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IMMUNOGENICITY DURING THE TREATMENT OF CHRONIC RHEUMATIC DISEASES: FOCUS ON TH9 LYMPHOCYTES

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Coordinatore: *Ch.mo Prof. Gaetano Thiene*
Supervisore: *Ch.mo Prof. Andrea Doria*
Co-Supervisore: *Ch.mo Prof. Piercarlo Sarzi Puttini*

Dottorando: *Rossella Talotta*

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Abstract

Background

Rheumatoid arthritis (RA) is an autoimmune chronic disease characterized by inflammation of peripheral joints with a various degree of systemic involvement. The pathogenesis is partly understood. Adaptive immunity plays indeed a pivotal role in inducing and maintaining the inflammatory process. Several cells belonging to the adaptive immune system have been associated to specific histological synovial patterns and clinical findings; among these, T helper (Th) lymphocytes have been exhaustively studied in RA due to their capability of producing cytokines and chemokines, migrating into articular sites and activating other immune or resident cells.

The pool of Th cells comprehends many subsets, each of which plays a precise role in inducing, tuning and repressing the immune response. Over the past twenty years, five distinct Th cell populations, properly named Th1, Th2, Th17, Th22 and Th9 cells, along with a counterpart of T cells with immune-repressive properties (T regulatory cells) have been described and characterized.

Th9 cells develop under stimulation with Tissue Growth Factor-beta (TGF-β) and Interleukin-4 (IL-4) either from naïve or primed Th lymphocytes. They prevalently synthetize IL-9, but, in vitro, the production of IL-10, IL-17, IL-21 and IL-22 has been also reported.

Th9 lymphocytes seem to be involved in the immunological responses underlying parasitic infections and allergic diseases. Neutralization of IL-9 worsens the symptomatic course of infestations while ameliorating the allergic manifestations. Some authors have demonstrated that Th9 cells take part to the pathogenesis of experimental autoimmune encephalomyelitis, systemic lupus erythematosus, systemic

sclerosis, psoriatic arthritis and RA. Th9 lymphocytes are increased in the bloodstream and in the synovial membranes of RA patients, being their percentage directly related to the degree of lymphoid organisation and to the production of autoantibodies, like anti-citrullinated peptide antibodies (ACPAs). However, it is unclear whether Th9 lymphocytes could be involved in the response to the therapy, or in the immunogenicity of biologic agents.

Aim

Primary objective: to evaluate the prevalence of Th9 lymphocytes in the peripheral blood of RA patients, assigned or not to an immunosuppressant treatment (including conventional drugs and infliximab), and to assess the immunogenicity of infliximab by detecting changes in Th9 percentages following an *in vitro* stimulation test.

Secondary objective: to compare the Th9-related immunogenicity of infliximab originator with that of its biosimilar compound (CT-P13, Remsima®), and to evaluate the influence of demographic and clinical features and concomitant medications on Th9 percentages.

Methods

We collected peripheral blood mononuclear cells (PBMCs) from 55 consecutive RA outpatients according to ACR/EULAR 2010 criteria and 10 healthy controls. We enrolled 15 subjects affected by RA not treated with immunosuppressive drugs, 20 patients successfully treated with branded infliximab, and 20 patients who discontinued branded infliximab due to adverse events or inefficacy. Allowed drugs included prednisone (≤ 10 mg/day), methotrexate (≤ 15 mg/week), sulphasalazine (≤ 3 g/day), hydroxychloroquine (≤ 400 mg/day) and, in the group of non responder patients, intravenous (i.v.) abatacept (10 mg/kg every 4 weeks), i.v. tocilizumab (8

mg/kg every 4 weeks), subcutaneous (s.c.) etanercept (50 mg once a week) and s.c. certolizumab pegol (200 mg every other week). The PBMCs were cultured with/without 50 μg/mL infliximab originator (Remicade®) or 50 μg/mL infliximab biosimilar (Remsima®), 50 μg/mL Human IgG1kappa and 50 μg/mL recombinant Human IgG Fc for 18 hours, and the percentage of Th9 cells was assessed by means of flow cytometry. Th9 lymphocytes were firstly identified as IFNγ-, IL-4-, IL-17-, IL-9-secreting CD4+ T cells, and, in a second time, as PU.1+, IRF4+, IL-9+ CD4+ cells. Furthermore, the markers CCR7 and CD45RA were used to distinguish naïve from memory IL-9-producer cells.

Results

In unstimulated condition, untreated RA patients showed the highest percentages of Th9 lymphocytes, either assessed according to cytokine or transcriptional profile, which was also higher in overall RA patients than in healthy controls.

The higher frequency of Th9 cells in RA patients was not associated with higher levels of anti-nuclear autoantibodies or other autoantibody subsets, or with a higher likelihood of experiencing an adverse event or lack of efficacy on infliximab treatment.

The percentage of PU.1+, IRF4+ Th9 cells, but not that of IL-9+, IFNγ-, IL-4-, IL-17- CD4+ cells, increased following the exposure to branded infliximab in the group of non responder RA patients, although these data were not confirmed after biosimilar infliximab exposure.

Furthermore, when IL-9 producing T cells were subdivided according to the expression of the markers CD45RA and CCR7, CCR7+, CD45RA- central memory and CCR7-, CD45RA- effector memory IL-9-producer lymphocytes increased in non responder RA patients after branded infliximab exposure, whereas CCR7+, CD45RA+

naïve and CCR7-, CD45+ terminal effector memory Th9 cells, although being more represented in RA patients than in healthy subjects, did not vary after drug stimulation.

In line with the previous experiment, the exposure to biosimilar infliximab did not induce an increase in the percentage of memory Th9 cell in non responder patients.

Conclusions

IL-9 levels are increased in RA patients, in whom this cytokine plays indeed a crucial role. Th9 cells are the major producers of IL-9, and their prevalence is higher in RA patients than in healthy subjects. According to our results, PU.1+, IRF4+ Th9 cells may be involved in orchestrating the immune response against the epitopes of branded infliximab; and this condition could rely on the recall and stimulation of both central and effector memory cells. On the other hand, biosimilar infliximab seems not able to activate these pools of cells. However, no significant difference was noticed in the PU.1+, IRF4+ Th9 cell percentages in Remicade®-responder patients after stimulation test either with biosimilar and branded infliximab, proving that *in vitro* both the two drugs seem to have a comparable efficacy.

Our results carry a novel point of view in the immunogenicity of anti-TNF agents, routinely based on the detection of anti-drug antibodies. However, since actual knowledge is still scarce, these data, highlighting a discrepancy between the Th9-driven immunogenicity of branded and biosimilar infliximab, indeed deserve further investigations.

- Chapter 1-

Immunogenicity of biologic drugs

1.1 Biologic drugs: properties, characteristics, beneficial and side effects

Biologic agents represent a revolutionary class of drugs, obtained by means of genetic engineering, commercially available since latest '90s. These drugs may be produced using the recombinant DNA technique that allows the introduction of a genetic sequence inside the genome of cultured cells, or by the development of *ibridomes*, consisting in murine B lymphocytes that share common aspects with neoplastic plasma cells, and are able to produce huge amounts of monoclonal antibodies reacting against specific antigens.

Specifically biologic drugs may be subdivided into 3 groups:

1) Proteins, obtained by means of recombinant DNA technique

2) Monoclonal antibodies, recognizing and neutralizing specific antigens (*ab*-ending)

3) Fusion proteins (*cept*-ending) that consist in soluble receptors, recognizing specific ligandos, joined to the fragment crystallizable region (Fc region) of an immunoglobulin G (IgG)

The last two classes include nearly the entire category of biologic drugs used for the treatment of rheumatic diseases.

Indeed the most important advantage in using these drugs is the high specificity in recognizing molecules usually involved in the inflammatory cascade, thus preventing further repercussions on other cells or organs.

Currently, rheumatologists have at disposition for the treatment of rheumatoid arthritis (RA) seven biologic drugs against the cytokine Tumor Necrosis Factor-alpha (TNFα) and therefore called anti-TNF agents; one directed against the B cells surface

antigen CD20; a receptor antagonist of the interleukin (IL)-1; an antagonist of the receptor of the IL-6; and a receptor fusion protein displaying a Cytotoxic T Lymphocyte Antigen-4 domain activity (CTLA4).

In the next years an increase in the availability of biosimilar drugs and drugs with other mechanisms of action (e.g. against IL-17) or with different molecular properties is expected.

These drugs have dramatically changed the prognosis of rheumatic diseases, leading to fair rates of clinical remission; however, the occurrence of adverse events such as infectious diseases or cancers arises the need for a strict surveillance of the patients undergoing this kind of treatment. Furthermore, there is 20-30% likelihood of non-response either based on a primary (genetic) resistance or on the development of an immune response directed against the drugs themselves, which may behave as antigens [1].

1.1.1 Anti-TNF agents

Anti-TNF agents include a class of biologic drugs directed against the cytokine TNFα and currently licensed for the use in RA, psoriasis and psoriatic arthritis (PSA), ankylosing spondylitis (AS) or non radiographic spondyloarthritis (SpA), intestinal bowel diseases (IBD), juvenile idiopathic arthritis (JIA) and uveitis. These compounds comprehend three entire monoclonal antibodies or moAbs (infliximab; adalimumab; golimumab), one fusion receptor protein (etanercept) and a FAB fragment conjugated to a pegylated substrate (certolizumab pegol).

All these agents share the capability of binding and neutralizing the cytokine TNFα, even though some differences occur in the molecular structures, the route and timing of administration, the spectrum of action, and the safety profile. TNFα is a cytokine notoriously associated to septic shock related to the exposure to lipopolysaccharide

(LPS) [2], but it also plays a prominent role in autoimmune diseases. TNFα belongs to the large super-family of TNF/TNF receptors (TNFRs) characterized by a trimeric structure and participating in the survival and activation of cells belonging to the immune system [3].

