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T cell subtypes in the management of osteoporosis with bisphosphonates and in the autoimmune reaction to collagen in rheumatoid arthritis.

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ABSTRACT

T lymphocytes with different T cell receptors are at the crossroad of inflammation and autoimmunity. We investigated the role of γδTCR and αβTCR T lymphocytes (γδT, αβT cells) in two model conditions represented by zoledronic acid (ZA)-induced acute phase reactions (APR) and in the immune reaction against collagen in rheumatoid arthritis (RA).

First, γδTCR T lymphocytes (γδTcells) are specifically activated by ZA infusion in the treatment of osteoporosis and is frequently associated with the onset of APR, possibly via 25-OH vitamin D (25-OHvD) levels. 50% of patients reported ZA-associated APR (APR+). APR+ cases had a higher percentage of central memory Th1-like γδTcells before treatment. One week after ZA infusion, a decreased percentage of central memory Th1-like γδTcells, an increase in the percentage and activation of effector memory Th1-like γδTcells, and an increase in Th17-like γδTcells were observed in the patients with APR. Lower 25-OHvD levels were significantly associated to APR, but no correlation was found between 25-OHvD level and γδTcell percentage or subsets. Second, αβTCR T lymphocytes (αβTcells) in RA recognize the DR4/DR1-restricted epitope 261-273 of the human type II collagen, whereas B cells recognize the epitope 359-369. We investigated the role of B and T cell epitopes and their post-translational modifications on the RA adaptive immune response. PBMCs from 5 HLA-DR4+ monozygotic twins, two HLA-DR4+ and one HLA-DR3+ healthy donor and synovial fluids (SF) from an HLA-DR4+ RA patient and HLA-DR3+ patient were used and cells cultured with the native form of the collagen T epitope (261-273T), its K264 carbamylated form (homocit261-273T), the native form of the collagen B epitope (359-369B), its R360 citrullinated form (cit359-369B) or the combination of the native and modified epitopes. We may conclude that the collagen T epitope 261-273 has a role in the pathogenesis of RA, but the carbamilation of this epitope dos not seem to be influent in the T cell response.

Key words. Osteoporosis, zoledronic acid, γδTCR T lymphocytes, rheumatoid arthritis, human collagen epitopes, citrullinations, carbamylation.

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INDEX

T lymphocytes with different T cell receptors are at the crossroad of inflammation and autoimmunity and the mechanism that lead to one road or the other are quite slight. In both cases, autoimmune and chronic inflammation, the triggers are represented by aberrant reactions of the human adaptive or innate immune systems. The immune response between two is practically identical, the immune system recognized the external antigens or the damage tissues trough TCR receptors present upon the T cells; the recognition actives the pro-inflammatory cytokines productions that generated an acute inflammation that in normal condition is auto-limited. T cell receptors TCR are the membrane receptors of all lymphocytes, they are generated through genic rearrangements in the thymus and they have the role to present the antigens to T cells between the link TCR-peptide-MHC. The maturation of T cells in the thymus is characterized by cells that achieved the propriety to recognize self-antigens and non-self-antigens. During the maturation is essential the weak affinity and specificity to self-antigens (negative selection) to complete the process of T cell maturation. Altered T cell maturation and/or an aberrant peripheral T cell homeostasis that characterized a chronic inflammation could have a role in the autoimmune condition and lead to expression of activation marker upon the effector T cells that prolong the chance to be in contact with self-antigens which could lead to a production of T and B cells self-reactive. In this project we want to investigate the role of γδTCR and αβTCR T lymphocytes (γδT, αβT cells) in two model conditions represented by zoledronic acid (ZA)-induced acute phase reactions (APR) and in the immune reaction against collagen in rheumatoid arthritis (RA).

gamma delta T cells

Osteoporosis: definition

Osteoporosis (OP) is a silent chronic condition where the bone become weak and prone to fracture. In OP, the bone mineral density (BMD) is reduced, bone microarchitecture deteriorates, and the amount and variety of proteins are altered. OP is considered “silent”

because it has a long preclinical asymptomatic course until the bone fracture with consequent irreversible damages.

During our life the bone is in continuous balance between the old bone degradation (osteoclastogenesis) and the new bone formation (osteoblastogenesis) and it takes 2-8 months to complete the coordinated process. The balance is slow down after the third decade, and in perimenopausal and early postmenopausal periods in women, where bone resorption predominates upon the osteoblastogenesis leading to a reduction of BMD. The normal bone formation process consists of several phases regulated by one mesenchymal cells that can originate 3 different bone cell types: osteoblast, osteocyte, and osteoclast. Osteoblasts are the immature bone cells, that synthetized various constituents of extracellular matrix,[1, 2] and are responsible of new bone formation: they are able to produce bone matrix. Osteocytes are senescent osteoblast located into the bone *lacuna and* have osteolytic propriety conferred by the presence in their lysosome of lytic enzymes.

Osteoclasts are large, multinucleated cells located on bone surface and are responsible for bone resorption; they belong to the monocyte-macrophage line and they remove mineralized bone matrix by producing lytic enzymes causing bone resorption [3].

Osteoporosis: signs and symptoms

The disease is classified as primary type 1, primary type 2. To the first type belongs the form of OP most common in women: the postmenopausal OP. Primary type 2 OP or senile OP occurs after age 75 and is seen in both females and males at a ratio of 2:1. Fractures are the most common consequence of the decreasing bone strength and frequently occur in the wrist, spine, shoulder and hip and can lead to further disability and early mortality and meaningful health care costs [4-7]. Several OP risks factors exist and are divided into *unchangeable* or *changeable* (table 1).

Table 1. The table shows a list of different OP risk factors divided into unchangeable and changeable categories.

Unchangeable risk factors	Comments
Sex	Women are much more affected than men (2:1) ratio

Age	Occurs in older subjects
Race	All races are involved but Asian and Caucasian descent increased the risk
Family history	Parent or sibling with osteoporosis increased the risk
Frame size	Small body frame corresponds to less bone mass
Changeable risk factors	Comments
Hormone levels	sex hormones (i.e reduction of estrogen level), thyroid problems (causing bone loss) and other glands (i.e. paratyhroid, adrenal gland)
Dietary factors	Low calcium intake, eating disorder and gastro-intestinal surgery
Steroids and other medications	Seizures, depression, gastric reflux, and cancer transplant rejection
Lifestyle choices	Sedentary lifestyle, alcohol consumption and tobacco use

Osteoporosis: diagnosis

The ideal steps to diagnose the OP are based on blood tests and bone densitometry.

The first level laboratory tests:

Blood calcium levels, 25-OH vitamin D (25-OHvD), thyroid tests (such as T4 and TSH to screen for thyroid disease), parathyroid hormone (PTH), follicle-stimulating hormone to check for menopause, testosterone to check for deficiency in men, protein electrophoresis (to identify monoclonal antibodies as in multiple myeloma that can break down bone), and alkaline phosphatase (as screening for other bone diseases as Paget's disease).

Tests measuring bone resorption are (before treatment and again after treatment):

- ✓ C-terminal telopeptide of type 1 collagen
- ✓ Urinary collagen type 1 cross-linked N-telopeptide
- ✓ Deoxypyridinoline Pyridinium Crosslinks Urinary hydroxyproline
- ✓ Tartrate-resistant acid phosphatase 5b

- ✓ Bone sialoprotein

Tests measuring bone formation are (before the start of treatment and periodically after treatment):

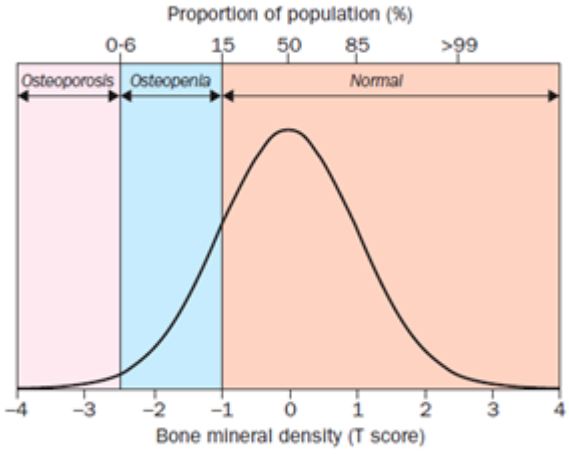
- ✓ Bone-specific alkaline phosphatase
- ✓ Osteocalcin
- ✓ Procollagen Type 1 N-Terminal Propeptide and C-terminal

OP criteria according to the World Health Organization (World Health Organisation. 1994) are based on the BMD evaluation of the proximal end of the hip or vertebrae in postmenopausal women, given as the T-score expressed as the number of standard deviations (SD); the baseline is the maximum bone mass (table 2 and figure 1).

Table 2. the table displays the T-score cut off expressed as the number of standard deviations (SD) and on the right is reported the status correlated to the T score level.

T score expressed in SD	Status
SD>-1	normal value
-1<SD<-2.5	osteopenia value
SD<-2.5	osteoporosis
SD<-2.5 and osteoporotic fracture	advanced osteoporosis.

Figure 1. the Gaussian distribution displays the different T-score expressed as bone mineral density (BMT) in correlation with different pathological conditions (osteoporosis and osteopenia) compared to the healthy condition.



The baseline is the BMD value for the relevant sex and age [8]; the diagnostic tests able to measure the BMD are:

- ✓ Dual-energy X-ray absorptiometry (DXA): it is used to measure BMD at the lumbar spine, hip, distal forearm, calcaneum and whole body. DEXA calculates the difference between bones and soft tissues measured by x-ray. The fracture risk is determined by the degree of bone mineralization and by bone size.
- ✓ Quantitative computed tomography (QCT): the results are 3D images it gives measurements evaluating sex-, age-, and ethnic-related differences in vertebral and femoral geometry, thereby providing new insight into the development of skeletal fragility buti s not used to predict risk of fractures.
- ✓ High resolution peripheral quantitative computed tomography (HR-pQCT): commercially available in the mid-2000, is a non-invasive, low-radiation method and requires specialized scanners, and measurements are limited to peripheral skeletal sites.
- ✓ Quantitative ultrasonography: it measures two parameters, the speed of sound (SOS) and the broadband ultrasound attenuation (BUA). It is not applicable in routine clinical practice.
- ✓ Magnetic resonance imaging (MRI): it is a non-ionizing method to assess bone microarchitecture; it generates 3D images of bone structure; however is not practical in the clinics.

Osteoporosis: epidemiology

Studies on worldwide OP prevalence highlight that the disease affects about 75 million people [9] of note 22.5 million are in particular postmenopausal women [10]. In the United States, the OP prevalence was more than 10 million people in 2002 reports [11] and, moreover, in the EU 2010 data reveal that 22 million of women and 5.5 million of men were affected by OP [12].

The annual incidence OP rates were available for most countries but data were underestimated because of the silent condition of the disease: OP incidence rate is calculated upon the new hip fractures that affect the OP patients, considered more devastating than any other type of

osteoporotic fracture. Worldwide data on 2006 declare that 1.75 million on new fractures annually arise; more data [12, 13] suggest that 9 million of new hip fracture/year rise worldwide and in particular 4.5 million are in Americas and Europe. The Newest report shows that the number of new fractures in 2010 in the EU was estimated at 3.5 million, comprising approximately 620,000 hip fractures, 520,000 vertebral fractures, 560,000 forearm fractures and 1,800,000 other fractures [5].

In Italy, the ESOPO (Epidemiological Study On the Prevalence of Osteoporosis), study conducted in 2001 shows osteoporosis prevalence in a random sample of 11,011 women and 4,981 men, in 83 Italian centers. The prevalence rate of osteoporosis in women 40-79 years old was approximately 18.5%, while men 60-79 years of age the rates were 10% [14].

More recent studies report a prevalence of osteoporotic fractures of about 34 % in a cohort of 4,000 women [15, 16].

The risk of overpass an osteoporotic fracture in 50-year old Caucasian woman is estimated to 17-23% and the men counterpart is estimated to 6-11% [13, 17]. The risk of clinical vertebral fracture decreases proportionally with the age [18].

Importantly, 1 in 5 persons die during the first year after a hip fracture, and 1/3 need nursing home or a caregiver after hospital discharge, and less than 1/3 reacquire their physical function before the fractures [19-22]. The latest data report that the number of deaths associated to fractures was estimated at 43,000; in particular, in women the 50% of fracture associated deaths were due to hip fractures, the 47% in men the 28% to clinical vertebral and the 39% in men, and the 22% to other fractures and the 14% in men [6, 20, 23, 24].

Table 3. Estimated number of fractures (in thousands) at the sites shown in men and women age 50 years or more in 2000 by World Health Organization (WHO) [25].

Region	Hip	Spine	Forearm	Humerus	Other	All sites	Percentage
Africa	8	12	16	6	33	75	0.8
Americas	311	214	248	111	521	1406	15.7
Southeast Asia	221	253	306	121	660	1562	17.4
Europe	620	490	574	250	119	3119	34.8

Eastern Mediterranean	35	43	52	21	109	261	2.9
Western Pacific	432	405	464	197	1039	2536	28.6
Total	**1627**	**1416**	**1660**	**706**	**3550**	**8959**	**100**

Data suggests that, in association with the aging of the world population, patients affected by OP will increase of 23% in the next 25 years [26], and, moreover, the OP underestimation, and insufficient diagnosis of the disease, result in the continuous increase of social problem of OP.

Data in 2005 report that the estimated direct cost of OP is 19 billion in the in the United States and expected to increase by 50% by 2025 [27-29]. In particular, 3.5 million/year hospital bed days are attributed to osteoporotic fractures and over 60,000/year nursing home admissions are attributed to hip fractures. In Europe the trends are similar, where the estimated cost of osteoporotic fractures was 36 billion euro in 2000 and is expected to increase to 77 billion euro by 2050 [30, 31].

The updated cost of OP, including pharmacological intervention in the Europe was estimated at €37 billion; in particular, costs of treating incident fractures represented 66%, Pharmacological prevention 5% and Long-term fracture care 29% [6]. **Table 4.**

Table 4. Yearly cost at long-term care facility (€, 2010). [6]

Country	Long-term care cost	Reference
France	31˙512	*[32]*
Germany	34˙534	*Long term care facilities in Germany: Accessed 24 May 2011; www.pflegeheimhaus-am-see.de, www.aphw.telebus.de, www.hausstiftstrasse.de and www.domicil-seniorenresidenzen.de*
Italy	50˙202	*[33]*
Spain	51˙786	*[34]*
Sweden	57˙247	*[35]*
UK	33˙756	*[36]*

Considering the expected increase in patients affected by OP, proportional to that of the economic factor, prevention and early intervention will be the key resolution.

Osteoporosis: management

The first european guide in the OP management was dated 1997, the update arrived in 2008 and in the 2012 the newest version and divided all therapeutic managements for OP into 2 classes:

1. Non-pharmacologic treatment options: calcium and vitamin D intake, weight-bearing exercise, and fall prevention;
2. Pharmacological treatments: bisphosphonates, amino-bisphosphonates, and hormone therapies (PTH and selective estrogen receptor modulators –SERM-), strontium ranelate, monoclonal antibody (Denosumab)

About the first class, calcium absorption normally decreases with advancing age. Calcium supplementation may prevent bone loss or even mildly increase BMD, and some data suggest that it may minimally reduce fracture risk [37-39]. However, in the daily clinical practice calcium is used in combination with the pharmacological therapies. The National Institutes of Health consensus conference guidelines suggest that women should optimize their elemental calcium intake to 1000 mg/d until menopause and increase it to 1500 mg/d thereafter. Men should optimize their calcium intake to 1000 mg/d until age 65, and then increase it to 1500 mg/d [40]. In addition, aging is associated with decreasing serum 1, 25-dihydroxyvitamin D levels. Vitamin D supplementation may reduce vertebral and non-vertebral fracture risk in vitamin D-deficient individuals; notions confirmed by a meta-analysis study which concluded that oral vitamin D supplementation of 700 to 800 IU/d appears to reduce the risk of hip and non-vertebral fractures in ambulatory or institutionalized aged persons, whereas a dose of 400 IU/d was insufficient for fracture prevention [41]. Sufficient vitamin D intake is necessary to maintain circulating serum levels of 1, 25-dihydroxyvitamin D adequate to stimulate calcium absorption [42, 43]. Vitamin D is a secosterol hormone that is present in humans in a cholecalciferol (vitD3) of vitamin D 2 exogenous (vitD2) form. The cholacalciferol is synthesized in the skin from the cholesterol metabolite 7-dehydrocholesterol under the influence of ultraviolet radiation. The exogenous form ergocalciferol is produced by ultraviolet irradiation of the plant sterol ergosterol and is

available through the diet. Both forms of vitamin D require further metabolism to be activated. In bone vitamin D causes bone resorption by mature osteoclasts, requiring cell recruitment and interaction with osteoblasts and the fusion of monocytic precursors to osteoclasts. Vitamin D also regulates the expression of several bone proteins, notably osteocalcin. In an epidemiological study in a cohort of older osteoporotic women, it has been demonstrated an inverse association between vitamin D repletion and the changes in BMD and the incidence of clinical fracture is 77% higher in vitamin D depleted women compare to normal vitamin D levels [44]. Vitamin D deficiency is extremely common problem and general levels of vitamin d status considered the patients with a baseline level of 25-OHvD above 30 ng/mL as normal vitamin D levels, with a level between 20 and 29 ng/mL they were considered vitamin D insufficient, and with levels below 20 ng/mL were considered deficient [45].

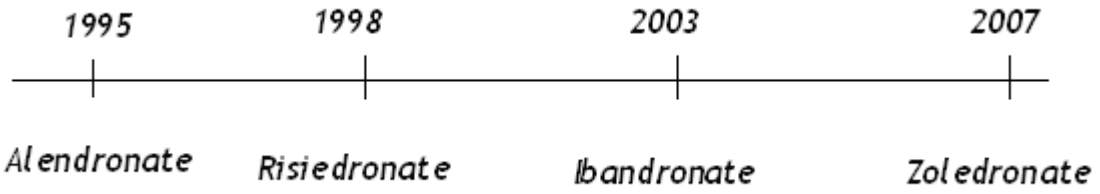
Recommendations include weight-bearing exercise in the form of walking, mild to moderate impact aerobics, and resistance exercises as tolerated. Regular exercise also increases muscle mass and strength, improves balance and coordination, and a lower risk of falls.

Regarding the second class, Hormone replacement therapy (HRT) may consist of estrogens alone or in combination with progestin. HRT slows bone turnover and increases BMD at all skeletal sites in early and late postmenopausal women [46, 47]. Epidemiological studies assessed that HRT decreases fragility fracture risk by 20 to 35 % [48-50] however, the few patients compliance in HRT tratments, lead to a reduction in efficacy.

SERM are synthetic molecules that are able to bind to estrogen receptors as estrogen agonists or antagonists depending upon the target organ. An example is the raloxifene (60 to 120 mg daily) that decreases the bone turnover (decrease in the BTM levels by 35%) and increases BMD by 2 to 3% at the lumbar spine and femoral neck [39, 51]. Another SERM is the bazedoxifene but is not yet approved for the prevention or treatment of OP.

The first bisphosphonates approved were: aledronate and risedronate at first for postmenopausal osteoporotic treatments and after for men osteoporosis **Figure 2**.

Figure 2. The table shows the timeline of osteoporosis bisphosphonates approved by Food and drug administration (FDA).

