human enzymes can only synthesize lipids up to palmitic acid (PA, 16:0), its structure impeding them to add up double bonds further than the 12$^{th}$ position of the carbon backbone, direct generation of linoleic acid (LA) (18:2n-6) or α-linolenic acid (ALA) (18:3n-3), (i.e. the precursors of AA and EPA/DHA respectively) from endogenous compounds is impossible and makes dietary intake as the only source for ω-3 and ω-6 PUFAs; besides, even though Fads and Elovls can synthesize these molecules, they still require dietary intake of LA and ALA. Most common food source of these essential lipids are represented by seed, nuts or vegetable oils (ALA, LA and AA) and fishes (EPA and DHA); diet-derived AL and ALA undergo sequential elongation and desaturation and generate AA, EPA and DHA which, along with the preformed amounts directly assimilated from food, are funneled through the biosynthetic pathways that give rise to autacoids and SPMs. **Fig 3.1** summarize the generation of bioactive PUFAs from essential lipid precursors.

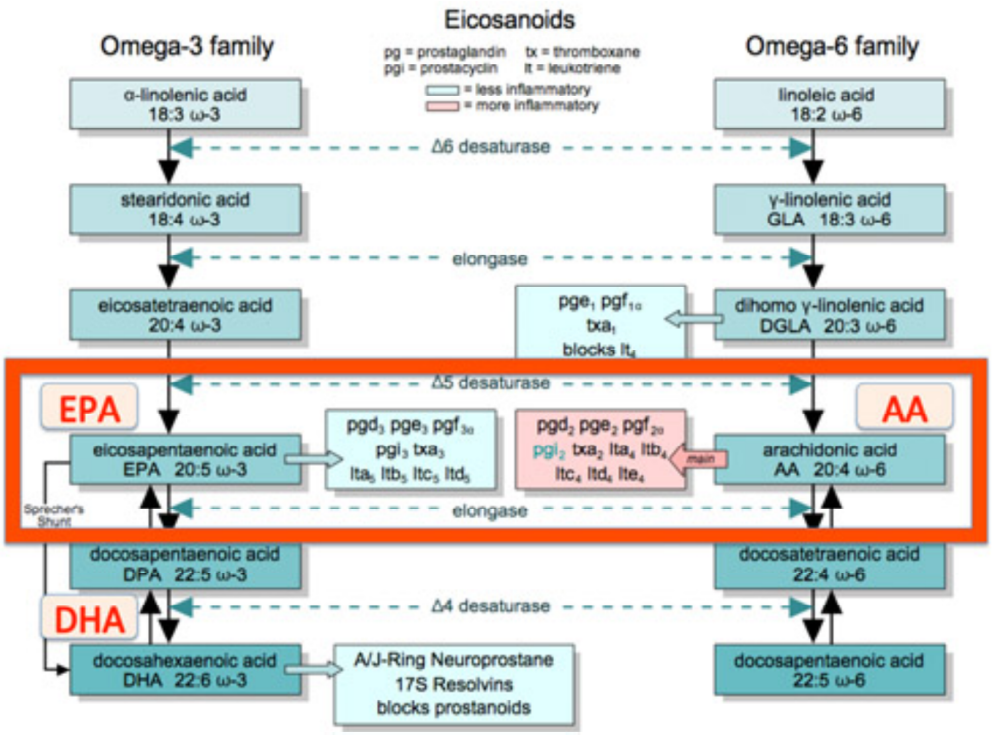



**Fig. 3.1** *Biosynthesis of the ω-3 and ω-6 PUFAs that serves as substrates for inflammation and resolution.* EPA, DHA and AA can either be directly assimilated through diet or synthesized by means of sequential Fads- and Elovl-mediated desaturation and elongation steps from their essential precursors, i.e. food-derived ALA and LA. Eventually, EPA/DHA and AA act as precursors for the molecules regulate inflammatory processes.

### *3.2. Biosynthesis of SPMs*

The lipid mediators class switch that occurs upon induction of resolution, initiates the biosynthesis of resolution-phase mediators (**Levy et al., 2001**). All SPMs are enzymatically produced by essential ω-3 and ω-6 PUFAs, such as AA, EPA and DHA, in a LOX-dependent manner and they start and drive the resolution phase (**Basil and Levy, 2016**). To date, six classes of SPMs have been identified: lipoxins, E-series and D-series resolvins, Maresins, protectins and the newly identified 13-series resolvins

(which seem to regulate resolution by appearing very early at the site of inflammation). Interestingly, aspirin-induced acetylation of cycloxigenases (COX), though inhibiting the production of eicosanoids, leads to the production of other byproducts that act as LOX substrates for the synthesis of other SPMs epimers, often referred to as aspirin-triggered SPMs (AT-SPMs), which share many, if not all, the pro-resolving properties with their classic SPMs cognate molecules. **Fig 3.2** shows the pathways through which SPMs are generated.

### *3.2.1. AA-derived SPMs: lipoxins*

Lipoxins (LX) are derived from AA, represent the lead class of SPMs and the first family of resolution-phase lipid mediators to be produced during the initiation of resolution (**Basil and Levy, 2016**). As an ω-6-derived SPMs, LX main source is represented by eosinophils, macrophages DCs and epithelial cells, which produce them through two main biosynthetic pathways: the first pathway involves the action of leukocytes and platelets, expressing respectively 5-LOX and 12-LOX, while the second one requires the catalytic activity of epithelial cell-, monocytes- and eosinophil-derived 15-LOX and leukocyte-derived 5-LOX (**Serhan and sheppard, 1990; Serhan et al., 2007**). Furthermore, aspirin-mediated covalent inactivation of COX2 leads to the production of 15-(R)-hydroxyeicosatetraenoic acid (15-(R)-HETE) which, as the epimer of the natural precursor of LXs, 15-HETE, acts as substrate for the 5-LOX-catalysed synthesis of AT-LXs (**Clària and Serhan, 1995**). Interestingly, since at the site of inflammation exists an inverse relationship between the levels of LX and leukotrienes (**Fredman et al., 2012; Fredman et al., 2014; Chiang et al., 2012**) and that they share AA as a common precur-

sor, it is possible that during the entire inflammatory process, cell membrane constantly provides a quote of arachidonic acid that is used in the first phase of inflammation to produce the prostanoids that fuel the acute phlogistic processes, and later on, during the lipid mediators class switch that opens the resolution phase, acts as a substrate for LX synthesis.

### *3.2.2. EPA-derived SPMs: E-series resolvins*

Biosynthesis of E-series resolvins (RvE) is initiated by the insertion of molecular oxygen in their essential fatty acid precursor, EPA. This process is usually performed by hypoxic endothelial cells while the enzymatic machinery that leads to the production of E-series resolvins can be either aspirin-dependent or independent (**Arita et al., 2005**). Indeed the synthesis of 18R-hydroperoxteicosapentaenoic acid (18(R)-HpEPE), the precursor of EPA-derived SPMs, can be catalyzed by acetylated COX2 or by cytochrome p450 (**Serhan, 2014**). Very interestingly, both mammal and bacterial cytochrome p450 can perform this step. (**Serhan et al., 2000**) Eventually, 18(R)-HpEPE is rapidly transformed into the final resolvin products, i.e. RvE1 and RvE2, through a multi-step pathway which involves the action of neutrophil-derived 5-LOX in the peripheral blood (**Arita et al., 2005; Pillai et al., 2011**). On the other hand, RvE3 is not generated through 5-LOX-catalyzed reactions, and is directly synthesized from 18(R)-HpEPE by the action of 12/15-LOX (**Isobe et al., 2012**)

### *3.2.3. DHA-derived SPMs*

DHA is precursor to the most heterogeneous group of SPMs known by far. This class includes D-resolvins, protectins and maresins. D-series resolvins synthesis starts off by 15-LOX-mediated conversion of DHA into 17(S)-hydroperoxy DHA (17(S)-HpDHA). From this point 17(S)-HpDHA fate depends on 5-LOX action and on which carbon is hydroxylated by its catalytic action: 7(S)-hydroperoxy-17(S)-HDHA is the precursor of RvD1, RvD2 and RvD5, while 4(S)-hydroperoxy-17(S)HDHA is used to generate RvD3, RvD4 and RvD6 **(Serhan, 2014).** Similarly to E-series resolvins, aspirin-acetylated COX2 can use the D-resolvin precursor, DHA, to generate 17(R)-HpDHA, which is then transformed to epimeric AT-D-resolvins in a 5-LOX-dependent manner (**Basil and Levy, 2016**). Alternatively, 17(S)-HpDHA can be converted to a 16,17-epoxy-docosatriene intermediate that in turn is converted to protectin D1. (**Mukherjee et al., 2004; Serhan et al., 2006**). DHA can be also channeled in other pathways, and be transformed into 14(S)-HpDHA by 12-LOX and, in turn, is converted to maresin 1 (**Serhan and Petasis, 2011**).

