1.TITLE PAGE

1.1.Title:

Gammadelta T cells in Crohn’s disease: a new player in the disease pathogenesis?

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1.3.Short Title:

Gammadelta T cells in Crohn’s disease.

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2.ABSTRACT

Crohn's disease (CD) is a chronic relapsing systemic disease affecting the gastrointestinal tract. An altered immune response to commensal intestinal bacteria takes place in genetically predisposed individuals, resulting in chronic inflammation in the gut. CD can be considered an immune deficiency condition and several alterations in the innate immunity mechanisms have been described in the recent years. Thus, the study of the immunological aspects of CD, specifically the role of lymphocytes, is a key element for understanding the pathogenesis of the disease.

Gammadelta T cells constitute only a small proportion of the lymphocytes that circulate in the blood and peripheral organs and they are present mainly in the epithelia, where they can constitute up to 50% of intraepithelial T cells (IELs) in the mucosa. Due to their lack of MHC restriction and their unique plasticity and immune regulating properties they are considered key cells in the first line of defense against infections and in wound healing. However, their clinical role in IBD, including CD, is largely unknown.

In this review, we attempt to address the possible involvement of gamamdelta T cells in the pathogenesis of CD, reviewing their role against infections and in inflammation, the current evidence in animal and human studies and hypothesis for their involvement in the disease.

Key words: Gammadelta T cells, Crohn's disease, pathogenesis.

3.MAIN TEXT

Crohn's Disease (CD) is a chronic recurrent inflammatory process that can affect any part of the gastrointestinal tract and may present associated extraintestinal manifestations(1, 2), leading to a significantly compromised quality of life for patients and high associated healthcare costs(3).

Despite the sharp increase in its incidence in recent decades(4) and the important advances made in understanding the mechanisms underlying CD, the etiopathogenesis of the disease remains largely unknown. It is currently considered a polygenic immune disorder due to multiple causes in which the following are involved: 1) individual genetic factors; 2) environmental factors; 3) intestinal flora (microbiome); and 4) immune response. A combination of these factors triggers an inadequate excessive immune response against the commensal flora in genetically predisposed subjects(5-8).

CD can be considered an immune deficiency condition(9-12). Several alterations in the innate immunity mechanisms have been reported in recent years, leading to the presence of micro-infections that are not efficiently solved by the immune system, thus perpetuating an exaggerated inflammatory immune response(13-21). Hence, the study of the immunological aspects of CD, and more specifically the role of lymphocytes, is a key element for understanding the pathogenesis of the disease.

γδ T cells constitute only a small proportion of the lymphocytes that circulate in the blood and peripheral organs and they are present mainly in the epithelia, where they can account for up to 50% of intraepithelial T cells (IELs) in the mucosa(22, 23). Due to their lack of MHC restriction and their singular plasticity and immunoregulatory properties they are considered key cells in the first line of defence against infections and in wound healing(22)(24-26). These unique features, their known participation in the pathogenesis of other autoinflammatory conditions(27-31) and the results of animal and human studies published

to date point to an important role of γδ T cells in the pathogenesis of CD(32-37), a condition characterised by the presence of recurrent infections and ulcerations in the gut (1) (2).

However, their clinical role in IBD, including CD, is still unclear. Our aim in this review is to examine the existing evidence on the role played by alterations in **gammadelta T lymphocytes (γδ T cells)** as a possible new pathogenic mechanism in CD, as these cells are essential to innate immunity in the first line of defence against infection in the mucosa, and play a key role in immunoregulation and tissue repair.

1.Innate immunity alterations in CD

There is a growing body of evidence from both basic and clinical research that suggests that CD would be essentially a primary immunodeficiency (PI) rather than an autoimmune process in the proper sense of the term. That is to say, patients with CD present a genetically determined flaw in their immune system (basically in their innate or unspecific immunity), with an identifiable phenotype which, as a result, can suffer unresolved recurrent infections and chronic "autoinflammation" with the secondary appearance of autoimmune phenomena or neoplasia. This aspect has been analysed in several recent reviews (9-12).

In fact, genetic alterations have been reported in mechanisms directly involved in the recognition (like the nucleotide-binding oligomerisation domain-containing protein 2, NOD2) (38, 39) and clearance of intracellular organisms and certain bacteria (like the autophagy related protein 16L -ATG16L- (40, 41) or the immunity-related GTPase M -IRGM-)(42, 43).

Moreover, the fact that the typical lesions occur in the areas of greater bacterial density (ileum and rectum), the improvement of the inflammation in areas without faecal transit or the presence of a greater number of intramucosal bacteria and granulomas, make the presence of recurrent infections a plausible mechanism for maintaining the inflammatory response (44, 45). Proof of the presence of adherent-invasive *Escherichia coli* (AIEC) in the colon and ileum(46) and in the granulomas of patients(47) would support this possibility.

In addition, there are other disorders in innate immune mechanisms that contribute to the appearance of recurrent infections and colonic inflammation in CD, such as: 1) alterations in the mucosal layer(13, 14); 2) alterations in the intestinal permeability(15-17); 3) dysfunction of Paneth cells (and the production of defensins)(18, 19) and of macrophage functioning(20, 21); or 4) alterations in the stress mechanisms of the endoplasmic reticulum(48, 49).

2.Gammadelta T cells

2.1.General aspects of γδ T cells

T cells can be divided into two large sub-populations according to the antigen receptor that they express on the surface membrane (T-cell receptor or TCR): alphabeta T cells (αβ T cells) and gammadelta T cells (γδ T cells) (50). TCR is a heterodimer that can be composed of two chains: α and β or γ and δ. Its expression is exclusive, that is, a T cell can only express one of these two phenotypic variants (αβ T cells or γδ T cells)(22, 51). This heterodimer is associated with the CD3 complex (T-cell marker) in the cell membrane(50).

Since the discovery of γδ T cells in 1984 by Saito et al.(52), there has been a growing interest in revealing the biological functions of this lymphocytes. Today, they are considered a key element in the first line of defence against invasive pathogens in the epithelia, as well as in the homeostasis of the immune response(22, 24).