TNFα is mainly synthesized by macrophages and monocytes in trans-membrane form (tmTNFα), part of which is then cleaved in soluble form (sTNFα) by a metallo-protease known as TACE. Both tmTNFα and sTNFα bind two receptors: TNFR1 and TNFR2. The first is expressed on almost all cells and mediates proliferative or pro-apoptotic signals through its TRAF Interaction Domain (TIM) and Death Domain (DD); the second, which has been less extensively characterised, is carried by nerve, endothelial and immune system cells and, as it does not have a death domain, seems to be mainly involved in the proliferative pathway [4,5]. The biological effects of TNFα on target cells (ranging from survival to apoptosis) explain many of the biological implications of anti-TNF treatment [6]. TNFα induces the activation of neutrophils, macrophages, monocytes, chondroclasts, osteoblasts, osteoclasts, synoviocytes, hepatic and endothelial cells, thus weaving a complex network that leads to synovial membrane hyperplasia, bone destruction and systemic inflammation. Most of these effects are induced by TNFα as a ligand, but tmTNF may also act as a receptor and carry pro-inflammatory signals inside the cells that bear it, including T cells, macrophages and Natural Killer (NK) cells. The individual anti-TNF drugs have different biological activities in contrasting the effects of tm and sTNFα. Being bivalent moAbs, infliximab, adalimumab and golimumab can bind simultaneously two different molecules of TNFα in both monomeric and trimeric form. Moreover, together with etanercept, they share a Fc that is responsible for antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC), which are mediated by monocytes or macrophages and NK cells after interactions

between the Fc and Fc receptors (FcRs). At the same time, the interaction with FcRII and FcRIII (which recognise very large immune complexes such as those containing bivalent moAbs) may also mediate a state of immune tolerance as a result of monocyte/macrophage hyper-production of tolerant cytokines, such as IL-10 [7]. Certolizumab pegol is devoid of a Fc and therefore unable to induce ADCC or CDC; however, it has greater avidity for tmTNF than the other anti-TNF agents, and may prevent the activation of cells by means of reverse signalling. Some authors have demonstrated that the interaction of anti-TNF moAbs with tmTNF may induce apoptosis via P53 and P21 activation, or the generation of reactive oxygen species (ROS), with golimumab having the weakest activity [8]. Anti-TNF agents can have different effects on cytokine production: some studies *in vitro* have found that the production of IL-6, IL-10 and IL-5 is increased in peripheral mononuclear cells incubated with infliximab but not with etanercept [9], and a study of RA patients found a rebalancing of IL-4 and interferon-gamma (IFNγ) in peripheral blood mononuclear cells (PBMCs) after exposure to infliximab [10]. The different biological properties of TNF-inhibitors may explain why moAbs seem to be more efficient in counteracting granulomatous diseases such as Crohn's disease or uveitis, and less safe during granulomatous infections such as tuberculosis [11,12,13].

Due to their mechanism of action, the treatment with anti-TNF agents has been mainly associated to the risk of developing opportunistic infections [14]. Another infrequent event is represented by the development of anti-nuclear antibodies (ANAs) or other autoantibodies [15], which may sometimes be associated with the clinical manifestations of connective tissue diseases. It is uncertain the role of anti-TNF agents in the induction and progression of cancer [16,17,18], therefore their use is currently not recommended in neoplastic patients.

1.1.2 Non anti-TNF biologic drugs

Beyond TNFα, other pathways are implicated in the inflammatory cascade operating in rheumatoid arthritis and have represented, therefore, valid goals for the development of biologic agents with a different mechanism of action.

This class of drugs includes:

- Anakinra, a receptor antagonist contrasting the signalling of IL-1, currently approved for the treatment of RA and autoinflammatory diseases
- Rituximab, a moAb directed against the B cells surface antigen CD20 [19] and licensed for the treatment of RA and small vessel vasculitis
- Abatacept, a fusion protein containing the CTLA4 domain attached to an IgG1Fc [20], that prevent the costimulation of T-lymphocytes and has been licensed for the use in RA and JIA patients
- Tocilizumab, a moAb targeting IL-6 receptors (IL-6Rs) that has been licensed for use patients with moderate to severe RA.

Non anti-TNF agents may therefore counteract the inflammatory cascade at various steps. Both tocilizumab and rituximab, and indirectly abatacept, may impede the activation of B lymphocytes. B cells act either as antigen presenting cells (APCs) and effector cells by secreting different cytokines, and thus directing the development of final T-helper (Th) phenotypes. B cells progressing to plasma cells can also produce antibodies whose biological effects range from antigen neutralisation to the activation of other cells through the interaction with FcRs, CDC and ADCC, and may favour the formation of immune complexes. This indicates that B lymphocytes are involved in almost all of the pathogenic phases of RA. CD20, is a trans-membrane calcium channel protein, expressed on plasma membranes from the stage of pro-B cells to that of mature B cells [21]. By binding CD20, Rituximab induces the death of B cells by means of CDC and ADCC, and may also induce apoptosis as

a result of an increase in the concentration of intracellular calcium ions and the activation of caspases, or alterations in mitochondrial trans-membrane potential and the generation of ROS [22].

IL-6 is a cytokine with a broad spectrum of action. It directly binds IL-6 receptors on myeloid cells, hepatocytes, synoviocytes, endothelial cells and osteoclasts, and the soluble form can create a complex with a trans-membrane protein called gp 130 that is found on the surface of many other cells [23]. By the activation of a complex intracellular pathway involving the phosphorylation of many protein kinases, IL-6 promotes Th2, Th follicular and Th17 lymphocyte survival, enhances the expression of adhesion molecules and the production of pro-angiogenic factors by endothelial cells, increases the expansion of synoviocytes and osteoclasts, and favours the synthesis of antibodies and the differentiation of megakaryocytes. Together with IL-1 and TNFα, it also significantly contributes to systemic inflammation as a result of the hepatic release of many acute-phase proteins, such as C-reactive protein (CRP).

The activation of T lymphocytes may be repressed by abatacept. By preventing the co-stimulatory mechanisms that occur during the activation of naïve T lymphocytes [24], abatacept induces clonal anergy and represses the transcription of genes encoding IL-2 and its receptor, CD25, CD68, cyclin D, and various cyclin-dependent kinases [25]. Abatacept acts at the beginning of the inflammatory cascade providing a negative signal that allows immune tolerance towards self-antigens in peripheral lymphoid organs. The CD80-CD86/CD28-CTLA4 complex acts in fact during the priming of naïve T cells, whereas other molecules such as ICOS or CD40 may be more important in the recall phases [26]. This condition is mirrored by the higher disease activity score on 28 joints (DAS28) remission rates in early RA patients than in long-lasting ones treated with abatacept for 1 year [27]. CTLA4 may also mediate a signal inside cells carrying CD80 or CD86 molecules, the effects of which include

the induction of tryptophan metabolism by the enzyme indolamine 2,3 dioxygenase (IDO) to the detriment of T cells [28], the reduced expression of adhesion molecules by endothelial cells [29], and impaired cytoskeletal organisation in macrophages [30].

Efficacy and safety data from registries and randomized controlled trials (RTCs) have shown a comparable profile between anti-TNF and non anti-TNF agents, although some slight differences may occur regarding the risk of opportunistic infections, autoimmune manifestations or cancer risk [31,32,33,34,35,36,37,38,39,40,41,42,43].

1.1.3 Biosimilar biologic drugs

According to World Health Organisation (WHO) definition, a biosimilar drug consists in a "biotherapeutic product that is similar in terms of quality, safety, efficacy to an already licensed reference biotherapeutic product" [44]. The use of biosimilar drugs has recently gained interest in consideration of the cost-sparing effect (about 20-30% saved compared to the reference product) and the patent expiry for many of the biologic drugs currently used for the treatment of rheumatic diseases. Currently, only infliximab (Remicade®) has expired its license, therefore the use of biosimilar drugs with the same therapeutic indications of infliximab has been licensed in USA and Europe, after successfully passing a careful examination on the comparability of the efficacy and safety profile.

In fact, before approving the commercialisation of a biosimilar product, the European Medicines Agency (EMA) requires a full report on physic-chemical characterization, *in vitro* and *in vivo* biologic activities of the new compound, and a demonstration of comparable efficacy and safety in patient population.

Two RCTs (PLANETRA and PLANETAS) have been conduced respectively on RA and AS patients in order to compare infliximab and its biosimilar CT-P13. Both the two trials led to the conclusion that biosimilar infliximab shares an overall comparable profile with the reference product [45,46]. According to these studies, EMA approved the extrapolation of the use of CT-P13 for each clinical indication for which infliximab has already been approved.

1.1.4 Recommendations for the use of biologic drugs in rheumatoid arthritis

The treatment of RA has been revolutionised since the introduction of biologic drugs that allow a fast symptoms remission, prevent disability and radiographic progression and ameliorate the prognosis. However, given the availability of several molecules with different targets, the current evidences for the best therapeutic strategy is still a matter of debate. Indeed RA patients should be early diagnosed and treated in accordance with the *treat-to-target* strategy [47]. The updated 2013 European League Against Rheumatism (EULAR) guidelines for the management of RA with synthetic and biological disease modifying anti-rheumatic drugs (DMARDs), suggest the use of biologic agents after the failure of a previous conventional line, without no preference for anti-TNF or non anti-TNF agents abatacept and tocilizumab as first or second line. The use of rituximab in first line may be taken in consideration in patients with a recent history of lymphoma or tuberculosis, whereas the use of anakinra has not been encouraged due to an inferior efficacy profile [48]. The 2015 American College of Rheumatology (ACR) guidelines approved the use of both anti-TNF and non anti-TNF agents in early (< 6 months) and established RA patients failing a previous conventional line, and suggest the swap to a non anti-TNF biologic in patients failing a previous anti-TNF agent, either in 2° or in 3° line [49]. Interestingly, both the

European and the American guidelines encourage the discontinuation of biologic therapies once the remission has been achieved and long maintained.

1.2 Generalities on the immunogenicity of biologic drugs

The immunogenicity of a drug consists in the faculty of inducing an immune-mediated response against the drug itself. This condition is related to the presence of epitopes in the molecular structure of the drug carrying an antigenic potential and capable, therefore, of activating specific T and B effector responses [15]. The biologic MoAbs are synthetized in murine cells and contain some foreign aminoacidic sequences that display a high antigenic potential. Therefore, these molecules undergo several modifications in order to minimize the antigenic burden due to allotypic components. Infliximab and its biosimilar compounds display the highest immunogenicity among other anti-rheumatic biologic agents, due to the 25% murine components in their structure, that may induce the formation of antibodies against the murine complementary and framework regions of the MoAbs [50]. The other biologic drugs, despite being modified and humanised, may retain variable percentages of immunogenicity, underlining the concept that other factors may influence the immunogenicity of a compound.

Some of these factors may belong to the patient whereas others depend on the drug itself [51].

Patients-related factors include the immunologic status, the genetic background, the age of the patient, the underlying disease and concomitant medications.