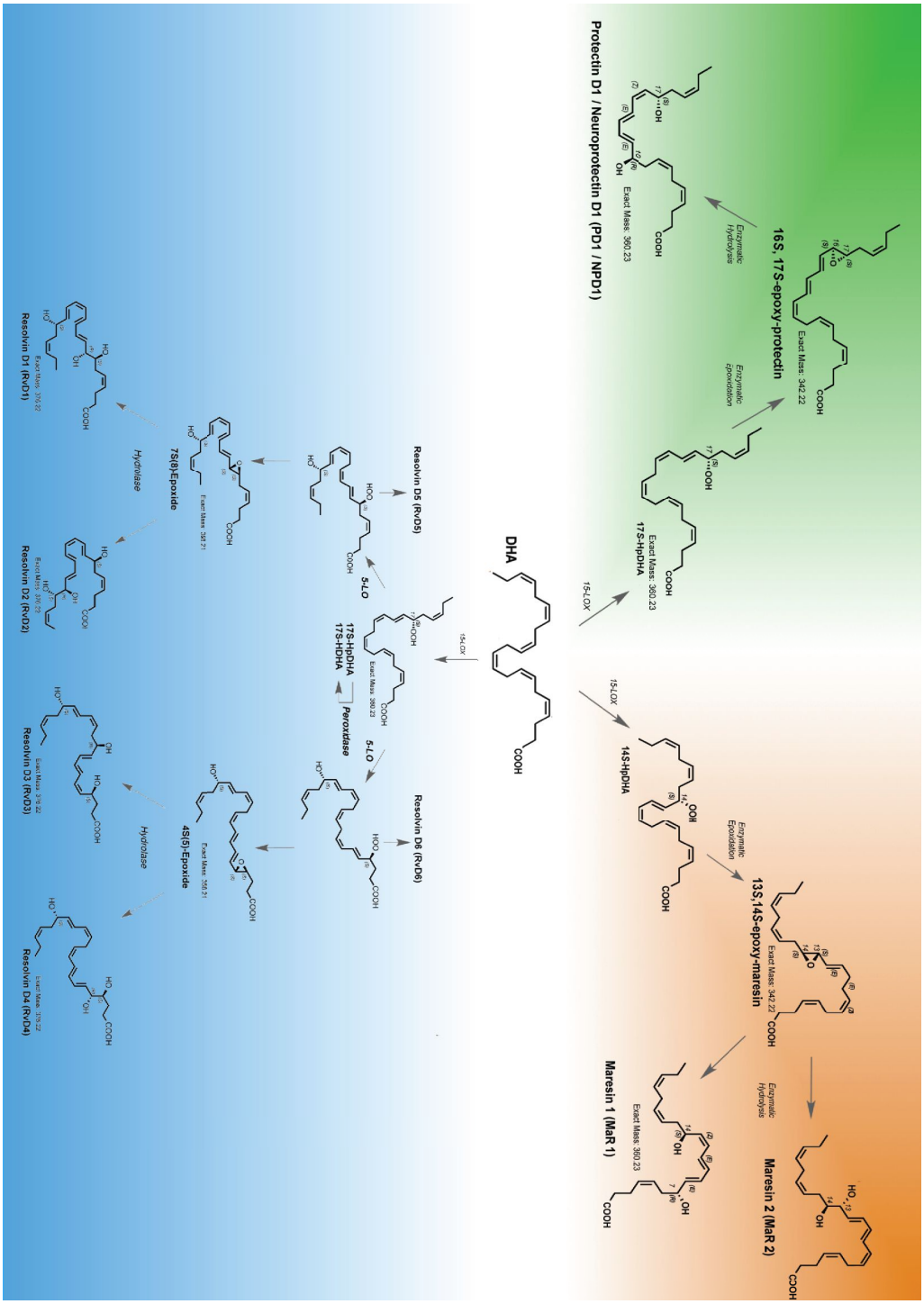



**Fig. 3.2** *Biosynthetic pathways of the main DHA-derived classes of SPMs: resolvins, maresins and protectins*

## 4. Immunomodulatory actions of SPMs

The mechanisms that lead to the regulation of the acute immune responses, along with the cells that trigger and drive them, evolved to face infections and ensure complete return to tissue function and homeostasis. Hence, the main role of SPMs main role is to limit and stop neutrophil accumulation, counter-balance the action of pro-inflammatory cytokines, while recruiting macrophages that promote the elimination of microorganisms and the removal of cellular debris and apoptotic cells (**Serhan, 2014; Maderna and Godson, 2009**). To date, we still possess an incomplete knowledge regarding the full array of receptors these molecules engage to exert their biological effects: the formyl peptide receptor 2 (FPR2), often referred to as ALX, and GPR32, are two G-coupled protein receptors (GPCR) that are able to bind PUFAs and PUFA-derivatives and have been identified as the mediators of lypoxin and RvD1 actions (**Chiang et al., 2006; Krishnamoorthy et al., 2012**) while GPR18, which is able to engage a great deal of endogenous lipid mediators, has only recently been established as the RvD2 receptor (**Chiang et al., 2015**). Other receptors known up to date to engage SPMs are the receptor R23 for chemerin (ChemR23) and the leukotriene B4 receptor BLT1, which are able to bind RvEs (**Arita et al., 2007; Ohira et al., 2009**), and the peroxisome proliferator-activated receptor (PPARγ), that has been proposed to mediate PD1 effects in a model of Alzheimer's disease (**Zhao et al., 2011**). To date, we still possess limited data on the specific receptor through which maresins work, even though in a recent paper they were reported to act on a model of neuropathic pain by blocking transient receptor potential V1 (TRPV1) currents (**Serhan et al., 2012**). Interestingly, the bulk of the knowledge gathered up until now, addressing the range of SPMs effects on immune system, focuses mostly on the effects on innate im-

mune cells; nonetheless, the many papers published so far regarding the effects of SPMs on pathophysiological functions that are regulated by the adaptive branch of immune system (**Basil and Levy, 2016**), limited as they may be, suggests that lymphocytes may be feasible targets for these lipids.

### *4.1. Immunomodulatory role of AA-derived SPMs*

LX main role is that of kick-starting resolution, interfering the function of neutrophils and other PMNs, while promoting the clearance of cellular debris and dead cells (**Basil and Levy, 2016**). $LXA_4$ and $LXB_4$ alongside with their aspirin-triggered epimers, inhibit neutrophil chemotaxis (**Papayianni et al., 1996; Bonnans et al., 2006**) and production of oxidative species (**Levy et al., 1999**); furthermore they inhibit macrophage-associated release of RNS, IFN-γ and IL-6 (**József et al., 2002; Schwab et al., 2007**) and DC-dependent production of IL-12 (**Aliberti et al., 2002**), while promoting monocyte chemotaxis (**Simoes and Fierro, 2005; Lee et al, 2010**), release of the anti-inflammatory cytokine IL-10 (**Schwab et al., 2007**) and inducing non-phlogistic phagocytosis of necrotic or apoptotic cells coming from acute inflammation (**Godson et al., 2000**; **Dalli and Serhan, 2012**). Conversely, scarce data exist on their role on lymphoid cells: they induce NK-dependent granulocyte apoptosis (**Barning et al., 2013**), modulate Breg-mediated responses (**Wang et al., 2015; Ramos et al., 2014**) and $T_H1$/ $T_H2$ balance (**Liao et al., 2013; Fahel et al., 2015**).

### *4.2. Immunomodulatory role of EPA-derived SPMs*

E-series resolvins are the only known SPMs that use EPA as a precursor and appear after the induction of resolution, alongside other SPMs. EPA-derived resolvins (e.g. RvE1, RvE2, RvE3) and, partly, their metabolites, display strong and pleiotropic anti-inflammatory and pro-resolving action by blunting superoxide production in neutrophils (**Hasturk et al., 2006**), while inhibiting leukotriene-dependent PMN chemotaxis (**Tjonahen et al., 2006; Isobe et al., 2013**), possibly by selectively inducing the expression of anti-adhesion molecules that interfere with trans-epithelial migration of granulocytes (**Campbell et al., 2007**). They also prevent the infiltration of other innate immune cells, such as eosinophils and mast cells, in experimental inflammatory models (**Kim et al., 2012**). Similarly to other SPMs, E-series resolvins also promote macrophage-mediated phagocytosis of apoptotic PMNs during acute inflammation (**Hong et al., 2008**; **El Kebir, PNAS 2012**), and reduce the release of pro-inflammatory cytokines such as IFN-γ and IL-6 (**Schwab et al., 2007**). They can also work by inhibiting DC migration (**Sawada et al., 2015**) and IL-12 release (**Arita et al., 2005**), and by inducing the development of DC phenotypes that trigger apoptosis of activated $CD4^+$ T cells (**Vassiliou et al., 2008**) as well as by engaging NK cells as mediators of their resolving actions in asthma models (**Haworth et al., 2011**). On the other hand, a number of authors have confirmed RvE ability to directly modulate adaptive immune cells: indeed, in mouse models of atopic dermatitis, RvE1 has been reported to reduce the recruitment of $CD4^+$ and $CD8^+$ cells, while limiting $CD4^+$-associated production of IFN-γ and IL-4 (**Kim et al., 2012**); furthermore, the ability of this lipid to ameliorate the clinical outcome and symptomatology in asthma as well as herpes simplex virus-induced stromal keratitis, has been demonstrated to reside in the inhibition of $T_H1$ and

$T_H17$ number and functons (**Haworth et al., 2008; Rajasagi et al., 2011**). Of note, RvE and its synthetic analogs are currently undergoing clinical trial in many chronic inflammatory diseases that involve eyes, lungs, intestine and kidney diseases (**Serhan and Petasis, 2011; Lee, 2012**).

### *4.3. Immunomodulatory role of DHA-derived SPMs*

D-series resolvins, maresins and protectins, as pro-resolving lipids coming from essential docosanoids, and similarly to other factors that intervene during resolution phase, elicit crucial effects on acute inflammation mechanisms; conversely, our knowledge of their action on the cellular and molecular processes involved in chronic inflammation are rather limited. All DHA-derived SPMs are strong modulators of neutrophil function: indeed RvD1 and its aspirin-triggered epimer (i.e. AT-RvD1), but also MaR1, were proven capable of limiting polymorphonuclear leukocytes infiltration in acute inflammatory disease models (**Sun et al., 2007; Bento et al., 2011; Norling et al., 2012; Abdulnour et al., 2015**) while RvD2 has been reported to modulate neutrophil mobility avoiding aberrant infiltration (**Kurihara et al., 2013**). Similarly, PD1 has been reported to fiercely thwart the tissue infiltration of neutrophils in many inflammatory models either by directly interfering with PMN transmigration (**Hong et al., 2003; Marcheselli et al., 2003; Bannerberg et al., 2005**) or by upregulating chemochine receptors (e.g. CCR5) on apoptotic neutrophils allowing them to leech chemoattractants from phlogistic milieu (**Ariel et al., 2006**).

Furthermore, DHA-derived SPMs elicit strong pro-resolving effects by acting on macrophages as well. As a matter of fact, D-series resolvins and protectins stimulate efferocytosis of apoptotic PMNs (**Rogerio et al.,**

**2012; Schwab et al., 2007**) and suppress the release of TNF-α (**Duffield et al., 2006**), in activated macrophages. Moreover, D-series resolvins and maresins can modulate macrophage metabolism, promoting their persistence at the inflammation site (**Winkler et al., 2016**) while skewing M1/M2 balance towards the M2 phenotype, which is crucial in debris clearance, tissue regeneration and release of anti-inflammatory cytokines (**Dalli et al., 2013; Titos et al., 2011**). On the other hand, the body of information we possess on DHA-derived SPMs ability to modulate adaptive immune cells, though encouraging, are extremely limited. Maresin 1 can promote the *de novo* formation of Treg cells, which, in turn, suppress innate responses by releasing TGF-β (**Krishnamoorthy et al., 2015**), while protectin D1, which is produced by PMNs under the influence of $T_H2$ cytokines, is able to hinder T cells migration and release of pro-inflammatory cytokines inducing their apoptosis at the same time (**Ariel et al., 2005**). RvD1 and other DHA-derived byproducts with pro-resolving activity, have also been recently reported to act on B and plasma cells by modulating differentially the antibody isotype switching, promoting the release of IgM and IgG antibodies (**Ramon et al., 2012**), while inhibiting IgE production (**Kim et al., 2016**).

## II. AIM

Resolution represents a pivotal process, which is prompted during acute inflammation in order to switch off phlogistic mechanisms and allow the return to functional homeostasis. In recent years pro-resolving lipid mediators, a novel class of bioactive molecules which are synthesized from essential polyunsaturated fatty acids such as AA, EPA and DHA, have been unveiled as master regulators of this process, revealing that resolution, just as inflammation, entrusts the regulation of its complex functions to a vast network of signature bioactive lipids. SPMs work by modulating innate immune responses and inhibiting neutrophil influx at the inflammation sites, while promoting non-inflammatory uptake of dying cells resulting from the inflammatory events by enhancing macrophage debris-clearing properties. However, the role of DHA-derived SPMs in chronic inflammation and on its main executors, the adaptive immune cells, is still unclear. Thus, in order to characterize the full extent of the immunoresolving actions exerted by these molecules on the inflammatory machinery, we sought to deepen the knowledge of their role in the functions of T lymphocytes, arguably the most important components of adaptive immune system and, to date, the immune cell population on which we possess the smallest amount of information. As a matter of fact, scientific literature still lacks a rigorous investigation of the effects elicited by these lipids on T cells. Furthermore, given the fact that RvE1 and two of its synthetic analogs are currently undergoing clinical investigation in a number of chronic diseases (e.g. inflammatory bowel disease, atherosclerosis and rheumatoid arthritis), the hypothesis that depicts DHA-derived SPMs as critical regulators of cell-mediated adaptive immunity can be considered fairly plausible. Thus, the aim of

this study was to investigate the potential effect of specific DHA-derived SPMs, namely RvD1, RvD2 and MaR1, on human T cells subsets (CD4 and CD8) by means of the sophisticated polychromatic flow cytometry, that allowed us to perform simultaneously analyze several phenotypic or functional parameters within the same cell population. Furthermore, we also set out to investigate whether these SPMs could affect the differentiation of the different T-helper subsets or to regulatory T cells from naïve CD4 naïve T cells and to unravel the potential involvement of the known receptors in SPMs-induced immunoregulatory effects. The understanding of DHA-derived pro-resolving lipids on these cell-mediated adaptive immunity might represent a promising starting point in postulating the possible involvement of these compounds in the pathogenesis of several lymphocyte-driven pathologies as well as the their use as potential therapeutic agents in many chronic inflammatory diseases.