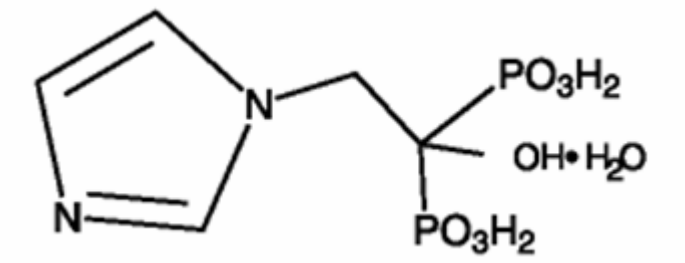


These drugs are synthetic analogues of inorganic pyrophosphate consisting of two phosphonate groups linked by non-hydrolysable phosphoesther bonds to a central carbon atom, to which are attached two covalently bonded side-chains (R1 and R2). This P–C–P backbone structure enables BPs to bind avidly to metal ions such as Ca2+ [52]and, as a result, BPs bind to bone mineral surfaces in vivo [53]. They can be administered orally (daily, weekly or monthly) or intravenously (quarterly or yearly). About cellular mechanisms, during the process of bones resorption, osteoclasts secrete protons, which decrease the affinity of BPs for calcium ions and cause the release of BPs from the bone surface; BPs are then endocyted by osteoclasts can stop osteoclast formation, promote osteoclast apoptosis, and inhibit osteoclast activity [54, 55]. BPs specifically, act on mature osteoclasts by interfering with adenosine-5′-triphosphate-dependent cellular processes: in vitro, some BPs have been shown to inhibit the formation of mature osteoclasts by inhibiting the fusion of pre-osteoclasts [56]. The presence in the chain of amine group divided the bisphosphonates into a class called nitrogen-containing BPs (N-BP). As N-containing BPs localize to bone surfaces, they are taken up by osteoclasts. The amino bisphosphonates (Alendronate, Ibandronate, Pamidronate, Risedronate, and Zoledronate) act by inhibiting farnesyl diphosphate synthase (FPPS), an enzyme in the mevalonate pathway, resulting either in accumulation of isopentynil-pyrophosphate (IPP) either in inhibition of mavelonate-metabolites synthesis and apoptosis [57-60]. FPPS is required for the prenylation of small GTPases such as Ras, Rab, and Rho, which is essential for the function of these proteins. Small GTPases regulate several cell processes that are important for osteoclast function. Regarding the pharmacokinetics, most circulating BPs (50%) accumulate in bone and excreted un-metabolized in the urine within 48 hours. When administered intravenously, BPs bound to bone are slowly

released back into the circulation during bone resorption and excreted in the urine over time. Because bisphosphonates are avidly bound to bone, a reservoir of drug accumulates after years of treatment and is gradually released over months or years and appears to result in a persistent anti-fracture benefit for some time after therapy is stopped (holiday drug) [61, 62]. The benefits are reported in several clinical trials and confirm that bisphosphonates have proven efficacy for prevention of bone loss due to aging, estrogen deficiency and glucocorticoid use; in particular, alendronate, risedronate and zoledronate have been shown in placebo- controlled trials to prevent fractures of the spine, hip and other non-vertebral sites [63-65].

We focus on zoledronic acid (ZA) the first intravenous N-BPs administrated annually. The way of administration solve the problem of adherence to OP treatment: a huge problem for the oral BPs characterized by a poor patients compliance that compromise the therapeutic benefits [66-69]. The single annual infusion of ZA might improve such efficacy in clinical practice. ZA (1-Hydroxy-2-imidazol-1-yl-phosphonoethyl) its structural formula is a white crystalline powder. Its molecular formula is $C_5H_{10}N_2O_7P_2 \bullet H_2O$ **(figure 3)** and its molar mass is 290.1 g/Mol.

Figure 3. Zoledronic acid (ZA)(1-Hydroxy-2-imidazol-1-yl-phosphonoethyl). (Manufactured by Novartis Pharma).



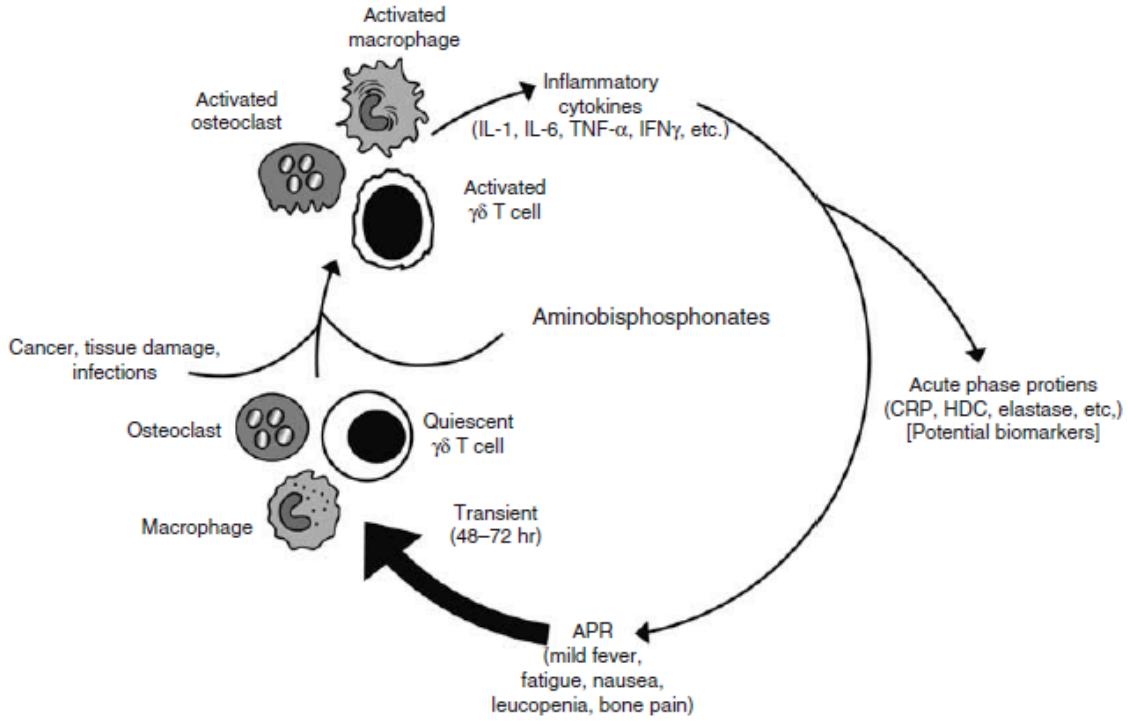
Several clinical trials on ZA describe the effect of drug versus placebo in cohort of post-menopausal OP women. In 2002, Reid IR conducts 1 year clinical trial in 351 OP divided into 3 groups: the first taken 4mg ZA year, the second group taken 2 mg/year ZA infusion, and the last placebo. The clinical trial suggested that ZA treated patients manifested higher adverse events compared to placebo group [70]. Similar results was described in 2007 from Kenneth W team in

bigger cohorts of women and also concluded that the ZA treated patients show few percentage of mortality after fractures compared to that of placebo group [71].

More recent studies highlighted the benefits of ZA in the increasing of BMD compared to placebo group, and a parallel reduction of fractures compared to other oral BP-s; those data reinforce the better patients adherence in the administration of ZA compared to the oral OP treatments [72-76].

The most common adverse events ZA-associated therapy reported in literature were transient fever, nausea, constipation, anemia and dyspnea. These symptoms are collectively called acute phase reaction (APR); they appear 24-48 hours later ZA infusion and solved in 3 days. In the literature, the frequency of APR ranges between 30% and 55% of the patients that for the first time were treated with intravenous N-BP [77, 78]. APR is caused by an increasing of pro-inflammatory cytokines production such as, of interleukin 6 (IL-6), tumor necrosis factor α (TNF-α), and interferon-γ (IFN- γ) released from cells activated by N-BP infusion [79-81]. The first who discussed the cellular mechanism was Kunzmann et al. in 1999 and suggested that N-BPs can stimulate a particular subset of T cells with a consequent release of cytokines production that can trigger to APR onset [82]. In particular, N-BPs was internalized in monocytes [83] or in dendritic cells [84] or act directly upon cells [85], next the FPP enzyme was inhibited and lead to the accumulation of metabolites of mavelonate pathway, such as isoprenyl-pyrophosphate (IPP) and dimetylallyl pyrophosphate (DMAPP) [86-89]. FPPS enzyme maintain in cell the isoprenoid lipids necessary for the post-translational modification of GTP binding proteins (Rho, Rac, Cdc42) those reaction are necessary for GTPase activity [90] important for the cytoskeletal rearrangements, vesicular activity in osteoclast [91-93].The accumulation of MVA metabolites lead to the activation of the γδTCR T lymphocytes (γδTcells) that after activation produce pro-inflammatory cytokines leading to APR [94, 95](figure 4).

Figure 4. The figure represents the hypothetical mechanism arise after aminobisphosphonates infusion or others (cancer tissuedamage, infections…) leading to the activation of the γδTCR T lymphocytes (γδTcells) that after activation produce pro-inflammatory cytokines leading to APR [77].



Gamma delta T cells:

T lymphocytes have two distinct CD3-associated antigen receptors leading to a two different T cell receptors (TCR). The main representative is the TCR bearing the α and ß chains (αßTCR), each chain having a variable genic region (V) and constant genic region (C); in V region there is the CDR3 region, one of the most variable region. The other counterpart, representing the 1-10% of the population, is the TCR bearing γ and δ chain (γδTCR). The main difference in between is the way of antigen recognition: αß T cells (αßTcells) predominantly recognize foreign and self-peptides presented by MHC class I and class II molecules [96, 97]; γδ T cells (γδTcells) seem to recognize non-peptidic antigens and lipids without necessary requiring MHC complex. Basically, in the MHC class I pathway, peptides generated from endogenous cytoplasmic proteins are transported into the endoplasmic reticulum and exposed; in the MHC class II pathway, peptides generated from extracellular proteins are processed and exposed. In contrast, superantigens do not require processing, because they are proteins that bind directly to MHC class II molecules on the cell surface and to y or δ chain of the TCR.

Studies in animal models identified γδ T cells in the skin, in the intraepithelial layers of the small intestine suggesting the role of γδ T cells in the first line of defense [98, 99] with a speed response against antigens. In the 1989 more studies investigated the role of γδ T cells in the immune response and pointed out that γδ T cells reacted, *in vitro* and in vivo, in mice and humans, to a series of small phosphorylated non-peptidic metabolites; they also proliferate in the course of bacterial and parasitic infections and mediate protective responses against these pathogens [100] among these has been reported to recognize mycobacterial antigens in a non-MHC restricted fashion and a tetanus toxoid (TT) peptide in the contest of an MHC-II molecule [101] Other specific ligands are presented in table 5.

Table 5. The table displays the ligands that specifically activated the γδ T cells.

Diseases associted to γδTcells	γδTcells ligand	References
Viral Diseases	Immunodeficiency virus-1, Cytomegalovirus (CMV), Epstein Barr Virus (EBV), and Human Herpes Virus (HHV)-8	[102-104]
Tuberculosis (TB)	Mycobacterium tubercolosis	[105]
Malaria	Plasmodium falciparum and with Plasmodium vivax	[106]
Local cutaneous leishmaniasis	Primary toxoplasmosis	[107]
Bacterial Infections	Salmonella, Shigella dysenteriae and Shigella flexnerii, Coxiella burnetii, Legionella micdadei,	[108, 109]
Cancer	solid tumor cancer patients, glioblastoma multiforme, nasopharyngeal carcinoma, squamous cell carcinoma, gastric cancer, renal cell carcinoma	[110, 111]
Immune-Mediated Diseases	Behcet's Disease, Rheumatoid Arthritis, Systemic Lupus Erythematosus, Takayasu Arteritis, Scleroderma,	[112, 113]
Auto-Immune Neurological Diseases	Multiple Sclerosis, Opsoclonus Myoclonus, Guillaine Barre Syndrome	[114, 115]
Atopic Diseases	Asthma	[116]
Gastrointestinal and Liver Diseases	Autoimmune hepatitis, Crohns Disease, Coeliac Disease, gastritis	[117]
Dermatological Disease	Psoriasis, atopic dermatitis	[118]
Miscellanea	Effect of bisphosphonates,	[119]

Based on all this notions, γδTcells could be considered as a "bridge" between innate and acquire responses. AßTcells and γδTcells arise from a common progenitor in the thymus and after maturation, while αßTcells exhibit two different surface markers: CD4 or CD8, the γδTcells show a double negative phenotype: CD4-CD8-. γδTcells have different subtypes in different tissues, the majority of peripheral blood γδTcells express Vγ9 Vδ2 [94] and are the 0.5-5% of the circulating T cells. In 2009, Ribot et al. reported that developing γδTcells in mice could be phenotypical separated into populations based upon CD27 expression and could be divided into naïve (CD45RA+CD27+) and memory (CD45RA-CD27-) subsets [120]. After in vitro antigen or cytokine stimulation, the central memory CD45RA-CD27+ (T_{CM}) phenotype shift to the effector phenotypes: effector-memory CD45RA-CD27- (T_{EM}) with a gradual loss of CD27 marker and a consequent cytokine production or to terminally differentiated CD45RA+CD27-(T_{EMRA}) with cytotoxic activity [121, 122]. The different phenotypes have different homing and effector functions. While the naïve and the T_{CM} are abundant in lymph nodes, the effector phenotypes are abundant in inflamed tissues. Moreover, naïve and $T_{CM\ cells}$ are able to proliferate in response to stimuli, instead of $T_{EM\ and}$ T_{EMRA} which are able to produce cytokines and to show cytotoxic activities [123].

Stimulation of γδTcells has received much attention regarding therapeutic treatments [124, 125]. In 1999 for the first time in *in vitro* experiment, it has been demonstrated that 4/10 patients had an APR after bisphosphonates treatment, and all 4 showed an increase in the percentage of γδTcells in peripheral blood [82]. Kunzmann and colleagues were the first to assign a role for γδTcells in the NBP-induced APR and after the discovery several *in vitro* and *in vivo* studies confirmed the data. T_{CM} γδTcells differentiate into T_{EM} γδTcells after N-BPs stimulation and then express receptors for the migration towards inflamed tissues and display immediate effector function as cytokine production, essentially IFNγ (Th1-like), and cytotoxicity. After 12 hours from N-BPs stimulation, both T_{CM} and T_{EM} γδTcells, express CD69, a marker of cell activation [126]. *In vitro* studies demonstrated a reduction of T_{CM} γδTcells and an expansion of the T_{EM} subsets, but limited *in vivo* data are available [127, 128]. Recently, N-BPs

were thought to activate Vγ9Vδ2 T cells by acting as agonists for the Vγ9Vδ2-TCR [85]. However Gober et al. demonstrated that ZA required internalization by Daudi cells to induce activation of γδTcell clones, thereby in conflict against a direct agonistic effect of ZA on the Vγ9Vδ2-TC [88]. The hypothesis of the indirect activation was then supported by studies that have clarified the role of monocytes in Vγ9Vδ2 T cell activation *in vitro* and using fluorescent N-BP analog-based approach, it has been showed that N-BPs are selectively internalized by peripheral blood monocytes in human PBMC cultures, with no detectable uptake into B- or T-lymphocytes. Interestingly, it has been demonstrated *in vitro*, that activation of human γδTcell by IPP leads to the association of the vitamin D receptor (VDR) [129]. VDR is a nuclear receptor of vitamin D, discovered in in 1974 and 1975 by Brumbaugh and Kream B.B: it is ubiquitous expressed in almost all immune cells, such as activated CD4+ and CD8+ T cells, B cells, neutrophils, antigen-presenting cells, as macrophages and dendritic cells [130, 131].

Arise a new concept of vitamin D, not only hormone, but a molecule with immuno-modulator activities. The new role of vitamin D in the immune system it has investigated through studies of vitamin D-deficient mice [132]. Furthermore, vitD3 has been associated to Th1-mediated autoimmune diseases in animal models for experimental allergic encephalomyelitis, systemic lupus erythematosus, and type I diabetes. In vitro studies revealed that VDR is expressed at low levels in naïve T cells, and the expression increased after activation; it was demonstrated *in vitro* that the activation of human γδTcells by IPP leads to the up-regulation of the VDR and that vitamin D negatively regulates several cytokines production [133-139]. The binding of vitD3 to VDR leads to the inhibition of IFN-γ production [140] and interleukin-2 (IL-2) in peripheral blood lymphocytes and T cell lines [141]. Furthermore, vitD3 inhibits the expression of inflammatory cytokines in monocytes, including IL-1, IL-6, TNF-α, IL-8, and IL-12 [142-144] and decreases the expression of MHC class II, CD40, CD80, and CD86 [145]. Interestingly, *in vivo*, but not *in vitro*, it has been demonstrated that the supplementation of vitD3 leads to an increased expression of IL-4 with a concomitant reduction of IFN-γ levels [146, 147]. Boonstra in 2001 revealed that supplementation of vitD3 breaks the balance between Th1- Th2 that, respectively, produce IFN-γ and IL-4, in favour of Th2 with a consequent reduction of IFN-γ and a parallel

increase of IL-4 [148]. In 2005 Bertoldo raised the question upon the association between 25-OHvD serum levels and the development of APR after the first infusion of N-BPs: 70% of the patients with APR had 25-OHvD levels below 30 ng/mL, and in the in the counterpart in whom APR did not happen, the 25-OHvD level was above 30 ng/mL. Low level of vitamin D arise the risk of APR after the ZA infusion [44]. Our group and other previous works have reported a higher risk to develop APR in response to N-BPs in patients with insufficient levels (≤30 ng/ml) of 25-OHvD confirmed the data expressed by Bertoldo (Crotti C et al submitted). Based on literature data, vitamin D can strongly modulate the γδTcell response, reducing the production of inflammatory cytokines after IPP stimulation [149] and increasing the activity of Th2 cells, and *in vivo* data suggesting that low level of vitamin D status may promote the APR [148]. Furthermore, several issues remain open, including that only a subgroup of patients develop APR and the *in vivo* role of γδTcells producing IL-17 (Th17-like) [150].

OSTEOPOROSIS: work hypothesis

Although the molecular mechanisms of APR related to N-BPs have been clarified, it is not completely solved the proportion between N-BPs-related APR and γδTcells: why only a minority of patients are subjected to APR after infusion of N-BPs?

We conducted a study to evaluate the number and phenotype of circulating γδTcells in relation to ZA-associated APR and 25-OHvD level to investigate:

- If the number or the γδTcells phenotype could be identified the patient that will arise APR after the first ZA infusion;
- If the 25-OHvD level could influence the number or the γδTcells phenotype and consequently the onset of APR.

Material and methods

Patients

We consecutively enrolled 52 patients (50-80 years old) affected by postmenopausal OP followed at the Rheumatology Unit of the Humanitas Research Hospital who received their first infusion of 5 mg intravenous ZA. Serum 25-OHvD levels were assessed one week before the ZA infusion. Patients previously treated with ZA or other amino-bisphosphonates, suffering from chronic renal failure with GRF values <30 mL /minute, other osteo-metabolic (Paget's disease, primary hyper-parathyroidism, osteomalacia), onco-haematological or rheumatologic diseases (arthritis, connective tissue diseases, vasculitis, other systemic autoimmune diseases) were excluded from the study.

The study has been conducted in accordance with the Declaration of Helsinki and its subsequent modifications. All patients provided written informed consent and the protocol was reviewed and approved by the local ethical committee.

Methods

Ten mL of venous blood were collected from the enrolled patients, just before ZA infusion, and they were used for flow-cytometer and ELISA analysis. One week after the treatment a second blood sample was obtained from 9 subjects and a phone call was used to record the occurrence or the absence of APR in all the patients. Patients experiencing APR were identified as APR+. One hundred µL of whole blood were immediately stained for cytometry analysis, the rest of the blood sample was used to obtain serum.

One hundred µL of fresh whole blood were diluted with 2mL of FACS buffer (2% FBS HBSS) and centrifuged at 400xg at 4°C for 5 minutes. Pellet was incubated for 10 minutes with 100µL of FACS buffer plus 1% IgG from human serum (Sigma Aldrich) in order to saturate FcγR sites on the cells. After a washing step, cell pellets were re-suspended in an appropriate volume of FACS buffer. The following mouse anti-human monoclonal primary antibodies were purchased from eBioscience: CD3 FITC, TCRγδ-PE, CD69-PERCP-Cy5.5, CD27-PE-Cy7, and IL23R-Alexafluor 700. Five µL of each antibody solution were used. Incubation for 30 minutes in the dark at 4°C followed. 2mL of 1x RBC Lysis Buffer (eBioscience) were added to each tube without washing the cells. After incubation for 10 minutes in the dark at room temperature cells were washed twice;

following the final centrifugation, samples were re-suspended in FACS buffer and acquired. Lymphocytes were gated according to their surface markers: γδTcells (CD3+γδTCR+), naïve γδTcells (CD3+γδTCR+CD27dimIL23R-), central memory Th1-like (CD3+γδTCR+CD27brightIL23R-), Th1/Th17-like (CD3+γδTCR+CD27+IL23R+), Th17-like (CD3+γδTCR+CD27-IL23R+), effector memory Th1-like (CD3+γδTCR+CD27nullIL23R-) (Caccamo, N.,2005; Sutton, C.E., 2012). To evaluate the activation in response to ZA infusion, γδTcells were gated with CD69 PERCP-Cy5. Data analysis was performed on a 5-laser cytometer (FACS Cytofluorimeter La Fortessa, BD) using Diva™ Software (BD Pharmingen).