The immunologic status conditions the response to an antigen and is often dictated by a favourable genetic background and amplified by the activity of the underlying disease. Polymorphic variants in the Major Histocompatibility Complex (MHC) may influence the presentation of epitopes to T lymphocytes, thus unbalancing the

immunologic tolerance. Previous infection in MHC-predisposed patients may trigger a cross-reaction with biologic drugs. *Andrick et al.* have supposed a molecular mimicry between the influenza hemoagglutinin antigen and anti-TNF moAbs in HLA-DR1 RA patients [52].

The age of the patient may also affect the immune response towards antigens, including drugs. Paediatric patients affected by JIA have a five-fold higher risk of developing anti-drug antibodies (ADAs) against infliximab compared to RA adult patients receiving intravenous (i.v.) infliximab 3 mg/kg plus methotrexate, and the latter patients have a two-fold higher risk of developing ADAs when compared to psoriatic arthritis patients receiving i.v. infliximab 5 mg/kg. These data show that the underlying rheumatic disease as well as the administered dose may also affect the immune response to a drug.

The concomitant use of methotrexate or other immunosuppressive drugs reduces the risk of ADAs. In a controlled randomized study on Crohn's disease patients, the premedication with i.v. hydrocortisone before the first infusion of infliximab approximately reduced the risk of ADAs from 42% to 26%, ameliorating also the clinical outcome. Similarly, in those patients, the concomitant use of azathioprine may reduce the formation of ADAs. However, in RA, steroid premedication and the use of other immunosuppressive drugs than methotrexate seem to be not successful in preventing immunogenicity [53].

The drug-related factors include the molecular structure, the route of delivery, the interval of administration, the exposure time, the cross-reactivity with previously used drugs, and the post-translational changes.

MoAbs are usually more immunogenic than receptor proteins. MoAbs directed against antigens sited on the cell surface are more immunogenic than those

recognizing a soluble ligando, perhaps due to the subsequent process of internalization of the complex and presentation to target cells.

The immunogenicity of a compound largely depends on the percentage of allotypic sequences on its molecular structure. Therefore, infliximab displays the highest immunogenicity among biologic drugs, as the murine sequences account for the 25% of the entire molecule. However, humanised moAbs, such as adalimumab, may have a surprisingly high immunogenicity, probably related to the post-translational modifications (glycosylation, deamidation, oxidation, formation of disulphide bridges, and protein folding) or to the formation of anti-idiotype antibodies. During the humanisation process, in fact, the complementarity determining regions (CDRs) of the variable framework maintain the original murine aminoacidic region and may represent a source of epitopes for CD4+ T cells [54].

The route of delivery and the interval between two consecutive administrations represent two other important factors that can influence the immunogenicity of a drug. Subcutaneous administration may paradoxically arise a higher amount of immune reject due to the abundance of antigen-presenting cells in the site of delivery. The intermittent exposure, as well as a long-term exposure, represents also an aggravating factor for the development of ADAs.

Cross-reactivity with previous drugs has gained a noteworthy interest in the switch among similar biologic drugs, like anti-TNF agents. There are no evidences that anti-TNF drugs belonging to different classes have cross-reactive epitopes [50]. On the contrary, it has been demonstrated that biosimilar compounds of infliximab may cross-react with the originator drug [55]. However, the immunogenicity of a biosimilar drug may not match entirely that of the original drug, mostly due to differences in post-translational modifications [56].

Finally, the assay chosen for the detection of ADAs may influence the amount of antibodies detected, been radio immunoprecipitation assay (RIA) a more robust but also a more expensive test than enzyme linked immunosorbent assay (ELISA). ADAs may belong to different isotypic classes, including IgE or IgM as demonstrated by *Vultaggio et al*. [57]. Moreover, the concentration of ADAs may fluctuate according to the time of serum collection. A specific ADAs limen in predicting the tolerability of infliximab is still controversial, although *Baert et al.* postulated that a serum concentration $\geq$ 8 μg/mL is associated with 2.4 RR of developing an adverse event [58,59]. Therefore, the serum concentration of the free drug, usually lower in patients developing ADAs towards a biologic treatment, has replaced the measurement of ADAs.

Furthermore, beyond the stimulation of B cells and the subsequent production of ADAs, the activation of other immunologic pathways in response to a biologic drug may account for an alternative point of view of the immunogenicity phenomenon. There are some reports in literature that focus on the activation of CD4+, CD8+ T cells, NK cells and monocytes in the skin biopsy specimens of rheumatic patients developing a cutaneous exanthema following the administration of i.v. infliximab [60]. In a recent study, we demonstrated that the addition *in vitro* of 50 μg/mL infliximab to PBMCs cultures from RA patients who had or not responded to infliximab, altered the balance Th1-Th17/T regulatory (Treg) lymphocytes in favour of Th1-Th17 cells in non responder patients and of Treg lymphocytes in responder ones [61]. Further studies are indeed requested to elucidate all the possible immunologic pathways, along with the related different clinical implications, involved in the immune response against a drug.

1.2.1 The immunogenicity of biosimilar drugs

According to WHO definition a biosimilar drug should have a comparable safety profile to the reference product (RP).

Being branded infliximab and CT-P13 biosimilar but not identical, many *in vitro* tests, such as antibody conformational assays, have been performed in order to assess and fully elucidate their immunogenicity. These experiments have shown that the immune-dominant structures are similar between the two compounds. The two RCTs PLANETRA and PLANETAS, respectively on RA and AS patients, have demonstrated a comparable percentage of anti-drug antibodies (ADAs), measured by means of electrochemiluminescence, both in the reference product and biosimilar arm (respectively 52.3% for CT-P13 and 49.5% for RP in RA, and 22.9% for CT-P13 and 26.7% for RP in SA) at week 54, and similar percentages were also maintained after switching from RP to CT-P13 in the extension phase at week 102 [45,46]. Similar results have been obtained in spontaneous trials on IBD as well as SpA patients, unless all these studies are characterized by a short period of observation [62,63,64]. *Ben-Horin et al.* have demonstrated that ADAs against infliximab from sera of IBD patients cross-reacted with CT-P13 but not with adalimumab *in vitro* [55]. Moreover, the authors found that in basal and unstimulated conditions, CT-P13 elicited a higher background signal than RP that became not significant only after monomer purification.

Original infliximab and biosimilar CT-P13 have a slightly different molecular structure. According to EMA report [65], CT-P13 shows less C-terminal lysine with a overall less basic charge [66], a higher amount of aggregates (however not exceeding 1% of the compound) and a higher amount of not-assembled forms than infliximab originator; furthermore, some differences in post-translational modifications such as a higher degree in oxidised and fucosylated variants have also been described. The

higher degree in fucosylation of CT-P13 has been particularly focused, according to the well-known interference of fucosyl residuals sited in the Fc with the FcγR (especially FCγRIIIa and FCγRIIIb) binding [67]. This condition, although apparently not affecting immunogenicity, may reduce the ADCC properties of the moAbs so that recently controlled fucosylation has become a goal in the field of onco-immunology in order to obtain the highest degree of ADCC with the lowest drug concentration [68]. EMA report assures that NK-dependent ADCC reactions, although seeming impaired *in vitro*, are restored *in vivo* by the coexistence of other serum factors. However fucosylation of Fc may also induce a change in steric conformation and in charge. It has been demonstrated, in fact, that infliximab displays at least 2 B cell epitopes in the Fc containing a glycosylated pattern [69]. Therefore, post-translational modifications may be at the basis of a different immunogenic profile between original and biosimilar drug.

- Chapter 2 -

The pool of T helper lymphocytes and T helper 9 cells

2.1 Generality on T helper lymphocytes and their role in rheumatoid arthritis

CD4+ T helper lymphocytes orchestrate the adaptive immune response to pathogens. Naïve CD4+ T cells interact by means of T Cell Receptors (TCRs) with APCs and are activated by costimulatory receptors and cytokines. According to the cytokine milieu, these cells may differentiate into many subsets. Originally it was supposed that CD4+ T cells could give raise to two different pools, named Th1 and Th2, the first involved in the defence against intracellular pathogens and the latter against extracellular ones. Nowadays it is well known that Th pools include other subsets, displaying both pro-inflammatory and anti-inflammatory properties [70].

Th1, Th2, Th17, Treg, Th9, Th22 and Th follicular cells represent CD4+ Th lymphocytes with different phenotypes, responsible for different immune responses.

Th1 lymphocytes differentiate in presence of IL-12, express the transcriptional factors T-bet, STAT1 and STAT4, and produce IFNγ, IL-2 and TNFα.

Th2 lymphocytes differentiate in presence of IL-4, express the transcriptional factors STAT6 and GATA-3 and produce IL-4, IL-5, IL-10 and IL-13.

Th17 cells require IL-6, Transforming Growth Factor-beta (TGFβ), IL-21 and IL-23 for their differentiation, express the transcriptional factor RORγt and produce mainly IL-17. This subset is highly expanded during autoimmune diseases and actively contributes to their pathogenesis.

Treg cells develop in response to TGFβ, express the transcriptional factor FOXp3 and produce IL-10 or TGFβ, repressing the inflammatory cascade and restoring the peripheral tolerance.

Th22 cells, developing following the stimulation with IL-6 and TNFα, represent a recently discovered subset that produces IL-22, IL-10 and TNFα with detrimental or favourable effects on inflammation. They express the transcriptional factor AHR and several fibroblast growth factors (FGFs).

Th follicular cells may develop from Th1, Th2, Th17 or Treg cells, express Bcl-6 and secrete IL-4 or IL-17 in the B-cell follicle, thus favouring the maturation of plasma cells and the production of autoantibodies.

Finally, Th9 lymphocytes designate another recently studied T helper subset, whose development is favoured by the combined action of TGFβ and IL-4. These cells express the transcriptional factors PU.1 and IRF4 and mainly synthesize IL-9. Th9 lymphocytes have been recently associated to the pathogenesis of rheumatic diseases.

T helper cells have a high plasticity and may differentiate from one subset to another one according to the cytokine milieu [71]. For example, Th1 cells may secrete IL-10 and Th2 cells may be induced to produce IFNγ or IL-9. Th17 cells may synthesize IL-4 and Treg cells may produce IL-17. These cells are therefore capable to reprogram their transcriptional factors to those owned at a naïve stage and re-differentiate into other subsets, prevalently during the early phase of differentiation.

2.2 Modification of T helper subsets according to concomitant treatment

The use of immunosuppressive drugs may influence the populations of CD4+ Th cells. Indeed many studies have reported an increased percentage of Th17 lymphocytes in the serum, synovial fluid and synovial membrane of patients with RA. Similarly, an increased number of Treg cells expressing both CD25 and FOXp3 has been reported in the synovial fluid and synovial membrane of patients with RA where they could counteract the inflammatory cascade [72]. Moreover, some authors have

described hybrid pools of CD4+ T cells, expressing both IL-17 and FOXp3 in the serum and synovial tissues of RA patients, underlining the concept that Treg cells may differentiate in a second moment into effector cells with perhaps more pathogenic properties than primary Th17 lymphocytes [73].