# III. MATERIALS AND METHODS

## 1. Materials

RvD1 (7S,8R,17S-trihydroxy-4Z,9E,11E,13Z,15E,19Z-docosahexaenoic acid), RvD2 (7S,16R,17S-trihydroxy-4Z,8E,10Z,12E,14E,19Z-docosahexaenoic acid), AT-RvD3 (4S,11,17R-trihydroxydocosa-5Z,7,9,13,15E,19Z-hexaenoic acid), MaR1 (7R,14S-dihydroxy-docosa-4Z,8E,10E,12Z,16Z,19Z-hexaenoic acid) were prepared by total organic synthesis as previously described (**Sun et al., 2007; Spite et al., 2009; Winkler et al., 2013; Serhan et al., 2009**).

## 2. Peripheral blood cells isolation and purification of naïve $CD4^+$ T-lymphocytes

Peripheral blood mononuclear cells (PBMCs) were isolated after venous puncture from healthy donors, and were separated by density gradient over Ficoll-Hypaque (Amersham Biosciences), as reported (**Chiurchiù et al., 2013**). CD4+ T Lymphocytes were purified by immunomagnetic depletion with the human CD4+ T Cell Isolation Kit II (Miltenyi Biotec), and were purified by means of negative selection through AutoMACS Pro Separator (Miltenyi Biotec). Briefly, non-naïve T cells (effector and memory T cells, NK cells, B cells, dendritic cells, granulocytes) were indirectly labeled using a cocktail of biotin-conjugated antibodies and anti-biotin magnetic Microbeads. Highly purified unlabeled naïve $CD4^+$ T cells ($CD4^+CD45RA^+CD27^+CD45RO^-$) were obtained by depletion of the magnetically labeled cells and had a purity of over 95%, which was constantly checked by flow cytometry.

*AutoMACS Pro Separator*

### 3. Flow cytometry

In order to measure the intracellular levels of cytokine, their release from T cells was inhibited by adding 1 μg/ml brefeldin A (Sigma-Aldrich), 5 hours before the end of stimulation (**Chiurchiù et al., 2013**). At the end of the incubation, cells were stained at cell surface with e780-conjugated anti-CD3 (eBiosciences), PerCP5.5-conjugated anti-CD4 (eBiosciences), Brilliant Violet-conjugated anti-CD8 (Biolegend), made permeable with Cytofix/Cytoperm reagents (BD Biosciences), and then stained intracellularly with Phycoerythrin-Cy7-conjugated anti-TNF-α (eBiosciences), Allophycocyanin (APC)-conjugated anti-IFN-γ (eBiosciences), Phycoerythrin (PE)-conjugated anti-IL-17 (eBiosciences), Brilliant Violet 421-conjugated IL-4 (eBioscience), anti-PercP5.5-conjugated anti-IL2 (Biolegend) and Fluorescein isothiocyanate (FITC)-conjugated anti-Granzyme B in 0.5% saponine, at RT for 30 min. In some experiments, cells were also stained at cell surface with PE-conjugated anti-FasL (Miltenyi Biotec) APC-conjugated anti-PD-1 (eBioscience) and PE-conjugated anti-CTLA-4 (Miltenyi Biotec). Intracellular cytokines were analyzed by flow cytometry in a FACS-Cyan ADP (Beckman Coulter), as reported (**Chiurchiù et al., 2013**). For each analysis, at least 300,000 events were acquired gating on Pacific Orange-conjugated Live/Dead negative cells. In some experiments PBMCs were pre-incubated for 30 min with anti-FPR2/ALX (2 μg/ml, clone FN-1D6-A1, Genovac, Freiburg, Germany), anti-GPR32 (2 μg/ml; clone GTX71225, GeneTex, CA, USA), prior to incubation with vehicle or 10 nmol/L RvD1 and to stimulation with Dynabeads CD3/CD28 T Cell Expander (one bead per cell; Invitrogen). The list of antibodies used for flow cytometry is detailed in **Table 1.**

*FACS CyAn ADP Analyzer*

### 4. Detection of apoptotic and necrotic cells

Apoptotic and necrotic cells were detected using Annexin-V-FITC and propidium iodide (PI) staining (eBioscience), respectively. Cells were washed twice in in PBS followed by re-suspension in Binding Buffer (Annexin-V Kit, eBioscience) and then incubated with 5µl of Annexin-V-FITC for 15 min at RT. Cell were then extensively washed with Binding Buffer and 5µl of PI was added and cells analyzed within 2 hours on a FACS-Cyan ADP (Beckman Coulter).

### 5. Proliferation Assay

CD3+ T cells were isolated through AutoMACS Pro Separator and 10X106 cells were labeled with CFSE at a final concentration of 5 µM for 10 min at 37 °C on a basculator. Then, cells were washed twice with PBS/10% FBS, suspended in culture media and analyzed immediately on a FACS-Cyan ADP (Beckman Coulter). Analysis of cells immediately after

CFSE labeling indicated a labeling efficiency higher than 99%. Cell proliferation was followed by flow cytometry at day 2 and 4 upon stimulation with Dynabeads CD3/CD28 T Cell Expander in presence or absence of 10 nmol/L RvD1, RvD2 and MaR1.

### 6. T helper cell differentiation assay

For $T_H1$ and $T_H17$ polarization of T cells, isolated naïve $CD4^+$ T cells were cultured in round bottom 96-well plates (Falcon) at a density of $5x10^4$ cells at 37°C in 200µl final volumes of X-VIVO 15 medium in presence of Dynabeads CD3/CD28 T Cell Expander (one bead per cell; Invitrogen) and under $T_H1$-, $T_H2$- and $T_H17$-polarizing conditions in presence of Rv(s) or MaR1 (10 nmol/L). The following human recombinant cytokines were used: for $T_H1$ polarization, 10ng/ml IL-12 and 20ng/ml IL-2 (Miltenyi Biotech); for $T_H17$ polarization, 10ng/ml IL-1β, 20ng/ml IL-6, 100ng/ml IL-23, 1ng/ml TGF-β (Miltenyi Biotech) and 20ng/ml IL-2 (Miltenyi Biotech). Cells were collected and washed extensively after 5 days and their viability was determined by trypan blue exclusion. Cells ($1x10^6$ cells/ml) were re-stimulated for 6h with 100nmol/L PMA and 1 µg/ml brefeldin A (Sigma-Aldrich) (for Flow Cytometry) or 24h with Dynabeads CD3/CD28 T Cell Expander (one bead per cell) (for ELISA and RT-PCR). For $T_H2$ polarization, naïve $CD4^+$ T cells were kept under polarizing conditions using 25ng/ml IL-4 (Miltenyi Biotec) and Dynabeads CD3/CD28 T Cell Expander (one bead per cell). Cells were extensively washed and re-stimulated with Dynabeads CD3/CD28 every 3 days with fresh medium plus IL-4 and 20ng/ml IL-2 (Miltenyi Biotec) up to day 12. After 12 days cells were collected for ELISA assay or qRT-PCR or re-

stimulated for 6h with 100nmol/L PMA for intracellular staining and 1 µg/ml brefeldin A (Sigma-Aldrich) (for Flow Cytometry); Cultures were supplemented with vehicle (Ethanol 0,1%), Rv(s) or MaR1 every day. In some experiments TH1, TH17 and iTreg were pre-incubated for 30 min with anti-FPR2/ALX (2 µg/ml), anti-GPR32 (2 µg/ml), prior to incubation with vehicle or 10 nmol/L RvD1 and to stimulation with CD3/CD28 beads as well as prior to restimulation.

### 7. Generation of induced Treg

Highly purified naïve CD4+ T cells were cultured in round bottom 96-well plates (Falcon) at a density of 5x104 cells at 37°C in 200µl final volumes of X-VIVO 15 medium in presence of Dynabeads CD3/CD28 T Cell Expander (one bead per cell; Invitrogen) and TGF-β (2 ng/ml; Miltenyi Biotec), in presence or absence of Rv(s) or MaR1 (10 nmol/L). Cultures were supplemented with SPMs every other day for 5 d.

### 8. ELISA assay

Cytokine content was determined by standard 2-site sandwich enzyme-linked immunosorbent assays (ELISA), using available commercial kits purchased from eBioscience for IFN-γ, IL-17, IL-4, IL-10 as previously reported (**Cencioni et al., 2010 PLoS One**).

### 9. qRT-PCR

Total RNA was extracted with an RNeasy Micro kit (Qiagen). A mixture containing random hexamers, oligo(dT)15 (Promega) and SuperScript II Reverse Transcriptase (Invitrogen) was used for cDNA synthesis. Transcripts were quantified by real-time quantitative PCR on an ABI PRISM 7900ht sequence detector (Applied Biosystems) with Applied Biosystems predesigned TaqMan Gene Expression Assays and Absolute QPCR ROX mix (Thermo Fisher Scientific). The following probes were used (Applied Biosystems, assay identification numbers in parentheses): T-bet (Hs00203436_m1), RORc (Hs01076112_m1), GATA-3 (Hs00231122), GPR32 (Hs01102536_s1) and FPR2 (Hs02759175_s1). For each sample, mRNA abundance was normalized to the amount of ribosomal protein L34 (Hs00241560_m1).

*7900HT Fast Real-Time PCR System*

### 10. SDS-page and Immunoblotting

Purified and polarized $T_H0$, $T_H1$, $T_H2$, $T_H17$ and iTreg cells were lysed with lysis buffer and cell homogenates were subjected to 10% SDS–PAGE (50 µg/lane) under reducing conditions. Gels were then electroblotted onto 0.45-µm nitrocellulose filters (Bio-Rad, Hercules, CA, USA) and were immunoreacted with primary anti-GPR32 polyclonal mouse antibody (1:500, GeneTex), anti-ALX/FPR2 monoclonal rabbit antibody (1:500, Genovac/Aldevron) or with anti-β-actin monoclonal mouse antibody (1:10000, Bio-Rad), and then with secondary goat-anti-rabbit polyclonal antibody (1:2000, Santa Cruz Biotechnologies, Santa Cruz, CA, USA) for GPR32 and goat-anti-mouse polyclonal antibody (1:2000 for ALX and 1:10000 for β-actin).

### 11. Statistical analysis

All data were expressed as means ± SD or SEM. Differences between groups were compared using Student's t test (two groups) or one-way ANOVA (multiple groups) followed by a post hoc Bonferroni test. The criterion for statistical significance was $p < 0.05$. All statistical analyses were performed with GraphPad Prism. The Pearson correlation coefficient was used to assess the significance of correlation between cytokines release and respective transcription factors expression. FACS analysis was performed using the Flowjo analysis program (Treestar, Ashland, OR).