Serum samples were processed with ELISA kits (R&Dsystem) for the detection of human IFNγ, human IL-17, and human IL-13 following the manufacturer's instructions. All samples were run in triplicate and the absorbance measured at 450 nm. Cytokine concentration was assessed with Soft-max Pro software.

Statistical analysis

The Mann-Whitney or Wilcoxon tests were used for comparisons between groups. Correlations were analyzed by Spearman correlation coefficient (R). The risk to develop APR in association with 25-OHvD levels was calculated with contingency table as odd ration (OR) and 95% confidence interval (95%CI). Statistical significance was defined as $p \leq 0.05$. Results were expressed as median and interquartile range. Statistical analyses were carried out using GraphPad PRISM for Windows.

Osteoporosis: results

We recorded that twenty-six/52 (50%) patients exposed to the first infusion of ZA developed APR three days after ZA infusion (APR+). We noted that the percentage of γδ T cells was not significantly different in APR+ patients compared to the APR- patients before ZA infusion (Table 6).

Table 6. The table displays the percentage of γδ T in all patients, in APR+ patients and APR- patients before the ZA infusion.

	All patients	APR +	APR -
number	52	26	26
age (years)	68 (62-77)	65 (60-73)	72 (64-78)
γδ T cells (%)	**4.1 (3.4-6.6)**	**3.8 (2.9-5.0)**	**5.7 (2.7-8.2)**

Furthermore, analyzing the γδT cell subpopulations, we observed a significant prevalence of central memory Th1-like population (cmTh1-like), the subset potentially targeted by ZA, that characterized APR+ patients with 48% (23.7-65.1) compared to APR- patients central memory Th1-like cells 24.5% (13.4-48.4); p>0.05 (table 7).

Table 7. The table shows the γδT cell subpopulations in all patients, in APR+ patients and APR- patients before the ZA infusion. Of note the cm Th1-like population higher representative in patients with APR experience.

	All patients	APR +	APR -
naïve γδ T cells (%)	7.4 (5.2-14.3)	10.2 (6.2-18.7)	6.1 (3.0-14.4)
cm Th1-like (%)	**34.1 (17.9-49.9)**	**48.0 (23.7-65.1)**	**24.5 (13.4-48.4)**
Th17-like (%)	14.9 (8.5-27.0)	12.0 (4.3-23.0)	18.6 (12.0-47.3)
Th1/Th17-like (%)	9.6 (7.3-15.4)	13.4 (6.1-17.3)	9.4 (6.3-19.6)
em Th1-like (%)	9.9 (4.1-12.3)	5.4 (3.9-11.2)	11.9 (3.6-37.9)

cm Th1-like: central memory Th1-like; emTh1-like: effector memory Th1-like.

Moreover, considering the subpopulation after the ZA intravenous infusion, in the patients that had an experience of APR, we observed a decrease in the central memory Th1-like subset from

35.7% (10.9-54.8) before the infusion until 25.9% (21.2-39.4) of central memory Th1-like subset after the ZA infusion. In the APR- patients we detected 48.4% (32.0-59.4) of central memory Th1-like subset ,before the treatment, and 53.1% (36.5-61.6) of central memory Th1-like subset after ZA infusion; however, the central memory Th1-like subset increase was not significantly relevant (table 8, figure 5,6).

In parallel, we observed a slight increase in the effector memory Th1-like subset in APR+ patients: from 6.6% (3.9-15.2) before the ZA infusion until 7.1% (5.2-8.2) after the treatment. In the patients APR-, instead, we detected 11.5% (6.1-10.0) of effector memory Th1-like subset before the infusion and 10.4% (5.2-12.3) of effector memory Th1-like subset, after the ZA treatment; the slight increase in the effector memory Th1-like in APR+ compared to APR- was not statistic significant ($p>0.05$) (table 8).

Notably, only the effector memory Th1-like cells in APR+ patients demonstrated a slight grade of activation after ZA infusion: 0% (0-41.8) before the treatment compared to 4.6% (1.3-25.5) after ZA infusion. In APR- patients, in the pre-treatment analysis, we detected 2.4% (0.7-31.7) of effector memory Th1-like cells and after the infusion we observed a similar percentage 2.9% (0-33.4) with no significant p value (table 8).

Interestingly, in APR+ patients, we observed an increase in the Th17-like population: from 15.9% (8.2-33.4) before the infusion, until 28.1% (25.6-42.4) after the treatment; in APR- we detected, before the ZA administration, 15.0% (7.0-33.7) of Th17-like population compared to 14.0% (7.9-26.5) after the infusion with a no significant p value table 8, figure 5,6 .

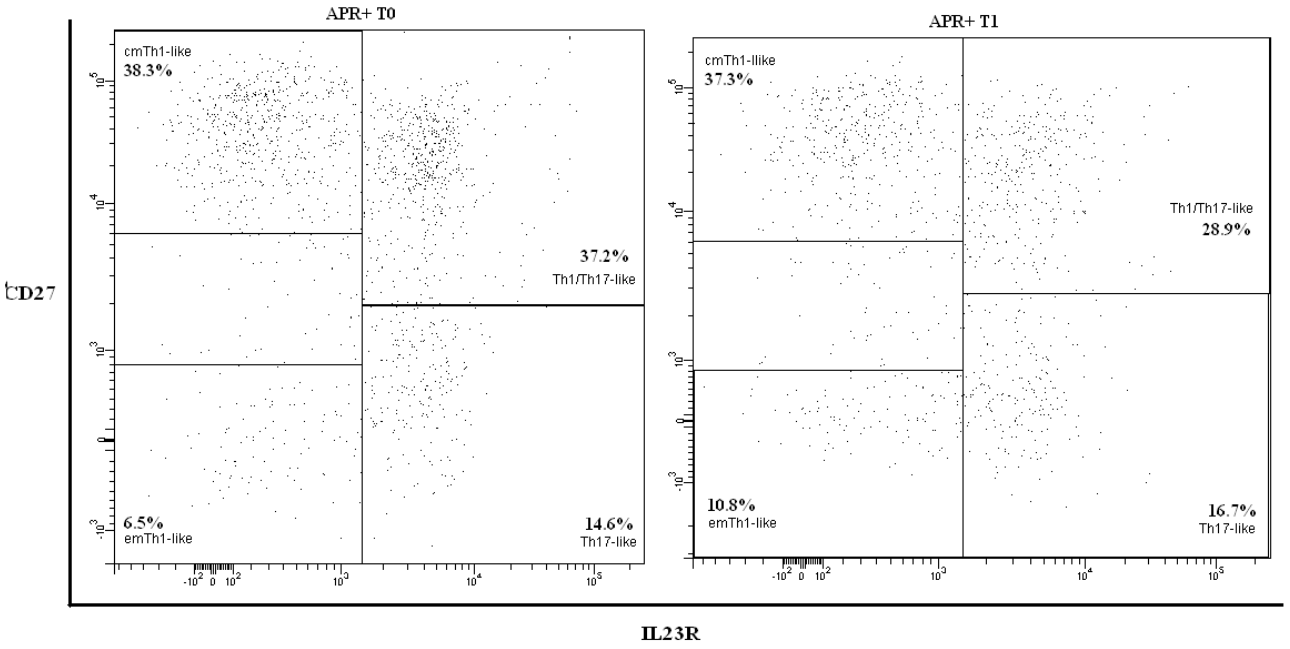
Table 8. The table displays in each cells the γδT cell and the subpopulations in APR+ patients and APR- patients before and after the ZA infusion.

Characteristics	APR +	APR -
patient number	5	4
γδ T cells (%) T0 vs T1	4.1 (3.0-5.0) vs 4.6 (1.9-7.6)	5.4 (2.8-7.3) vs 5.4 (3.7-9.3)
naïve γδ T cells (%) T0 vs T1	5.2 (0.9-10.8) vs 5.1 (1.8-17.1)	3.0 (1.4-5.9) vs 3.5 (2.1-4.2)

cm Th1-like (%) T0 vs T1	35.7 (10.9-54.8) vs 25.9 (21.2-39.4)	48.4 (32.0-59.4) vs 53.1 (36.5-61.6)
Th17-like (%) T0 vs T1	15.9 (8.2-33.4) vs 28.1 (25.6-42.4)	15.0 (7.0-33.7) vs 14.0 (7.9-26.5)
Th1/Th17-like (%) T0 vs T1	13.4 (3.1-25.7) vs 10.9 (4.0-23.1)	10.8 (9.4-19.6) vs 15.0 (11.5-20.8)
em Th1-like cells (%) T0 vs T1	6.6 (3.9-15.2) vs 7.1 (5.2-8.2)	11.5 (6.1-10.0) vs 10.4 (5.2-12.3)
activated γδ T cells (%) T0 vs T1	11.0 (2.3-27.8) vs 11.1 (4.9-38.0)	6.6 (2.7-36.7) vs 5.6 (1.8-34.1)
activated cm Th1-like (%) T0 vs T1	0 (0-11.3) vs 0.9 (0-17.0)	0.6 (0.3-15.3) vs 0 (0-10.9)
activated Th17-like (%) T0 vs T1	25.8 (3.8-53.8) vs 20.6 (2.6-59.4)	30.5 (10.1-54.9) vs 26.8 (14.6-53.2)
activated Th1/Th17-like (%) T0 vs T1	30.9 (12.9-66.7) vs 29.5 (19.2-36.6)	15.9 (8.8-53.8) vs 13.7 (4.3-50.2)
activated em Th1-like (%) T0 vs T1	0 (0-41.8) vs 4.6 (1.3-25.5)	2.4 (0.7-31.7) vs 2.9 (0-33.4)

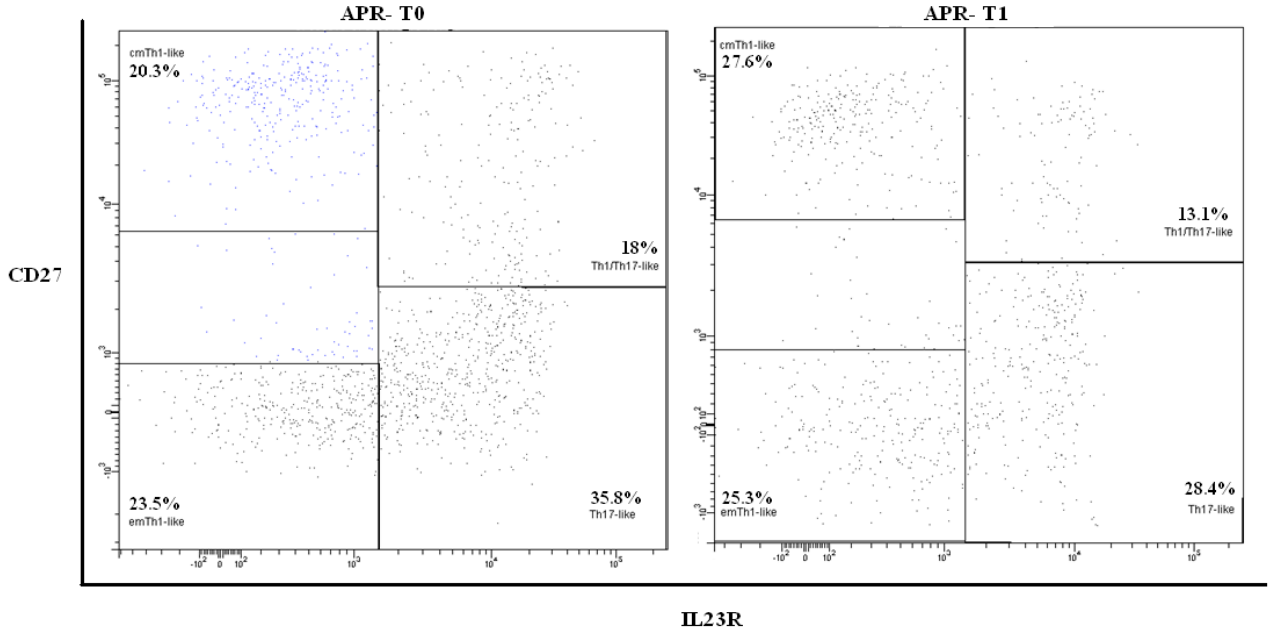
cm Th1-like: central memory Th1-like; emTh1-like: effector memory Th1-like.

Figure 5. APR+ patients had higher peripheral blood central memory Th1-like γδ T cells before ZA infusion and showed an increase in effector memory Th1-like γδ T cells and Th17-like cells after ZA infusion.



cm Th1-like: central memory; em Th1-like: effector memory; CD27 and IL23R subset marker; T0: pre-treatment; T1:post-treatment.

Figure 6. In APR- patients had similar percentage of effector memory Th1-like cells , cm Th1-like Th1/Th17-like with a parallel decrease of Th17-like population after the infusion.



cm Th1-like: central memory; em Th1-like: effector memory; CD27 and IL23R subset marker; T0: pre-treatment; T1:post-treatment.

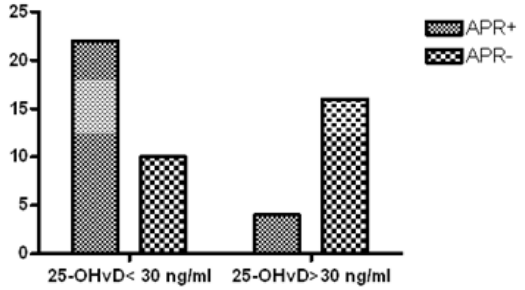
Furthermore we processed sera, in order to validate the cytokine production with ELISA tests in our cohort of patients, but no significant differences were detected in serum IL-17, IFNγ, and IL-13 cytokine levels before and after ZA infusion or in relation to APR (data not shown).

Significantly lower 25-OHvD levels were observed in APR+ compared to APR- patients: in details 22.9 (16.9-28.2)ng/ml was calculated in the patients that had experienced of APR compared to 40.5 (20.8-47.1) ng/ml characterized the APR- patients; the difference is statistically significant with a p value of 0.005 (table 9). In our cohort, 32 patients had insufficient 25-OHvD levels (≤30 ng/mL), 22 of whom had APR (table 9), which is comparable with the results found in literature. 25-OHvD levels were than correlated to APR experienced, in order to evaluate if the levels of 25-OHvD level was considered protective or not; we found that patients with 25-OHvD levels ≤30 ng/mL had a significantly higher risk to develop APR compared to patients who had adequate levels of 25-OHvD. in details, the odd ratio was estimated to 8.6 IC 95% 2.3-33.8 with a significant p-value= 0.0006 (figure 7).

Table 9. The table shows the frequencies of 25-OHvD inadequate levels (≤30 (ng/mL) and sufficient level in all patients, APR+ and APR- patients.

	All patients	**APR +**	**APR -**
25-OHvD levels (ng/mL)	26.7 (18.7-42.3)	22.9 (16.9-28.2)	40.5 (20.8-47.1)
25-OHvD ≤30 (ng/mL)	32 (61.5%)	22 (84.6%)	10 (38.5%)

Figure 7. The odd ratio and contingency table show that a lower 25-OHvitD levels increases the risk of APR



P value	**0.0006**
Odds ratio	8.6
95% confidence interval	2.335 to 33.16

Data analyzed	APR+	APR-	Total
25-OHvD ≤30 ng/ml	22	10	32
25-OHvD >30 ng/ml	4	16	20
Total	26	26	52

The correlation analysis shown that, there was no significant association between 25-OHvD and γδ T cell percentage and between 25-OHvD and each γδ T cell subpopulations. Moreover we investigated the several cell populations percentages based on the insufficient and on the adequate 25-OHvD levels. We found that the percentages of γδ T cells, central memory Th1-like, and effector memory Th1-like were slightly higher in the patients with insufficient 25-OHvD levels; in details: γδ T cells: 5.8% (2.9-7.4) in the insufficient 25-OHvD levels condition and 3.8% (2.5-4.6) in the sufficient 25-OHvD levels; the central memory Th1-like shown 37.7% (14.4-59.4) in the deficient 25-OHvD levels compared to the adequate one with 34.1% (16.7-55.6) percentages; the effector memory Th1-like displayed 10.6% (4.1-22.7) in the lacking 25-OHvD levels conditions compared to 6.6% (2.9-15.0) with the appropriate 25-OHvD levels; but this did not reach statistical significance (table 10).

Table 10. the table displays the percentages of γδ T cells and subpopulation in two categories of our cohort (25-OHvD ≤30 ng/mL and 25-OHvD >30 ng/mL).

Characteristics	25-OHvD ≤30 ng/mL	25-OHvD >30 ng/mL
number	32	20
γδ T cells (%)	5.8 (2.9-7.4)	3.8 (2.5-4.6)
naïve γδ T cells (%)	6.1 (4.7-10.2)	11.0 (4.7-22.1)
cm Th1-like (%)	37.7 (14.4-59.4)	34.1 (16.7-55.6)

Th17-like (%)	13.9 (8.0-24.6)	19.0 (4.8-42.5)
Th1/Th17-like (%)	9.1 (6.3-15.4)	12.0 (5.5-23.0)
em Th1-like cells (%)	10.6 (4.1-22.7)	6.6 (2.9-15.0)

cm Th1-like: central memory Th1-like; emTh1-like: effector memory Th1-like.

Osteoporosis: discussion and conclusions

OP is a growing healthcare problem worldwide, particularly when associated with chronic disease, and bisphosphonates such as ZA have significantly changed our approach to these patients [151]. Nonetheless, ZA is burdened by the risk of APR which significantly impacts the adherence to treatment. For this reason, it is important to identify biomarkers that allow predicting the occurrence of APR, similar to what suggested for vitamin D. We herein investigated the changes in γδcells and vitamin D levels in this clinical setting.

Memory T cell populations comprise central memory T cells, which are restricted to the blood and secondary lymphoid tissues, and effector memory T cells, which migrate between blood, spleen and peripheral tissues. These two memory populations are the main players of host defense and auto-immunity in αß T cells, but also in γδ T cells. Once the antigen is eliminated, these cells persist in the memory pool to react promptly in case of antigenic rechallenge. Our data suggest that central memory γδ T cells differentiate into effector cells after ZA stimulation, confirming what has been reported in vitro when ZA, generally in combination with IL-2, is used to expand γδ T cells for cancer immune-therapy [152, 153] . In line with this view, patients with a greater pool of central memory γδ T cells in the peripheral blood have a higher chance to develop APR upon phosphoantigen stimulation. It has to be clarified why approximately 50% of patients do not manifest such ZA-associated APR. The protective effect of higher 25-OHvD levels together with a lower number of central memory γδ T cells could explain the absence of APR in these patients, even if we could not find a direct correlation between 25-OHvD and γδ T cell percentages in our small cohort of patients. We cannot exclude that the protective role of 25-OHvD is due to its immune-modulatory effect on macrophages [154], which

act as antigen presenting cells for γδ T cells during ZA stimulation [155], or to its action favoring a Th2 shift instead of Th1 polarization [148]

The main γδ T cell population is the γ9δ2 T cells and up to 80% of these are polarized to produce IFN-γ upon activation as default. In murine models the fate of γδ T cells is decided in the thymus and depends on the signaling through TCR and CD27 for Th1-like γδ T cells, and on cytokines as lymphotoxin or TGF-β for Th17-like γδ T cells. In adulthood, Th1-like γδ T cells continue to require triggering of both TCR and the co-stimulation molecule CD27 to differentiate in Th1-like effector γδ T cells and to produce IFN-γ [123]; while Th17-like γδ T cell activation and expansion seem to depend on cytokine milieu and antigen stimulation, but it is still debated if TCR engagement or costimulation are required [156], considering the absence of CD27. It has been hypothesized that 2 types of Th17-like γδ T cells exist: thymocyte-derived "natural" Th17-like γδ T cells and "inducible" Th17-like γδ T cells [157]. The former are localized in peripheral tissues, they express IL23R and they are fast producing IL-17 (24 hours), probably without requiring TCR engagement [158]; the latter derive from the uncommitted naïve pool, after the antigen-specific stimulation they express Th-17 marker as IL23R and they produce IL-17 in 60 hours [145]. Considering the timing of APR (24-36 hours) we think that "natural" Th17-like cells could expand during the course of APR thanks to the macrophage activation by ZA and the consequent production of cytokines such as IL-1 and IL-23. While it has been described that central memory γδ T cells can down-modulate CD27 and acquire an effector memory Th1-like phenotype following phosphoantigen activation, it has never been proved that they can express IL23R and then produce IL-17 after ZA stimulation. We can also hypothesize that increased Th17-like cells can further contribute to APR together with effector memory Th1-like cells.