Treatment may influence the amount of T effector and regulatory cells. The use of corticosteroids in RA, for example, seems to reduce the Th2 responses while does not affect the Th17 ones, perhaps due to a multi-drug resistance [72]. Although methotrexate seems to have no effect in healthy subjects, in RA patients it may down-regulate Th17 responses and up-regulate Treg ones. Methotrexate reduces the transcription of IL-17 messenger RNA from PBMCs of RA patients in a dose-dependent manner, although it does not seem to influence the percentage of IL17+, CD4+ T cells [74]. The concomitant use of methotrexate and steroids in an *in vivo* experiment on RA patients reduced the percentage of Th17 cells sorted as CCR4+, CCR6+, CD4+ lymphocytes [75]. Similarly, hydroxychloroquine (HCQ) may interfere with the production of some cytokines such as IL-17, IL-6 and IL-22. *Oh et al.* have reported that in healthy subjects the use of sulphasalazine and leflunomide may reduce the expression of FOXp3 in Treg cells, repressing the inhibitory properties on CD25-, CD4+ T effector lymphocytes, without affecting their whole number, whereas methotrexate and infliximab do not [76]. On the contrary in RA patients other authors have reported that infliximab induces an increase in Treg cells, while reducing Th17 cells, especially in responder patients. The neutralisation of TNFα may variously affect the Th subsets. TNFα favours the development of Th17 lymphocytes and some *in vitro* experiments with infliximab have proved efficacious in reducing Th17 effector differentiation [77]. However, in a study on healthy subjects the administration of i.v. infliximab 10 μg/mL depressed the production of IFNγ by Th1 cells, with minor effects on Th17 cells [78]. In RA patients infliximab may have an opposite effect,

increasing the ratio IFNγ/IL-4 in responders and depressing Th2 responses [10]. In AS patients, following a 6 week treatment with infliximab, some authors have observed a discrepancy between the improved clinical outcomes and the T cell subsets, with a persistent prevalence of Th2 and Th17 lymphocytes at 6 weeks of therapy [79]. Contradictory results have reported with adalimumab. A recent work on psoriatic patients has reported a reduced concentration of circulating Th1, Th17 and Th22 lymphocytes following a 12 week treatment with adalimumab [80]. Etanercept seems not to influence the percentage of Th17 lymphocytes with contrasting results on CD4+, CD25+, FOXp3+ T cells. According to some authors, etanercept but not adalimumab nor infliximab normalizes the percentage of Th2 cells after 4 weeks of treatment [75]. No certain results are available on abatacept either regarding Th17 or Treg cells. In line with other conventional and biologic drugs, tocilizumab reduces the number of Th17 cells and induces an increase in Treg cells.

2.3 Th9 lymphocytes

Th9 cell, discovered in 2008 by *Veldhoen et al*., are a T helper cell subset developing from primary naïve T helper lymphocytes or from primed T helper 2 lymphocytes in presence of IL-4, TGFβ, OX40 and PU-1 [81]. The main cytokine secreted is IL-9, but, *in vitro*, these cells may also produce IL-10, IL-17, IL-21 and IL-22 [82]. It has been demonstrated that Th2 cells, Th17 cells, Treg cells, innate lymphoid cells (ILC), mast cells, NKs are capable of releasing large amount of IL-9 [83]. However, Th9 cells are a distinct class of CD4+ T lymphocytes, identified by a peculiar set of transcriptional factors (PU.1, IRF4). The role played by Th9 cells has been investigated *in vitro* and *in vivo*. Th9 lymphocytes seem to be involved in the immunological responses underlying parasitic infections as well as allergic diseases [84,85]. A consistent number of reports has evidenced that these cells are highly

represented in the airways of asthmatic subjects and in the skin of people suffering from atopic dermatitis [86,87]. Recent studies on melanoma carcinogenesis have evidenced that Th9 lymphocytes enhance the intra-tumor expression of chemokines and their receptors such as CCL20 and CCR6, thus promoting the local recruitment of dendritic cells and anti-tumor CD8+ T lymphocytes [88].

The role of Th9 cells in rheumatic diseases is less characterized. According to some studies, IL-9 may contemporarily favour the differentiation of Th17, acting like IL-6 or IL-21 in the presence of TGFβ, and inhibit the apoptosis of Treg cells, by enhancing the expression of STAT5 (*Fig.1*). Mice knock-out for IL-9R gene develop a more aggressive form of experimental autoimmune encephalomyelitis when compared to wild types, mainly due to an over-expansion of the Th1 lineage [89]. Similar results have been obtained in a recent report that analysed the cytokine interplay in human skin samples and murine models of atopic dermatitis, finding an indirect regulation of IL-9 on IFNγ expression [90]. On the other hand, IL-23 and IL-21 represent inhibiting cytokines for the development of Th9 lineage. IL-9 mRNA and IL-9R have been found significantly increased in gut specimens from ulcerative colitis patients, where this cytokine may activate neutrophils and epithelial cells, delaying ulcers healing [91]. In the gut, Th9 cells are involved in the defence against parasitic infections and an increase in this T helper subset may represent a link between dysbiosis and autoimmune inflammatory bowel diseases [84]. One study on connective tissue diseases reported increased serum levels of IL-9 in systemic sclerosis but not in systemic lupus erythematosus or dermatomyositis, being inversely correlated with the degree of lung fibrosis [92]. This result may be interpreted in the light of the pleiotropic effects of IL-9, promoting at the same time the expansion of Treg and Th17 lymphocytes [93].

To our knowledge, there are actually few studies on the involvement of IL-9 and IL-9 producer cells in RA. One study analysed the serum cytokine profile by means of multiple suspension array in six RA patients undergoing a treatment with rituximab after failing a previous therapy line with infliximab. The authors found increased levels of IL-9 at baseline and at follow-up times in responder patients and concluded that IL-9 may be considered as a predictive marker of response to rituximab [94]. Another study on 44 subjects showed an increased serum concentration of IL-9 in first-degree relatives of RA patients, which was associated to a higher ratio of autoantibody positivity, such as rheumatoid factor (RF) or anti-citrullinated peptide antibodies (ACPAs) [95]. A recent report on RA, PSA and osteoarthritis patients showed an augmented concentration of IL-9 in blood and synovial fluid of RA and PSA subjects than in osteoarthritis ones. Furthermore, the treatment of magnetically sorted synovial and blood CD3+T cells with recombinant IL-9 induced their expansion only in RA and PSA samples, underlining the hyper-expression of IL-9R on cells coming from patients suffering from autoimmune arthritis [96]. Finally, an elegant histological study on synovial membrane biopsies from RA patients, evidenced an augmented expression of Th9 lymphocytes in the most aggressive forms of disease and an association between these cells and the organisation of the lymphoid centres or the synthesis of ACPAs [97]

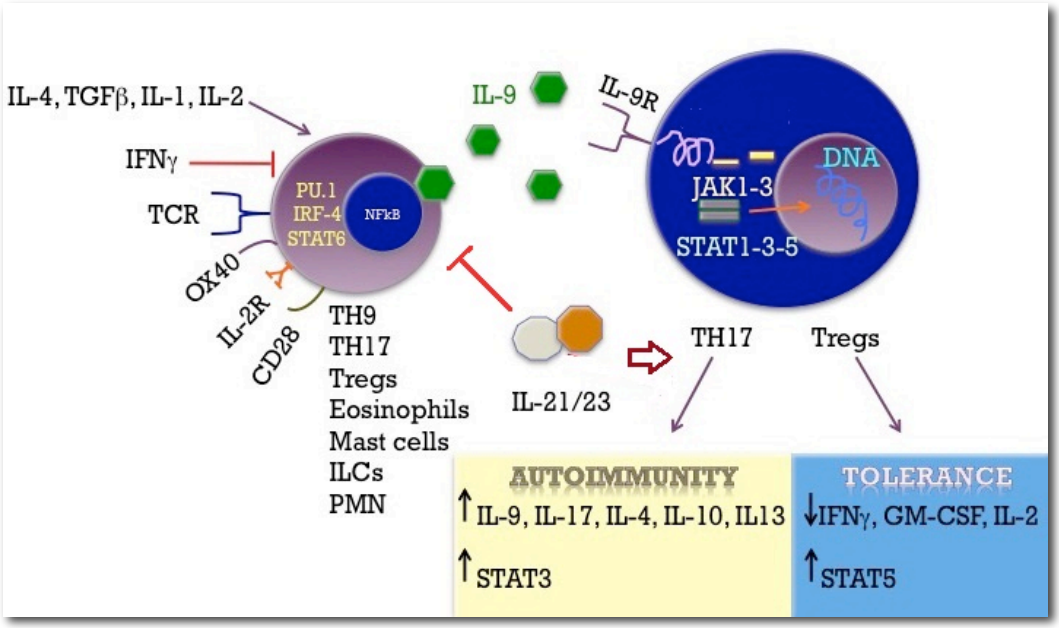

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Fig.1. The network of IL-9 and IL-9 producing cells. Th9 lymphocytes are induced following the stimulation with IL-4, TGFβ, IL-1, IL-2 and the interaction of TCR, OX40 and CD28 with their ligandos; on the contrary, IFNγ, IL-21 and IL-23 inhibit their differentiation. Th9 cells express specific transcriptional factors (PU.1 and IRF4) that favour the activation of NFkB. Beyond Th9 lymphocytes, Th17 cells, T regulatory cells, eosinophils, mast cells, innate lymphoid cells (ILCs) and polymorphonucleates (PMN) may produce IL-9. IL-9 interacts with its receptor (IL-9R), composed by a specific α- and a common γ-chain, thus activating an intracellular pathway that promotes the transcription of specific genes responsible for the survival of either Th17 (involved in autoimmunity) and T regulatory cells (involved in immune-tolerance).

