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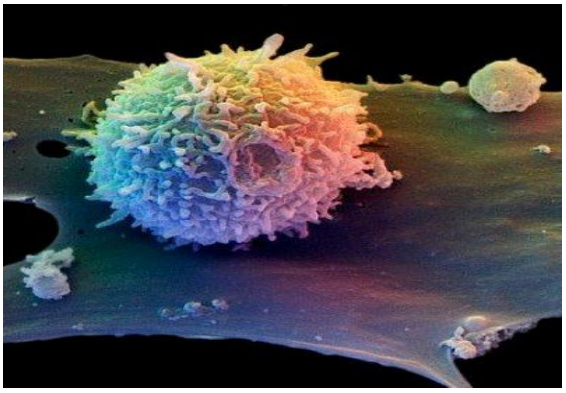
Transglutaminase type II role in dendritic cell differentiation and function



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By three methods we may learn wisdom: First, by reflection, which is noblest; second, by imitation, which is easiest; and third by experience, which is the bitterest.

Confucius

To my mom,

Thank you for being my biggest support in life and my conscience helping me always get on the right path. I dedicate this work to you.

Mojoj majci,

Hvala Ti što si moja najveća podrška u životu, i moja savest usmeravajući me uvek na pravi put. Ovaj rad posvećujem Tebi.

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Abstract

Dendritic cells (DCs) play an essential role in the endotoxic shock and their profound depletion occurs in both septic patients and septic mice. Type II transglutaminase (TG2) knock out ($TG2^{-/-}$) mice are more resistant to the endotoxic shock induced by LPS. Here, we aimed to understand the cellular and molecular basis of this effect analyzing the role of the enzyme in DC maturation and function. We show that TG2 is drastically up-regulated during the final functional maturation of DCs consequent to LPS treatment. In keeping with this finding, the inhibition of the enzyme cross-linking activity determines the impairment of DC function highlighted by wide phenotypic changes associated with a reduced production of cytokines (IL-10, IL-12) after LPS treatment and a lower ability to induce IFNγ production by naïve T cells. The in vivo analysis of DCs obtained from $TG2^{-/-}$ mice confirmed that the enzyme ablation leads to an impairment of DC maturation and their reduced responsiveness to LPS treatment. In fact, a marked decrease in DC death, TLR4 down-regulation and impaired up-regulation of MHC II and CD86 were observed in $TG2^{-/-}$ mice. Taken together these data suggest that TG2 plays an important role in regulating the response of DCs to LPS and could be target in a promising strategy for treating endotoxin-induced sepsis.

Introduction

Immune system is a complex of a variety of tissues and cell types, fixed and mobile throughout the body, working together in rapid recognition of harmful antigens and/or tissue injury and developing a proper response in order to eliminate or neutralize the danger. Immunity is divided into two interdependent parts determined by the speed and specificity of the reaction – innate and adaptive immunity (Medzhitov, 2007).

Innate immunity is an ancient, evolutionary conserved system consisting of components ready to act prior to exposure to antigens. It is designed to prevent the entry of and/or rapidly eliminate antigens such as pathogens, toxins, or other foreign materials. The skin, mucosal secretions, and stomach and intestinal pH are physical/chemical barriers that form the first line of defense. When an infectious agent evades these barriers and invades the host, cellular innate immune defense come into play that includes natural killer (NK) cells, granulocytes, macrophages and dendritic cells (DCs). Unlike the more sophisticated adaptive immune system, the innate system cannot distinguish subtle differences among different foreign antigens. However, it relies heavily on a limited number of highly conserved cellular receptors termed pathogen-recognition receptors (PRRs) which detect pathogen associated molecular patterns (PAMPs) - common molecular structures associated with many classes of pathogens (Medzhitov, 2007; Kawai and Akira, 2010). This allows the discrimination between infectious non-self and non-infectious self antigens. Innate immunity is the first line of defense and it acts in the first few hours and days after exposure to infectious agent. If an antigen exposure is sufficiently large and involves a virulent pathogen, the innate immune system then serves as a trigger for the adaptive immune system to mount a response.