αβ T cells are the largest population in peripheral blood, where they account for up to 95% of the circulating T cells, and respond exclusively against antigens that are processed and presented by antigen-presenting cells (APC) in molecules of the major histocompatibility complex (MHC). Their response is therefore restricted and delayed(22).

There are three fundamental characteristics that differentiate γδ T cells from αβ T cells (*Figure 1*):

1) γδ T cells are mainly located in the mucosa – between the epithelial cells – accounting for up to 50% of the intraepithelial lymphocytes (IEL) and acting as the first line of mucosal immune defence; they are scarce in peripheral blood (PB) and the secondary lymphoid organs(22, 23). During infections, however, the proportion of γδ T cells in PB may increase considerably, to the extent where they can make up to 40-60% of the circulating T cells(53, 54). This increase can remain for up to four months following some infections(55).

2) γδ T cells recognise proteins directly without the need for prior antigenic processing by APC or their presentation in the MHC molecules(56, 57). They are also able to recognise a wide range of non-peptide ligands such as viral proteins(58), bacterial superantigens - such as staphylococcal enterotoxin A -(59), lipid antigens(60), heat shock proteins (HSP)(61), or MICA/B molecules (MHC-class I related molecules) inducible by cell stress(62).

3) γδ T cells are powerfully stimulated directly by phosphorylated microbial metabolites or phosphoantigens(63, 64). Among these, their most powerful activator capable of stimulating γδ T cells at nanomolecular concentrations is HMBPP (E-4-hydroxy-3-methyl-but-2-enyl-pyrophosphate), a sub-product of the synthesis of isoprenoid by the non-mavelonate pathway synthesised by bacteria and protozoa(60). They are also activated at much higher concentrations by isopentenyl pyrophosphate (IPP), a derivative produced by eukaryotic cells via the mavelonate metabolic pathway(60, 65). Aminobisphosphonates (ABP) such as alendronate, pamidronate or zoledronate (currently used in clinical practice for the treatment and prevention of osteoporosis) as well as alkylamines have been employed for the in vivo and in vitro activation of these lymphocytes, since they increase the intracellular concentration of secondary PA by inhibiting the enzyme of the mavelonate pathway farnesyldiphosphate synthase(66-68). Some synthetic phosphoantigen derivatives such as bromohydrin pyrophosphate BrHPP (Phosphostim®), have been successfully used to stimulate γδ T cells ex vivo(69).

These specific characteristics and their great plasticity enable them to offer a swift response against a wide range of antigens, making γδ T cells a key element in the defence against infections, tumors, and in the regulation of the immune response in many chronic and autoimmune inflammatory diseases(70, 71). They are thus considered to be "bridge" cells between innate (or unspecific) and acquired (or specific) immunity(72).

In fact, and most important for clinical practice, several lines of research are currently using these cells in antineoplastic and anti-infectious immunotherapy with promising results.(67, 73).

2.2.Subtypes and phenotypic classification

Gammadelta T cells are the first T cells to develop in vertebrates and the first to appear in the foetal thymus(22). They make up the greater sub-population of T cells during the first year of life, which is suggestive of their key role in neonatal protection while the IgA protective system is still not fully developed(71, 74).

Gammadelta T cells can be classified into two main populations, according to TCR expression: Vδ1+ and Vδ2+ (also known as Vγ9Vδ2 because this is its predominant phenotype) (22).

Vδ1+ are predominant in epithelia: skin (Vγ5δ1), intestine (Vγ8δ1) and genitourinary tract (Vγ6δ1)(28, 75, 76). They usually express the marker CD8+(77), present characteristics of motility and migration to the mucous membranes (adherence, emission of long filopodia, etc.)(78, 79) and play a crucial role in epithelial regeneration(80).

Vδ2+ (Vγ9Vδ2) are the predominant subtype in peripheral blood(81) and possess a greater cytotoxic capacity of the natural killer (NK) and antibody-mediated types(82, 83). These features made them most suitable for immunotherapy against cancer (67, 84). Recent studies reveal a significant presence of a third subtype, Vδ3+, in the intestinal mucosa.(85).

Most of the peripheral γδ T cells are double negative for CD4 and CD8 (CD4-CD8-)(23).

However, intestinal γδ T cells (γδ IELi) frequently express the marker CD8+ (50% of γδ intraepithelial lymphocytes), and the homodimer CD8αα, an extrathymic differentiation marker, is commonly expresse(86).

A variable percentage of peripheral γδ T cells express CD28 (co-stimulator of αβ T cells)(87), CD40L (which entails its capacity to interact with B lymphocytes)(88), or NK cell receptors such as NKG2D (a mediator of cytolytic activity)(89).

In recent years other populations of γδ T cells have also been reported, such as IL-17-producing γδ T cells, which seem to play an essential role in the pathogenesis of certain autoimmune diseases(28, 90), and δ2 CD56+ TL, with a greater cytolytic and anti-infectious capacity(91).

2.3.Specific actions: cytotoxicity, immunoregulation and tissue repair.

In recent years other specific functions of γδ T cells that reinforce their key role in immunoregulation and tissue regeneration have been reported(71, 92) *(Figure 2)*:

1) Direct cytotoxic action against infected or tumorous cells via the secretion of perforins and granzyme B(93), independent of antibodies(94). They are even capable of opsonising and engulfing infected cells directly(95).

2) Secretion of proinflammatory cytokines, above all Th1 type, which are essential for controlling intracellular viruses and bacteria (such as IFN-γ or TNF-α), or extracellular bacteria and fungi (such as IL-17)(96, 97).

3) Activation of the immune response locally, thereby promoting the maturation of dendritic cells and the anti-infection response of macrophages and NK cells(98, 99) and increasing the capacity to resist invasion of epithelial cells(100).

4) Stimulation and regulation of innate and acquired immunity. γδ T cells cells can regulate other cells involved in the innate immunity response by producing immunosuppressant cytokines like TGF-β or IL-10(70). Furthermore, they can migrate to the secondary lymphoid organs and collaborate with B lymphocytes in the production of antibodies via the production of CXCR5 (CXC chemokine receptor 5)(101, 102); regulate the activity of αβ T cells(103) (in fact mice with γδ T-cell deficiency present an exaggerated response to αβ T cells); or eliminate regulatory T cells (Treg)(104).