- Chapter 3-

Th9 lymphocytes in rheumatoid arthritis: focus on immunogenicity during the treatment with anti-TNF agents

3.1 Rationale

Since Th9 lymphocytes have been associated to the pathogenesis of RA and other autoimmune diseases, we wondered whether these cells might also be involved in the immune response against biologic drugs. This consideration arose from an our previous study demonstrating that infliximab may repress or induce the proliferation of Th17 and Th1 lymphocytes when added *in vitro* to PBMCs from RA patients respectively responding or not to the therapy [61]. Along with these results, we also observed in the same experiment an overgrowth of CD25+, FOXp3+, CD4+ Treg cells in RA patients who achieved a good response under infliximab treatment, but not in those who had discontinued the treatment, where these cells were on the contrary reduced following the *in vitro* addition of infliximab. Th9 cells may be considered as connectors between autoimmunity and immune-tolerance, favouring at the same time Th17 and Treg responses. Th9 lymphocytes are associated to the production of antibodies and, in RA, to the titres of ACPAs and RF. The immunogenicity of a drug is mostly based on the production of antibodies against the epitopes of the drug itself. Accordingly, Th9 lymphocytes may help B cells in the production of ADAs, or tune Th17 or Treg drug-specific responses. To our knowledge, no study has still attempted to elucidate the role of Th9 cells in the immunogenicity of biologic drugs used for the treatment of RA.

According to the molecular structure and the high number of immunogenic epitopes, the i.v. anti-TNF moAb infliximab was considered the most suitable candidate for our intent and therefore chosen as both original and biosimilar product for the experiment.

3.2 Aim of the study

The aim of this study can be subdivided into the following objectives:

1) Primary objective: to evaluate the prevalence of Th9 lymphocytes in the peripheral blood of RA patients, assigned or not to an immunosuppressant treatment (including conventional drugs and infliximab), and to assess the immunogenicity of infliximab by detecting changes in Th9 percentages following an *in vitro* stimulation assay.
2) Secondary objective: to compare the Th9-related immunogenicity of infliximab originator and its biosimilar compound (Remsima®). Other secondary endpoints included the association between the variation in the Th9 cell percentage and the demographic and clinimetric features of the cohort, the use of concomitant drugs or the reason of infliximab discontinuation.

3.3 Methods

3.3.1 Population

We enrolled 55 consecutive RA outpatients according to ACR/EULAR 2010 criteria [98]. Patients were recruited from another previous study aiming to explore the Th1/Th17-driven immunogenicity of infliximab (Remicade®) [61]. This cohort included 15 subjects affected by RA not concurrently treated with immunosuppressive drugs, 20 patients successfully treated with branded infliximab, 20 patients who had switched or swapped from branded infliximab to other biologic drugs due to adverse events or inefficacy, and a matched control group of 10 healthy subjects. Patients and controls were consecutively enrolled for six months from June 2013 to December 2013.

Concurrent infections, atopic dermatitis, haematological disorders, a concomitant or recent treatment with leflunomide or cyclosporine, and vaccinations in the previous 2 months represented exclusion criteria, since these drugs or medical conditions could variously affect the Th cell pool [99,100,101,102,103].

The protocol was approved by our Local Ethic Committee and conduced in accordance with the Declaration of Helsinki. An informed consent was obtained from each participant before entering the study.

3.3.2 Laboratory

After giving an informed consent, 18 mL peripheral blood from each subject was collected into Vacutainer tubes containing EDTA (Becton Dickinson; Rutherford, NJ, USA). PBMCs were isolated by centrifugation on lymphocyte separation medium (Cedarlane Laboratories, Burlington, NC, USA). The number and viability of PBMCs were determined by an automatic cell counter, ADAM-MC (Digital-Bio, NanoEnTek Inc., Corea). PBMC viability was typically > 98%. Cell cultures were performed in RPMI 1640 plus Penicillin, Streptomycin, L-Glutamine and 10% pooled Human AB Serum (all from Euroclone, Siziano, Italy). PBMCs, at a concentration of 1×10^6 cells/mL were incubated for 18 hours with culture medium alone or in the presence of 50 μg/mL infliximab (Remicade®, Janssen Biologics, Leiden, Netherlands) or its biosimilar (Remsima®, Celltrion Healthcare, Budapest, Hungary), 50 μg/mL Human IgG1kappa (Sigma-Aldrich, Saint Louis, Mo, USA) or 50 μg/mL recombinant Human IgG Fc (R&D Systems, Minneapolis, MN, USA). PWM (Lectin from *Phytolacca Americana*; 1 μg/mL Sigma-Aldrich), was used as a positive control to evaluate the responsiveness of PBMCs. To facilitate co-stimulation, 1 μg/mL anti-human CD28 (R&D Systems, Minneapolis, MN, USA) was added to the cell cultures. Brefeldin A (10 μg/mL; Sigma-Aldrich) was added after the first three hours in order to inhibit cytokine secretion.

The percentage of Th9 lymphocytes was determined by flow cytometric analysis. In a former analysis, Th9 lymphocytes were identified as IFNγ-, IL-4-, IL-17-, IL-9 secreting CD4+ T cells [104]. In a second time, for a more precise identification of the Th9 subset, the co-expression of IL-9 and transcriptional factors PU.1 and IRF4 (which are required for

IL-9 production and Th9 development) was assessed. The percentage of OX40 expressing, IL-9 secreting CD4+ T cells was measured as well. IL-9 production by naïve CD4+ T lymphocytes (CCR7+, CD45RA+), central memory CD4+ T lymphocytes (CCR7+, CD45RA-), effector memory CD4+ T lymphocytes (CCR7-, CD45RA-), and terminal effector memory (TEMRA) CD4+ T lymphocytes (CCR7-, CD45RA+) was also evaluated. A concentration of infliximab of 50 μg/mL was chosen after setting a titration test with increasing concentrations of the drug and on the basis of the infliximab median serum concentrations one hour after infusion (peak serum concentration: 39.9-219.1 μg) [105]. The following antihuman monoclonal antibodies (mAbs) were used: CD4 PE-Cy7, IFNγ FITC and CD45RA FITC (Beckman Coulter Milan, Italy); IL17 A PerCP-Cy5.5 (Biolegend, San Diego, CA, USA); IRF4 PerCP-eFluor® and CD134 (OX40 FITC) (eBioscience, Diego, CA, USA); IL-9 APC, IL-4 PE, CCR7 PE, and PU.1 PE (R&D Systems). To evaluate the percentage of IL-9 secreting-PU.1 and IRF4 expressing-CD4+ T lymphocytes, PBMCs were incubated 15 minutes with the mAbs for the detection of cell surface antigens. Then cells were permeabilized 30 minutes at 4 °C with the Fixation/Permeabilization buffer (eBiosciences), and further stained for 30 minutes at 4°C with the antibodies for the detection of intracellular transcription factors and IL-9. For the evaluation of IL-9 production by naive and memory CD4+ T cells, PBMCs were incubated 15 minutes at RT with the mAbs for the detection of cell surface antigens and fixed with 1% PFA 15 minutes at 4 °C. Then cells were permeabilized with Saponin (Sigma) and stained with the antibodies for the detection of intracellular cytokines. Following a 45-minute incubation in ice, the cells were fixed with 1% PFA. Lymphocyte population was gated based on the basis of forward and side scatter properties, and further gated for CD4 expression; at least 20,000 events were acquired within the CD4 gate. The samples were acquired using a Gallios flow cytometer and data were analysed using Kaluza software (both Beckman Coulter).

3.3.3 Statistical analysis

As data were normally distributed, procedures were based on parametric analyses. Comparisons between the different groups were performed using unpaired Student's T Test for unequal variances with a two tailed P value. A multivariate analysis was carried to detect any interference of demographic characteristics and therapeutic regimens on Th9 percentages. Significance was set at $P < 0.05$. Statistical analysis was performed using GraphPad Prism Software (GraphPad Software, San Diego, CA, USA) and SPSS version 24.0 (International Business Machines Corporation, New York, NY, USA).

3.4 Results

3.4.1 *Baseline demographic and clinical assessment*

In the cohort of drug-naïve RA patients (14 Caucasian subjects and 1 Chinese subject), 12 were females, mean age ± standard deviation (SD) at the time of enrolment was 54.8 ±16.2 years, mean disease duration ± SD was 2.3 ± 3.9 years, ACPAs were detected in 5 patients, FR in 7 patients and ANAs in 2 patients. At the time of enrolment mean ± SD CRP-DAS28 was 4.65 ±1.0. All these patients were taking anti-inflammatory and analgesic drugs at need.

In the cohort of infliximab-good responder RA patients (19 Caucasian subjects and 1 Hispanic subject), 16 were females, mean age at the time of enrolment was 61.3 ± 12.2 years, mean disease duration 13.4 ± 7.2 years, ACPAs were positive in 15 patients, FR in 11 patients and ANAs in 12 patients. Anti-double stranded DNA (dsDNA), anticardiolipin (ACLAs) and anti-extractable nuclear antigen antibodies (ENAs) were detected respectively in 3, 1 and 1 patient. Patients have been treated with infliximab (Remicade®) for a mean ± SD of 8.3 ± 3.9 years, maintaining a good control on RA activity (mean ± SD CRP-DAS28 at the time of blood sampling 2.5 ±1.0). Concomitant medications consisted in prednisone (2.5-10 mg/day), methotrexate (5-15 mg/week) and hydroxychloroquine (200-400 mg/day) respectively in 8, 20, 3 patients.

In the cohort of infliximab-non responder RA patients (19 Caucasian subjects, 1 Indian subject) 15 were females, mean age at the time of enrolment was 57.0 ± 12.2 years, mean disease duration 18.1 ± 9.5 years, ACPAs were positive in 15 patients, FR in 15 patients and ANAs in 17 patients. Anti-dsDNA, ACLAs and anti-ENAs were detected respectively in 2, 3 and 2 patients. At the time of enrolment 13 patients were treated with i.v. abatacept 10 mg/kg every 4 weeks, 5 patients with i.v. tocilizumab 8 mg/kg every 4 weeks, 1 patient with s.c. etanercept 50 mg once a week, and 1 patient with s.c. certolizumab pegol 200 mg every other week, as second (8 patients), third (8 patients) or fourth (4 patients) biologic line. Patients had been treated with infliximab (Remicade®) for a mean ± SD of 2.4 ± 1.9 years, having discontinued the drug since a mean ± SD of 8.0 ± 2.5 years due to inefficacy (11 cases) or adverse events (mostly allergic or infusion reactions, 9 cases). Mean ± SD CRP-DAS28 at the time of enrolment was 2.9 ± 0.8. Concomitant conventional drugs included prednisone (2.5-10 mg/day) in 14 cases, methotrexate (5-15 mg/week) in 9 cases and hydroxychloroquine (200-400 mg/day) in 5 cases.

Demographic characteristics are resumed in table 1.