# IV. RESULTS

## 1. Pro-resolving lipid mediators modulate CD8+ and CD4+ T cell responses

Specialized pro-resolving mediators (SPMs) exert a plethora of effects, mostly on innate immune cells, with the aim of resolving inflammation (**Serhan, 2014**). We hypothesized that these bioactive resolving lipids could also affect the immune responses of cells belonging to the adaptive immunity. To test this hypothesis, initial studies were performed to assess whether increasing concentrations of RvD1, RvD2 and MaR1 (ranging 1-100 nmol/L) affected the production of TNF-α from human T lymphocytes, the main cells of adaptive immunity. The dose-response of the SPMs is reported in **Fig 1C**, testing them for their ability to modulate TNF-α production from PMA/Ionomycin-activated CD4+ and CD8+ T lymphocytes (**Fig 1A** and **B**). Both CD4+ and CD8+ T cells, when activated with PMA/Ionomycin, produced high amounts of intracellular TNF-α, which was dampened upon pretreatment with all SPMs. The different compounds inhibited TNF-α production in a dose-dependent manner and substantially reduced the cytokine at doses as low as 10.0 nmol/L. The lower dose tested (1.0 nmol/L) only showed a slight and non-significant reduction of TNF-α production from both T cell subsets (**Fig 1C**). For this reason, in all experiments to be reported in this study, SPMs effects were evaluated using 10.0 nmol/L as the lowest efficacious concentration. These initial data suggest that SPMs might indeed be effective in modulating adaptive immune responses. T lymphocytes play a central role in cell-mediated immunity either against pathogens or for

the maintenance of immunological tolerance. Thus, we next ascertained the possible impact of SPMs (at 10 nmol/L) on the production of the specific cytokines that characterize the main pro-inflammatory T cell subsets, i.e. cytotoxic CD8+ T cells and T helper-1 ($T_H1$) and T helper-17 ($T_H17$) cells. Indeed, activated CD8+ T cells produced high amounts of TNF-α and IFN-γ, which were strongly inhibited by all SPMs (**Fig 2A**). Furthermore, the production of IFN-γ and IL-17 from activated CD4+ T cells was also blunted by pretreatment with SPMs (**Fig 2B**), although only RvD1 and MaR1 were significantly effective on IFN-γ, whereas IL-17 was equally reduced by all SPMs. Since the cytokine profile of human T cells may be differently determined, depending on the assay and conditions used, we further investigated the immunomodulatory role of RvD1. RvD2 and MaR1 using a more specific and physiological stimulus for activating T cells, i.e. polyclonal activation with anti-CD3 and anti-CD28. In **Fig 3** we show that T cells cytokine production after stimulation with the anti-CD3/CD28 was almost identical to that of the following PMA/ionomycin stimulation, even if intracellular levels of cytokines were inferior, as already reported (**Han et al., 2012**). In particular, RvD1, RvD2 and MaR1 significantly reduced the capability of CD8+ T cells to produce TNF-α and IFN-γ (**Fig 3A**) and that of CD4+ T cells to become producers of TNF-α, IFN-γ and IL-17 (**Fig 3B**), suggesting that SPMs might regulate $T_H1$ and $T_H17$ responses.

**2. SPMs regulates IL-2 production from T cells and preserves their viability**

Such immunomodulatory activity of RvD1 and MaR1 on T cell responses was also paralleled by a concomitant reduction of the crucial growth factor IL-2 compared to IL-2-producing and proliferative CD8+ and CD4+ activated T cells (**Fig 4A**). This effect was not due to an induction of cell death, as assayed by annexin-V staining that was used as a marker for apoptosis in combination with PI in order to distinguish between apoptotic and necrotic cells. As expected, resting T cells were all annexin-V and PI negative, whereas anti-CD3/CD28-activated T cells showed a ~20% of total apoptotic cells (annexin-V positive) (**Fig 4B**). Almost no variation in early apoptosis (annexin-V and PI negative), late apoptosis (annexin-V and PI positive), or necrosis (PI positive and annexin-V negative) could be detected in RvD1-, RvD2- and MaR1-treated activated T cells, either on the total CD3+ T cell population (**Fig 4B left panel**) or in CD8+ or CD4+ cells (**Fig 4B right panel**). These data were also confirmed by analyzing the surface expression of the pivotal cell death receptor FasL (**Fig 4C)**, as well as that of CTLA-4 and PD-1, two of the most important inhibitory receptors on T cell surface (**Tab 2 and 3**), inasmuch as we found that none of the tested SPMs modified the expression of none of the aforementioned proteins. Given the evidence of the role that these bioactive lipids carry out on IL-2 production, we further sought to ascertain if such an effect was reflected by a change in T cell proliferation rate. CFSE staining performed on $CD3^+$ lymphocytes showed that SPMs elicited only a slight, though not significant decrease in cell replication, in respect with stimulated cells, strongly suggesting, alongside with the other data we gathered, that an important part of the

immunomodulatory role played by DHA-derived SPMs on adaptive immunity is achieved by directly blunting T cell effector functions (e.g. cytokine release) rather than modulating the expression of their suppressive receptors or influencing their viability.

### 3. SPMs critically affect $T_H1/T_H17$ and Treg differentiation

$T_H1$ and $T_H17$ subsets in peripheral blood are both derived from naïve CD4+ T cells upon antigen stimulation and specific skewing cytokines (**Raphael et al., 2015**). Since all tested SPMs dampened the inflammatory response of TNF-α- and IFN-γ-producing $T_H1$ cells and of IL-17-producing $T_H17$ cells from peripheral blood mononuclear cells, we next investigated whether RvD1, RvD2 or MaR1 were able to directly affect their differentiation from naïve CD4+ T cells into $T_H1$ and $T_H17$ lineages. Furthermore, given the technical limitations commonly encountered in studying $T_H2$ cells from PBMCs, we also sought to investigate any potential effect exerted by SPMs on the development of $T_H2$ cells. To this aim, a standard naïve CD4+ T cells differentiation assay was performed in the presence of polyclonal stimulation with anti-CD3/CD28, and specific polarizing cytokines in presence of RvD1, RvD2 or MaR1, as schematized in **Fig 5A.** Upon specific polarizing conditions, highly purified CD4+ T cells displayed significant amounts of intracellularly produced and extracellularly released IFN-γ ($T_H1$), IL-4 ($T_H2$) and IL-17 ($T_H17$), as compared to non-polarized cells ($T_H0$) (**Fig 5B** and **C**). Furthermore, $T_H17$ cells produced also low levels of IFN-γ, while $T_H1$ cells produced very low levels of IL-17, confirming previously reported data showing that the $T_H17$ cytokine profile overlaps with that of $T_H1$ cells (**Weaver et al.,**

**2007**). The treatment with RvD1, RvD2 or MaR1 significantly affected $T_H1$ and $T_H17$ polarization, while being substantially unable to exert any considerable effect on $T_H2$ development or function. Notably, RvD1 and RvD2 significantly reduced $T_H1$ and $T_H17$ generation, acting on both intracellular production (**Fig 5B**) and extracellular release (**Fig 5C**) of IFN-γ from $T_H1$ cells and of IL-17 and IFN-γ from $T_H17$ cells. On the other hand, the overall effect of MaR1 on $T_H1/T_H17$ differentiation was less prominent and was particularly significant on $T_H17$ cells, where the inhibitory effect on IL-17 production and release was greater than on IFN-γ from $T_H1$ cells (**Fig 5B** and **C**). Next, to address whether $T_H1$, $T_H2$ and $T_H17$ polarization was associated with the acquisition of their typical features, we also measured the mRNA encoding for the transcription factors known to be critical for their differentiation, T-bet, GATA-3 and RORc, respectively. As expected, polarized $T_H1$, $T_H2$ and $T_H17$ cells displayed the highest expression of their specific transcription factors. The presence of RvD1, RvD2 or MaR1 during $T_H1/T_H17$ polarization determined a decrease of T-bet in $T_H1$ cells and of RORc in $T_H17$ cells, with a stronger effect exerted by RvD1 and RvD2 compared to MaR1 (**Fig 5D**). Again, all tested SPMs were incapable to affect $T_H2$ polarization, causing no appreciable variation in the expression of GATA-3 (**Fig 5D**). These findings support a pivotal role for SPMs also in hindering *de novo* $T_H1/T_H17$ differentiation. On the light of the role of SPMs in resolving inflammation and of regulatory T cells (Treg) as the most important cell subset involved in modulating and maintaining self regulation of the immune system, we also wondered whether SPMs could also affect the generation of induced Treg cells (iTreg). This cell subset develops from naïve CD4+ T cells upon antigen stimulation and TGF-β (**Yamagiwa et al.,**

**2001**). To this aim, highly purified naïve CD4+ T cells were cultured under Treg-inducing conditions in presence of RvD1, RvD2 and MaR1, all at 10 nmol/L (**Fig 6A**). We found that all SPMs synergized with TGF-β in skewing iTreg differentiation by significantly boosting the expression of Treg pivotal transcription factor Foxp3 (**Fig 6B**). RvD1, RvD2 and MaR1 were also proven capable of enhancing iTreg suppressive features inducing a significant increase of CTLA-4 expression as well as by enhancing the levels of IL-10 released by these cells, with MaR1 being slightly more efficient compared to D-series resolvins (**Fig 6B).** Interestingly, the production of granzyme B was not affected by SPMs, suggesting that these molecules might rather modulate their regulatory abilities instead of their ability to kill activated T cells. These findings confirm recently reported data on the role of MaR1 in augmenting de novo generation of Tregs in mice (**Norling et al., 2012**), and support a critical role for D-series SPMs and MaR1 in modulating the balance between TH1/TH17 and iTreg cells during inflammation. Taken together, these findings confirm recently reported data on the role of pro-resolving lipids in augmenting *de novo* generation of Tregs in mice (**Krishnamoorthy et al., 2015**), and support a critical role of SPMs in modulating the balance between $T_H1/T_H17$ and iTreg cells during inflammation.