5) They can act as antigen-presenting cells. They can process and present antigens of pathogens (infected cells, viruses) to other immune cells, such as αβ T cells(105, 106).

6) Epithelial regeneration and wound healing: by stimulating the production of hyaluronic acid by epithelial cells (which is deposited in the extracellular matrix and attracts monocytes and macrophages)(25) or by means of the production of epithelial growth factors – such as insulin-like growth factor (IGF-1) or keratinocyte growth factor 1 and 2 (KGF-1, KGF-2)(26).

2.4.The role of γδ T cells in defence against infections

There is strong evidence for the fundamental role of γδ T cells in the immune response against pathogens (excellent reviews elsewhere) (24).

The antiviral effects of γδ T cells has been confirmed in several acute and chronic viral infections, both by attack on infected cells and by the production of antiviral cytokines, especially interferon gamma (IFN-γ)(107, 108). A number of studies have confirmed the protective role of γδ T cells in infection by the influenza virus(109), human immunodeficiency virus (HIV) (110, 111) cytomegalovirus (CMV) (112), or West Nile virus(113). They also play a role in the control of infection due to *Epstein Barr* virus (EBV) (110) and in the suppression of tumour growth induced by human polyomavirus (HPV)(114).

Also, although there is a decrease in the population of Vγ9Vδ2 in peripheral blood in patients with chronic infection by hepatitis C virus (HCV)(115), it has been suggested that the infiltration of activated Vγ1 lymphocytes into hepatic tissue could accelerate disease progression(116).

γδ T cells are capable of recognising a number of bacterial antigens and of triggering a rapid immune response(76). There is evidence of their participation in the regulation of the immune response in a number of bacterial infections, including salmonellosis, brucellosis, legionellosis, tularemia, listeria and infections by *Escherichia coli*(24). Infection by *Mycobacterium tuberculosis*, produces an expansion and activation of Vγ9Vδ2 T cells(117) and mice lacking γδ T cells undergo an increased and sustained granulomatous inflammatory response following tuberculosis infection via aerosol(118) reinforcing their importance in the pathogenesis of the disease. A recent study showed a deficit of γδ T cells in the PB of patients with sepsis, with a significant correlation between low levels and mortality(119).

These cells have a protective role against other pathogens like malaria (120), *Toxoplasma Gondii* ileitis (121), *Trypanosoma cruzi* hepatic infection via IFN-γ (122) or against *Cryptosporidium parvum* (123). Their action against fungi has also been demonstrated by an early and significant increase in γδ T cells following infection by *Encephalitozoon cuniculi*(124).

3.The role of γδ T cells in intestinal inflammation:

3.1.Preclinical studies

There is a large body of evidence of the protective role of γδ T cells in intestinal inflammation in several murine models (32-34). This is consistent with their role against mucosal infections, tissue repair, and regulation of the immune response. They play a role in oral tolerance(125) and can enhance IgA-mediated responses (126) or contribute to tissue healing by means of the production of KGF(127) (128). In addition they can exert protective

immunoregulatory effects – such as producing TGF-β (32), decreasing the expression of MHC II molecules(128) or supressing αβ T-cell activity (129) (*Table 1*).

Tsuchiya et al. and Chen et al. used dextrane sulfate sodium DSS-induced colitis models to test the anti-inflammatory effects of γδ T cells, showing that large numbers of γδ T cells (but not αβ T cells) were found in the intestinal damaged areas, and were able to protect from the colitis by stimulating tissue repair by means of KGF expression and controlling infiltration by neutrophils(32, 130). Other studies used colitis induced by intrarectal TNBS (2,4,6-trinitrobenzene sulphonic acid) to show that the passive transfer of αβ T cells (and not γδ T cells) was capable of inducing colitis(131).

Studies by Hoffmann and Kühl et al. demonstrated the protective role of the inflammation of γδ T cells in different murine models of IBD. The same researchers also used a TNBS-induced colitis model to prove that depletion of γδ T cells with monoclonal antibodies (and not αβ T cells or T-helper cells) increased the severity of colitis and even mortality(36). This aggravation of the lesions with increased mortality was corroborated in later work(132). They also used TNF ΔARE/+ mice that present a transmural ileitis similar to CD with extraintestinal manifestations (arthritis) showing that γδ T cells depletion caused a histological aggravation – without statistical significance -and lower levels of TGF-β– (132).

A later study confirmed their role as a protector against colitis in two murine models of ulcerative colitis (DSS and IL-2 k.o. mice), and a distinct (although non-significant) histological improvement in a model similar to CD (TNF ΔARE/+). The authors showed that γδ T cells are also capable of controlling the production of IFN-γ by αβ T cells and of stimulating epithelial regeneration(37).

Two key studies by Inagaki-Ohara et al. and Hoffmann et al. opened up the possibility of using γδ T cells as immunological therapy in IBD after showing that their selective transfer improves inflammation and even decreases mortality in mice, through the diminished production of TNF-α and the increase in IL-10 and TGF-β (33) (35).

Nevertheless, although several preclinical studies have revealed the importance of γδ T cells as agents protecting against inflammation, there is no common agreement on their pro- or anti-inflammatory role, as inflammation-inducing effects have also been reported in some murine models of colitis(133).

Simpson et al. were the first to suggest that γδ T cells might contribute to intestinal inflammation, showing that their infusion in mice lacking these cells (tg∑ 26 mice) was capable of producing colitis via a Th1 response and the production of IFN-γ (134). In the same line, others showed proinflammatory effects of these cells in TCRα -/- mice (135).

Recent reports have offered evidence of the existence of a specific subtype of IL-17A-producing γδ T cells with proinflammatory actions in immunocompromised mice (136). This phenomenon had already been confirmed earlier in models of autoimmune encephalomyelitis(97) and in collagen-induced arthritis. This subtype of γδ T cells could aggravate IL-17-mediated colitis by lack of suppression by Treg(137).