Adaptive immune response is characterized by extraordinary specificity and long-lasting memory to antigens so that the second time the same invader is encountered a more rapid and intense response can occur. There are two types of specific immune responses: humoral and cell mediated. Humoral immunity is mediated by B Lymphocytes that produce antibodies (IgG, IgM, IgA, IgE) and is important in combating acute bacterial infections (Strugnell and Wijburg, 2010). Cellular immunity is especially important in combating intracellular organisms, performing tumor surveillance, mediating transplant rejection, and fighting fungal and viral infections. It also mediates the hypersensitivity reactions. Cellular immunity is mediated by T Lymphocytes which are processed in the thymus. T lymphocytes are classified according to surface receptors, with cytotoxic T cella

bearing the CD8 receptor and the T helper cells the CD4. CD4 are further subdivided in at least two lineages: Th1 and Th2 (Diebold 2008) which secrete different cytokines which can influence the clinical manifestations of any immune response. The Th1 is associated with production of interleukin 2 (IL-2) and Interferon-gamma (IFNγ) and may have a greater role in T cell proliferation and inflammation while the Th2 is associated with the production of IL-4, -5, -6 and -10 and may be more involved in B cell development and proliferation (Steinman and Hemmi, 2006).

A key role in the generation of adaptive immune responses is ascribed to dendritic cells (DCs) that are professional antigen presenting cells (APCs) and the most potent stimulators of T lymphocytes that have never before encountered an antigen - naïve T cells. DCs represent a certain messenger between the innate and adaptive immunity by providing lymphocytes with the full information about the nature of the pathogen (Steinman and Hemmi, 2006) (Figure 1).

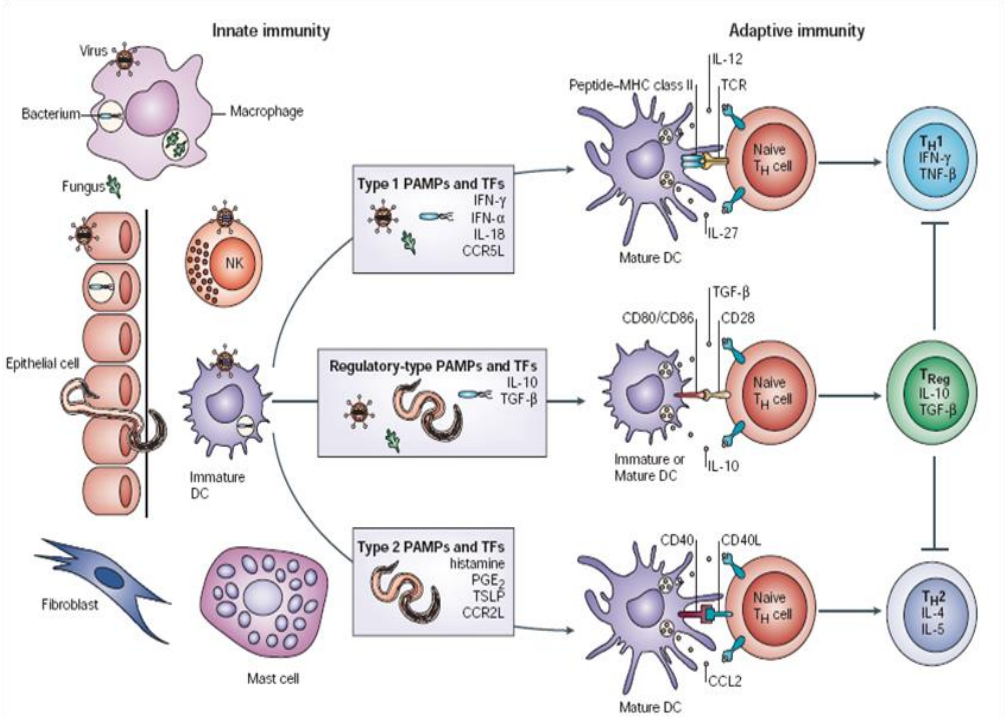

Figure 1. **Schematic representation of the DCs as a link between innate and adaptive immunity.** Following a microbial encounter, immature DCs downregulate phagocytic activity and increase the efficiency of antigen processing and presentation to prime T cells. Moreover, activated DCs express, with strictly defined kinetics, cytokines and chemokines for activation and control of innate and acquired immune responses. At later time points, DCs reach the final stage of maturation and express high levels of peptide-MHC complexes and costimulatory molecules at their surface. They migrate to lymph nodes and activate T-cell responses (late adaptive response) (Kapsenberg, 2003).