It is still debated whether Th17-like γδ T cells can also produce IFNγ as αβ T cells [156, 159]. We herein observed a small percentage of γδ T cells expressing CD27 and IL23R, markers of Th1-like γδ T cells and Th17-like γδ T cells, respectively. Moreover, we observed a decrease in the Th1/Th17-like γδ T cell population as for central memory pool in patients with APR. Indeed, we cannot exclude a down-modulation of CD27 in the Th1/Th17-like γδ T cells contributing to the increase in the Th17-like pool. These data support the hypothesis that γδ T cell commitment or

differentiation is a plastic mechanism and that there is some flexibility within central memory γδ T cell population [160].

No differences were observed in serum IL-17 or IFNγ cytokine levels, in relation to Th1-like and Th17-like changes, before and after ZA infusion or in relation to APR, probably because the samples were available some days after APR occurrence when the acute reaction had already been resolved. Moreover, other inflammatory cytokines are involved in ZA-associated APR, as TNFα, IL-1, and IL-6 but we did not evaluated their concentration [155].

In conclusion, our data suggest that differences in the γδ T cell memory pool together with 25-OHvD levels can both influence the occurrence of ZA-associated APR. Moreover, an adequate supplement of the vitamin D pool may be appropriate to prevent APR in ZA-treated patients thus reducing the access to healthcare for the symptoms of APR and possibly maximizing the treatment adherence and response to ZA.

alpha-beta T cells

Rheumatoid arthritis: definition

Rheumatoid arthritis (RA) is an autoimmune disease affecting synovial joints. The chronic inflammation of the synovia cause: pain, stiffness, swelling, and limited movements and function of the involved joints. The main joints involved are: the small joints of the hands and feet. RA leads to the destruction of articular cartilage and bone erosions. RA is a systemic disease, often involving internal organs as lungs, pericardium, pleura, sclera, and is also associated to skin nodular lesions [161].

Because its unpredictable course, RA classification criteria were developed in order to standardize the diagnosis between different centers. The most used classification criteria are the 1987 American College of Rheumatology (ACR; formerly the American Rheumatism Association) criteria [162]. Several updates occurred during the years, the last was published in

2012. RA inflammatory activity cannot be measured using one single variable, so several parameters have been used for this purpose, such as joint counts, acute phase reactants, global assessment scales, pain, fatigue, duration of symptoms, and serology. The new classification criteria are summarized in table 11

Table 11. The 2012 classification criteria of RA are recapitulated by joint distribution, serology, symptoms duration, and acute phase reactants.

JOINT DISTRIBUTION (0-5)	**points**
1 large joint	0
2-10 large joints	1
1-3 small joints (large joints not counted)	2
4-10 small joints (large joints not counted)	3
>10 joints (at least one small joint)	5
SEROLOGY (0-3)	
Negative RF* AND negative ACPA**	0
Low positive RF* OR low positive ACPA**	2
High positive RF* OR high positive ACPA**	3
SYMPTOM DURATION (0-1)	
<6 weeks	0
≥6 weeks	1
ACUTE PHASE REACTANTS (0-1)	
Normal CRP*** and normal ESR****	0
Abnormal CRP*** or abnormal ESR****	1

*Rheumatoid factor; ** anti-citrullinated protein antibody; *** C reactive protein; **** Erytrocytes sedimentiation rate

If the total score is equal or major than 6, the patients may have diagnosis of RA.

Other scores have been used to complete the monitoring of the disease activity such as, Disease Activity Score (DAS), the DAS using 28 joint counts (DAS- 28) [163], the Simplified Disease Activity Index (SDAI) [164]and the Clinical Disease Activity Index (CDAI) [165]. The parameters of each activity score have been represented in the table 12.

Table 12. the table displays the comparison of several parameters in the different disease activity scores: SDAI, CDAI, and DAS28

Elements	SDAI	CDAI	DAS28
Number of swollen joints	Simple count (0-28)	Simple count (0-28)	Simple count, square root transformed (0-1.48)
Number of tender joints	Simple count (0-28)	Simple count (0-28)	Simple count; square root transformed (0-2.96)
Acute phase reactants	CRPin mg/dL (0.1-10.0)	– –	ESR, log transformed (0.49-3.22)**
Patient global health	–	–	VAS in mm (0-1.40)**
Patient global disease activity	VAS in cm (0-10.0)	VAS in cm (0-10.0)	–
Evaluator global disease activity	VAS in cm (0-10.0)	VAS in cm (0-10.0)	–
Total index	No immediate scoring due to CRP; simple calculation possible (0.1-86.0)	Immediate scoring possible; simple calculation possible (0-76.0)	No immediate scoring due to ESR; calculator required (0.49-9.07)

The DAS28 is calculated using an external standard of RA disease activity, and combines information from swollen joints, tender joints, the acute phase response and general health into one continuous measure, from 0 to 10, of rheumatoid inflammation. The level of disease activity can be interpreted as low (DAS28 < 2.4), moderate (2.4 < DAS28 < 3.7), or high (DAS28 > 3.7) [166]. A DAS28 < 1.6 corresponds to remission according to the American Rheumatism Association (ARA) criteria [163].The SDAI score was created to simplify the measurement during the clinical practice, ESR was change in CRP which is one of the most unfailing measures of the acute phase response. The level of disease activity can be interpreted as low (SDAI ≤ 11), moderate (SDAI ≤ 26), or high (SDAI > 26) [164]. The remission has a cut off of SDAI ≤3.3 according to the American Rheumatism Association (ARA) criteria [163]. The CDAI score was created in order to overpass the missing laboratory test results during the patient visits. the

remission was detected with CDAI≤2.8, the low disease activity has CDAI≤10, the moderate activity has CDAI≤22 and the high disease activity has CDAI>22 [165].

The introduction of new scores such as the SDAI and the CDAI was not aimed at competing with the DAS28, which remains the most extensively validated activity index for RA, but they were created in order to satisfy the several clinical practice conditions [165].

Rheumatoid Arthritis: diagnosis

The diagnosis of early stage of RA is the goal, but the missing of disease-specific early-onset features, makes the investigation of the diagnosis challenging [167]. Methods to recognize RA signs and symptoms include clinical assessment, imaging and laboratory tests [168].

X-ray are the technologies to detect the joints damage calculated by scores. Magnetic resonance imaging and ultrasound can support visualisation of early signs, but are not considered the principal strategy [167, 169].

The laboratory tests consist in the detection of rheumatoid factor (RF) and anti-CCP antibody, but the first test is non-specific and has low sensitivity in patients with early stage of RA [170, 171]. For instance, RF is negative in between 20% and 30% of RA patients [172]. Finally, synovial fluid analysis could exclude other possible causes of inflammatory arthritis, such as gout [167].

Rheumatoid Arthritis: epidemiology

The disease affected all races of all over the world, it could be occur any time during our life, but the age predominantly affected is about 40-50 years; RA is a female predominant disease with a ratio of 1:3 female vs men [173].

In 1995 in the Rochester Epidemiology Project, the RA prevalence in women was 7.7 per 1000 compared with 4.4 per 1000 among men. Recent prevalence data, in the 2005 showed an increased prevalence among women (9.8 per 1000), differently from male (4.1 per 1000) [174]. In the general population, the prevalence of RA is to range from 0.5-1.0% [175, 176]. Data about

the incidence of RA report that the peak among people aged 65-74 years was 89 per 100,000 (all estimates age-adjusted to 2000 US population) [177]. Recent data showed from 1995 to 2007, an increase in the diagnosis of 2.5% each year among women, but there was a small decrease (0.5%) among men [178]. Epidemiological study in 2014, show that five million adult Italian people, have RA with a prevalence of 0.41 % and an incidence of 35/100,000, with no differences between Northern and Southern regions or urban and rural areas "with a female/male ratio declining from ca. 3.3 during the second–third decade of life to less than 2 at later ages" .
The mortality rate of RA in 1997 was about 22% of all deaths due to arthritis and other rheumatic conditions [179, 180]. In the latest data the rate increased among people with diagnosed RA in comparison with the general population. Deeply, 40% of all deaths in RA are attributable to cardiovascular complications, including ischemic heart disease and stroke. In particular, among RA patients, the presence of RF and/or anti-CCP are potential markers of premature mortality [181].

Rheumatoid Arthritis: Signs and symptoms

Often the onset of RA is asymptomatic or could affect the patients with nonspecific symptoms such as astenia, inappetence and mild fever. Commonly, patients report gradual joint pain associated with generalized persistent morning joint stiffness. In most cases early RA onset involves multiple joints, in particular the smaller joints of hands and feet. With the progression of the disease knees, ankles, elbows, hips and shoulders can be affected. RA, as systemic disease, could lead in the 40% of the cases to extra-articular involvement and the 15% of those are severe. Rheumatoid nodules represent the 20-30% of systemic manifestations and are localized next to the peri-articular area or in the lungs, pleura and meninges. The nodules are often asymptomatic, but they could be infected or broken. Another extra-articular manifestation is the vasculitis which characterized the patients with severe RA and could lead to neuropathy and skin ulcers. The pulmonary involvement, males associated, leads to pleuritis, interstitial fibrosis, nodules, pneumonia. Episcleritis and scleritis usually occur in patients with RA of long

duration with rheumatoid nodules. 15-20% of patients with RA may develop a secondary Sjögren's syndrome. Felty's syndrome is typical of chronic RA and is associated with splenomegaly, neutropenia, and occasionally anemia and thrombocytopenia. Another RA associated disease is OP, because RA leading to a decrease of BMD proportionally to a parallel increase of RA disease activity. Finally, lymphomas, in particular those of B cells are associated with RA [182].

Rheumatoid Arthritis: management

The goals in the RA treatments are:

- the decrease of inflammatory state;
- the inhibition or the slowdown of RA progression;
- the preservation or the recovery or the joints function;
- the prevention or the correction of the joints deformity.

Basically, the therapeutic options adopted go in parallel with patients behaviour: control of physical activity, to maintain a moderate activity to prevent muscular atrophy and articular rigidity; the diet, allows the overloading the joints. The therapeutic options include non – biologic and biologic immunomodulators.

non-biologic therapies:

- Glucocorticoids: including prednisone and methylprednisone used for their anti-inflammatory and immunosuppressant proprieties [183].
- Methotrexate: a folic acid analogue approved in 1970 for RA management is still considered the main treatment for moderate to severe RA. Sometimes methotrexate could be administered in combination with other therapies if the response is not sufficient or inadequate [184, 185].
- Sulfasalazina: is a combination of anti-bacterial and anti-inflammatory compounds and was the first that showed efficacy in the RA treatment in the 40’s. In the today

clinical practice is not considered the first choice, but is well tolerated by the patients [186].

- Leflunomide: proved in 1998 for RA treatment, equal efficacy compared to methotrexate but higher side effects (15%) such as rash, alopecia, diarrhea and transaminase elevation; moreover, its active form, the teriflunomide, persist in sera patients for up to 2 years [187].
- Cyclosporine: in the past was indicated for active RA or non-responder to methotrexate, but in the clinics is overpass by the biologic therapies [188, 189].
- Cytotoxic agents: such as azathioprine and cyclophosphamide approved for the treatment for active RA, but their efficacy were found to be inferior to methotrexate [190].

Biologic therapies:

- TNF inhibitors: such as Etanercepr, Infliximab, Adalimumab, Certolizumab, Golimumab; they act reducing the inflammation by the inhibition of TNF activity [191, 192];
- CTAL4 inhibitor: such as Abatacept, is a fusion protein that leading to the inhibition of the second signal of T-cell activation [193];
- IL1 ß inhibitor: such as Anakinra, is a recombinant human IL-1 receptor antagonist and inhibits inflammation and cartilage degradation [194];
- IL6 inhibitor: such as Tocilizumab, a humanized monoclonal antibody that blocks the pro-inflammatory cytokine [195];
- B cell depletory: such as Rituximab, a chimeric monoclonal antibody targeting the CD20 [196];
- Newest: such as Baricitinib (anti-JAK1-JAK2) mediates signal transduction for variety of cytokines involved in inflammatory conditions; is in Phase III development [197];

All biologic treatments have several common side effects such as the re-activation of latent infections (i.e.Tubercolosis) or malignant process, leading to the monitoring of the patients.

Rheumatoid Arthritis: pathogenesis

The RA is a multifactorial disease of unknown etiology, consequently exists a contribution of several factors, such as genetic, environmental and immune system factors acting together in the onset of RA. Twin studies are a useful tools to investigate the contribution of genetics in the pathogenesis of RA, because of their identical genetic background shows how other factors can interfere with the onset of the disease. Several studies on twins demonstrate that the concordance rate of monozygotic twin is 15-30% and among dizygotic is 5% [198].The low concordance rate in an hypothetical identical background suggests a greater contribution of the environmental factors; one of the greater environmental risk is represented by the smoke; for instance an old twin study, demonstrated that in a total of 13 monozygotic twin pairs discordant for RA, the RA twins in the 92.3% were smokers [199].

The most significant genetic contribution was the association of RA with the class II molecules (HLA), especially DR4 and DR1 alleles, suggesting that the disease is associated with the binding of an auto-antigen to predisposing HLA-II molecules [200]. The HLA –II are expressed on the surface of B lymphocytes, denditric cells and monocytes, and are divided in several alleles: HLA-DR, -DP and –DQ. HLA alleles have different variants and are genetically inherited by both parents (Abbas AK, Lichtman AH. Fondamenti di immunologia. Edizione Piccin; 2010). The HLA molecules are constituted by two chains, the alpha (DRα) and the beta (DRβ) chain, anchored together to build the membrane receptor upon antigen presenting cells. The receptor presents the peptide antigens through the binding pocket to T lymphocytes then processed and next leading to the immune response.

In particular, HLA-DR molecules are clustered on chromosome 6 and encode for various alleles and the DR4 and DR1 have been investigated in several studies with different ethnic groups [201, 202] and regarding RA disease, it has been demonstrated that the HLA-DR4 occurred in 70% of RA patients compared with ~30% of controls. In different ethnic groups the predominant alleles

vary: *0401 and *0404 are the predominant RA associated alleles in Caucasians; *0405 in Japanese and *0101 in Israeli Jews. Association with DRB1*09 has been described in Chilean RA patients, Japanese and more recently UK Caucasians [203]. In particular, the third hyper-variable region of DR ß -chains, from amino acids 70 through 74 [204], constituted by glutamine-leucine-arginine-alanine- alanine (QKRAA) aminoacids, is called shared epitopes (SE) and is present in the peptide binding pocket of the HLA-DR [205, 206]. The presence of SE on the surface of the antigen presenting cells could influence both, peptide binding to HLA and T cell presentation leading to RA susceptibility. In this contest, several hypothesis have been postulated: the SE shapes the TCR repertoire permitting the escape from tolerance or the survival of autoreactive clones; the SE is involved in the molecular mimicry with a pathogen or fails to bind an arthritogenic peptide, leading to an inadequate tolerant immune response [207]. Recently, it was observed that the SE alleles are present in most of the anti-CCP+ RA patients, and not in anti-CCP–negative RA [208]. The HLA polymorphism predicts also the course of RA symptoms: the alleles carrying this nucleotide sequence DRB1*0401, *0404, *0405, *0408, *0101, *0102, *1402, *09 and *1001 are associated with greater disease severity, positive IgM rheumatoid factor and greater degrees of joint deformity [200, 209]. In contrast, there are other alleles that are negatively associated with RA and therefore provide a protective role: DRB1*0103, *0402, *0802, *1302. Consequently, the use of HLA typing for screening the RA patients could be useful to provide valuable markers for the prognosis of joint destruction in RA and to decide the appropriate treatment.

More recent studies show other non –HLA genes involved in the RA pathogenesis; for instance, in anti-CCP+ patients, Begovich et al in the 2004, in a case-control association study, discovered a SNPs on PTPN22 gene which are associated with RA [210]. PTPN22 encodes a 110-kD cytoplasmic protein tyrosine phosphatase that negatively regulates T and B cells [211];the association was weaker in anti-CCP negative patients. Other association are reported: FCGR are Fc fragment of IgG receptors and several SNPs are detected to be involved in RA. Anti-CCP+ RA patients showed an overexpression of variant 187-Ile (rs1050501: a substitution of isoleucine with the threonine) and seemed to regulate negatively B cell responses [212]. Another variant rs396991 created by

the sostitution of valine with the phenylalanine (158 V/F) has been involved in Dutch and British anti-CCP+ RA patients [213]. PTPRC, is an enzyme of the protein thyrosine phosphatase, its variant has been associated to RA and seems to regulate T- and B- antigen receptor signalling [214]. CTLA4, is a surface protein on T cells and seems to inhibit the co-stimulator signals leading to a negative regulation of T cells; a variant of CTLA4 has been studied in European cohort and has been demonstrated the association of gene to anti-CCP+ RA patients [215]. PADI4, is a peptidyl arginine deaminase responsible for conversion of arginine residues into citrullinated residues, is an obiquitinous enzyme expressed in most of nucleated cells and several variants has been associated to RA Asian patients, but the association has not yet confirmed in the European cohort [216]. TNFAIP3 belongs to NF-kB pathway, inhibiting the TNF-receptor and consequently the effects; recent GWAS studies have been demonstrated the association of TNFAIP3 to RA [217]. The variants of IL-6 signal transducer (IL6ST) and the ankyrin repeat domain (ANKRD55) seems to be nearly located and it has been demonstrated to be involved in RA susceptibility [218]. Malysheva, in 2008 has been found that beta-2-adrenergic receptor (ADRB2) could be involved in RA disease: in particular the study has been show how the ACPA positive RA patient show a particular polymorphism on ADRB2, a substitution of a glycine to arginine [219]. The allograft inflammatory factor 1 (AIF1) is involved in several autoimmune and inflammatory condition and in particular in RA it seems to be associated to the disease with a specific variant which is subjected to a polymorphism Arg15Trp [220].

CCR6 is a chemokinine involved in RA susceptibility (even CCL21), is expressed by immature dendtritic cells and memory T cells [221]. The interferon regulatory factor 5 (IRF5) is a ligand of several tool like receptors and plays an important role in innate and adaptive immunity; IRF5 seems to be associated to anti-CCP- patients [222].

BLK, the B lymphoid tyrosine kinase is involved in B cell signalling and development and a SNPs it has been associated to RA anti-CCP+ patients [216]. CLEC16 encodes for a C-type lectin, the polymorphisms associated are found in several autoimmune diseases, such as type-1 diabetes, multiple sclerosis, Addison's diseases and juvenile arthritis; a particular SNPs is associated with anti-CCP- RA patients [223].

Among environmental factors associated with RA, the discovery in most of case was conducted through a retrospective and prospective case-control studies, in order to highlight the influence of lifestyle, behaviour and the environment in the onset of disease. In literature the most important environmental risk highlighted during the years are: occupational exposure to silica, reproductive and hormonal factors, infections, dietary factor such as antioxidant, proteins and iron, alcohol, moreover, pollution and cigarette.

The most well-known risk factors among occupational exposure that is considered associated to RA, is the silica dust. Epidemiological study in the cohort of granite workers hired between 1940 and 1971 and followed up until the end of 1981 demonstrated that exposure to silica may contribute to an immunosuppressant phenomenon, leading to increase bacterial and viral infections of the respiratory tract. The risk was demonstrated in 2004 to be highest in the longest time of exposure [224]. Studies revealed that, silicosis patients are subjected to several pulmonary complications such as tuberculosis, emphysema, secondary bronchitis, and lung cancer [225]. Silicone contains silicon together with carbon, hydrogen, and oxygen; some common forms include silicone oil, silicone grease, silicone rubber, and silicone resin and medical use of silicone (particularly the gel form, includes bandages and dressings, breast implants) and a variety of other medical applications [226]. Studies on the pathogenesis mechanisms involved macrophages and peripheral T cells and silica is a selective macrophage toxin in all species examined leading to an inhibition of their function against bacterial and viral infections [227]. More recent studies suggest that silica can activate peripheral T cells: an observational study reveals that the inhibition of Treg was reduced in silica patients compared to healthy donors [228]. Another occupational exposure was discovered in the painter category: famous artists, during the history, have been affected by several autoimmune diseases, an observational study in the 1988, was conducted considering Rubens, Renoir, Dufy, all 3 had RA, compared to 8 artists without rheumatic diseases. “Artists can be heavily exposed to paint pigments and other harmful substances” and has be suggested that could be part in the development of their rheumatic diseases. The chemical substance, used by painters, were mercury sulphide, cadmium sulphide, arsenic sulphide, lead, antimony, tin, cobalt, manganese,

and chromium. Artists today are more safe from harmful substance, but “heavy metal contamination in food and drinking water exists and experience from the occupational exposure of old masters is still relevant”[229].