The main preclinical studies of γδ T cells in animal models are shown in *Table 2*.

3.2.Role of γδ T cells in Crohn's disease: studies in humans

Several groups have conducted studies on γδ T cells in peripheral blood (PB) and in the intestinal mucosa of patients with CD in an attempt to explain their role "in situ" in humans *(Table 3).*

3.2.1.Serum values of lymphocytes and γδ T cells in Crohn's Disease

The vast majority of studies of lymphocytes in PB in patients with CD show the presence of significant lymphopenia, with heterogeneous results as regards the circulating γδ T cells.

The first evidence of the presence of lymphopenia in patients with CD was reported in the 1970s. Using antiserum against human thymocytes and a rosette assay utilising ram erythrocytes, respectively, Strickland et al. and Sorensen et al. were the first to report a decrease of T cells in the PB of patients with CD and normal levels in patients with UC(138)

(139). The authors point to differences in the pathogenesis of the two diseases as a possible explanation for this. Yet these differences have not been confirmed in later studies(140).

Selby et al. employed monoclonal antibodies for the first time to identify lymphocyte sub-populations in 54 patients with IBD (28 UC and 26 CD), showing a significant decrease of T cells both in CD and in active UC (with lower levels of both CD4+ T cells and CD8+ T cells in CD with respect to controls) (140). This decrease in circulating T cells in patients with CD was confirmed in a later study that included 43 patients(141). A recent study of circulating lymphocytes in a broad sample of patients with CD confirmed the presence of lymphopenia regardless of the clinical activity and the use of treatment(142).

The impact of the presence of lymphopenia in CD and its possible effect on the appearance of secondary autoimmune phenomena (which might explain, for example, the appearance of extraintestinal manifestations) must be taken into account(143). The association between lymphopenia and autoimmunity is well known in other autoimmune diseases -such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) or Sjogren's disease-, and the induction of lymphopenia is used in several animal models of autoimmunity (143) (144) (145). Sustained lymphopenia can give rise to a peripheral homeostatic lymphocyte proliferation (PHLP) to maintain the peripheral T cell population stable, with the subsequent loss of diversity of the TCR repertoire and the appearance of autoreactive effector T cells(143). Two animal studies conducted in mice, on autoimmune diabetes(146) and autoimmune pancreatitis models(147) support this hypothesis. It has recently been suggested that states of lymphopenia could also alter the balance between regulatory T cells (Treg) and effector T cells (Tef), thereby inducing states of autoreactivity via IL-2 and IL-21(148) (149). However, two studies were including a total of 40 patients with CD did not find any significant lymphopenia(150) (151).

Several previous studies analyse the serum values of γδ T cells in CD. Giacomelli et al. observed a significant increase in γδ T cells in serum in CD only in activity, especially by

expansion of the subtype Vδ1+ in a small sample of nine patients(151). Bucht et al. also found higher values of γδ T cells (with a relative increase in the subtype Vδ1+) in three patients with CD with respect to controls(152), a finding that was confirmed in a later study carried out by the same group(153).

We published recently a study comparing the serum levels of different lymphocyte subpopulations in 40 patients with CD and 40 controls. Results showed the presence of lymphopenia and a pronounced deficit of γδ T cells in the patients in all the subtypes studied (CD4+ γδ T cells, CD8+ γδ T cells and CD56+ γδ T cells), especially significant in the CD8+ γδ T cells subset, which was independent of disease activity (remission or active disease) and of the treatments used(142). These data established for the first time the possible existence of an immune disorder in CD that affects this sub-population and contributes to the appearance of recurrent infections, thus corroborating the data obtained in experimental studies in mice(32-34). This deficit of γδ T cells in PB has recently been confirmed in a later study conducted by our group involving a larger sample of 102 patients with CD with respect to controls (186).

A recent study showed similar results, with significantly lower levels of Vδ2+ T cells in 12 paediatric patients with moderate CD without any immunosuppressant treatment in comparison with controls with irritable bowel syndrome (IBS) (154). Despite presenting fewer circulating Vδ2+, a selective depletion of the subtype CD27+ (related with Th1 response and TNF-α producers) was observed, which was found to be increased at the same time in the biopsies of colon tissue(154). In addition, the same study showed the selective ablation of Vδ2+ cells in azathioprine treated patients.

Similar results have been reported in patients with CD undergoing treatment with infliximab, who presented lower levels of γδ T cells in blood than the controls (1.6% of the total number of T cells vs. 2.6% in controls), although without reaching any statistically significant differences(155).

It is striking that none of the studies published reported an increase in the total number of γδ T cells (or of the subtype Vδ2+) in PB in patients in activity. It is known that this population increases significantly following infections, with values that can reach up to 40-50% of the total T cells(156). Hence, a secondary expansion could be expected in the active phases of the disease (in which there is an increase in permeability and recurrent micro-infections). This lack of expansion of the γδ T cells in active CD is consistent with the deficiency observed in these studies and would support the notion of an altered immune response of the γδ T cells in CD(155) (157). In fact, preliminary data from our series suggest an inverse relationship between the γδ T cell values and the clinical and endoscopic disease activity(186).

3.2.2.Study of γδ T cells in the intestinal mucosa in Crohn's Disease

Three studies have confirmed a decrease of γδ T cells in the intestinal mucosa of patients with CD(158) (152) (159).

Fukushima et al. were the first to observe a significant decrease of γδ T cells in the mucosa in CD. The intraepithelial lymphocytes (IEL)/γδ T cell ratio was 13% in CD vs. 36% in controls, and that of the lamina propria lymphocytes (LPL) was 4% in CD vs. 15% in controls. Most of the LPL were CD4(-)CD8(-)(158).

Another study showed as well a decrease in γδ T cells (sub-population Vδ1+) in the inflamed mucosa with respect to the healthy mucosa in four patients. The authors interpreted that these findings may be due to the destruction of the epithelial barrier or to a local expansion of γδ T cells in the inflamed mucosa(152).