**4. SPMs immunoregulatory effects on T cells are mediated by GPR32 and ALX/FPR2 receptors**

In order to verify whether SPMs-induced effects were associated to a higher response of these CD4+ naïve-derived T cell subsets and to ascertain the molecular mechanisms behind the immunomodulatory role of these lipid mediators on T cells, we sought to investigate the involvement of

SPMs receptors in the effects we observed. Since we still possess limited information regarding the full spectrum of receptors engaged by the different classes of SPMs, our study had to be restricted to the only two known receptors known to be able to bind D-series resolvins, i.e. GPR32 and ALX/FPR2. As a matter of fact, receptors for MaRs have yet to be identified. In **Figure 7 (left panel)** we show that $T_H1$ cells displayed the highest expression of both GPR32 and ALX/FPR2 compared to $T_H0$ cells, whereas $T_H17$ cells and $T_H2$ showed similar levels of GPR32 and lower levels of ALX/FPR2 compared to $T_H0$ cells. Furthermore, iTreg cells expressed very low levels of both receptors. Interestingly, the levels of GPR32 were significantly higher in all the T cell subsets compared to ALX/FPR2, whereas iTreg cells showed a similar expression. Since mRNA expression might not indicative of the actual expression into protein of a certain transcript, we also investigated their protein expression and immunoblotting analysis demonstrated that all T helper subsets express both GPR32 and ALX/FPR2, representing to our knowledge the first detailed evidence of SPMs receptors expression on T cells. In particular $T_H1$, $T_H17$ and iTregs showed higher and comparable levels of GPR32 respect to $T_H0$, whereas the expression of such receptor in $T_H2$ was similar to that of $T_H0$ control group (**Fig 7A, right panel).** Conversely, ALX/FPR2 showed no differential expression and remained unchanged among all T cell subsets (**Fig 7A, right panel**). Since these two G protein-coupled receptors are the only known receptors for RvD1 (**Chiang et al., 2006**; **Krishnamoorthy et al., 2012**), we next sought to verify their possible role as mediators of the observed effect of this pro-resolving lipid on T lymphocytes. Interestingly, pre-incubation with anti-GPR32 or anti-ALX/FPR2 neutralizing antibodies (alone or in combination) abrogated the suppressive activity of

RvD1 on $T_H1$ and $T_H17$, as well as its enhancing activity on iTreg lymphocytes. In particular, the single neutralization of either GPR32 or ALX/FPR2 significantly blunted the inhibitory action of RvD1 on both PBMC-derived (**Fig 7B**) and *de novo* generated $T_H1$ and $T_H17$ cells (**Fig 7C**), whereas the inactivation of both receptors was additive, completely restoring the intracellular levels of IFN-γ and IL-17 as to those of activated T cells (**Fig 7B** and **C**), suggesting that these two receptors might work in synergy to mediate the effects of RvD1. GPR32 and ALX/FPR2 also seem to work together as mediators of the immunoregulating effect of RvD1 on PBMC-derived $CD8^+$ CTLs. Conversely, iTregs showed a differential involvement of these receptors, as only the neutralization of GPR32 could significantly revert the expression of Foxp3 (**Fig 7C**), whereas the neutralization of ALX/FPR2 did not exert any effect.

## V. FIGURES AND TABLES

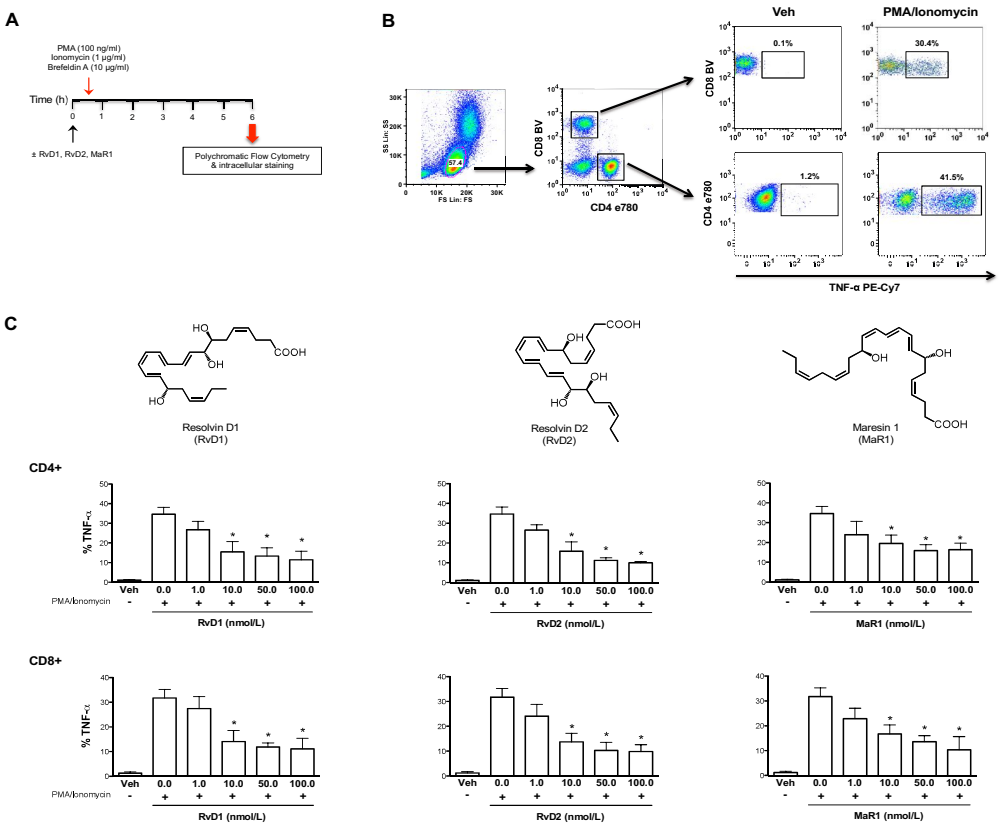



**Fig.1** *Dose-dependency of SPMs treatment on TNF-α production in PMA/Ionomycin-stimulated CD4⁺ and CD8⁺ T cells derived from PBMCs.* **(Panel A and B)** PBMCs were incubated for 30' with vehicle (0,1% Ethanol) or 10nmol/L RvD1, RvD2 or MaR1, prior to stimulate them with 100ng/ml PMA, 1µg/ml Ionomycin and 10µg/ml Brefeldin-A. After 6 hours of stimulation, cells were collected and stained intracellularly with TNF-α antibody. The intracellular levels of TNF-α were assayed by means of polychromatic flow cytometry. Lymphocytes were firstly gated according to physical parameter, and the expression of TNF-α in vehicle- and PMA/Ionomycin-treated cells was measured based on their positivity for CD4 or CD8. All data are expressed as mean ± SEM. PBMC= Peripheral blood mononuclear cell; Veh= Vehicle; PMA= phorbol myristate acetate; TNF-α= Tumor necrosis factor-α; Rv= Resolvin; MaR1= Maresin 1.

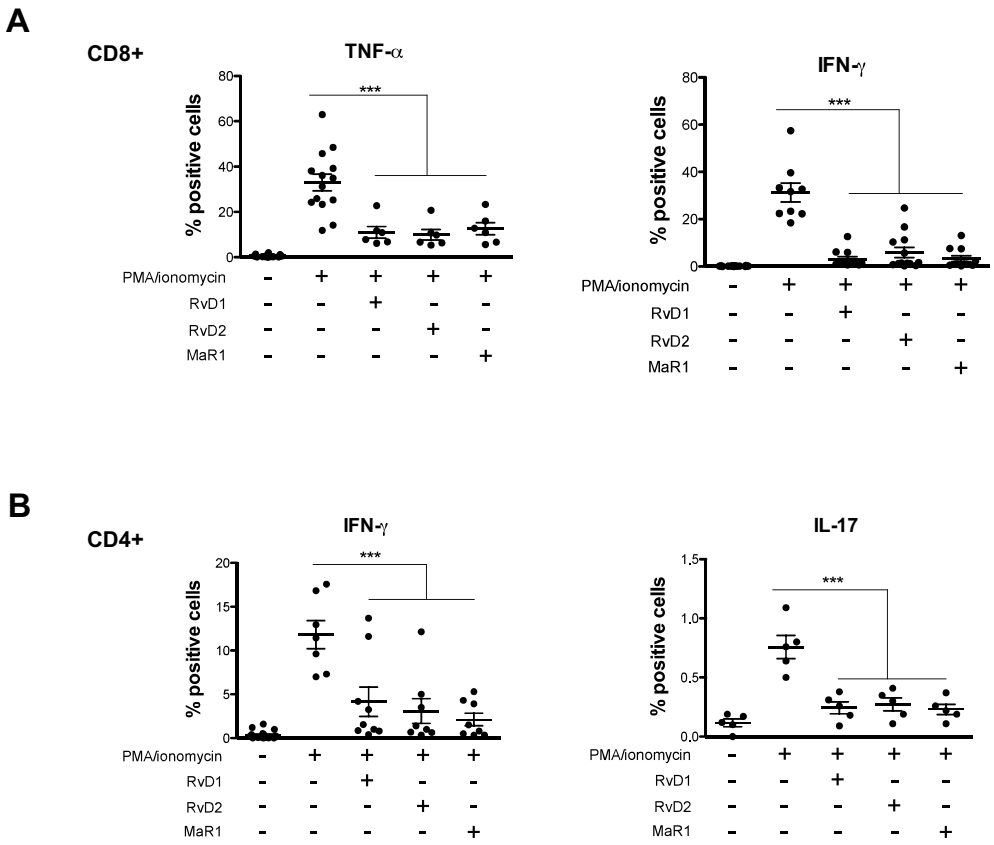



**Fig.2** *Effect of SPMs-treatment on cytokine production in PMA/Ionomycin-stimulated CD4⁺ and CD8⁺ cells derived from PBMCs.* PBMCs were incubated for 30' with 10nmol/L RvD1, RvD2 or MaR1 prior to stimulate them with 100ng/ml PMA, 1µg/ml Ionomycin and 10µg/ml brefeldin-A. After 6 hours of stimulation, cells were collected and stained with TNF-α, IFN-γ and IL-17 antibody. Using polychromatic flow cytometry, lymphocytes were firstly gated according to physical parameters and then on their positivity for CD4 or CD8. $CD8^+$ cells were assayed for the intracellular levels of TNF-α and IFN-γ **(Panel A)** while $CD4^+$ cells for those of IFN-γ and IL-17 **(Panel B).** All data are expressed as mean ± SEM. PBMC= Peripheral blood mononuclear cell; TNF-α= Tumor necrosis factor-α; IFN-γ= Interferon-γ; IL-17= Interleukin-17; Rv= Resolvin; MaR1= Maresin 1.