Variables	Healthy controls	Treatment-naïve RA patients	RA patients responding to IFX	RA patients not responding to IFX
No.	10	15	20	20
Mean age ± SD, years	43.9 ± 8.3	54.8 ±16.2	61.3 ± 12.2	57.0 ± 12.2
Mean disease duration ± SD, years	/	2.3 ± 3.9	13.4 ± 7.2	18.1 ± 9.5
F/M	4/6	12/3	16/4	15/5
ACPA +	/	5	15	15
RF +	/	7	11	15
ANA +	/	2	12	17
Anti-dsDNA Ab+	/	0	3	2
Anti-ENA Ab+	/	0	1	3
ACLA/LAC +	/	0	1	2
Prednisone (2.5-10 mg/day)	/	/	8	14
Methotrexate (5-15 mg/week)	/	/	20	9
Hydroxychloroquine (200-400 mg/day)	/	/	3	5
NSAIDs	/	14	as needed	as needed

Table 1. Demographic characteristics of the population included in the study. RA: rheumatoid arthritis; IFX: infliximab; SD: standard deviation: F: females; M: males; ACPA: anti-citrullinated-protein antibodies; RF: rheumatoid factor; ANA: anti-nuclear antibodies; anti-dsDNA: anti-double stranded DNA antibodies; anti-ENA: anti-extractable nuclear antigen antibodies; ACLA: anticardiolipin antibodies; LAC: lupus anticoagulant; NSAIDs: non-steroidal anti-inflammatory drugs.

3.4.2 Th9 pool at baseline

We conducted two separate experiments for the evaluation of Th9 cells. According to *Schlapbach et al*. [104], Th9 cells were initially isolated as IFNγ-, IL-4-, IL-17-, IL-9-secreting CD4+ T lymphocytes. At baseline, this pool was mostly increased in drug-naïve RA patients than in the other groups, with a more pronounced difference when compared to healthy controls ($p<0.01$) than to the group of infliximab responders ($p<0.05$); *fig. 2*.

In a second part of the experiment, we aimed to select Th9 cells according to the lineage-specific transcriptional factors since other immune cells may produce IL-9. To increase the specificity of our analysis we decided to evaluate PU.1 and IRF4, that represent the transcriptional factors associated with the Th9 pool; moreover, the costimulatory molecule OX-40, which could selectively drive the Th differentiation toward a Th9 phenotype while repressing both Treg and Th17 cells development [106], was considered in a separate experiment.

At baseline, the percentage of IFNγ-, IL-4-, IL-17-, IL-9+, CD4+ Th lymphocytes was significantly concordant with that of IL-9+, PU.1+, IRF4+, CD4+ Th cells ($p=0.01$). When identified by the expression of PU.1 and IRF4, Th9 cells were increased in drug-naïve patients compared to healthy controls and infliximab responder patients ($p<0.01$); *fig.6*. When sorting Th9 cells according to the expression of the costimulatory marker OX40, we found that OX40+ IL9-producing CD4+ T cells were increased in the group of drug-naïve RA patients, infliximab-responder and non responder patients compared to healthy controls; *fig. 11*.

The higher frequency of Th9 cells in RA patients was not associated with higher levels of ANAs or other autoantibody subsets, the duration of the disease, the CRP-DAS28 score at the enrolment time, neither to the reason of infliximab discontinuation or the number of previous biologic drugs administered to the non responder patients. Moreover, a multivariate analysis did not evidence a significant influence of concomitant conventional or biological treatment on Th9 percentages at baseline, although the heterogeneity of biological therapies and the limited number of cases could have indeed represented a bias in the statistical evaluation.

In conclusion, either evaluated by the solely production of IL-9 or by the concomitant expression of transcriptional or costimulatory factors, Th9 cells were mostly increased in unstimulated conditions in untreated RA patients with the highest difference when

compared to healthy controls than to the other RA groups. These results highlight that the activation of Th9 cells is a distinctive character of RA and may be restored by the concomitant treatments.

3.4.3 Effects of infliximab (Remicade®) on T helper 9 pool

The stimulation of IFNγ-, IL-4-, IL-17-, IL-9-secreting CD4+ T cells with 50 μg/mL original infliximab did not induce a variation in the percentage of these cells in any of the group of patients and controls, *fig.2*. These provisional results have been recently published [107]. On the other hand, PU.1+, IRF4+ Th9 cells increased following the stimulation with branded infliximab only in the group of non responder patients, *fig.3*. This different behaviour may be related to the heterogeneity of IL-9 producing CD4+ cells, which may include at some extent also regulatory cells. When sorted for specific transcriptional factors, Th9 cells were distinguished from other T cell subsets and this may account for the discrepancy in the results. No differences before and after infliximab exposure were noticed among OX40+, IL-9+, CD4+ T cells *(fig.11)* and these results may be explained in light of the widespread expression of OX40 on Th cell pool [106].

We further wondered whether Th9 lymphocytes could be activated following a specific stimulus on Th memory cells from patient who had discontinued the treatment in the past for inefficacy or an adverse event. Following an antigenic stimulation, in fact, central memory CCR7+, CD45RA- T cells may migrate from lymph nodes to peripheral tissues, lose the molecule CCR7 and transform into CCR7-, CD45RA- effector memory T cells that display immediate effector functions [108,109]. Furthermore, in case of a protract low-dose antigen stimulation, these cells may be able to re-express the molecule CD45RA and acquire surveillance functions with less pronounced effector properties (TEMRA). Therefore, we subdivided IL-9+, CD4+ T cells according to the expression of the surface molecules CCR7 and CD45RA that allow the discernment among naïve,

central memory, effector memory and TEMRA cells. All these pools of cells were increased in untreated RA patients compared to the other groups. Following the addition of infliximab, IL-9+, CCR7+, CD45– central memory cells and IL-9+, CCR7-, CD45– effector memory cells but not naïve Th9 cells were increased only in the group of non responder patients, underlining that this pool of cells may account for the modification in PU.1+, IRF4+, IL9+, CD4+ T cell percentage in the previous experiment; *figg.4,5,8,9*. On the contrary, the percentage of TEMRA lymphocytes did not vary, due presumably to the scarce proliferative activity that characterizes this pool of cells; *fig. 10*.

3.4.4 Remicade® versus Remsima® effects on T helper 9 pool

We repeated the same experiment replacing the branded drug with biosimilar compound CT-P13 (Remsima®). In this case we directly evaluated the pool of PU.1+, IRF4+ Th9 cells, being the transcriptional markers more specific than the secreted cytokines for the determination of the Th9 lineage. Like the experiment with original infliximab, in both basal and stimulated conditions, we did not observe a significant variation in the percentage of PU.1+, IRF4+ Th9 cells in the group of healthy controls, untreated and responder RA patients. However, in the group of non responders, the increase in the percentage of both PU.1+, IRF4+, IL9+, CD4+ T cells and of central and effector memory IL-9+, CD4+ T cells, which was observed following the addiction of infliximab originator, did not reach the significance following the addition of the biosimilar compound, *figg.6,8,9*. Similarly to originator infliximab, the exposure to biosimilar CT-P13 did not induce a significant modification in naïve, TEMRA and OX40+, IL9+, CD4+ T cell percentages in any of the groups of patients; *figg.7,10,11*.

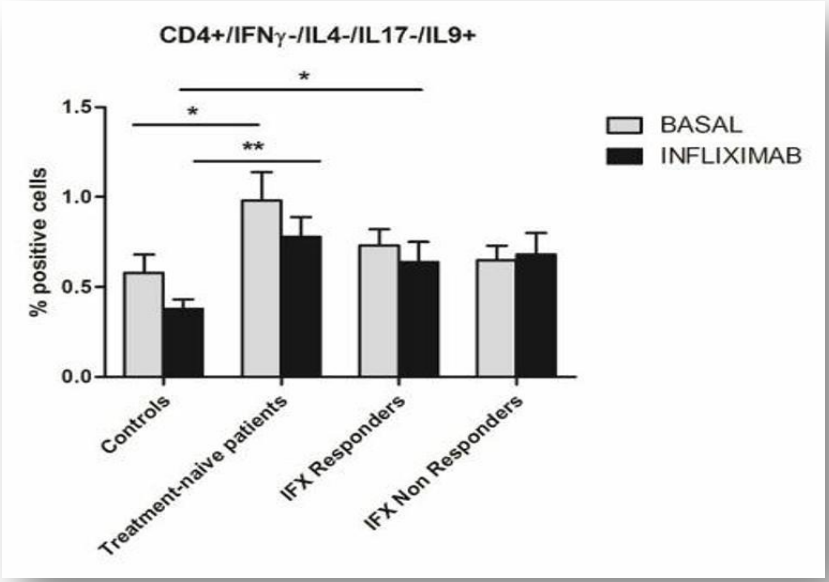

Fig.2. Percentages of IFNγ-, IL-4-, IL-17-, IL-9+, CD4+ T lymphocytes in the 4 groups before and after the addition of infliximab (Remicade®); *p<0.05, **p<0.01.

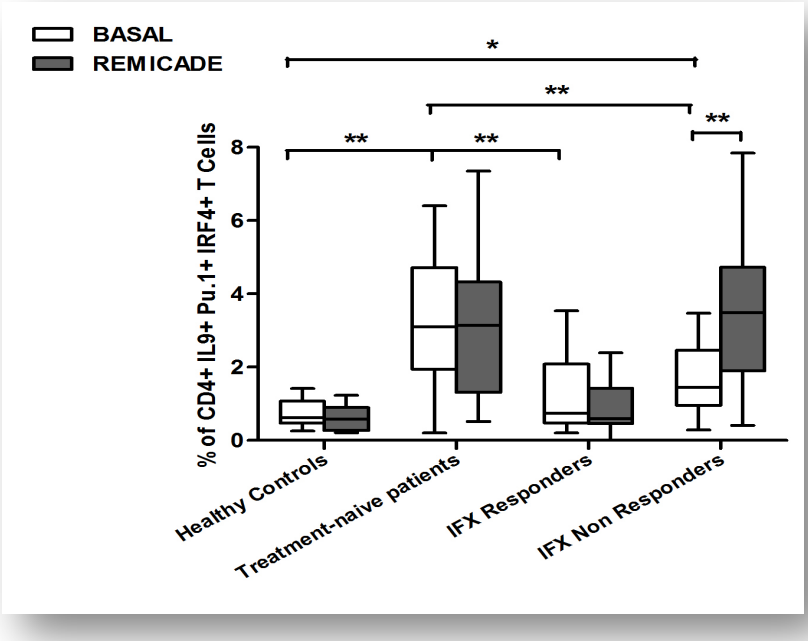

Fig.3. Percentages of PU.1+, IRF4+, IL-9+, CD4+ T lymphocytes in the 4 groups before and after the addition of infliximab (Remicade®); *p<0.05, **p<0.01.