Lee et al. reported a significant decrease in the sub-populations CD3+, αβ TCR+, and γδ TCR+ in the intestinal mucosa of patients with CD with respect to controls. A significant decrease in γδ T cells was also observed in patients with CD compared to patients with UC, which would suggest a different role of this sub-population in the two diseases(159). Another study found no significant differences in the proportion of γδ T cells and the LPL in 14 patients with ileal CD(160). Conversely, other authors found an increase in Vδ1+ γδ T cells

capable of producing IFN-γ and interacting with fibroblasts in inflamed areas with respect to non-inflamed areas in a broad series of surgical samples of CD, UC and diverticulitis(161).

Another recent study explored for the first time the molecules responsible for the intestinal migration of γδ T cells in the PBof patients with CD and active UC. It was shown that both the γδ T cells of healthy subjects and active IBD express the molecule β7, responsible for migration to the intestine, and that patients with IBD present an increased expression of CCR9 (corresponding chemokine receptor-9), a specific marker of migration to the small intestine(162). These data agree with those of the studies carried out by McCarthy et al. that showed that activated Vδ2+ γδ T cells display enhanced gut-homing potential and can populate the human intestinal mucosa, thereby confirming that recruitment does indeed take place from PB to the inflamed areas(162) (157).

4.Gammadelta T cells in clinical therapy

γδ T cells have been used in several clinical trials, especially as anti-tumour immunotherapy. γδ T cells possess a powerful cytotoxic capacity independent of MHC that enables them to act against haematopoietic and solid tumours(67). In fact, γδ T cells have been isolated from the intratumoral lymphocytes (ITL) of patients with colorectal, kidney, prostate, ovarian and lung tumours(163).

Ligands expressed in tumour and non-healthy cells (such as NKG2D or MIC/B)(164, 165) would bind the TCR activating thereby their anti-tumour action by means of the secretion of IFN-γ and TNF-α, cytotoxins such as granzyme B and perforins(93, 94), or by stimulating antibody-dependent cytotoxic activity(166, 167).

Two strategies have been tried to use γδ T cells as anti-tumour immunotherapy: 1) transfer of *ex vivo* activated autologous *Vγ9Vδ2*; and 2) induction of the expansion of Vγ9Vδ2 *in vivo*(67, 168).

The anti-tumour action of *ex vivo* activated autologous Vγ9Vδ2 transfer has been shown to be well tolerated and effective, especially in advanced tumours, such as renal cancer(169) (170). The other alternative with promising results is to induce the expansion of Vγ9Vδ2 in vivo. The use of zoledronate and low doses of IL-2 in patients with metastatic breast cancer and metastatic prostate cancer was well tolerated and produced a sustained activation and maturation of γδ T cells, which also correlated with better clinical responses(171) (165).

Research is currently being conducted to explore the activation of γδ T cells through the use of dendritic cells (DC), since these are able to increase their cytotoxic and anti-tumour capacity(172).

Although there is less clinical experience in this regard, γδ T cells have also been used successfully in the treatment of some infectious diseases. Activated γδ T cells can improve immunocompetence in antiretroviral-naive HIV-infected patients, thus opening up new therapeutic possibilities in the infection(173). In another phase II study, repeated treatment with phosphoantigens reduced the viral load by up to 50% in patients with chronic infection by the hepatitis C virus (HCV), probably due to the increased production of IFN-γ.

Currently there are still not any clinical studies testing these cells in intestinal inflammation diseases in humans, like IBD.

5.The role of γδ T cells in CD: friend or foe?

The singular characteristics of γδ T cells, their great plasticity and the possibility of their use in immunotherapy has stimulated their study in the pathogenesis of CD. Yet, their role as a protector or inducer of intestinal inflammation in the disease today remains insufficiently clear.

There is good evidence on the protective role of γδ T cells in intestinal inflammation in several murine models, in line with their protective capacity against infections, in the regeneration of the mucosa tissue and as a regulator of immunity (32-36). The fact that the

depletion of γδ T cells in induced colitis aggravates the lesions and that their transfer produces a histological improvement and increased survival in mice is suggestive of their antiinflammatory role(35) (33).

Other studies, however, point to their possible proinflammatory role(134) (135), especially of the IL-17-producing subtype of γδ T cells in CD(136). This phenomenon has previously been reported in models of autoimmune encephalomyelitis(97) and in collagen-induced arthritis(174).

Apparently contradictory phenomena, such as the capacity of murine γδ T cells to produce IFN-γ(134) and to inhibit the production of IFN-γ by αβ T cells(37), need to be investigated further in order to understand the plasticity of these cells in the different contexts of infection or inflammation.

Another aspect to be taken into account is the heterogeneity of the murine models used in the experimental studies of IBD and their reproducibility in such a heterogeneous disease as CD. In this sense, Kühl et al. suggest the possibility that the mice models utilised in many studies were not valid for studying the behaviour of γδ T cells. They argue that α-chain deficient mice perhaps produce γδ T cells that are altered or which have a greater capacity for cytolysis or IFN-γ production(132) (175). This would explain their proinflammatory effect in some studies, since, as has been shown, the development and correct functioning of γδ T cells is partly dependent upon αβ T cells(176).

Some data suggest that there may be a primary deficiency of this cell type in patients with CD, in line with other previously reported defects in the innate immune defence mechanisms in the disease(13-19) (21) (48) (177). In fact, several studies have reported lower values of γδ T cells in the PB of these patients(142) (155) (154), independently of the clinical activity and the use or not of specific treatment(142). The presence of low levels of IL-7 (one of their most powerful stimulants) in patients with CD both in remission and with active disease(119) with a tendency to return to normal values in remission(178) would support the hypothesis

that this γδ T deficiency is characteristic of the disease and could have clinical consequences, favouring the appearance of recurrent infections that could perpetuate the intestinal inflammation.

In this regard, it is to note the recent report of an opportunistic infection by an intracellular micro-organism of the fungi family, Microsporidia, in patients with CD(119), which may take advantage of this defect in cell immunity and, specifically, in γδ T cells(179) (124). In fact, this study also showed an inverse correlation between IgE anti-Encephalitozoon (a subspecies of microsporidia) antibodies and γδ T cell levels, especially the subtype CD8+ γδ T cells(142).