As a component of the innate immune system, DCs reside in most peripheral tissues and organs (skin, lungs, and other mucosal tissues) - major portals of microbial entry, where they perform a sentinel-like function continuously sampling their external environment for foreign antigens. Under physiological conditions, DCs exist in an immature state primed for antigen uptake and processing. Pathogen recognition (via receptors such as the toll-like receptor (TLR) family), together with the production of proinflammatory signals by other innate immunity cells and/or damaged tissue cells, initiates a maturation process which transforms the DCs into efficient T-cell stimulators. During maturation, DCs up-regulate expression of molecules essential for the activation of T cells, such as major histocompatibility complex (MHC) molecules and the costimulatory molecules CD80 and CD86. DCs then leave the peripheral tissues and migrate to the draining lymph nodes where they encounter T and B lymphocytes from circulating lymph and blood and activate them. Activated T and B cells then leave the lymph nodes and find their way back to the site of inflammation based on the thorough information delivered by DCs. At the site of pathogen invasion adaptive immune components (activated T and B cells) then call on the innate immune system to provide the professional phagocytes (e.g. macrophages and neutrophils) and specialized granulocytes (e.g. eosinophils and basophils) necessary to engulf small pathogens and contain larger parasites. In this way the appropriate innate and adaptive system interactions provide efficient protection to the host against invading pathogens.

Given their pivotal role as immune-surveillance against the microbial infections and as central link between innate and adaptive immunity, DCs are receiving increasing scientific and clinical interest in becoming the key weapon in the therapies based on the host response (cancer, autoimmunity and many other pathological conditions).

Morphological and phenotypic features of DCs

DCs were first visualized as Langerhans cells (LCs) in the skin in 1868, then in 1973 Steinman and Cohn described them as the main cells of immune system. They found a population of striking dendritic-shaped cells in the spleen. Shortly after, it became clear that DCs existed in all lymphoid and most non-lymphoid tissues. Main characteristic of mature DCs are numerous thorn-like cytoplasmatic processes – dendrites, whose length can reach up to several hundreds of micrometers (Steinman and Nussenzweig, 1980). Dendrites serve to increase surface to volume ratio of DC thereby providing very close contacts with immune-

competent cells. Immature DCs don't have these dendrites but instead they are characterized by veils - wide non-spiked cytoplasmatic protrusions (Figure 2).

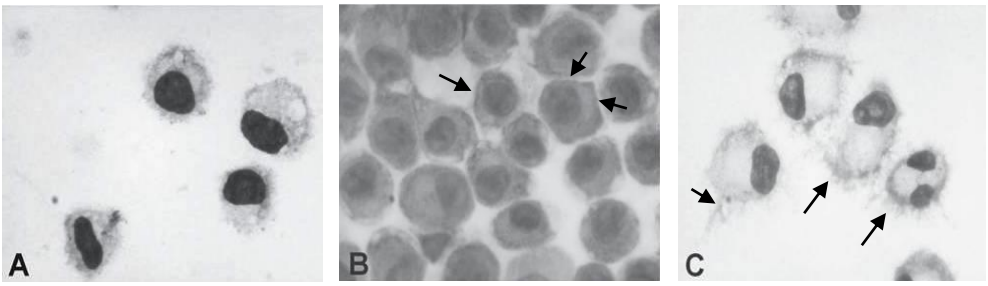

Figure 2. **Morphological changes of DC in presence of maturation stimuli.** A) CD14 precursor monocytes with smooth membrane surface; B) immature DC with slightly pronounced veil-like structures on their membrane; C) mature DC with numerous dendritic membrane forms.