Nutritional factors of interest in the aetiology of RA have included antioxidants [230] fatty acids from oils, alcohol [231], coffee [232], red meat with proteins and iron [233], and vitamin D [234]. Antioxidant therapy have demonstrated promising results in experimental RA models and in human, for instance, products of free radical oxidation have been identified in the synovial fluid of inflamed rheumatoid joints, and several studies revealed low level in the plasma of patients with RA [235], which supports the theory that inflammation is mediated by free radical activity and they could be act with a protective role against oxidative damage. The antioxidants are vitamin E vitamin C and selenium and recently it has been demonstrated that antioxidants intake for 12 weeks reduced significantly serum hs-CRP and DAS-28 score [236, 237]. Little association was reported in coffee and tea, in particular, catechins of green tea seems to be protective against RA by acting together with antioxidant vitamins (i.e., vitamins C and E) and enzymes [i.e., superoxide dismutase (SOD) and catalase][230]. The study by Linos et al. (1999) confirmed that higher intakes of olive oil reduced the risk of developing RA (OR 0.39 (95% CI 0.19, 0.82) [238]. Alcohol is thought to have effects on both the hormonal and immunologic systems, a recent study found that the moderate alcohol consumption was associated with RA, peculiar the case of beer, that seems to be protective in RA onset [239].

RA is a female disease and the incidence is higher near the menopausal period suggesting an involvement of the sex hormones in the onset of the disease [240]; male investigations reported the low testosterone concentrations in male RA patients [241]. Consequently, the sex hormones oestrogen, androgen and prolactin have all been proposed as having a role in the onset of RA. The levels of oestrogen to androgen in synovial fluid are elevated in both male and female RA patients [242]. Since the publication by Hench, in 1983, several studies have confirmed the spontaneous improvement of RA during pregnancy and an increased risk of flare *post-partum* [243, 244]. In a retrospective analysis, Oka and Vainio compared “100 RA patients who had experienced at least one pregnancy after disease onset with 100 RA patients who had had no

further pregnancies after onset" [245]. No association of the pregnancy has been found on functional outcome, disease activity or erosive arthritis in long-term follow-up. Few evidences were highlighted about the influence of oral contraceptives or hormone replacement therapy on the risk of rheumatoid arthritis [175].

Although many types of bacteria and viruses have been associated to RA onset; the candidates for infectious triggers in human RA included mycoplasma, cytomegalovirus, rubella virus, human parvovirus, Epstein-Barr virus, Proteus, and a recent interest in mycobacteria. It has been proposed that synovial and adjacent soft tissue inflammation may be initiated by a number of microbial factors, including bacterial DNA, CpG motifs, heat shock proteins, and lipopolysaccharides.

There are reports about Epstein-Barr virus, cytomegalovirus, parvoviruses and bacteria and in particular their products: the heat-shock proteins. EBV DNA/RNA have been detected in PBMCs, saliva, synovial fluid, and synovial membranes [246] 10-fold higher frequencies in RA patients compared to healthy controls [247].

Mycoplasmas cause chronic RF seropositive erosive arthritis in some animal models are associated with rheumatoid factor. Ebringer has developed the hypothesis that Proteus mirabilis may trigger RA, but few evidence confirm the data [248]. Albani, et al suggested that Escherichia coli heat shock protein (dnaJ) displayed the QKRAA amino acid sequence present in the HLA-DRB1 shared epitope [249]. Indeed, DNA from a wide variety of organisms, including Hemophilus, Bordetella, and Acinetobacter were isolated from synovial fluid from patients with RA [250]. Emerging evidences in environmental trigger for RA regarding the microbiome, defined as "the ecological communities of commensal, symbiotic, and pathogenic micro-organisms that literally share our body space"[251]. The idea was that oral or intestinal micro-organisms were associated with the development of RA; the intestinal microbioma was tested in animal models of RA-like disease from the 70's, and data suggested that a particular intestinal microbiota, in particular certain Gram-negative enterobacteria attributing either protective or proarthritogenic roles is associated to inflammatory arthritis in animal [252]. Many studies recently published, show epidemiological associations between the presence of periodontal

disease (PD) and RA [253]. In particular, Porphyromonas gingivalis, a periodontopathic bacterium, has been recognized as a possible link between periodontitis, peptide citrullination, autoantibody formation and joint inflammation [254]. Prospective clinical trials demonstrated that RA was associated to experience moderate to severe PD compared with healthy subjects; moreover there was evidence that RA patients had deeper periodontal pockets (OR=2.47) and greater severity of periodontitis (OR=2.27) [253]. In a recent case-control study that involved 57 RA patients and 52 healthy donors, RA patients show a positive association (OR=8.05) with PD [254].

Several cohort and case-control studies show that cigarette smoking is associated with an increased risk of RA [231, 255, 256]; many of these studies demonstrated an association only for seropositive RA. Smoking is associated with the production of RF, and smokers are more prone to show extra-articular manifestations such as rheumatoid vasculitis, nodules and lung disease [257].The increased risk from smoking is related to duration rather than intensity of smoking, and may remain for several years after smoking cessation. Padyukov et al recently described a gene–environment interaction between smoking and shared epitope that provides risk (odds ratio (OR)=2.8 (95% confidence interval (CI), 1.6 to 4.8)) [258].

In the 2005, two large cohorts from the USA and Europe study showed that HLA-DRB1 alleles were only a risk factor for RA in people who have anti-CCP [208]. Smoking has been confirmed to be a risk factor for positive anti-CCP and positive RF antibodies in the presence of SE in patients with RA [259].

Alpha-beta T cells:

RA is characterized by synovial inflammation rich in activated T and B cells, activated macrophages, and other antigen-presenting cells. Antigen-presenting cells interact with T cells through the TCR–HLA complex in the presence of co-stimulation and B cells can act both as antigen-presenting cells and as antibody-producing cells. Moreover, macrophages activated by

signals from T cells and by immune complexes, produce pro-inflammatory cytokines which contribute to perpetuate the inflammatory condition [161, 260].

In joints tissue of RA patients the most representative cell that play the immune response are T cells that express the alpha beta receptor [261]; the αβT cells are CD4+ or CD8+ and could act as helper T cells and have cytotoxic proprieties, respectively. In the inflamed RA joints, αβT cells interacts with HLA DR1/DR4 and unknown epitopes and lead to cytokines production. Based on this notion it has been detected different αβT cell phenotypes that secrete different cytokines: Th1, Th2, Tregs, and Th17.

- ✓ Th1 cells express T-bet, use STAT1 and STAT4, and produce IFN-γ, IL-2, and IL-6, if stimulated by IL-12. Those cytokines are responsible to stimulate macrophages, lymphocytes, and PMNs in the destruction of bacterial pathogens. Moreover they contribute to the development of cytotoxic lymphocytes (CTL & NK cells) that are responsible for the cell-mediated immune response against viruses and tumor cells. Due to the pro-inflammatory cytokines production, Th1 cells are investigated in the cellular immune responses in host defense systems for intracellular microbial agents and viruses, and autoimmune disorders.

- ✓ Th2 cells express GATA-3, use STAT6, and produce anti-inflammatory cytokines as, IL-4, IL-5, and IL-13. Th2 cells are involved in humoral-mediated immunity, with the production of antibodies in response to extracellular pathogens. IL-4 is able to down-regulate IFN-γ, IL-1, IL-6, and TNF-α production of mononuclear cells of RA patients [262]. Il-4 production leads to an expansion of Th2 cells phenotype [263] and it has been demonstrated that a continuous administration of IL4 produce a suppression of collagene-induce arthritis in animal models [264].

- ✓ Tregs cells express CD25 (IL-2Rα), cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) and glucocorticoid-induced tumour necrosis factor receptor family-related protein (GITR), use Foxp3 and produce IL-10 and TGF-β [265]. Tregs can be divided into two class: the naturally arising cells (nTreg) that are generated by the thymus are CD4+CD25-

cells and, after stimulation, in presence of co-stimuli, promote the inducible Treg (iTreg) which are generated in periphery CD4+CD25+FoxP3+ [266]. Tregs play a protective role in RA and other autoimmune diseases, and are investigated into two compartments: the peripheral blood and the inflammatory site (synovial fluids).

- ✓ Recently arise a new αβT cells phenotype characterized by the use RORγδ and STAT3 and the expression of IL-17A and IL- 17F, as well as IL-22 and IL-21 (which lead to B cell differentiation) called Th17 cells [267]. The Th17 cells are stimulated by IL-23, produced by dendritic cells, and is considered as an important effector T-cell subset in diseases such as RA, multiple sclerosis, and inflammatory bowel disease. Animal models of arthritis show that IL-17 inhibition has anti-inflammatory proprieties and protects animals from bone and cartilage destruction [268].

Old RA theories pointed out the role of Th1 subset as the one be associated to RA because its presence in the inflamed joint and because of IFN-*γ* production as because its implication in other autoimmune diseases [269]. However, in recent studies it has evaluated the importance of TH17 cells in autoimmunity, when it was discovered that the p40 subunit of the pro-inflammatory cytokine IL-12 can not only dimerize with the p35 subunit to form IL-12, but also with p19 to create IL-23 [270]. Furthermore, several animal models had demonstrated the non-exclusivity of Th1 associaton in RA; for instance, mice lacking Th1 cytokine IFN-*γ* tended to have enhanced susceptibility to disease. Moreover, deficiency of the Th17 cytokines it caused safety, led to the notion that Th17 cells are the chief contributors to autoimmune tissue inflammation, however in animal model, the lacking of th17 did not always give a defense from autoimmune disorders.

This notions suggest a more complex story: a broken balance of Th1/Th2 and Th17/Treg cells may be responsible for the onset and the progression of RA: with a pro-inflammatory role due to Th1 and Th17 cells and, in the other counterpart, a protective role thanks to Th2 and Treg cells [271]; Moreover, the cytokines milieu is involved in every stage of RA disease through

maintaining inflammatory synovitis to promote autoimmune disorders leading to a more complex network.

T cell differentiation and localization are another main theme in the investigation of RA T cell response. In particular, the presence or the absence of certain chemokine receptors could be predictive of homing of cells and moreover the specific cytokines milieu could be predictive the Th-subset response.

Briefly, naïve T cells circulating in the blood, express CCR7 and are localized in the lymphoid organs, T cells will be activated by recognition of MHC-peptide complexes on the APC, proliferate and differentiate into effector or memory T cells . Based on the presence or the absence of the chemokine receptor CCR7 it has been demonstrated that T cells could migrate to peripheral tissues (effector cells) or could localize in the lymphoid organs (memory cells). T effector (CCR7-) can exert their function instantly upon antigen contact, instead of the memory cells (CCR7+) that, after antigen contact, they have to proliferate, expand and after acquire the effector function. Moreover, memory T cells can be divided into central and effector memory; the central memory that express CCR7 are in lymphoid organs and produce IL2 upon stimulation and the effector memory produce IFN-γ and IL4 [272].

So, in order to develop an autoimmune response, it is required an activation of T naive in lymphoid organs and migration, orchestrated by chemokines and cytokines, of the effector T cells to the site of inflammation. It takes place the antigen presentation by professional APC and part of effector T cells produce an activation-induced cell death, a part of effector produce an immune response, and only a small part of the antigen specific memory T cell stain for a long time in lymphoid organs waiting for a second antigen presentation.

Rheumatoid Arthritis: autoantibodies and candidate biomarker

RA, as an autoimmune disease, leads to the production of various autoantibodies (auto-Ab), easily detectable in serum and for this reason they help to understand pathological mechanisms and constitute biological markers of the disease. Several studies postulated different self-

antigens as candidates for RA including collagen type II (COLIAII), filaggrin, fibrinogen/fibrin, and vimentin [207]. COLIAII is a potential candidate as self-antigen for RA for several reasons; in the 70's, in several animal models of arthritis commonly used to investigate the mechanisms of synovitis, such as collagen-induced arthritis or adjuvant arthritis in rodents, lymphocytes reactive against COLIAII have been identified in the peripheral blood and joints of patients with RA [273, 274]. Moreover, anti-collagen antibodies can induce arthritis in mice lacking both B and T cells. Injection of collagen into the knee joints of collagen-immunized rabbits induced experimental arthritis [275]. In 1971, Steffen showed anti-collagen antibodies in RA synovial fluids, and cartilage of RA patients, verifying the autoantibody property of these antigen [276]. Moreover in 1973, he analyzed 238 RA sera and 50 control confirming the anti-collagen antibodies presence in about 60% of 100 arthritis sera and about 9% of 400 age- and sex-matched control sera patients [277]. Furthermore, it was demonstrated in 1974 that collagen and IgG created the immune complex in RA synovial fluids [278]. All these findings indicate a possible pathogenic role of collagen in RA. The anti-CCP autoantibodies recognize, as antigenic target, citrullinated epitopes that are present in different proteins and generated by enzymatic digestion; the key element are the PAD enzymes, which convert arginine to citrulline. Citrulline is an amino acid derived from a post-translational modification and is present in some human proteins leading to a conversion from a basic amino acid into neutral, changing the protein conformation [279].Increased citrullination is not specific of RA, but is a result of cell damages or uncontrolled apoptosis processes and occurs regularly with any environmental stress, (including in alveolar macrophages in cigarette smokers. The citrullination may lead to the creation of neo-epitopes triggering the immune response with the production of anti-CCP and the onset of synovitis [280]. Because COLIAII is a major and specific molecule in joints, it is reasonable to think it as a possible target of citrullination in RA. In 2004 it has been demonstrated that the major T cell epitope on type II collagen can be glycosylated [281]; moreover, the addition of the specific peptide glycosylated in the model of CII-immunized DBA/1 mice has been associated to an increase of severity of RA and the production of collagen specific antibodies [282].

In RA, antibodies against carbamylated proteins (anti-CarP) can be detected: Anti-CarP have been found in the serum of 45% of RA patients, so the presence of anti-CarPA partially overlapped with the presence of anti-CCP, but interestingly anti-CarP was detected also in 20% of the anti-CCP- RA patients and are associated with more severe prognosis [283].

In detail, the concept of carbamylation (homocitrullination) rose many years ago in the context of uremia and has been more recently associated to RA. Carbamylation is a post-translational, mediated by cyanate, leads to the change of the amino acid into the homocitrulline (Hcit); Hcit residues affect the charge distribution, changes in protein molecular weight and isoelectric point and are associated to loss of enzymatic activity. This reaction can be mediated *in vivo* by myeloperoxidase (MPO). MPO has been investigated as a potential trigger factor for atherogenesis and inflammation [284], because are abundant in neutrophils, monocytes and macrophages, they seem to act through antimicrobial activity against pathogens leading to production of free radicals [285]. Homocitrulline is an amino acid that highly resembles citrulline; moreover, it was shown that collagen can be carbamylated *in vivo*. In 2010, it has been demonstrated that, Hcit-immunized mice became susceptible to arthritis induced by the intra-articular injection of Cit-peptides [286]; moreover, in animal models, it has been shown that Hcit proteins are present in the RA joint and that they may affect T-cell triggering and autoantibody formation in rodents [287]. Recently, in humans, the anti-CarP antibodies, which recognize carbamylated proteins, can be detected in sera of RA patients. Even if citrulline and homocitrulline are similar, anti-CarP and anti-CCP represent two different and independent autoantibody families and they do not necessarily cross-react with the other modification.

Potential trigger of autoimmunity response in rheumatoid arthritis:

In COLIAII two different B and T cell epitopes have been identified. In humans the most important COLIAII B cell epitope is the peptide 359-369 (CII359-369)-ARGLTGRPGDA [288], while T cells preferentially recognize the DR4/DR1-restricted COLIAII peptide 261-273 (CII261-273)-AGFKGEQGKGEP [273]. The sequences of both peptides contain a large number of lysine residues

that are often post-translationally in vivo, as citrullination, homocitrullination, and glycosylation. The T peptide has lysine sites and could be carbamylated (homocitrullinated) or glycosilated; the B peptide has Arginine sites and could be citrullinated.

It has been shown that during joint inflammation, collagen is post-translationally modified and cleaved with release of many peptides, among which CII261-273 and CII359-369 and that such peptides can be exposed to the immune system in their native and modified forms [289].

An interesting hypothesis regarding the potential mechanism that could explain the breakdown of self-tolerance for joint collagen, leading to the development of both humoral and cellular immunity in RA, is based on the linked recognition of CII359-369 and CII261-273 and obviously on the MHC class II haplotype DR1/DR4. COLIAII undergoes degradation by different enzymes leading to the production of smaller COLIAII fragments being picked up by antigen presenting cells and presented by HLA-DR1/DR4. T cells can recognize peptides derived from the core regions of proteins presented in the class II HLA, while B cells can recognize surface epitopes on the same proteins. In general, B cells could act as an antigen presenting cell that recognize a surface epitope through its B cell receptor, internalize the whole protein or a large part of its, cleave it and present on HLA class II the internal epitope recognized by a T cell. In the case of COLIAII, B cells specific for CII359-369 could be activated by this epitope and, at the same time, could present CII261-273 bound to DR4/DR1 to the specific T cells. T cells, besides producing pro-inflammatory cytokines, could then help B cells in anti-collagen auto-antibodies production.

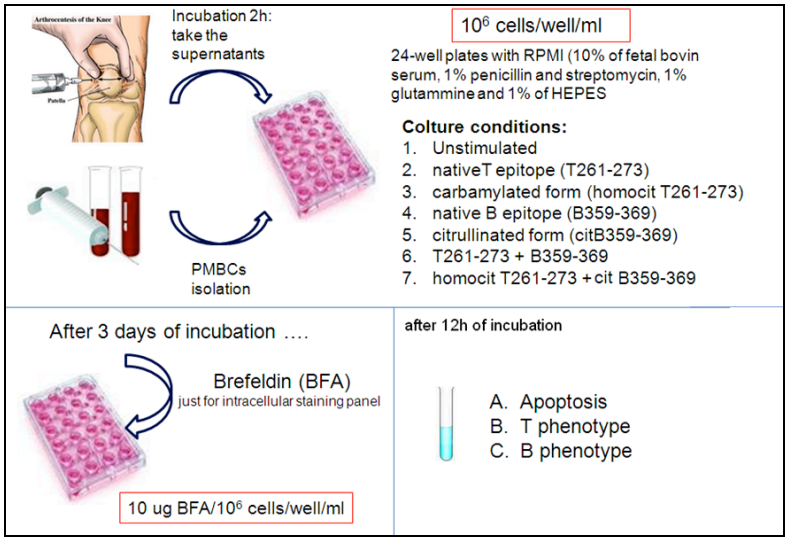
Rheumatoid Arthritis: work hypothesis

In this contest arise our work hypothesis and we want to investigate the role of B and T cell collagen epitopes and their post-translational modifications on the adaptive immune response in a set of RA patients compared to healthy controls.

Material and methods:

We enrolled six female donors (48.3 ±18.3 years) from rheumatology unit constituted from a pair of monozygotic twin HLA-DR4+ discordant for RA, two unrelated HLA-DR4+ RA patients, one HLA-DR3+ RA patients, and one HLA-DR3+ healthy control. PBMCs have been obtained from peripheral blood of 5 donors (HLA-DR4+ twins, two HLA-DR4+ and one HLA-DR3+ healthy donor) and synovial fluids (SF) have been taken from two of donors (HLA-DR4+ RA patient and HLA-DR3+ patient). We processed PBMCs : 1x10^6 cells have been cultured in 24-well plates with RPMI (10% of fetal bovin serum, 1% penicillin and streptomycin, 1% glutammine and 1% of HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)) for 3 days with 20 ug/mL of the native form of the collagen T epitope (261-273 aa AGFKGEQGKGEP), its K264 carbamylated form (homocitT), the native form of the collagen B epitope (359-369 aa ARGLTGRPGDA), its R360 citrullinated form (citB) or a combination of the native and modified epitopes or without peptides (figure 8).