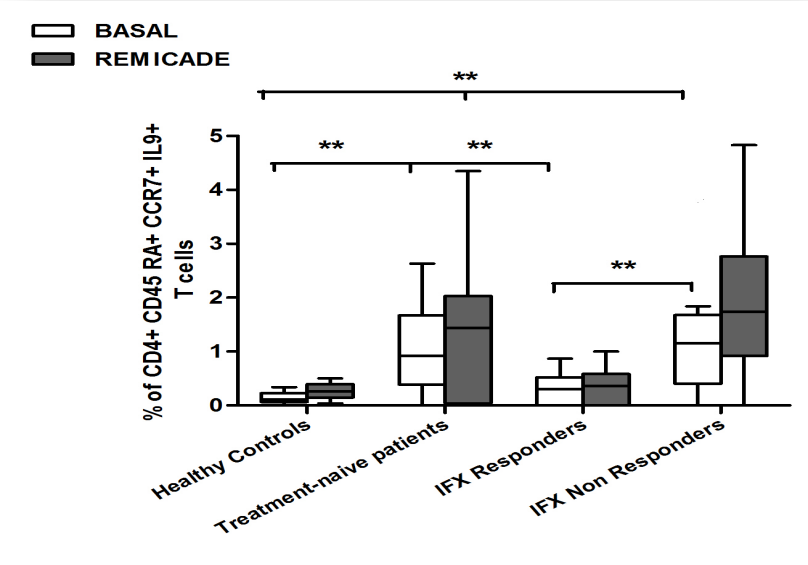

Fig. 4. Percentage of CD45RA+, CCR7+, IL-9+, CD4+ (naïve) T cells at baseline and after exposure to infliximab (Remicade®); **p<0.01.

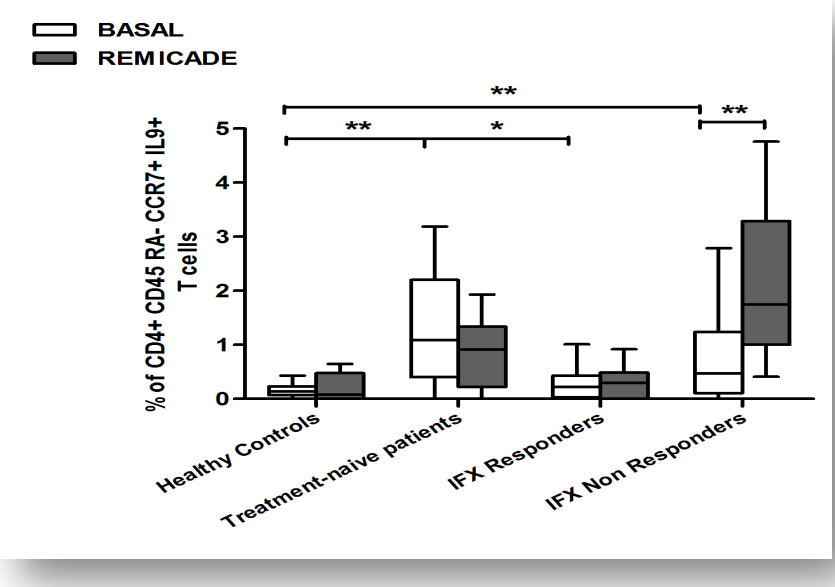

Fig. 5. Percentage of CD45RA-, CCR7+, IL-9+, CD4+ (central memory) T cells at baseline and after exposure to infliximab (Remicade®); *p<0.05; **p<0.01.

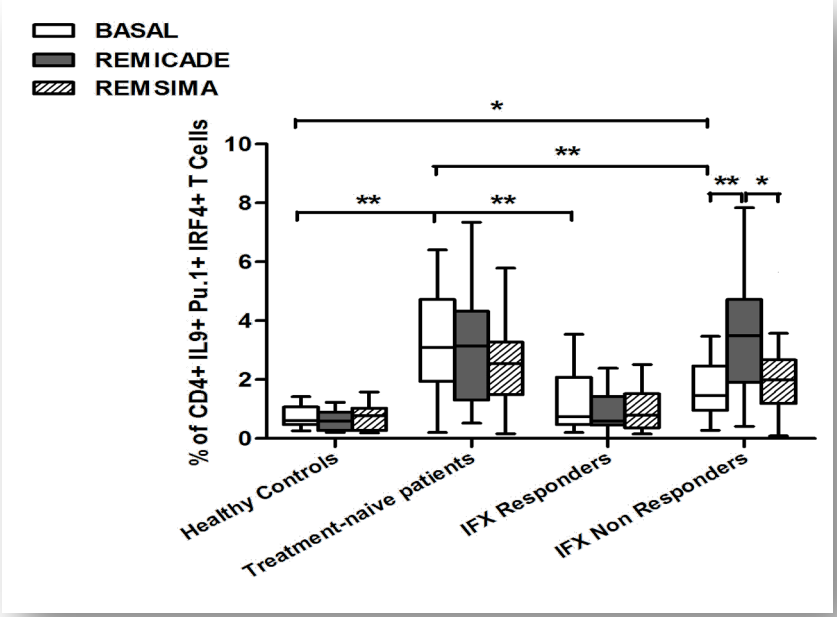

Fig. 6. Percentage of PU.1+, IRF4+, IL-9+, CD4+ T cells at baseline and after exposure to branded and biosimilar infliximab; *p<0.05, **p<0.01.

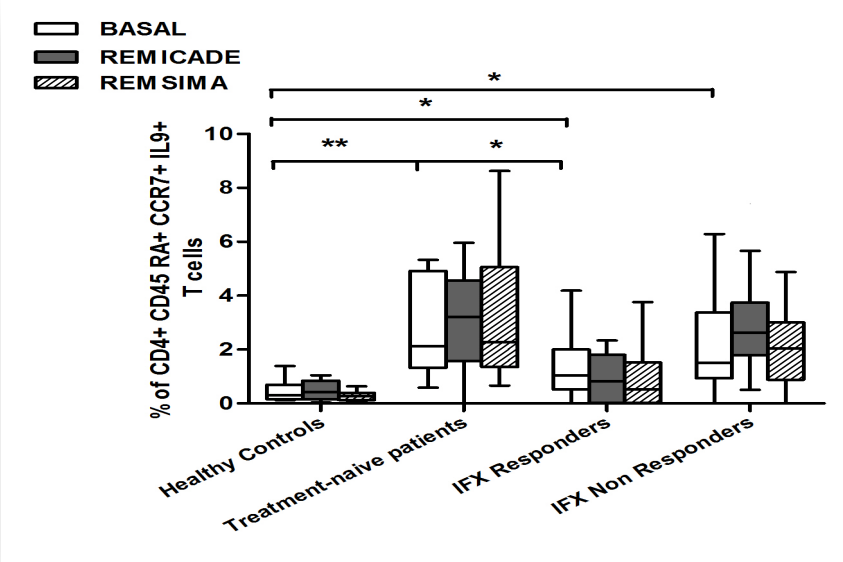

Fig. 7. Percentage of CD45RA+, CCR7+, IL-9+, CD4+ (naïve) T cells at baseline and after exposure to branded and biosimilar infliximab; *p<0.05, **p<0.01.

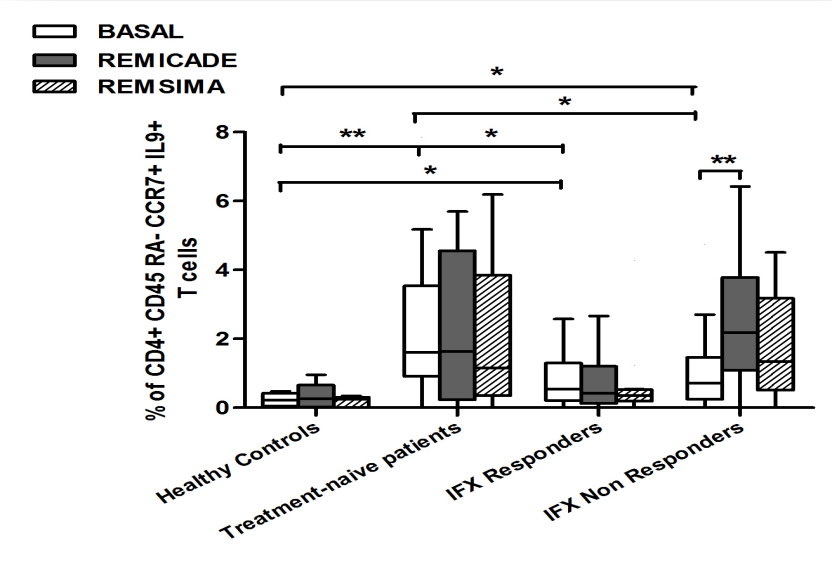

Fig. 8. Percentage of CD45RA-, CCR7+, IL-9+, CD4+ (central memory) T cells at baseline and after exposure to branded and biosimilar infliximab; *p<0.05, **p<0.01.

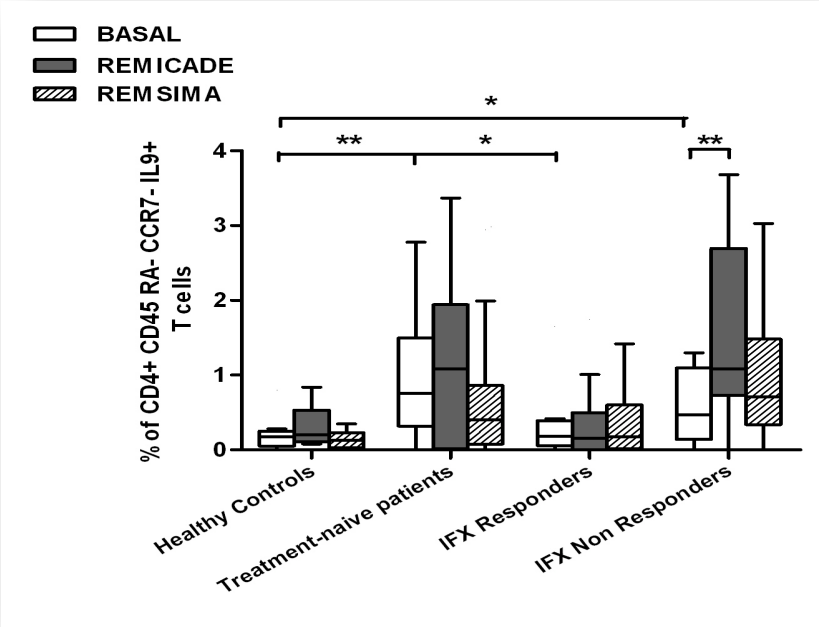

Fig. 9. Percentage of CD45RA-, CCR7-, IL-9+, CD4+ (effector memory) T cells at baseline and after exposure to branded and biosimilar infliximab; *p<0.05, **p<0.01.