In contrast, other authors noted an increase in γδ T cells in patients with CD, especially in activity, and of the subtype Vδ1+ (151-153). In line with this, McCarthy et al. provided evidence of a selective depletion of the subtype CD27+ (related with Th1 response, and with high expression of intestinal migration β7 integrins), which was simultaneously increased in colon biopsies(157). Thus, they propose the attractive hypothesis that, despite having a γδ T-cell deficiency in PB (at least in paediatric CD patients), some cells with a high degree of activation (capable of producing TNF-α, IL-17A and stimulating the production of IFN-β by αβ T cells) managed to migrate to the inflamed areas in the intestine(157).

Although no clear data are available about the clinical correlation of these alterations of γδ T cells in PB, a recent study conducted by our group in a comprehensive sample of patients with CD reveals an inverse correlation between the serum values of γδ T cells (and not αβ T cells) and clinical and endoscopic activity (own data, not published). This would reinforce the relation between γδ T-cell deficiency and the more severe forms of the disease, and a possible predicting role of its determination.

There is currently no general agreement about the location and the role of γδ T cells in the intestinal mucosa in CD, which makes it more difficult to interpret their role on a local level. Some authors detected a decrease in the γδ T-cell population in the biopsies of patients compared to controls, especially in the ileum(158) (159). Others, however, found no

significant differences in the percentage of IEL or of γδ+ TCR LPL(160), or even observed an increase of them in colon biopsies, although without reaching statistical significance(180). Nevertheless, most of the studies showed a decrease in γδ T cells in the inflamed vs. non-inflamed mucosa, although in a very limited number of patients(152) (161).

This contradiction between the pro- and anti-inflammatory functions of γδ T cells is not exclusive to CD, since similar phenomena have been reported in other chronic inflammatory diseases such as multiple sclerosis(27) (28), Behcet's disease(29) or systemic lupus erythematosus(30) (31).

For example, IL-17-producing γδ T cells can aggravate and induce inflammation in models of rheumatoid arthritis(181), psoriasis or ankylosing spondylitis(182), whereas, in contrast, this sub-population has been shown to play a protective role in the development of diabetes in NOD mice via the production of TGF-β (183). Likewise, it has been shown that γδ T cells can perform an anti-tumour function or favour tumoral growth in different immunological contexts(73).

Another possible explanation for these discrepancies is the possibility that the different subtypes of γδ T cells may have distinct actions and effects. The subtype Vδ2+ is predominant in PB(81), and has a greater cytotoxic capacity of both the natural killer (NK) and antibody-mediated types(82) (83). The Vδ1+ subtype predominates in the epithelia(75), has a lower cytotoxic capacity and plays a crucial role in epithelial regeneration(78-80).

A relative increase in Vδ1+ γδ T cells has been demonstrated not only in patients with CD(180) (152), but also in the inflamed mucosa of patients with UC, with a direct relationship between their number and the severity of the inflammation(184). It appears that the Vδ1+ subtype may play an important role in chronic inflammation in the epithelia, since similar findings have been reported in active coeliac disease(185), in the skin lesions of leprosy(187) or in the synovial tissue of patients with rheumatoid arthritis(75).

Some authors also point to the possibility that exposure of the different types of γδ T cells in different cytokine micro-environments in inflamed tissues would produce activation of distinct genes, with opposing effects. That is to say, the diverse interactions of γδ T cells with the microbiota, epithelial cells and other cells in the immune system (such as macrophages) would result in a different immune response.

6.Conclusions and future directions.

Crohn's disease is a chronic recurrent systemic disease affecting any part of the gastrointestinal tract, typically the ileocecal area. An altered immune response to commensal intestinal bacteria takes place in genetically predisposed individuals, resulting in chronic inflammation. Several alterations in the innate immunity mechanisms have been reported in recent years, leading to the presence of micro-infections that are not efficiently solved by the immune system, leading to chronic inflammation.

γδ T cells constitute only a small proportion of the lymphocytes that circulate in the blood and peripheral organs and they are present mainly in the epithelia, where they can account for up to 50% of intraepithelial T cells (IELs) in the mucosa. Their lack of MHC restriction and their singular plasticity and immunoregulatory properties makes them key cells in the first line of defence against infections and in wound healing. These unique features, their known participation in the pathogenesis of other autoinflammatory conditions and the results of animal and human studies published to date point to an important role of γδ T cells in the pathogenesis of CD. However, their clinical role in IBD is still not fully understood. Some animal studies indicate that they may play a protective role against colitis, while others have shown a possible deleterious role. In humans, there is evidence that patients with CD present a γδ T-cell deficiency in PB, with possible clinical consequences. Other studies, however, have identified proinflammatory phenotypes that could contribute to intestinal inflammation.

These contradictory effects must be studied in depth in order to know the biology of this type of lymphocyte, its interaction with the microbiota, the epithelial cells and its relation with other innate and acquired immunity cells. A systematic study of the different sub-populations of γδ T cells (Vδ1+, Vδ2+, Vδ3+), as well as their different phenotypes (IL-17+γδ, γδ Tregs, etc.), within the different clinical contexts of a heterogeneous disease like CD will allow some of these key issues to be solved.

The study of the relations between the values in serum and in the mucosa (both inflamed and healthy), together with the migration mechanisms, their possible prognostic value, their correlation with opportunistic infections, the effect of treatments and their role in the different phenotypic forms of CD (including the extraintestinal manifestations) will help to define better their true importance in the pathogenesis and clinical symptoms.

Furthermore, we could also speculate on the possibility of using immunoregulatory treatments to modulate their action and improve the propensity to present infections, by optimising the immune response in the intestinal mucosa in CD. In fact, clinical trials have already been conducted that show that manipulating these cells in immunotherapy against cancer is effective and well tolerated, thereby opening up a new possible therapeutic alternative in the future for CD patients.

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8.FIGURE LEGENDS

Fig 1. Main differences between αβ T cells and γδ T cells*.*

Fig 2. Immunoregulatory functions of γδ T cells.