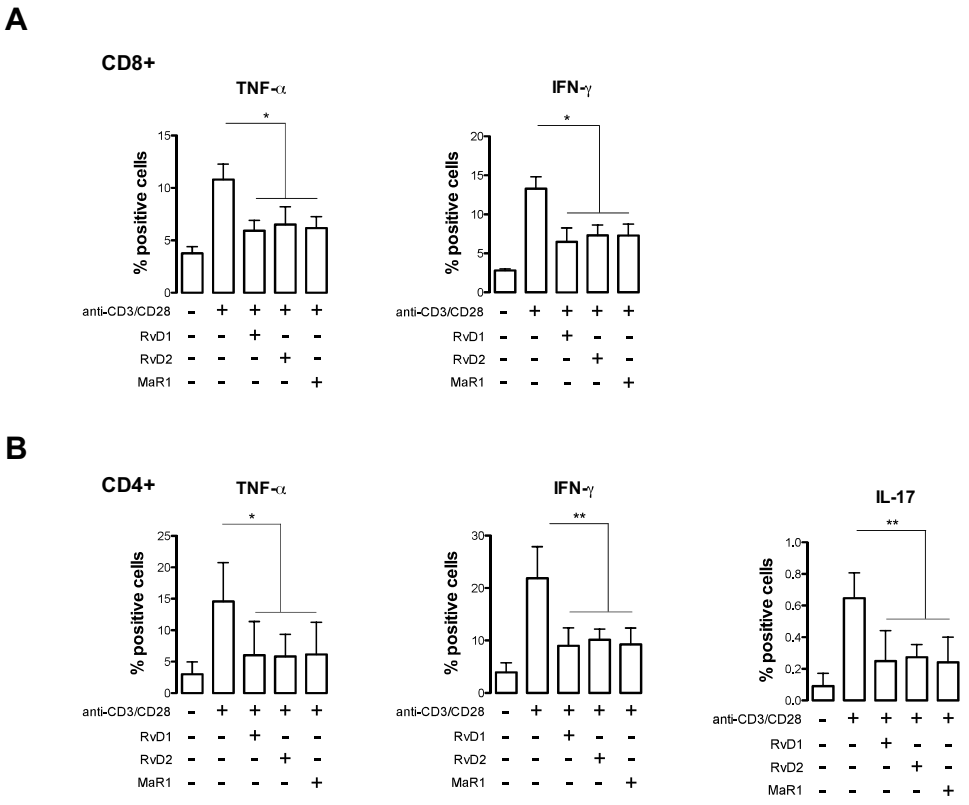



**Fig. 3** *Effect of SPMs-treatment on cytokine production in anti-CD3/CD28-stimulated CD4⁺ and CD8⁺ cells derived from PBMCs.* PBMCs were incubated for 30' with 10nmol/L RvD1, RvD2 or MaR1 prior to stimulate them with anti-CD3/CD8-coated Dynabeads (1µl/50.000 cells) and brefeldin-A, in order to simulate APC-dependent B7-like simulation. After 8 hours of stimulation cells, cells were collected and stained with TNF-α, IFN-γ and IL-17 antibody. Using polychromatic flow cytometry, lymphocytes were firstly gated according to physical parameters and then to their positivity for CD4 or CD8. $CD8^+$ cells were assayed for the intracellular levels of TNF-α and IFN-γ **(Panel A)** while $CD4^+$ cells for those of TNF-α, IFN-γ and IL-17 **(Panel B).** All data are expressed as mean ± SEM. PBMC= Peripheral blood mononuclear cell; TNF-α= Tumor necrosis factor-α; IFN-γ= Interferon-γ; IL-17= Interleukin-17; Rv= Resolvin; MaR1= Maresin 1.

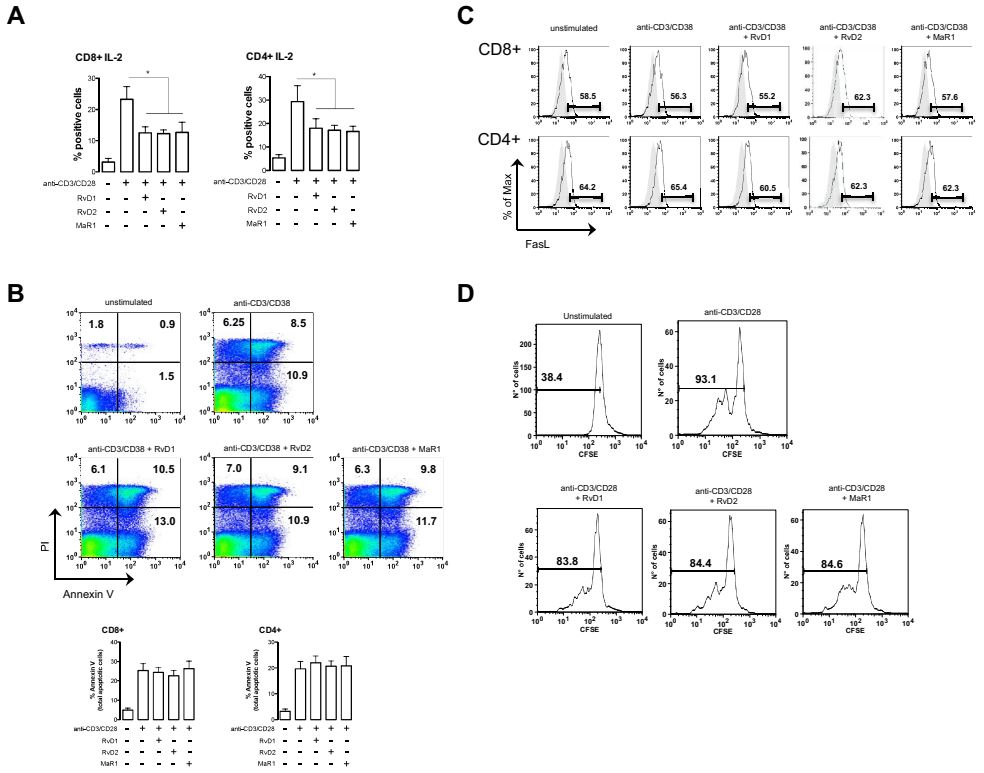



**Fig. 4** *Effect of SPMs treatment on IL-2 production, cell proliferation and cell death in anti-CD3/CD28-stimulated lymphocytes* **(Panel A, B and C)** PBMCs were incubated for 30' with 10nmol/L RvD1, RvD2 or MaR1 prior to stimulate them with anti-CD3/CD8-coated Dynabeads (1µl/50.000 cells) and brefeldin-A, in order to simulate APC-dependent B7-like stimulation. After 8 hours, cells were collected and stained with IL-2 antibody or annexin V and PI. Intracellular levels of IL-2 or positivity to annexin V and/or PI was assayed by means of polychromatic flow cytometry. **(Panel D)** In an independent set of experiments, purified $CD3^+$ lymphocytes were stained with CFSE prior to stimulate them with anti-CD3/CD8-coated Dynabeads (1µl/100.000 cells), and treated each day either with vehicle (ethanol 0,1%) or 10nmol/L RvD1, RvD2 or MaR1 as detailed in materials and methods. After three days, cells were collected and cell proliferation was assayed measuring CFSE positivity by means of polychromatic flow cytometry. In all experiments, lymphocytes were firstly gated according to physical parameters and then, the different subsets were discriminated based on their positivity for CD4 or CD8. All data are expressed as mean ± SEM. PBMC= Peripheral blood mononuclear cell; IL-2= Interleukin-2; FasL= Apoptosis stimulating fragment ligand; PI= Propidium Iodide; CFSE= Carboxyfluorescein succinimidyl ester; Rv= Resolvin; MaR1= Maresin 1.

**Fig 5**

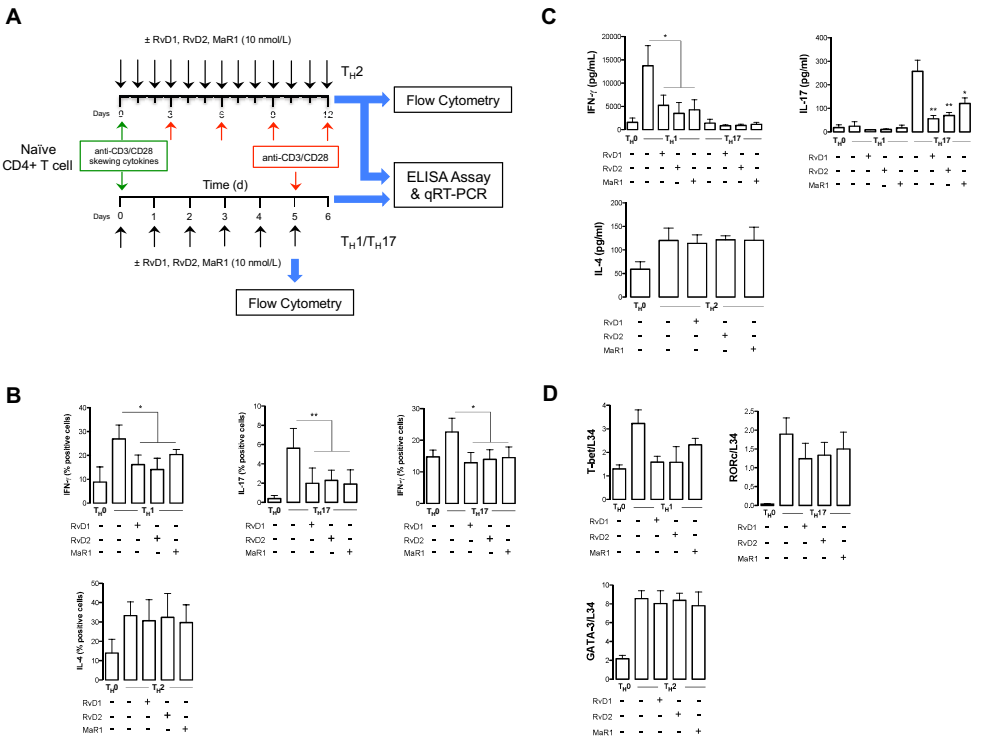



**Fig. 5** *Effect of SPMs treatment on CD4⁺ naïve lymphocyte-derived T helper cells.* **(Panel A)** Purified naïve T cells were treated with anti-CD3/CD28-coated dynabeads (1µl/100.000 cells) and $T_H1$, $T_H2$ or $T_H17$ skewing cytokines. They were treated each day either with vehicle (ethanol 0,1%) or 10nmol/L RvD1, RvD2 or MaR1 as detailed in materials and methods. **(Panel B)** At the due time, an aliquot of lymphocytes was collected and stained intracellularly with IFN-γ, IL-4 and IL-17 antibodies in order to evaluate $T_H1$, $T_H2$ and $T_H17$ polarization by means of polychromatic flow cytometry. **(Panel C)** ELISA assay was performed in order to measure the levels of IFN-γ, IL-4 and IL-17 released by polarized $T_H1$, $T_H2$ and $T_H17$ respectively. (**Panel D)** Relative mRNA expression of T-Bet, GATA3 and RORc in $T_H1$, $T_H2$ and $T_H17$ respectively performed by means of qRT-PCR. All data are expressed as mean ± SEM. $T_H$= T helper; IFN-γ= Interferon-γ; IL-4= Interleukin-4; IL-17= Interleukin-17; Rv= Resolvin; MaR1= Maresin 1.

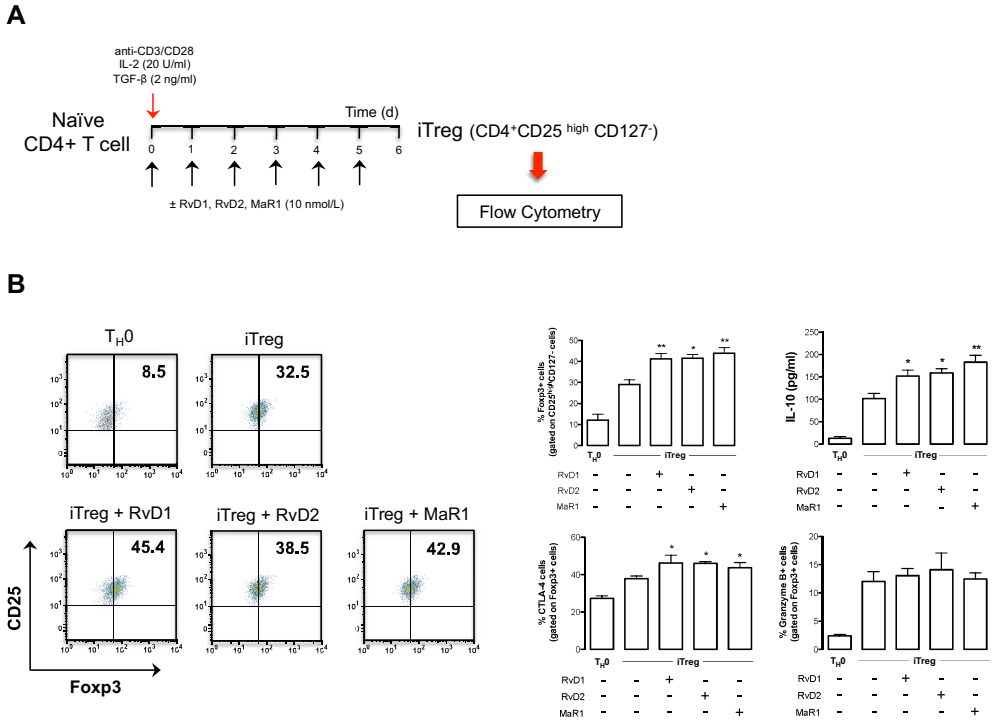



**Fig. 6** *Effect of SPMs treatment on induced T regulatory cells.* **(Panel A)** Purified naïve T cells were treated with anti-CD3/CD28-coated dynabeads (1µl/100.000 cells) and Treg skewing cytokines. They were treated each day either with vehicle (ethanol 0,1%) or 10nmol/L RvD1, RvD2 or MaR1 for six days prior to being collected along with their supernatants. **(Panel B)** Using polychromatic flow cytometry, Treg cells were characterized as $CD4^{+}$, $CD127^{-}$, $CD25^{high}$ cells, and were then assayed for the expression of Foxp3. CTLA-4 and granzyme B expression was measured by gating on $Foxp3^{+}$ cells. IL-10 production was evaluated by means of ELISA assay. All data are expressed as mean ± SEM.