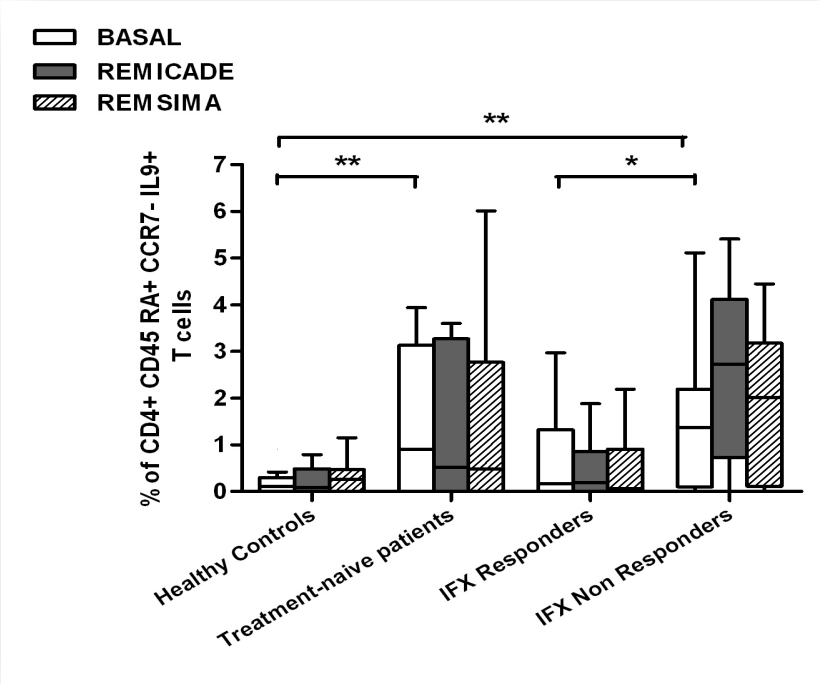

Fig. 10. Percentage of CD45RA+, CCR7-, IL-9+, CD4+ (terminal effector memory) T cells at baseline and after exposure to branded and biosimilar infliximab; *p<0.05, **p<0.01.

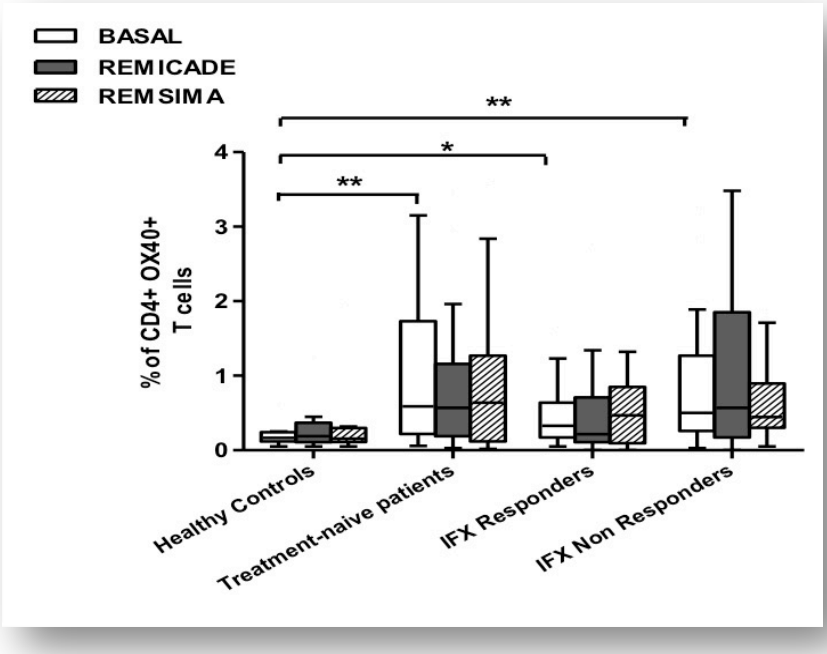

Fig. 11. Percentage of OX40+, IL-9+, CD4+ T cells at baseline and after exposure to branded and biosimilar infliximab; *p<0.05, **p<0.01.

3.5 Discussion

In our work we aimed firstly to evaluate the percentage of Th9 lymphocytes in peripheral blood of treated and untreated RA patients, compared to a group of healthy controls. Secondly, we performed an immune-stimulation assay *in vitro* in order to detect a possible relationship between Th9 cells and the outcome of a biologic therapy (infliximab). Thirdly, we aimed to demonstrate a comparable immunogenic profile between branded and biosimilar infliximab, according to the Th9-driven immune response.

The immunogenicity of a drug depends in fact on the presence of B and T specific epitopes contained in the primary aminoacidic sequence or developing during the post-translational modifications. Since now, the production of ADAs has represented the most speculated mechanism of a drug-induced immune response. The production of ADAs may be responsible for the development of adverse events or a progressive lack of efficacy. Biologic drugs have indeed ameliorated the course and the prognosis of rheumatic patients, however a not negligible amount of failure on treatment is reported. The immunogenicity of biologic drugs, especially chimeric molecules like infliximab, may partly rely on the induction of ADAs. However, we and other authors have demonstrated that other immune pathways, like the antigen-specific activation of Th1 or Th17 cells may represent an alternative way for rejecting a biological treatment. Th9 cells represent a T helper cell subset developing either from naïve or primed Th2 lymphocytes in presence of IL-4, TGFβ and OX40 and are characterized by the transcriptional factors PU-1 and IRF4 [81]. These cells represent the main source of IL-9, although other cells, including Th2 cells, Th17 cells and Treg cells may become specialised in the production of this cytokine [82,83]. IL-9 is capable of activating various cells including both Th17 and Treg lymphocytes [93]. Therefore, according to the local microenvironment, Th9 lymphocytes may contemporaneously orchestrate the immune response toward

autoimmunity/inflammation or tolerance. IL-9 and Th9 cells are increased in inflammatory arthritis, connective tissue diseases, autoimmune colitis and autoimmune encephalomyelitis [91,92,94,95,96,97]. In line with these data, our results showed an increased percentage of Th9 cells, either assessed by cytokine or transcriptional phenotype, in the peripheral blood of RA patients when compared to healthy controls. These cells were particularly elevated in untreated patients, while the treatment with conventional and biologic drugs smoothed their percentage. Following the stimulation with original infliximab, cytokine-sorted Th9 cells did not show any variation in any of the group examined, however, when sorted according to the transcriptional profile, PU.1+, IRF4+ Th9 cells increased in the group of non responder patients. These results were confirmed when the experiment was repeated with both central and effector memory IL9+ CD4+ T cells, but not with naïve or TEMRA IL9-producer CD4+ T cells. The discrepancy in the behaviour of Th9 cells assessed by cytokine or transcriptional profile may be explained in light of the pleiotropic pool of IL-9 producer cells (including also Treg cells); therefore we chose PU1+, IRF4+ Th9 as most specifically representative for the subsequent experiments. PU1+, IRF4+ Th9 cells may increase following an antigenic stimulation with infliximab in those patients who discontinued the treatment, perhaps following the recall and the activation of central and effector memory Th9 cells. When we assessed the response of PU.1+, IRF4+, IL9+ CD4+ lymphocytes to biosimilar infliximab we did not find any significant variation in their percentages from baseline in any of the 4 groups, although a trend was noticed for central and effector memory cells. Therefore, in the group of non responder patients, transcriptional factor-sorted Th9 cells increased following the exposure to original but not biosimilar infliximab. This discrepancy may be related to several conditions. Firstly, we observed a trend to an increased percentage of overall, central memory and effector memory Th9 lymphocytes following biosimilar infliximab exposure, although the significance was not reached; this

may depend on the scarce number of the enrolled patients (n.20). Furthermore, our experiments were carried using one single batch either for Remicade® and Remsima®, despite several structural differences may occur from one batch to another of the same drug. Finally, the epitopes recognized by Th9 cells on original infliximab may not correspond to those belonging to the biosimilar compound, due to some differences in the post-translational motifs, like the pattern of glycosylation. Moreover, although the current research including RCTs and spontaneous reports has demonstrated a comparable profile of immunogenicity between biosimilar and branded infliximab [45,46,55,62,63,64], all these studies have addressed the production of ADAs that may depend on different immunogenic properties and biologic pathways. Contrary to the group of non responder patients, no significant difference between branded and biosimilar infliximab was found after stimulating PU.1+, IRF4+, IL9+ CD4+ T lymphocytes from responder patients, and this may perhaps account for a more prominent role of Th9 cells in Remicade®- versus Remsima®-treated patients, although baseline percentages of these cells did not significantly differ between responder and non responder patients. However, both responder and non responder patients had received a treatment with Remicade® but not with Remsima®, therefore the clinical and biological effects of the biosimilar drug *in vivo* are not available in our cohort.

3.6 Conclusions

IL-9 levels are increased in RA patients, in whom this cytokine plays indeed a crucial role. Th9 cells are the major producers of IL-9, and their prevalence is higher in RA patients than in healthy subjects. According to our results, PU.1+, IRF4+ Th9 cells may be involved in orchestrating the immune response against the epitopes of branded infliximab; and this condition could rely on the recall and stimulation of both central and

effector memory cells. On the other hand, biosimilar infliximab seems not able to activate these pools of cells.

These data carry a novel point of view in the immunogenicity of anti-TNF agents, routinely based on the detection of ADAs. The paradoxical activation of Th9 pool following the exposure to infliximab may contribute to the underlying inflammation, thus determining a progressive lack of efficacy or the development of adverse effects. However, no significant difference was noticed in the PU.1+, IRF4+ Th9 cell percentages in Remicade®-responder patients after stimulation test either with biosimilar and branded infliximab, proving that *in vitro* both the two drugs seem to have a comparable efficacy. The discrepancy between the Th9-driven immunogenicity of branded and biosimilar infliximab in the group of non responder patients, observed in our experiment, may be attributed either to methodology, either to the presence of dissimilar epitopes on the two compounds or to the involvement of other T cell subsets, like Treg lymphocytes, therefore deserving further investigations.

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