Figure8. The figure shows the summary of method described above.



For intracellular cytokine analysis 10 ug of brefeldin has been added in the last 12 hours of culture. After the stimulation, cells have been analyzed by flow cytometry (CD3-APC-H7, CD4-PE-Cy7, CD8-Alexa700, CD19-HZN V500) for phenotype (CD45RO-PE-Cy5, CCR7-PE-Cy7, CD45RA-HZN V500, CD24- PE-Cy7, CD1d-PE, and CD5-Alexa700), activation (CD154-APC,CD69-PERCP-Cy5.5), apoptosis (annexin-FITC, 7-AAD), and (INFγ-FITC, IL17- HZN V450, IL4-PE, IL10-PE-CF594, TNFα- FITC, IL6-APC) cytokine polarization. All fluorochromes have been purchased from

BioLegend. Briefly, cells were transferred from well to facs tube and diluted with 1 ml of FACS buffer (BioLegend); facs tubes were centrifuged 5 min at 400 xg and then the supernatants were removed. The cells were stained first for extracellular staining at 4°C with CD3-APC-H7, CD4-PE-Cy7, CD8-Alexa700, CD45RO-PE-Cy5, CCR7-PE-Cy7, CD45RA- HZN V500, and CD154-APC, for T panel and with CD19-HZN V500 CD24-PE-Cy7, CD1d-PE, CD5-Alexa700, and CD69-PERCP-Cy5.5 for B panel. Facs tubes were then incubated 30min at 4°C in dark condition. FACS buffer (2ml) were added at facs tubes; facs tubes were centrifuged 5 min at 400 xg and the supernatants removed. The facs tubes were resuspended in 1 ml of citofix fixation buffer (BioLegend) and then incubated 20min at room temperature (RT) in dark condition. The facs tubes were resuspended in 1ml of perm/wash buffer (BioLegend) and the incubated 20 min at RT in dark condition. The cells were stained for intracellular staining at RT with IFNγ-FITC, IL17- HZN V450, IL4-PE, IL10-PE-CF594 for T panel and with, TNFα- FITC, IL6-APC, and IL10-PE-CF594 for B panel. Facs tubes were then incubated at RT for 30min in dark condition. Facs tubes were resuspended in 2mL of perm/wash buffer and the centrifuged 5 min at 400 xg and then the supernatants were removed. Facs tubes were resuspended in 500 uL of perm/wash buffer and then acquired at LS Fortessa flow cytometer (BioLegend).

Surnatants and cells of different culture conditions have been also stored for further cytokine validation analysis by ELISPOT and/or RT-PCR analysis.

Rheumatoid arthritis: results

Collagen T epitopes induced CCR7 overexpression:

We observed a significant increase in CCR7 expression on CD4 T cells with the T epitopes stimulation compared to not stimulated cells in both twins (figure 10). No significant differences were found between the native T epitope and the modified form (figures 10 and .11, tables 13 and 14). CCR7 was over-expressed not only in the central memory subset, but also in the effector memory counterpart (figures 11 and 12). The simultaneous stimulation with the B cell epitopes did not further increase CCR7 expression (tables 13 and 14, figure 10). We observed a

weak CCR7 up-regulation also in the circulating memory T cell compartment of the DR4 positive inactive long-standing RA patient (patient 3) and in the synovial fluid T cells of the DR4 active RA patient (patient 2) (tables 13 and 14). No increase in CCR7 expression was found in the peripheral blood T cells of DR3 healthy control and in the synovial fluid T cells of DR3 active RA patient (patient 3) (tables 13 and 14).

Table 13. Mean intensity of fluorescence of central memory CD4 T cells.

	no peptide	T	homocitT	T + B	homocitT +citB
PB DR4 H twin	1765	6125	5069	5210	5931
PB DR4 RA twin	1028	5799	6162	6137	5792
PB DR4 RA pt 1	2037	2172	2109	2230	1896
SF DR4 RA pt 1	847	1030	1018	963	1037
PB DR4 RA pt 2	1001	1278	1278	1347	1371
SF DR3 RA pt 3	611	606	617	680	635
PB DR3 H control	1971	1873	1985	2346	2286

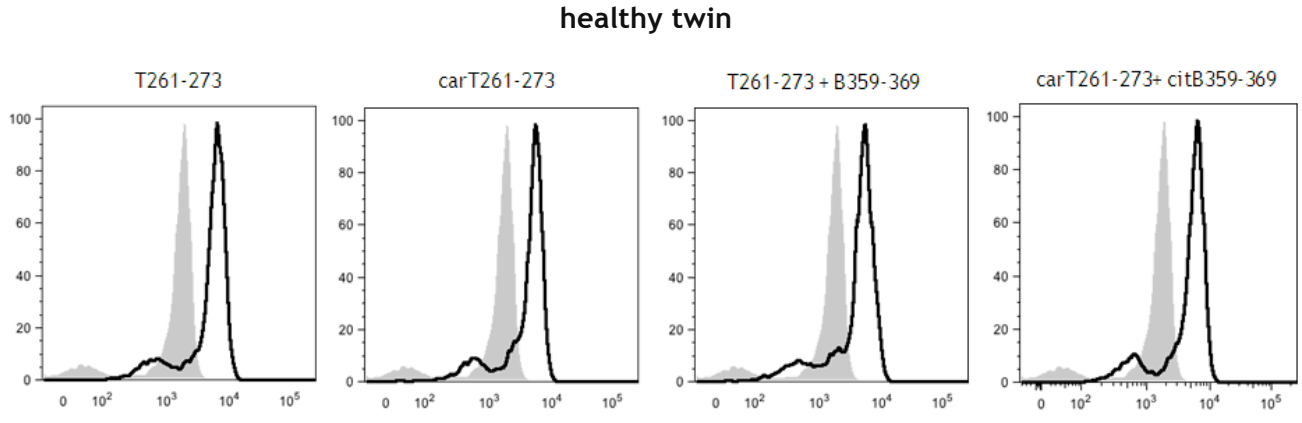
PB: peripheral blood; RA: rheumatoid arthritis; H: healthy; pt: patient; SF: synovial fluid

Table 14. Mean intensity of fluorescence of effector memory CD4 T cells

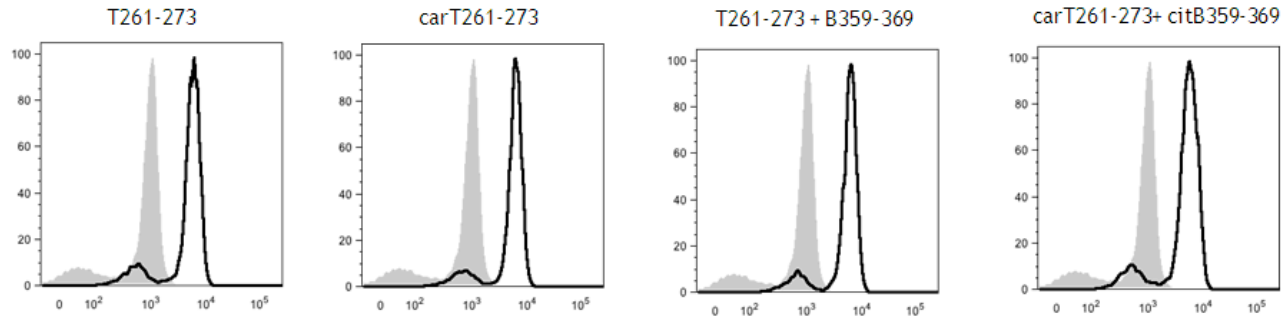
	no peptide	T	homocitT	T + B	homocitT + citB
PB DR4 H twin	57	656	619	586	651
PB DR4 RA twin	71	581	683	691	596
PB DR4 RA pt 1	508	593	603	582	555
SF DR4 RA pt 1	202	207	209	208	217
PB DR4 RA pt 2	291	419	408	427	418
SF DR3 RA pt 3	224	222	237	266	212
PB DR3 H control	257	259	254	260	255

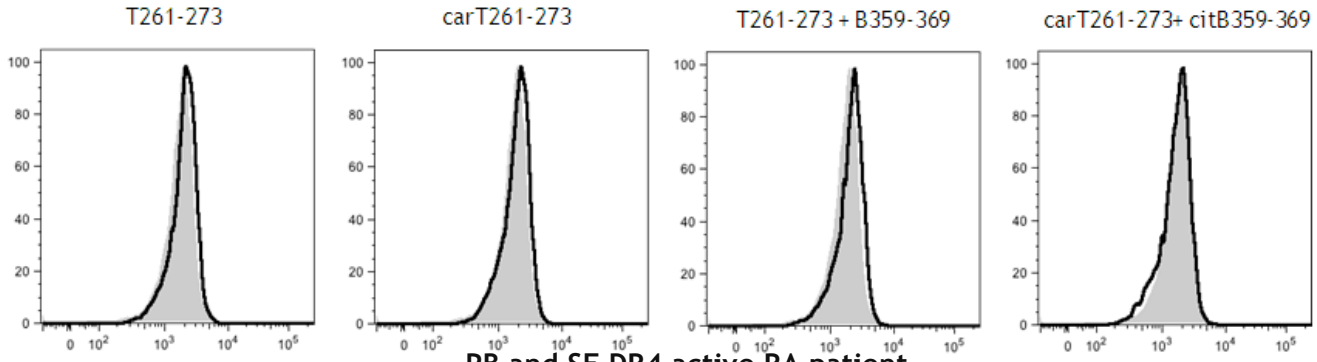
PB: peripheral blood; RA: rheumatoid arthritis; H: healthy; pt: patient; SF: synovial fluid

Figure 10. CCR7 expression in CD4 T cells with the different epitope stimulations

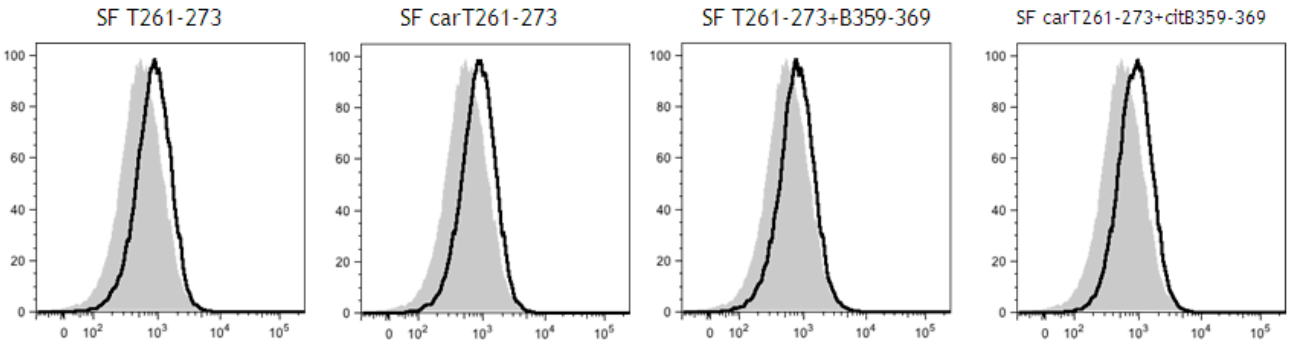


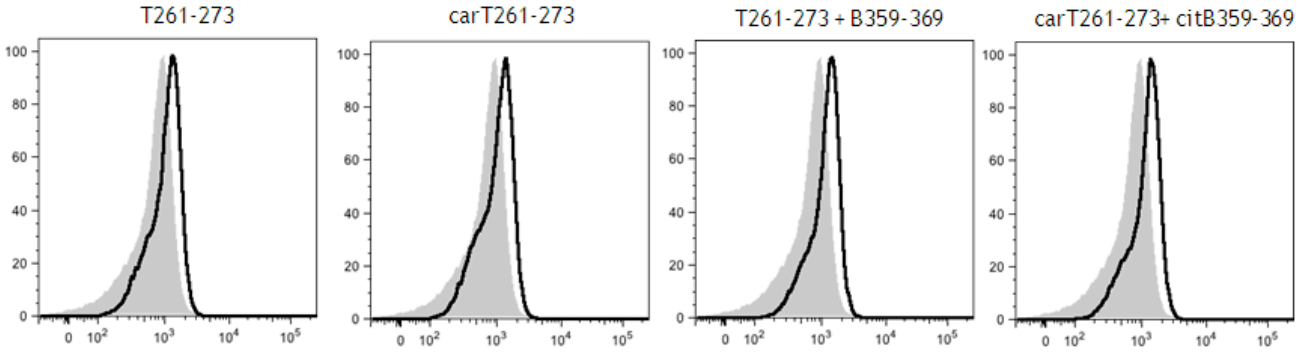
RA twin





PB and SF DR4 active RA patient





DR4 inactive RA patient

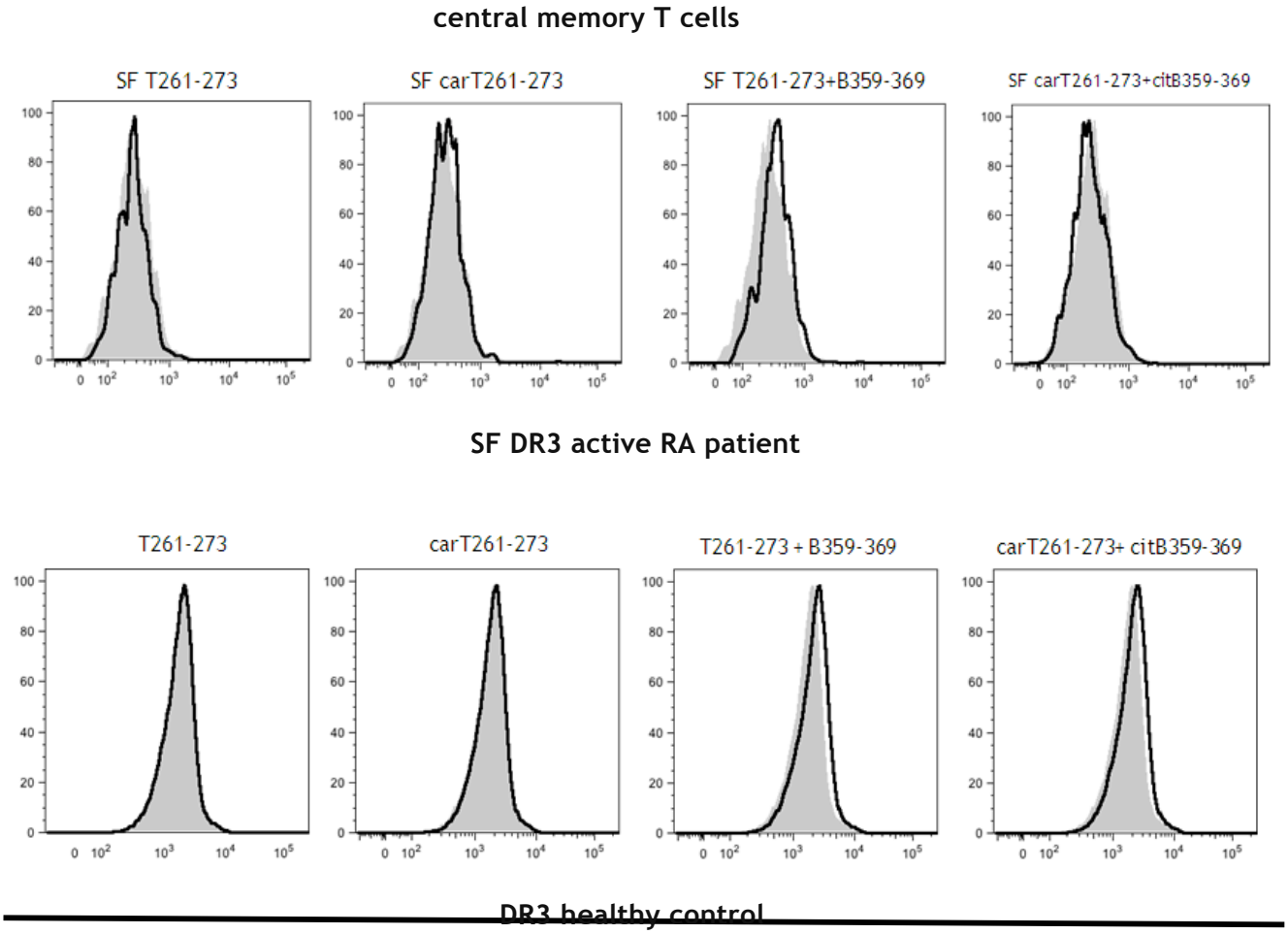
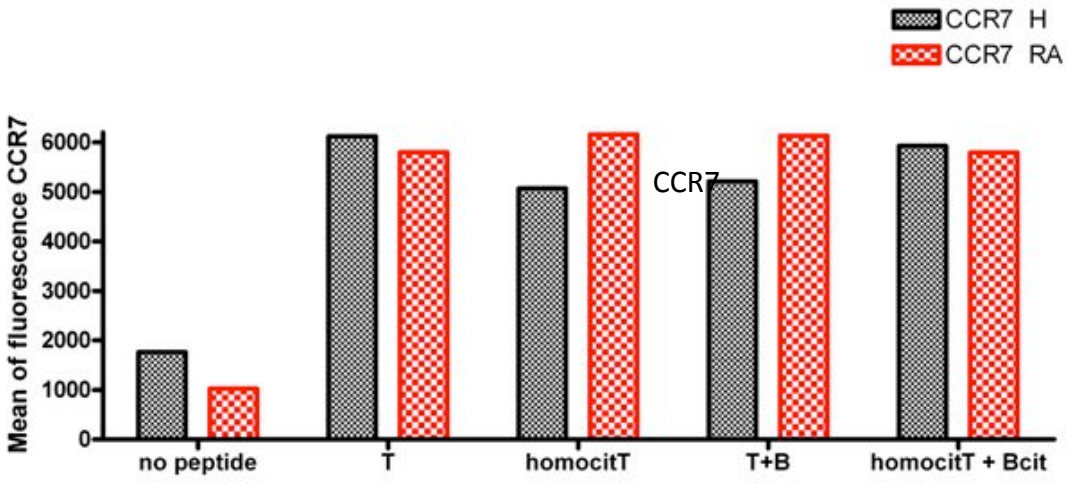
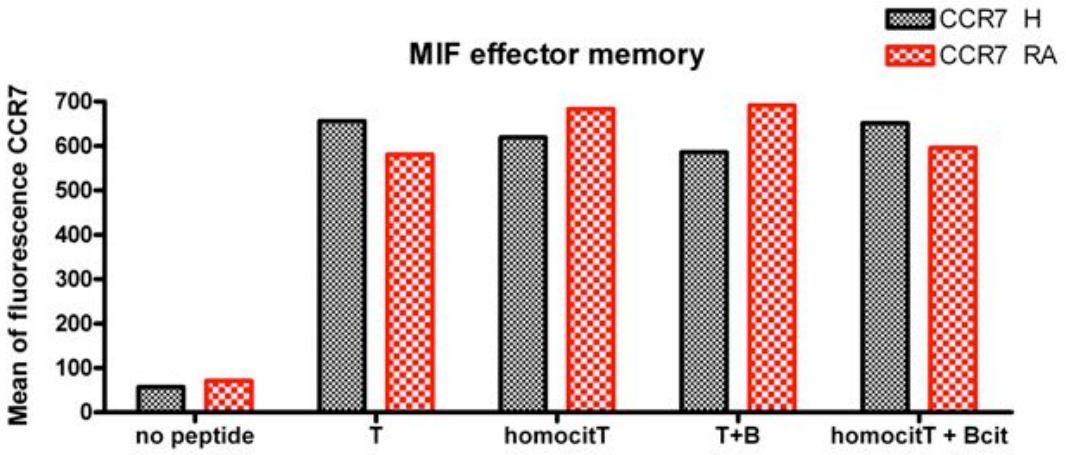