DC are characterized by strong expression of main histocompatibility complex molecules (MHC) class I and II. The MHC proteins act as "signposts" that serve to alert if foreign material is present inside a cell by displaying fragmented pieces of antigens on the antigen presenting cell's surface (Blanchard and Shastri, 2010; Landsverk et al., 2009). This peptide-MHC complex binds T cell receptor (TCR) on the surface of T cells (Abbas et al., 1996). Next, DCs constitutively express co-stimulatory molecules CD86, CD80 and CD40. The co-stimulatory signal is antigen non-specific and is needed for the proper T cells activation. Co-stimulatory molecules of APC interact with the specific receptors on the T cells of which the best characterized is CD28 that binds to CD86 and CD80 on DCs (Steinman et al., 1997). Also, expressed on the surface of DCs is a number of adhesive molecules such as CD11a, b and c, CD15s, CD18, CD29, CD44, CD49d, CD50 and CD54 which are a prerequisite for complete T cell activation.

Heterogeneity of DC subsets

Often opposing roles now ascribed to DCs (initiation of immune response vs. tolerance and autoimmunity) cannot all be carried out at once by the same cell, so different sets of DCs that perform different functions should exist. And in fact DCs are a very heterogeneous group of leukocytes (Coquerelle and Moser, 2010) but although many attempts to classify DCs subsets were carried out by phenotypical

and functional analysis in the past years, the complete model has not yet been established.

Distinct subtypes were initially more evident among mouse DCs than among human DCs, because of the ready availability of different murine lymphoid tissues and the expression on mouse DCs of markers not present on human DCs.

In mice, at least two distinct pathways of DC development have been identified: lymphoid and myeloid DCs differ in phenotype, localization, and function. Both subsets express high levels of CD11c, class II major histocompatibility complex (MHC), and the costimulatory molecules CD86 and CD80. Lymphoid DCs are localized in the T cell–rich areas of the periarteriolar lymphatic sheaths (PALS) in the spleen and lymph nodes (Steinman et al., 1997; Leenen et al., 1998). In contrast, myeloid DCs are in the marginal zone bridging channels of the spleen (Steinman et al., 1997) but can be induced to migrate to the PALS under the influence of proinflammatory signals such as lypopolysaccharide LPS (De Smedt et al., 1996) or parasite extracts (Reis e Sousa et al., 1997). To date, the most reliable marker for distinguishing these two subsets is CD8a, which is expressed as a homodimer on the lymphoid DCs, but is absent from the myeloid subset (Shortman and Heath, 2010). Other markers such as DEC-205 and CD1d are expressed at higher levels on lymphoid DCs. Myeloid origin of DCs comes from myeloid-committed precursors that give rise to both granulocytes/monocytes and myeloid DCs under the influence of granulocyte/macrophage colony-stimulating factor (GM-CSF) (Jacobs et al., 2008).

T-cell markers CD4 and CD8 are expressed on mouse DCs and are useful for segregating subtypes. Other markers that are useful for segregating mouse DC subtypes include CD11b (the integrin αM chain of Mac-1) and the interdigitating DC marker CD205 (the multilectin domain molecule DEC205, originally known as NLDC-145).

The heterogeneity of DCs in humans is reflected at four levels: the precursor populations, anatomical localization, function and final outcome of immune response (Banchereau et al., 2000). The analysis of human blood DCs is significantly limited by its scarce distribution (less than 1%) and the absence of specific markers. There are at least two subsets of DCs in peripheral blood: $CD11c+CD123^{low}$ myeloid DCs and $CD11c-CD123^{high}$ plasmacytoid DCs (Olweus et al., 1997). Some studies (MacDonald et al., 2002) suggest that the CD16, CD1b/c and BDCA-3 DCs expressed CD11c are of myeloid origin, while the $CD123^{+}$ and $CD34^{+}$ DCs are lymphoid cells and hematopoietic progenitors, respectively. The level of heterogeneity reflected by anatomical localization comprises skin epidermal LCs, dermal (interstitial) DCs (intDCs), splenic marginal DCs, T zone interdigitating cells, germinal-center DCs, thymic DCs, liver DCs, and

blood DCs. The circulating DCs are either precursor DCs, drained from bone marrow to peripheral tissues, or antigen-captured DCs, drained from peripheral to lymphoid tissues.