This figure shows a representation of the main intestinal epithelial cells, and the intraepithelial location of γδ T cells (black square) and their main immunoregulatory functions. γδ T cells can exert direct cytotoxic action against infected or neoplastic cells (93) (94) and are capable of opsonising and engulfing infected cells (95) . They can stimulate αβ T cells for the production of Th1 or Th17 cytokines, or supress their activation by secreting TGF-β or IL-10(70). In addition, γδ T cells can activate the immune response in a local environment by promoting the maturation of dendritic cells and stimulating macrophages, neutrophiles and NK cells (98, 99), as well as migrate to the secondary lymphoid organs and collaborate with B lymphocytes in the production of antibodies (101)(102). Epithelial regeneration and wound healing is actively promoted by γδ T cells by stimulating the production of hyaluronic acid by epithelial cells (25) and epithelial growth factors (26).

9.TABLES

Table 1. Main antiinflammatory immunomodulator effects of γδ T cells in preclinical studies.

Antiinflammatory functions of γδ T cells
• Immunoregulation and protection of the mucosa in infections(103) • Capacity to repair epithelial tissue via the production of KGF(127) • Stimulation of IL-10 and TGF-β production(32) (35) • Decreased expression of MHC II(128) • Role in oral tolerance following the administration of antigens(125) • Enhancement of IgA-mediated responses(126) • Role in suppressing the proinflammatory effects of αβ T cells(128)

***Table 2*.** Main preclinical γδ T-cell studies in murine colitis models**.**

Animal Studies	Publ. year	Mouse model	Protective / Pro-inflammatory	Main results
Szczepanik M. et al. (131)	2000	TNBS	**PROTECTIVE**	Passive transference of αβ T cells and not γδ T cells induce colitis.
Hoffmann J.C. et al. (36)	2001	TNBS	**PROTECTIVE**	Depletion of γδ T cells with mAb (and not αβ T cells) ameliorated the colitis and reduces mortality.
Chen Y. et al. (32)	2002	DSS	**PROTECTIVE**	γδ T cells accumulate in the inflammed areas and colaborate in tissue repair through KGF.
Kühl A.A. et al. (132)	2002-2003	TNF ΔARE/+	**PROTECTIVE**	Depletion of γδ T cells aggravates colitis and increases IFN-γ production.
Tsuchiya T. et al. (130)	2003	DSS	**PROTECTIVE**	γδ T cells control the migration of neutrophils.
Inagaki-Ohara K. et al. (33)	2004	Cδ-/- / TNBS	**PROTECTIVE**	γδ T cells deficient mice develop colitis. Transfer of γδ T cells ameliorated TNBS induced colitis.
Kühl A.A. et al. (37)	2007	DSS/ TNF ΔARE/+ / IL-2 ko	**PROTECTIVE**	Depletion of γδ T cells aggravates colitis. Increased mortality after early depletion in IL-2 ko mice.
Hoffmann J.C. et al. (35)	2008	TNBS/IL-10 tg	**PROTECTIVE**	γδ T cells transfer ameliorates TNBS induced colitis and prolonged survival.

Simpson S.J. et al. (134)	1997	tgε 26	**PRO-INFLAMMATORY**	γδ T cells infusion produces colitis.
Kawaguchi-Miyashita M. et al. (135)	2001	TCR α -/-	**PRO-INFLAMMATORY**	Elimination of γδ T cells ameliorates colitis.
Do J.S. et al. (136)	2011	TCR βδ (-/-)	**PRO-INFLAMMATORY**	IL17 + γδ T cells transfer induces a Th17 diferenciation of colitogenic lymphocytes and induces colitis.

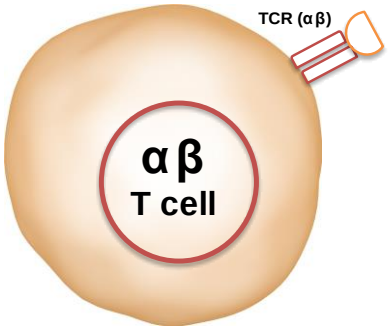
Table 3. Main human γδ T cell studies in Crohn's disease.

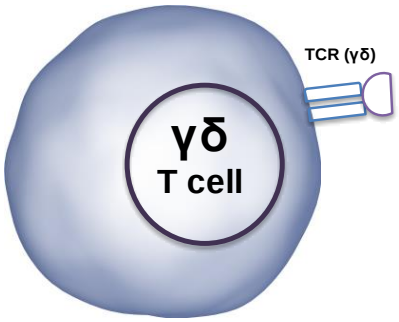
Human Studies	Publ. year	γδ T in PB/mucosa	Number of patients and disease location	Main results
Trejdosiewicz LK. et al. (180)	1991	Mucosa	**6 (colonic)**	Increased γδ T cells percentage in 3 out of 6 CD patients (not statistically significant). Predominance of Vδ1+ subtypes
Fukushima K et al. (158)	1991	Mucosa	**10 (8 ileocolonic, 1 ileal, 1 colonic)**	Reduced ratio of IEL and LPL γδ T cellsin CD, particularly in the ileum.
Cuvelier CA. et al. (160)	1992	Mucosa	**14 (ileal)**	No changes in IEL/LPL γδ T cells proportion in CD.
Giacomelli R. et al. (151)	1994	Blood	**9 (7 ileal, 2 ileocolonic)**	Increased γδ T cells numbers in PB only in activ CD.
Bucht A. et al. (152)	1995	Blood/Mucosa	**4 (3 colonic, 1 ileocolonic)**	Increased proportion of γδ T cells in PB of CD (increased Vδ1+). Decreased γδ T cells in healthy mucosa compared with inflammed.
Söderström. et al. (153)	1996	Blood	**16 (N/A)**	Increased proportion of γδ T cells in PB in CD.
Lee H.B. et al. (159)	1997	Mucosa	**5 (N/A)**	Decreased γδ t cells percentage in CD compared with UC and controls.