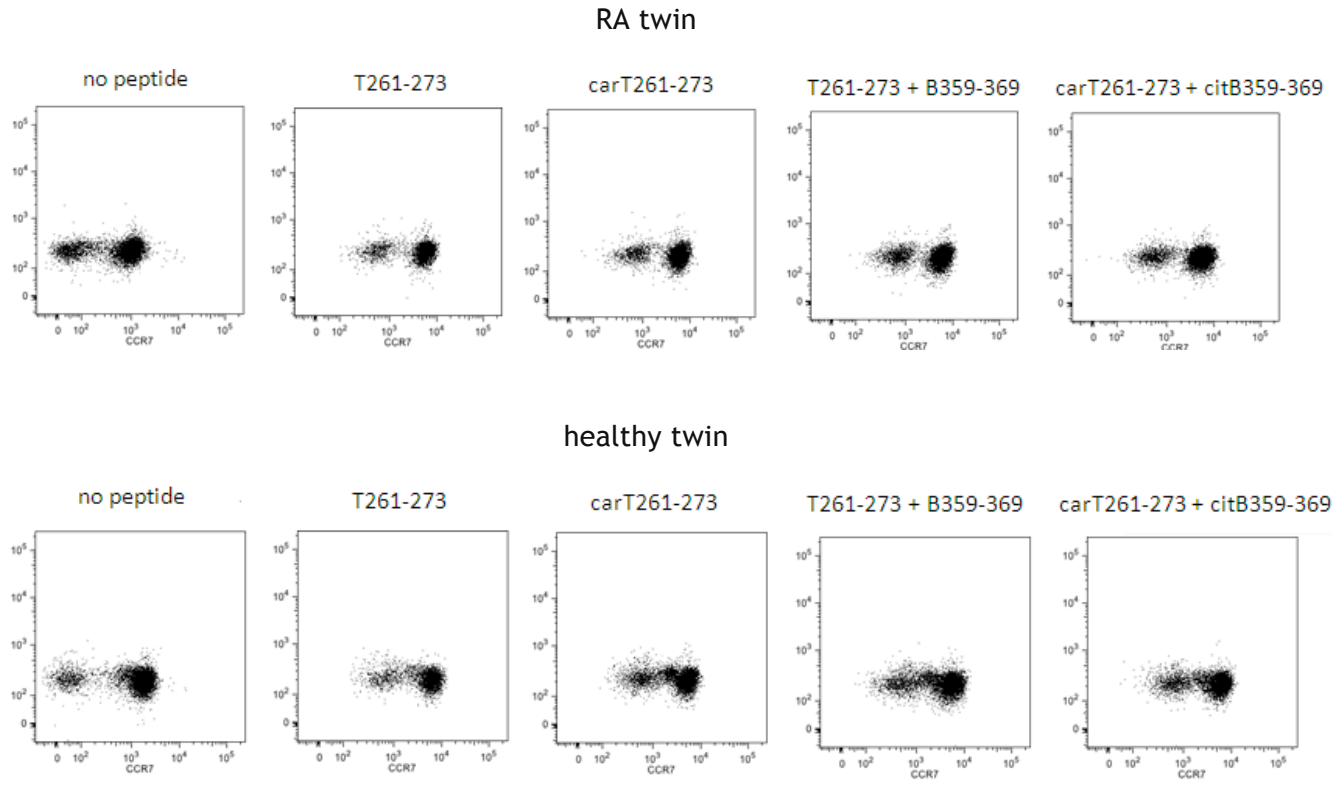
Figure 11. Mean of fluorescence of CCR7 in CD4 central memory and effector memory T cells in the RA and healthy twins





effector memory T cells

Figure 12. CCR7 over-expression after T epitope stimulation in RA and healthy twins.



CD4 T cell response in the DR4 RA and healthy twins:

The stimulation with the collagen T epitopes induced an antigen-mediated activation of CD4 T cells only in the RA twin (table 15, figure 12). No significant differences were observed using the

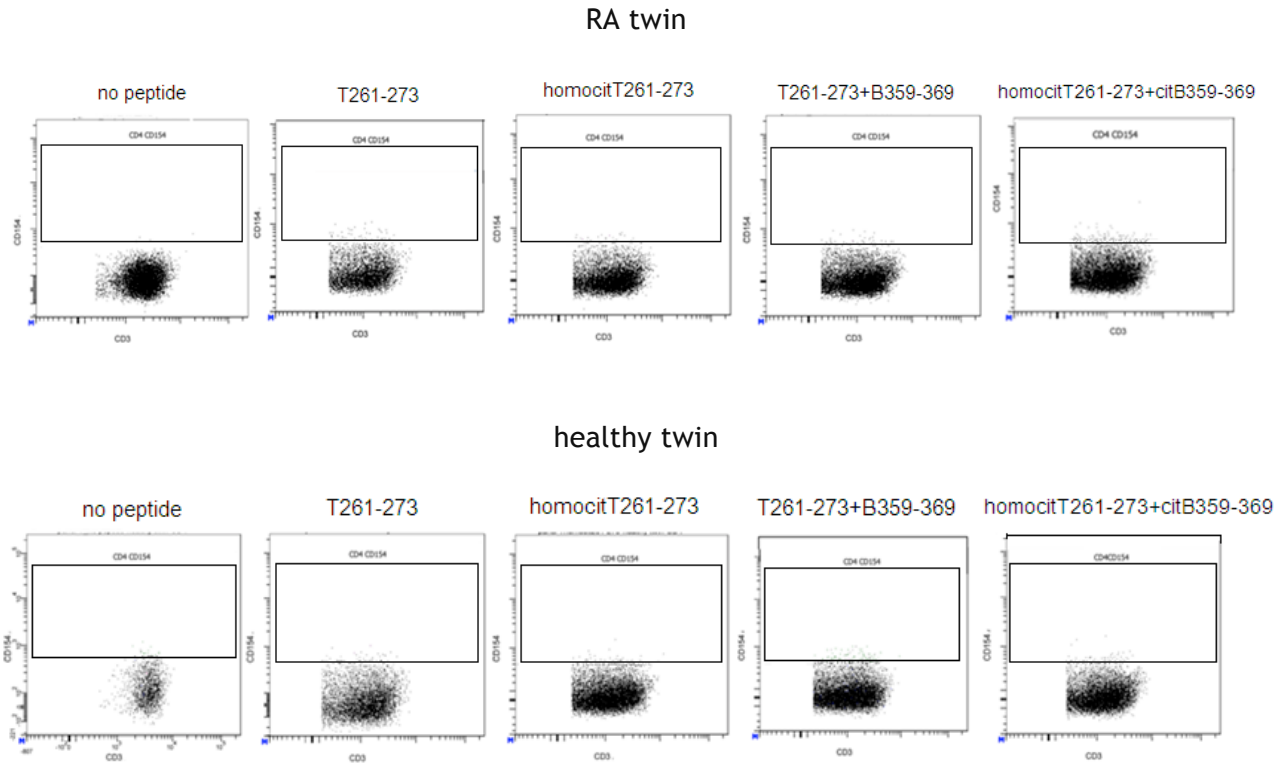
native or the modified form of the T epitopes or their combination with the B epitopes (table 15). The stimulations with the native or modified B epitopes alone was not able to induce CD154 expression (data not shown).

Table 15. Antigen-activated CD4 T cells

CD3CD4CD154%	no peptide	T	homocitT	T + B	homocitT + citB
PB DR4 H twin	0.3	0.6	0.5	1	0.5
PB DR4 RA twin	0.5	3.2	2.4	2.5	2.5
PB DR4 RA pt 1	1.5	2	1.3	0.6	0.6
SF DR4 RA pt 1	0.8	1.1	1.1	0.8	0.8
PB DR4 RA pt 2	0.1	0.1	0.1	0.1	0.2
SF DR3 RA pt 3	2.8	2.8	0.2	1.3	3
PB DR3 H control	0.5	0.8	0.5	0	1.3

PB: peripheral blood; RA: rheumatoid arthritis; H: healthy; pt: patient; SF: synovial fluid

Figure 12. Antigen-activated CD4 T cells in RA and healthy twin .



Interestingly. in the RA twin the antigen-activated CD4 T cells did not exhibit a Th1 phenotype, but weakly expressed only IL-17 (tables 16 and 17, figure 13). A greater IL-17 expression was found in the synovial fluid of both DR4 and DR3 RA patients. A further increase was observed

after the stimulation, that was higher in the case of DR3 RA patient and modified T epitope (table 17).

Figure 13. CD4 Th1 and Th17 phenotypes with T and B epitope stimulation

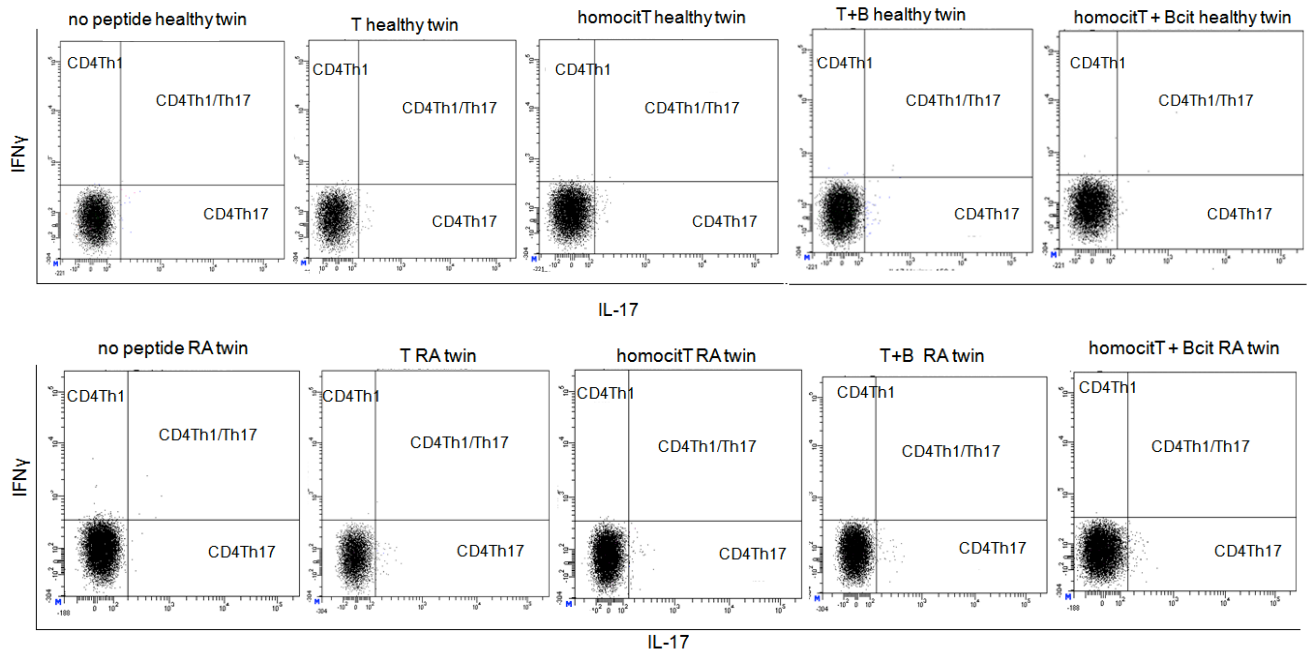


Table 16. Th1 CD4 T cells

CD3CD4Th1	no peptide	T	homocitT	T + B	homocitT + citB
PB DR4 H twin	0.2	0.4	0.7	0.2	0.5
PB DR4 RA twin	1.3	0.1	0.2	0.3	0.1
PB DR4 RA pt 1	0	0.1	0.2	0.2	0.2
SF DR4 RA pt 1	0.2	0.3	0.3	0.3	0.3
PB DR4 RA pt 2	0.7	0.3	0.2	0	0
SF DR3 RA pt 3	0.1	0.2	0.1	0.1	0.1
PB DR3 H control	2.7	1.6	4.6	2.7	4.9

PB: peripheral blood; RA: rheumatoid arthritis; H: healthy; pt: patient; SF: synovial fluid

Table 17. Th17 CD4 T cells

CD3CD4Th17	no peptide	T	homocitT	T + B	homocitT + citB
PB DR4 H twin	0.5	0.4	0.3	0.3	0.1
PB DR4 RA twin	0.4	0.6	0.4	0.3	0.5
PB DR4 RA pt 1	0.3	0.3	0.3	0.1	0.2
SF DR4 RA pt 1	1	1.1	1.5	1.5	1.4
PB DR4 RA pt 2	0.1	0	0	0	0
SF DR3 RA pt 3	2.2	2.3	3.9	2.5	6.1
PB DR3 H control	0.1	0.1	0.1	0.2	0.1

PB: peripheral blood; RA: rheumatoid arthritis; H: healthy; pt: patient; SF: synovial fluid

Table 18. Th1/17 CD4 T cells

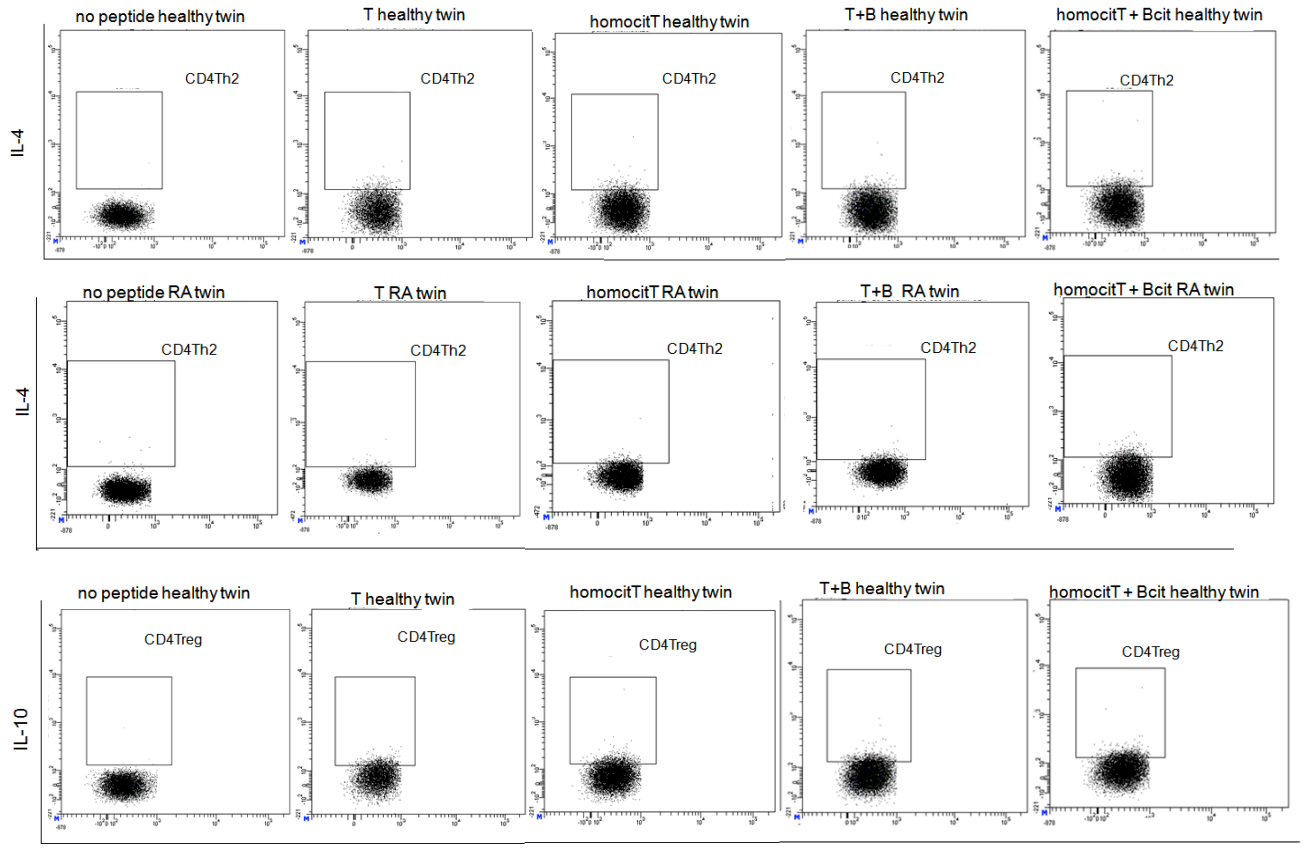
CD3CD4Th1/17	no peptide	T	homocitT	T + B	homocitT + citB

PB DR4 H twin	0	0	0	0	0
PB DR4 RA twin	0	0	0	0	0
PB DR4 RA pt 1	0	0	0	0	0
SF DR4 RA pt 1	0	0	0	0	0
PB DR4 RA pt 2	0.2	0	0	0	0
SF DR3 RA pt 3	0	0	0	0.1	0
PB DR3 H control	0	0	0.1	0.1	0.2

PB: peripheral blood; RA: rheumatoid arthritis; H: healthy; pt: patient; SF: synovial fluid

Both native and modified T epitopes and the combinations with the B epitopes induced a similar Th2 response in the RA and healthy twins (tables 19 and 20, figure 14). An over-expression of IL-10 was also observed, but it was greater in the healthy twin compared to RA twin (tables 19 and 20, figure 14).

Figure 14. CD4 Th2 and Treg phenotypes with T and B epitope stimulation



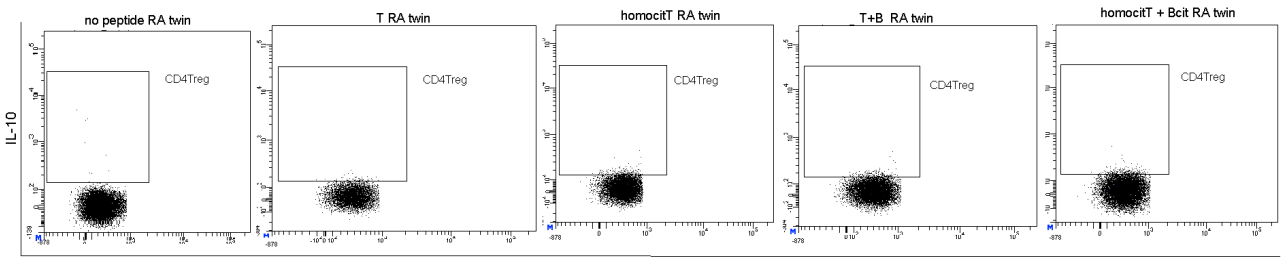


Table 19. Th2 CD4 T cells

CD3CD4Th2	no peptide	T	homocitT	T + B	homocitT + citB
PB DR4 H twin	0	2.9	2.3	1.9	3.7
PB DR4 RA twin	0.1	1.3	3	2.7	2.9
PB DR4 RA pt 1	1.3	0.3	0.3	0.1	0.4
SF DR4 RA pt 1	0.7	1	1.6	1.7	0.8
PB DR4 RA pt 2	0.3	0.1	0.1	0.1	0.1
SF DR3 RA pt 3	1.1	1	2.2	2.5	4.2
PB DR3 H control	1.5	0.3	0.2	0.1	0.2

PB: peripheral blood; RA: rheumatoid arthritis; H: healthy; pt: patient; SF: synovial fluid

Table 20. Treg CD4 T cells

CD3CD4Treg	no peptide	T	homocitT	T + B	homocitT + citB
PB DR4 H twin	0	8.9	7.5	7.7	9
PB DR4 RA twin	0.2	0.9	2.3	1.5	2.3
PB DR4 RA pt 1	0.8	0.6	3.8	0.1	1
SF DR4 RA pt 1	1	1.3	2.8	3	1.7
PB DR4 RA pt 2	0.4	0.7	0.2	0.1	0.2
SF DR3 RA pt 3	0.5	0.7	1	1.1	2.1
PB DR3 H control	1.6	1.4	0.6	0.3	0.4

PB: peripheral blood; RA: rheumatoid arthritis; H: healthy; pt: patient; SF: synovial fluid

CD8 T cell response in the DR4 RA and healthy twins:

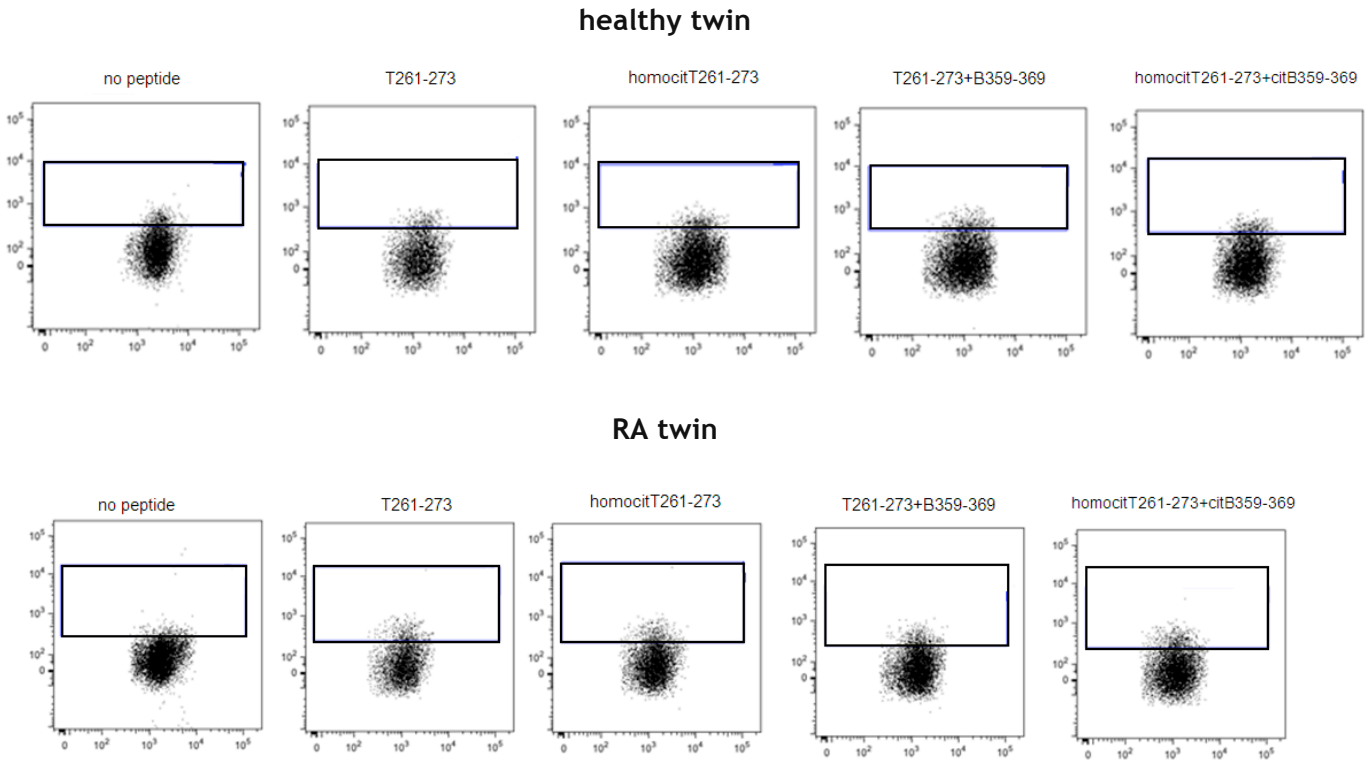
CD154 expression on CD8 increased on circulating T cells of RA twin and, slightly, in the synovial fluid T cells of both RA patients (table 20, figure 14).