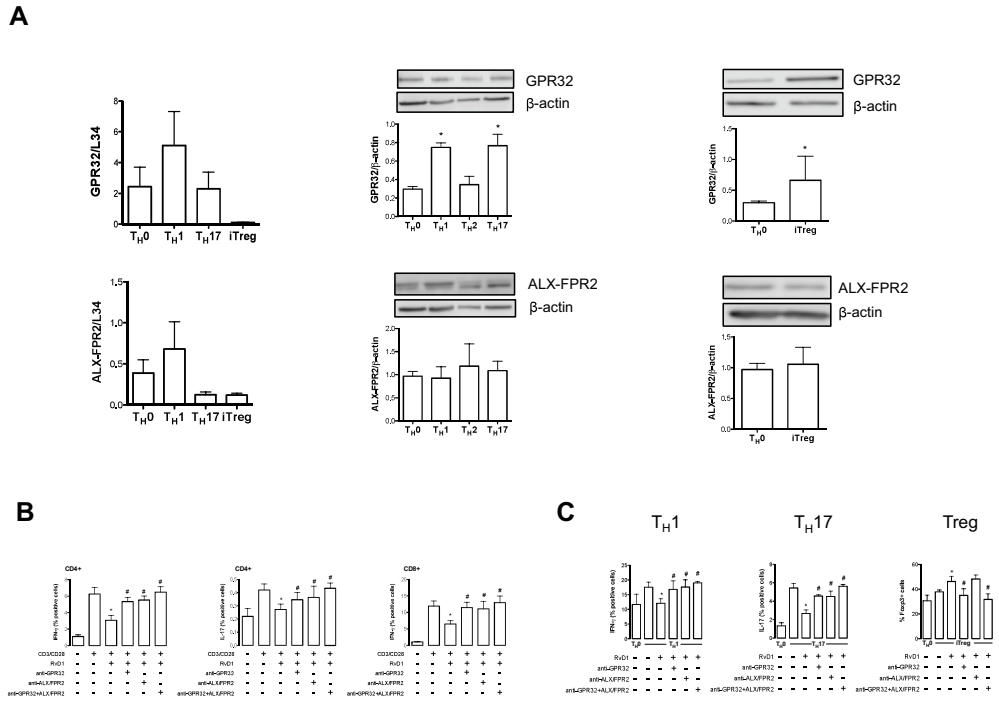



**Fig. 7** *Neutralization of GPR32 and ALX/FPR2 reverts the effects of RvD1 on T cells.* **(Panel A)** Relative mRNA (left) and protein expression (right) of GPR32 and ALX/FPR2 in CD4 naïve T cell-derived T helper cells. **(Panel B)** Intracellular production of TNF-α, IFN-γ and IL-17 in $CD4^+$ and $CD8^+$ lymphocytes. PBMCs were incubated either with anti-GPR32, anti-ALX/FPR2 or both antibodies, prior to treat them with 10nmol/L RvD1 and stimulate them with anti-CD3/CD28 beads. After 8 hours, cells were stained intracellularly with TNF-α, IFN-γ and IL-17 antibodies. Cytokine production was assayed by means of polychromatic flow cytometry. Lymphocytes were firstly gated according to physical parameters and then discriminated based on the expression of CD4 or CD8. **(Panel C)** Intracellular production of IFN-γ, IL-17 and Foxp3 in polarized $T_H1$, $T_H17$ and iTreg respectively. Purified naïve T Cells were incubated with skewing cytokines, in presence or absence of anti-GPR32/ALX antibodies, and were treated each day with RvD1. After 6 days, cells were stained intracellularly with IFN-γ, IL-17 or Foxp3 antibodies and assayed by means of polychromatic flow cytometry. All data are expressed as mean ± SEM. GPR32= G-coupled protein receptor 32; FPR2= Formyl peptide receptor 2; TNF-α= Tumor necrosis factor-α; IFN-γ= Interferon-γ; IL-17= Interleukin-17; $T_H$= T helper; Treg= Regulatory T cell; RvD1= Resolvin D1.

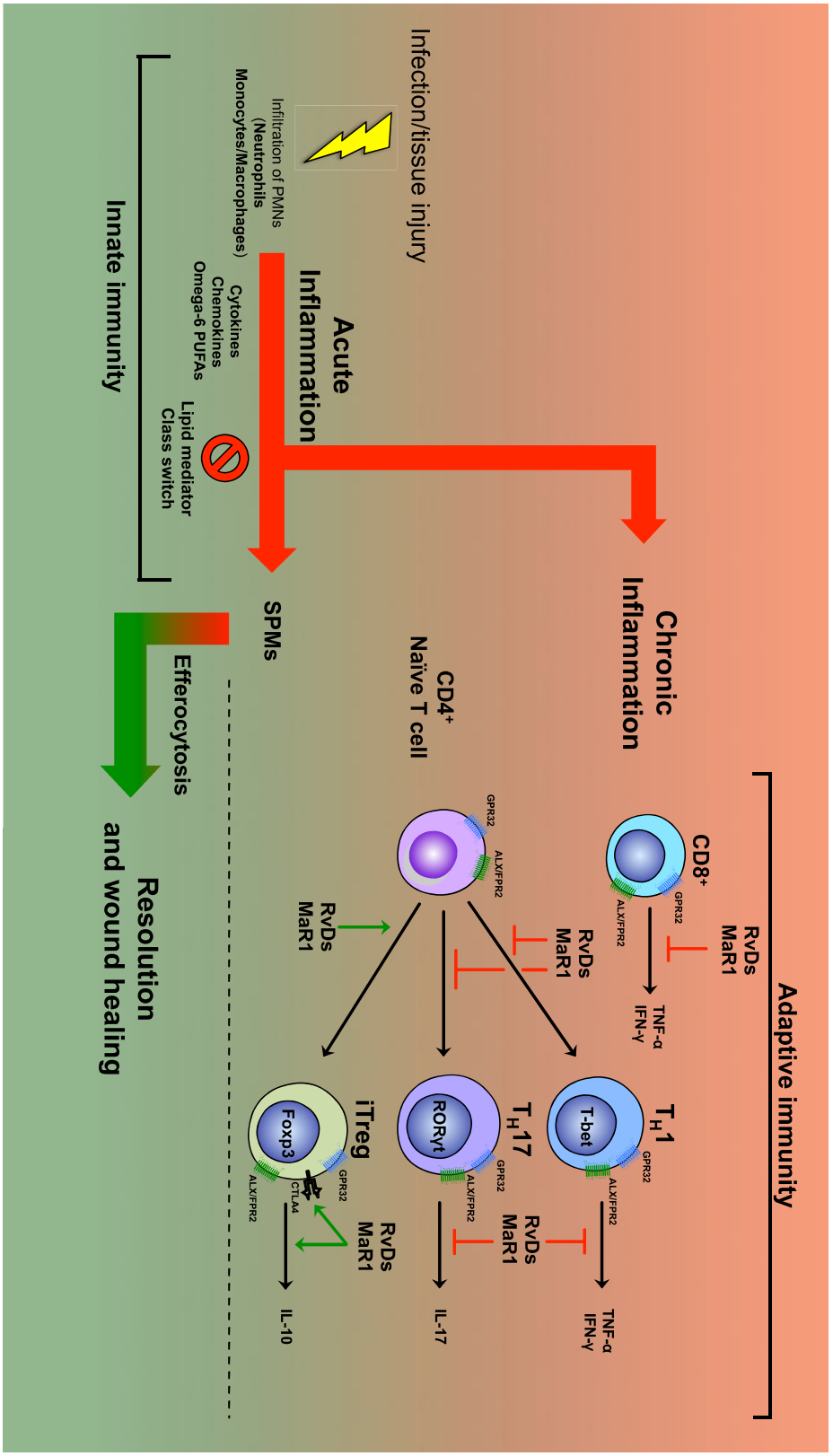


**Fig. 8** *Immunomodulatory effects elicited by SPMs on adaptive immune cells.* During acute inflammation, PMNs invade the site of inflammation and produce vast amounts of pro-inflammatory cytokines, chemokines and ω-6-derived lipid mediators (e.g. prostaglandins, leukotrienes, thromboxanes) that drive inflammation. Upon induction of resolution phase of inflammation, PMNs undergo a dramatic change in the lipid mediators they synthesize and start producing SPMs instead of prostanoids. These compounds act mostly by modulating innate immune responses, stopping neutrophil recruitment, promoting macrophage-mediated efferocytosis, removal of debris and return to tissue homeostasis. However we found that SPMs can also act on adaptive immune system by acting on T cell-dependent functions: indeed RvD1, RvD2 and MaR1 inhibit the development of $T_H1$ and $T_H17$ lymphocytes and the release of pro-inflammatory cytokines from $T_H1$, $T_H17$ and $CD8^+$ CTLs as well as enhancing all Treg-dependent suppressive functions in a GPR32- and/or ALX/FPR2-dependent fashion.

| Antibody | Manufacturer | Dilution |
|---|---|---|
| CD4 e780 | eBiosciences (San Diego, CA) | 1:100 |
| CD8 Brilliant Violet | Biolegend (San Diego, CA) | 1:100 |
| FasL PE | Miltenyi Biotec. (Bergisch Gladbach, Germany) | 1:60 |
| PD-1 APC | eBioscience | 1:50 |
| CTLA4 PE | Miltenyi Biotec. | 1:60 |
| TNF-α PE-Cy7 | eBiosciences | 1:100 |
| IFN-γ APC | eBiosciences | 1:100 |
| IL-17 PE | eBiosciences | 1:50 |
| IL-2 PercP5.5 | Biolegend | 1:50 |
| IL-4 BV421 | Biolegend | 1:50 |
| Granzyme FITC | eBioscience | 1:30 |