McVay LD. et al. (161)	1997	Blood/Mucosa	**3 (colonic)**	Increased proportion of IEL and LPL γδ T cells in the inflammed mucosa of CD patients vs non inflammed.
Andreu-Ballester J.C. et al. (142)	2011	Blood	**40 (N/A)**	Decrease γδ T cell numbers in PB of CD. Especially CD8+γδ subset.
Mann E.R. et al. (162)	2012	Blood/Mucosa	**15 (4 colonic, 5 ileal, 4 ileocolonic)**	Increased expression of CCR9 in γδ T cells of CD patients.
McCarthy N.E. et al. (154)	2015	Blood/Mucosa	**12 (pediatric, N/A)** **12 (N/A)**	Decreased Vδ2 in the PB of CD (selective depletion of CD27+Vδ2) vs IBS controls. No differences in Vδ2 numbers in PB in CD vs healthy controls. Increased β7+Vδ2 “gut tropic” in CD.

10.FIGURES

Figure 1.





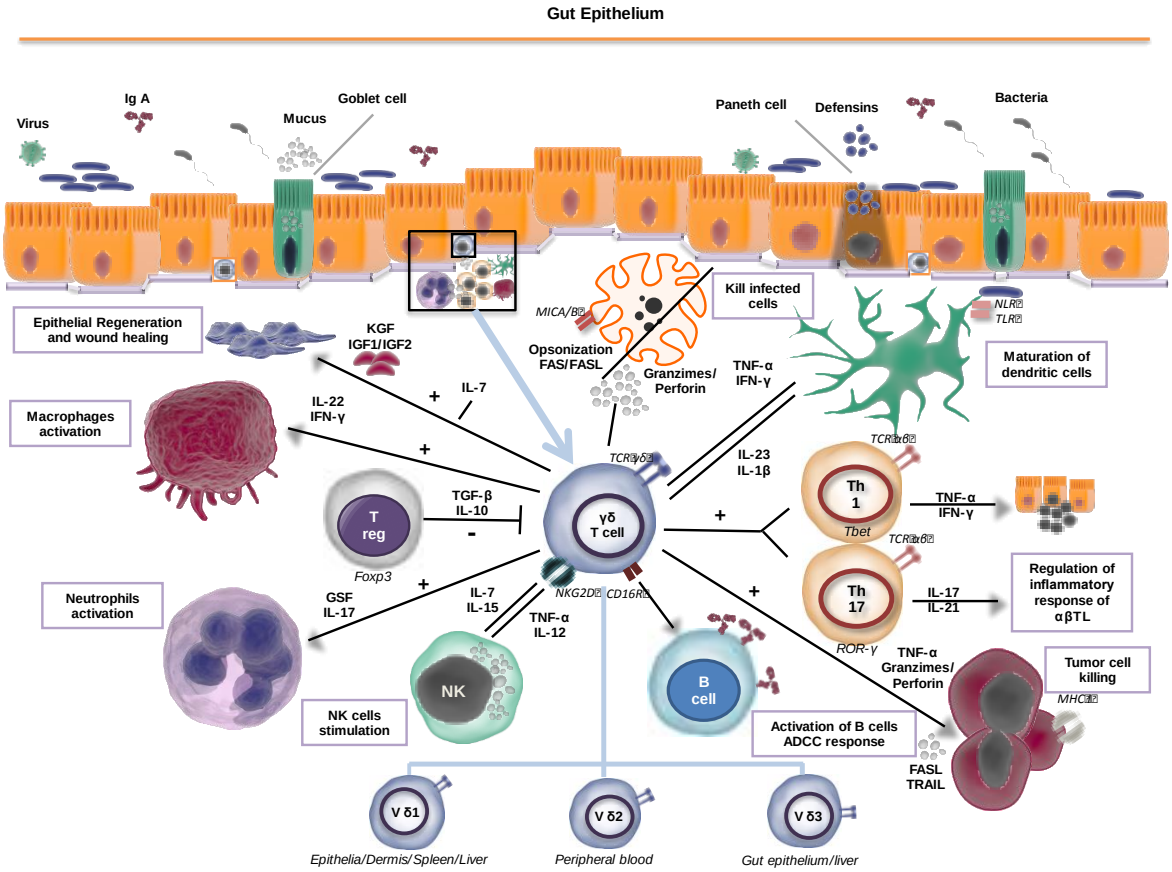
- "Classic" T cells
- Adaptative immunity
- Retarded response
- 95% in the peripheral blood

- MHC restricted (CD4+ MHC I /CD8+ MHC II)
- Recognise prosessed peptides presented by APC
- Majority express CD4 or CD8
- High TCR diversity

- First described 1984 by Saito et al.
- Innate immunity
- Quick response (first line of defense)
- 3-5% in the peripheral blood (50% of the IEL in the gut mucosa)
- Not MHC restricted (direct recognition)
- Recognise unprosessed peptides, viral proteins, lipids etc.
- Majority in PB are double negative (CD4-CD8-)
- Restricted TCR diversity

***Abbreviations**: TCR:T cell receptor, MHC: major histocompatibility complex, APC: antigen presenting cell, PB: peripheral blood, IEL: intraepithelial lymphocytes.*

Figure 2.


Gut Epithelium
Virus
Ig A
Mucus
Goblet cell
Paneth cell
Defensins
Bacteria
Epithelial Regeneration and wound healing
KGF
IGF1/IGF2
Kill infected cells
MICA/B
Opsonization
FAS/FASL
Granzimes/
Perforin
NLR
TLR
Maturation of dendritic cells
TNF-α
IFN-γ
Macrophages activation
IL-22
IFN-γ
IL-7
TCRγδ
IL-23
IL-1β
T reg
TGF-β
IL-10
γδ T cell
Th 1
Tbet
TCRαβ
TNF-α
IFN-γ
Foxp3
Th 17
ROR-γ
IL-17
IL-21
Regulation of inflammatory response of αβTL
Neutrophils activation
GSF
IL-17
IL-7
IL-15
TNF-α
IL-12
NKG2D
CD16R
NK
B cell
TNF-α
Granzimes/
Perforin
Tumor cell killing
MHC
NK cells stimulation
Activation of B cells ADCC response
FASL
TRAIL
V δ1
V δ2
V δ3
Epithelia/Dermis/Spleen/Liver
Peripheral blood
Gut epithelium/liver