Table 20. Antigen-activated CD8 T cells

CD3CD8CD154%	no peptide	T	homocitT	T + B	homocitT + citB
PB DR4 H twin	5.4	4.5	4	3.7	3.3
PB DR4 RA twin	1.9	6.5	5	4.7	5.4
PB DR4 RA pt 1	1.6	1.7	0.3	0.4	0.3
SF DR4 RA pt 1	0.4	2.1	4.4	2.1	5.1
PB DR4 RA pt 2	0.1	0.1	0	0.1	0.2
SF DR3 RA pt 3	6.4	8.6	6.2	8.5	7.5
PB DR3 H control	0.1	0.1	0.3	0.1	0.6

PB: peripheral blood; RA: rheumatoid arthritis; H: healthy; pt: patient; SF: synovial fluid

Figure 15. Antigen-activated CD8 T cells in healthy and RA twin



Interestingly, collagen T epitopes were also able to induce IL-4 and IL-10 expression in CD8 T cells in both RA and healthy twin (tables 21-25, figure 16).

Table 21. IFN-γ producing CD8 T cells

CD3CD8Th1	no peptide	T	homocitT	T + B	homocitT + citB
PB DR4 H twin	0.7	1	1.5	0.5	1.6
PB DR4 RA twin	0.7	0	0	0.1	0
PB DR4 RA pt 1	0.1	0	0.1	0.1	0.1
SF DR4 RA pt 1	0.4	0.8	0.6	0.7	0.6
PB DR4 RA pt 2	1	0.4	0.6	0	0.1
SF DR3 RA pt 3	0	0.4	0	0	0.2
PB DR3 H control	0.3	0.2	0.4	0.3	0.5

PB: peripheral blood; RA: rheumatoid arthritis; H: healthy; pt: patient; SF: synovial fluid

Table 22. IL-17 producing CD8 T cells

CD3CD8Th17	no peptide	T	homocitT	T + B	homocitT + citB
PB DR4 H twin	1.1	0.6	0.5	0.5	0.5
PB DR4 RA twin	0.8	0.5	0.4	0.5	0.7
PB DR4 RA pt 1	0.3	0.3	0.2	0.1	0.1
SF DR4 RA pt 1	0.2	0.2	0.6	0.5	0.6
PB DR4 RA pt 2	0.5	0	0	0	0
SF DR3 RA pt 3	3.4	4.7	5.9	4.7	7.9
PB DR3 H control	0.2	0.2	0.1	0.4	0.1

PB: peripheral blood; RA: rheumatoid arthritis; H: healthy; pt: patient; SF: synovial fluid

Table 23. IL-1/17 producing CD8 T cells

CD3CD8Th1/17	no peptide	T	homocitT	T + B	homocitT + citB
PB DR4 H twin	0.1	0.1	0.1	0	0.1
PB DR4 RA twin	0.1	0	0	0	0
PB DR4 RA pt 1	0	0	0	0	0
SF DR4 RA pt 1	0	0	0	0	0
PB DR4 RA pt 2	0.6	0	0	0	0
SF DR3 RA pt 3	0.3	0.6	0	0	0.8
PB DR3 H control	0.1	0	0.1	0.1	0.2

PB: peripheral blood; RA: rheumatoid arthritis; H: healthy; pt: patient; SF: synovial fluid

Table 24. IL-4 producing CD8 T cells

CD3CD8Th2	no peptide	T	homocitT	T + B	homocitT + citB
PB DR4 H twin	0.2	2.1	1.5	1.3	2
PB DR4 RA twin	0.3	2.4	5.8	6.2	5.7
PB DR4 RA pt 1	1.4	0.2	0.1	0.1	0.2
SF DR4 RA pt 1	0.4	0.7	1.8	1.8	1
PB DR4 RA pt 2	0.1	0.1	0	0.1	0.1
SF DR3 RA pt 3	2.8	3	4.7	3.1	7.1
PB DR3 H control	1.5	0.4	0.3	0.2	0.3

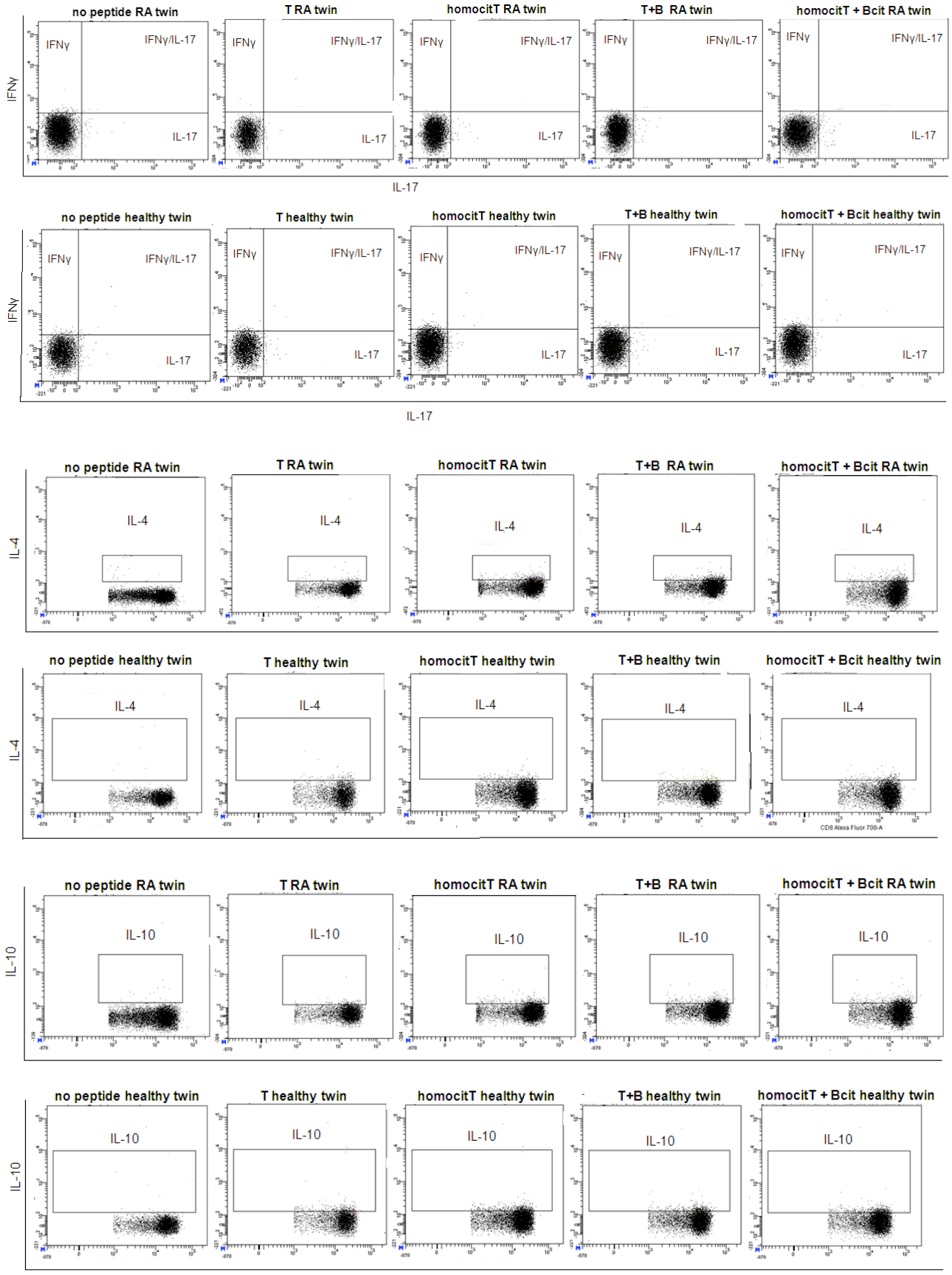
PB: peripheral blood; RA: rheumatoid arthritis; H: healthy; pt: patient; SF: synovial fluid

Table 25. IL-10 producing CD8 T cells

CD3CD8Treg	no peptide	T	homocitT	T + B	homocitT + citB
PB DR4 H twin	0.3	6.9	6.6	6.1	7.7
PB DR4 RA twin	0.2	3.4	5.2	6.8	6.7
PB DR4 RA pt 1	3.5	0.7	3.5	0.1	0.8
SF DR4 RA pt 1	1	1.3	0.9	3.6	2
PB DR4 RA pt 2	0.7	0.4	0.5	0.4	1
SF DR3 RA pt 3	2.3	2.1	2.6	1.2	5.2
PB DR3 H control	4.1	1.4	0.6	0.5	0.5

PB: peripheral blood; RA: rheumatoid arthritis; H: healthy; pt: patient; SF: synovial fluid

Figure 16. IFN γ, IL-17 and IFN γ/IL-17 producing CD8 T cells with T and B epitope stimulation.



Activation and cytokine production of B cells:

An increased activation of B cells was observed with all the conditions of stimulation in DR4 patients and control, but not in DR3 patients (Table 26). The activation of B cells was associated to an overexpression of both IL-6 and IL-10 in the healthy twin, while in the RA twin a decreased IL-10 expression was observed (tables 27 and 28).

Table 26. Activation of B cells with collagen epitopes

CD19CD69	no peptide	B	citB	T + B	homocitT + citB
PB DR4 H twin	16	21.4	36.7	47.1	45.6
PB DR4 RA twin	17.3	29.9	24.5	24.4	22.5
PB DR4 RA pt 1	7	9.3	10.9	8.4	13.2
SF DR4 RA pt 1	42.3	43.8	42.2	40	46.4
PB DR4 RA pt 2	22.7	23.4	39.9	32.4	58.7
SF DR3 RA pt 3	47.2	38	28.4	12.5	34.6
PB DR3 H control	13.4	11	11.7	10.2	11.8

PB: peripheral blood; RA: rheumatoid arthritis; H: healthy; pt: patient; SF: synovial fluid

Table 27. IL-6 producing B cells

CD19IL-6	no peptide	B	citB	T + B	homocitT + citB
PB DR4 H twin	3.7	13.7	7.2	8.2	6.8
PB DR4 RA twin	8.1	10.8	5.2	2.5	16.2
PB DR4 RA pt 1	1.9	1.2	3	2.2	4.3
SF DR4 RA pt 1	2.7	4.7	3.4	2.6	3
PB DR4 RA pt 2	6.6	13.6	21.7	8.4	23.2
SF DR3 RA pt 3	11.2	10.4	8.4	0.6	8.3
PB DR3 H control	3.3	6.3	3.4	5.3	3.8

PB: peripheral blood; RA: rheumatoid arthritis; H: healthy; pt: patient; SF: synovial fluid

Table 28. IL-10 producing B cells

CD19IL-10	no peptide	B	citB	T + B	homocitT + citB
PB DR4 H twin	1	1.7	5.7	4.9	5.7
PB DR4 RA twin	3.5	1.2	0.4	0.6	6.8
PB DR4 RA pt 1	3	3.5	4.3	3.4	7.3
SF DR4 RA pt 1	16	17.6	15	11.4	12.5
PB DR4 RA pt 2	10.1	3.9	10.4	2.9	13
SF DR3 RA pt 3	18.2	18.7	15.8	14.3	9.5
PB DR3 H control	13.4	9.7	7	7	8.2

PB: peripheral blood; RA: rheumatoid arthritis; H: healthy; pt: patient; SF: synovial fluid

Apoptosis of T and B cells after collagen epitope stimulation:

In RA twin a decrease in spontaneous T cell apoptosis was observed after epitope stimulation, which was significant with the native T epitope and in the CD4 T cells (Tables 29-31).

Apoptosis of CD154+ T cells and CD69+ B cells was not analyzed because of the low number of events. Synovial fluid cells were not shown due to the high percentages of and necrotic cells.

Table 29. Apoptosis of CD4 T cells

Annexin CD3CD4	no peptide	T	homocitT	T + B	homocitT + citB
PB DR4 H twin	7.8	6.5	4.5	5.3	5.6
PB DR4 RA twin	5.1	1.7	2.7	3.3	2.8
PB DR4 RA pt 1	4.4	4.6	4.5	5.5	5.4
PB DR4 RA pt 2	36.8	26.7	27.8	33	28.1
PB DR3 H control	31.3	29.8	36.9	31.7	34.9

PB: peripheral blood; RA: rheumatoid arthritis; H: healthy; pt: patient; SF: synovial fluid

Table 30. Apoptosis of CD8 T cells

Annexin CD3CD8	no peptide	T	homocitT	T + B	homocitT + citB
PB DR4 H twin	15.5	15.1	9.8	14.8	12.2
PB DR4 RA twin	21.9	16.0	15.4	16.1	12.8
PB DR4 RA pt 1	28.3	29.2	25.9	25.5	25.8
PB DR4 RA pt 2	37.7	21.8	23.1	26	19.2
PB DR3 H control	37.8	32.9	36.7	29.8	37

PB: peripheral blood; RA: rheumatoid arthritis; H: healthy; pt: patient; SF: synovial fluid

Table 31. Apoptosis of B cells

Annexin/PICD19	no peptide	B	citB	T + B	homocitT + citB
PB DR4 H twin	18.4	17.1	16.3	13.1	20.7
PB DR4 RA twin	22.8	18	16.1	19.6	14.6
PB DR4 RA pt 1	10.9	9.6	10.9	11.2	10.6
PB DR4 RA pt 2	27.9	24.7	25.9	28.6	28.8
PB DR3 H control	56.4	51.1	53.4	54.1	55.2

PB: peripheral blood; RA: rheumatoid arthritis; H: healthy; pt: patient; SF: synovial fluid

Rheumatoid Arthritis: discussion and conclusions

In this project we focused on the role of B and T cell collagen epitopes and their post-translational modifications on the adaptive immune response using peripheral blood and synovial fluid cells and creating an *in vitro* model that mimicking the RA immune response against the peptides of the collagen type II.

Both native and modified collagen T epitopes were able to activate memory T cells in DR4 subjects as suggested by CCR7 over-expression. Moreover, collagen T epitope-mediated CCR7 over-expression seems to be independent of TCR specificity, but DR4-restricted. CCR7 is a chemokine receptor, responsible of secondary lymphoid organ homing trough high endothelial venules in response to CCL19 and CCL21, it distinguishes effector memory T cells (CCR7-) from central memory T cells (CCR7+). A brief up-regulation of CCR7 can be observed after a TCR

engagement and it is thought to be useful for lymph node homing by central memory T cells or to achieve the site of chronic inflammation by effector memory T cells [272]. This phenomenon can be produced after TCR antigen stimulation or anti-CD3 ligation, can be sustained up to 96 hours, is independent of co-stimulation or IL-2 [272], and it cannot be induced by PMA/ionomycin stimulation [290]. CCR7 over-expression in all different stimulation, with no particular differences from the native to the modified forms, may explain the immunodominant propriety of collagen type 2 that lead to a general differentiation of T cells, that acquire effector properties.

Moreover it has been evaluated the contribution of the collagen T epitopes in the activation of CD4 T cells in twins, in order to establish if the T epitopes induced an antigen-mediated activation of CD4 T cells. Previously, it has been reported that CD154 up-regulation varied between patients with different peptides stimulation and the T cells specifically activated are able to produce cytokines in response to peptide stimulation [291]. Our results show that only in the RA twin both native and modified T epitopes were able to induce a specific antigen-mediated activation, suggested by CD154 (CD40L) expression, suggesting that only in the RA twin TCR collagen-specific T cells are present in the memory compartment.

Then, it has been studied if CD4 specific activations lead to a particular cells phenotypes; Th1 cells producing IFN-γ, Th17cells producing IL-17, Th1/Th17 cells producing both IFN-γ and IL-17, Th2 cells producing IL-4, and Treg cells producing IL-10. Interestingly, both collagen T epitopes did not induce a Th1 response, but only a weak IL-17 production. In both twins it has been observed an increase of Th2 and Treg response either with the native T epitope or with modified T epitope, but it was greater in the healthy twin.

In the CD8 T cell compartment we observed CD154 over-expression in RA twin, but the cytokine balance was not altered between RA and healthy twins. Data in literature confirm our results suggesting that increased levels of pro-inflammatory cytokines in peripheral blood but almost in synovial fluids may be caused by the interaction of CII-reactive T cells with cell present the site of inflammation, and the interactions may stimulated or increased the activation of IL-17 and/or IFN-γ and T cell–derived cytokines perpetuating the inflammation in the synovia [292]. Of note,

since the twins have identical genetic background, may be other mechanisms (epigenetic?) define the pathogenic phenotype (IL-17) or the protective one (IL-10) in the RA and in the healthy twin, respectively.

Regarding the analysis in B cells we cannot conclude that B epitope in native and in citrullinated form induce a specific activation of B cells, because we observed a general increase of the activation maker in all conditions of stimulation. Of note, the native epitopes may cause induction of B-reg that are IL-10 producing B cells in the healthy twin [294]. However, as for CD4 T cells, the healthy twin showed a greater protective response (IL-10) compared to RA twin. Our results are in line with the murine collagen-induced arthritis (CIA) models project, in which it has been investigated the therapeutic effects of anti-IL-4, anti-IL-10, given intraperitoneally before or after the onset of CIA; the results confirm the role of IL-10 in the natural suppression of arthritis expression [293].

Furthermore, the apoptosis investigations reveal that the collagen T epitope stimulation was able to increase CD4 T cells survival in RA twin. This observation could suggest that in healthy twin the collagen T epitopes are recognized as non-self-epitopes, and as defense reactions, the activated CD4 cells undergo to apoptosis.

We may conclude that the collagen T epitope 261-273 has a role in the pathogenesis of RA, but the carbamylation of this epitope does not seem to be influent in the T cell response.

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