**Table 1** *Fluorochrome-conjugated antibodies used to characterize the immunophenotype of T cells, $T_H1$, $T_H2$, $T_H17$ and Treg and for surface and analysis of intracellular cytokine production*

| | Unstimulated | anti-CD3/CD28 | anti-CD3/CD28 + RvD1 | anti-CD3/CD28 + RvD2 | anti-CD3/CD28 + MaR1 |
|---|---|---|---|---|---|
| CD8+ | 59,3 ± 7,8 | 57,7 ± 5,8 | 58,9 ± 9,3 | 59,1 ± 8,8 | 58,7 ± 8,8 |
| CD4+ | 57,4 ± 8,9 | 63,1 ± 9,2 | 61,6 ± 9,1 | 55,6 ± 4,9 | 61,4 ± 6,3 |

**Table 2** *PD-1 expression expressed as Mean Fluorescence Intensity in T cells*

| | Unstimulated | anti-CD3/CD28 | anti-CD3/CD28 + RvD1 | anti-CD3/CD28 + RvD2 | anti-CD3/CD28 + MaR1 |
|---|---|---|---|---|---|
| CD8+ | 79,4 ± 8,1 | 78,6 ± 9,5 | 82,5 ± 4,3 | 85,7 ± 7,5 | 82,7 ± 9,6 |
| CD4+ | 81,2 ± 6,7 | 84,1 ± 5,7 | 79,3 ± 9,6 | 81,4 ± 7,7 | 77,2 ± 7,7 |

**Table 3** *CTLA4 expression expressed as Mean Fluorescence Intensity in T cells*

## VI. DISCUSSION

Since their first identification 15 years ago, SPMs have been shown to act as true initiators of resolution programs of acute inflammation by reducing granulocyte trafficking and the production of cytokines and reactive oxygen species, as well as by lowering the magnitude of the overall inflammatory response (**Serhan, 2014**). Although the role of SPMs is highly associated with the resolution of acute inflammation operated by cells of innate immunity, it is becoming increasingly clear that these bioactive lipids might take part also in the control of chronic inflammation possibly acting on cells of adaptive immunity. However, no evidence of a direct role of these resolving mediators on the distinct adaptive cell populations has been undertaken. Thus, in the present study, we have unprecedentedly investigated the adaptive immune responses to the SPMs. Cytotoxic CD8+ T (CTL) cells and CD4+ T-helper ($T_H$) cells are responsible for the orchestration of the adaptive immune response. CTL cells eliminate neoplastic, infected or damaged cells mainly through the release of cytotoxins (perforin, granzymes and granulysins) and potentiate innate immune responses (macrophages and NK cells) through the release of cytokines (IFN-γ, TNF-α) (**Barry and Bleackley, 2002**). The ability of SPMs to dampen cytokines from CTL cells suggest that the role of these bioactive lipids in resolving inflammation not only is exerted directly by clearing and blunting the responses of those innate immune cells during acute inflammation once they completed their function, but is also exerted indirectly by avoiding further recruitment or activation of innate cells, in order to avoid instauration of chronic inflammation or immune-mediated damage. Furthermore, CTL cells are also able to prime naïve and restimulate experienced CD4+ T cells to release great amounts

of helper cytokines (**Romagnoli et al., 2013**), suggesting a possible role for SPMs in regulating this novel functional interaction between CD8+ and CD4+ T cells. $T_H$ cells are arguably the most important cells in adaptive immunity, as they are required for almost all adaptive immune responses. They develop from naïve CD4+ T cells and differentiate into different $T_H$ subsets after encountering with foreign or auto- antigens. $T_H1$ cells, which produce interferon IFN-γ, are the immunity effectors against intracellular microbes; $T_H2$ cells, which secrete interleukin IL-4, IL-5 and IL-13, activate the response for clearance of helminthes and in hypersensibility-triggering immune responses, while $T_H17$ cells, producers of IL-17A and IL-17F, protect mucosa from any bacterial or fungal infection (**Steinman, 2007; Raphael et al., 2015**). However, persistent or uncontrolled $T_H$ cell responses are often associated with pathological states and tissue damage. In particular, excessive and/or abnormal $T_H1$ and $T_H17$ cell responses are involved in chronic inflammation and mediate several autoimmune diseases, including multiple sclerosis rheumatoid arthritis, and psoriasis (**Korn et al., 2009**). Our data reveal that SPMs, not only can directly modulate the inflammatory responses of already existing and activated $T_H1$ and $T_H17$ cells by hindering the production and release of effector cytokines, but they can also critically prevent their generation from naïve CD4+ T cells acting on their transcription factors-induced activation programs. Additionally, SPMs are able to enhance the differentiation of CD4+ T cells into Treg cells while boosting their immunomodulating abilities. This is of particular importance since this regulatory cell type, which typically serves to modulate and deactivate the excessive immune responses, plays an important role in preventing the over-activation of $T_H1$ and $T_H17$ cells. This result

is in line with the very recent report of MaR1 in engaging Treg cells in mice to promote resolution of lung inflammation (**Krishnamoorthy et al., 2015**). Although further studies are needed to verify the existence of an indirect modulation of $T_H1$ and $T_H17$ cells by a SPMs-mediated sustained induction of Treg cells, these findings suggest that SPMs might modulate inflammatory responses through pleiotropic mechanisms on distinct cells of the adaptive immunity. This hypothesis is conceivable, also on the light of the very recent discovery that $T_H17$ cells transdifferentiate into Treg cells during resolution of inflammation (**Gagliani et al., 2015**), where SPMs might be the possible executioners of such $T_H17$ instability and plasticity. Our evidences also fit with recent papers reporting a role for E-series resolvins in modulating antibody production in plasma cells (**Ramon et al., 2012**; **Kim et al., 2016**) as well as regulating $T_H1$ and $T_H17$ effector function in animal models of inflammatory diseases (**Haworth et al., 2008; Rajasagi et al., 2011**) and support the vision that portrays pro-resolving lipids as key regulators of adaptive immunity. On the other hand, $T_H2$ lymphocytes were substantially inert to SPMs treatment, in that none of the pro-resolving lipids we assayed was able to exert any sort of influence on the generation of mature effector cells or on cytokine release. Surprising as they may seem, these results are aligned with recent papers reporting that, in $T_H2$-driven pathologies and models, DHA-derived SPMs might ameliorate clinical outcome by acting on different targets than $T_H2$ cells (**Barnig et al., 2013; Basil and Levy, 2016**), in order to modulate immune responses; this is supported by the fact that in these works, neither RvD1 nor PD1 could act on the levels of IL-4, $T_H2$ signature cytokine, in mouse models of asthma, while only being able to dampen IL-5 and IL-13 production,

(**Levy et al., 2007; Rogerio et al., 2012**); notably it's interesting to point out that, unlike D-series resolvins, EPA-derived SPMs can ameliorate the clinical phenotype of animal models of atopic dermatitis and asthmatic and asthmatic airway inflammation by influencing $T_H2$ function (**Aoki et al., 2010**; **Kim et al., 2012**), thus suggesting that, in these lymphocytes, SPM-mediated modulation might rely on an intricate mechanism that involves differential action of these immunoresolving lipids. The recent identification of SPMs in secondary lymphoid organs (lymph nodes and spleen) (**Colas et al., 2014**), where most of naïve-to-effector or iTreg differentiation happens, provides an in vivo relevance of our findings. Moreover, the important role for DHA-derived SPMs in the modulation of adaptive immune system is perfectly plausible on the light of the fact that all T cell subsets we analyzed expressed GPR32 and ALX/FPR2, which are known to bind RvD1; interestingly enough, however, while GPR32 was only upregulated in newly generated T cells that were able to respond to SPMs action, namely $T_H1$, $T_H17$ and iTreg, the expression of ALX/FPR2 remained constant in all tested populations: on one hand, in $T_H1$ and $T_H17$ subsets, both receptors revealed to be involved in RvD1 signaling, independently of their expression, while GPR32 appeared to be the only master regulator behind the effects of RvD1 on iTreg cells; conversely, $T_H2$, which failed to be modulated by SPMs, displayed no variation in the expression of any of these receptors, in respect with their $T_H0$ precursors; these evidences might imply that the differential changes in the expression of GPR32 or ALX/FPR2 during polarization, while being characteristic of each subsets, don't necessarily reflect the dependency of $T_H$ cell response to SPMs on that particular receptor. Very interestingly, RvD1 receptors were found to be expressed

on T cell surface regardless of their ability to intercede for RvD1 immunomodulatory effects; moreover, in all *de novo* generated T lymphocytes we analyzed, the amounts of expressed GPR32 and ALX/FPR2 were always at least comparable to those found in $T_H0$ lymphocytes, where, conversely, they were able to modulate polarization. This might imply that in SPMs-non-responsive cells such as $T_H2$, or in iTregs, where ALX/FPR2 seems to be fairly expressed even without mediating RvD1 actions, these GPCRs are kept inactive, possibly through covalent modifications or by reorganizing their membrane location. Taken together, the data we collected represent, to date, the first report of the expression of SPMs-engaging receptor on T helper population, as well as of their role as mediators of the effects of these immunoresolvent lipids on cell-mediated adaptive immunity; the picture depicted is a rather intricate one and implies that the mechanisms behind the immunomodulatory purposes of SPMs are convoluted and, probably, varying significantly between T cell subsets. Surely, the characterization of the full list of SPMs-binding receptors, and their expression pattern, will be essential to unravel the full array of modulating actions these pro-resolving compounds elicit on adaptive immune cells. Finally, the fact that SPMs preserve cell viability whilst modulating the pro-inflammatory response is of note, not only because it rules out the possibility that the observed immunomodulation of T cells is caused by SPMs-induced cell-death rather than a direct modulation on cell signaling, but it also represents a safe approach to avoid autoimmune reactions without affecting protective immune responses. The effects elicited on adaptive immunity by all the SPMs we tested, though displaying little variability, seem to actually follow a recurring scheme, since they all modulate T cell effector func-

tions toward resolution. As a matter of fact, SPMs are temporally regulated and each class is specifically produced by distinct innate immune cells (which also follow a temporal recruitment during inflammation) and as such, they are likely to oversee self-limitation of acute and chronic inflammation by acting in a time-dependent fashion on the different effectors that appear along the resolution of the different inflammatory processes (**Serhan, 2014; Basil and Levy, 2016; Winkler et al., 2016**). Of note, such redundancy of SPMs mirrors the well-known role of their pro-inflammatory counterpart, i.e. AA-derived eicosanoids, whereby a huge network of lipid mediators regulates a relatively limited number of functions. Furthermore, this suggests that molecular redundancy is indeed a well-conserved feature that evolution managed to shape in order to ensure cell- and time-specific regulation of physiological mechanisms (**Nowak et al., 1997**). As a matter of fact, current clinical research is increasingly directed to the possibility of interfering with the function of these $T_H$ cells, and the finding that natural endogenous compounds like SPMs exert a suppressive – but not cytotoxic – effect also on cells with a central role in the induction of autoimmunity, represents a promising beginning for a new avenue of research. Overall, this is the first evidence that SPMs act on cells of the adaptive compartment of immunity, being able to suppress highly pro-inflammatory T-lymphocytes, while potentiating *de novo* generation of regulatory T-lymphocytes, thus possibly acting on the balance between pathogenic $T_H1/T_H17$ and tolerogenic Treg cells, which is typically altered during chronic inflammatory diseases. This study, summarized in a simplified model in **Fig 8**, further extends the original paradigm whereby the SPMs found in the resolved exudate not only stimulate signs of resolution - ending acute inflamma-

tion and restoring homeostasis - but also can truly signal to adaptive immunity, eventually inhibiting chronic inflammation.

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