Circulating blood DC are rare (they account for less than 1% of human peripheral blood mononuclear cells (PBMC) and are difficult to maintain in culture. Although in vivo expansion of blood DCs by administration of the hemopoietic growth factors Flt-3 ligand and GM-CSF can be used to increase the yield of isolated cells (Pulendran et al., 2000), most experimental and clinical studies currently rely on the *in vitro* development of DC-like cells from CD34 progenitor cells or blood monocytes (Liu and Nussenzweig, 2010). Commonly, monocytes are cultured for 5–7 days with GM-CSF and IL-4 to generate immature DCs that have to be activated for another 2–3 days with microbial, proinflammatory, or T cell-derived stimuli to obtain mature DCs with full T stimulatory capacity. There is increasing evidence that maturation of DCs from monocyte precursors may also be relevant *in vivo*, although the conditions under which it occurs and the exact time span required for the differentiation process are not known. However, experimental data indicate that the kinetics of DC differentiation from monocytes under physiologic conditions may not be reflected by current protocols for the *in vitro* development of DCs. Monocytes may give rise to a subset of DCs during infection or inflammation, when high levels of proinflammatory mediators such as TNFα, IL-1β, PGE2, and IFNγ are produced (Santini et al., 2000).

DCs in the regulation of immune response

DCs reside within the tissues in an immature state characterized by high endocytic activity and low T-cell activation potential. Immature DCs constantly sample the surrounding environment engulfing both self and non-self antigens by phagocytosis, by forming large pinocytic vesicles in which extracellular fluid and solutes are sampled, or by macropinocytosis. Also, they express receptors that mediate adsorptive endocytosis. Macropinocytosis and receptor-mediated antigen uptake make antigen presentation so efficient that picomolar and nanomolar concentrations of antigen are sufficient, much less than the micromolar levels typically employed by other APCs (Banchereau and Steinman, 1998). Inside DCs captured antigens get degraded into small peptides and presented on membrane surface in complex with MHC molecules. DCs have the ability to distinguish pathogens from harmless antigens by using pattern recognition receptors (PRRs) such as the toll-like receptors (TLRs). These receptors recognize specific chemical

structures found on subsets of pathogens (Kumar et al., 2009). Once DCs encounter a potential pathogen, they become activated into mature DCs. Maturation process is characterized by up-regulation of co-receptors on their surface such are CD86, CD80 and CD40 thereby greatly enhancing the ability to activate T-cells. Also, a chemotactic receptor CCR7 gets up-regulated and this enables DC to travel through the blood stream to the spleen or through the lymphatic system to a lymph node where they will act as antigen-presenting cells (activate helper T-cells and killer T-cells as well as B-cells by presenting them with antigens derived from the pathogen, alongside non-antigen specific co-stimulatory signals). Additionally, locally activated DCs recruit other immune-competent cells at the spot of pathogen invasion orchestrating in that way co-operation between different components of immune system (Figure 3).

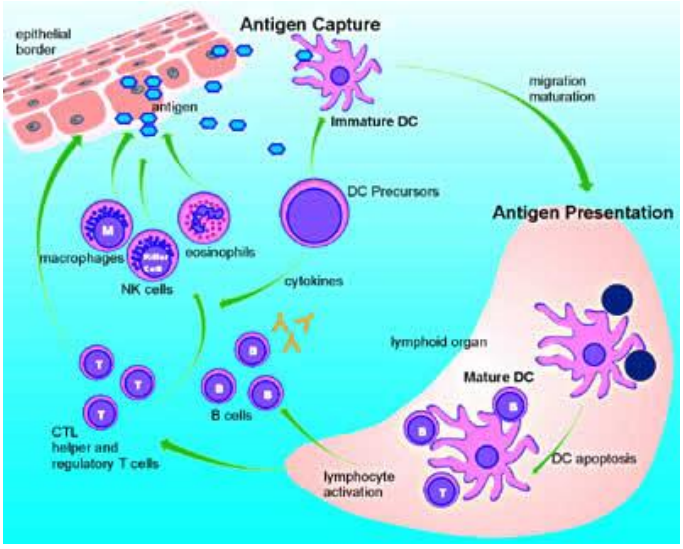

Figure 3. **Life cycle of DCs.** Antigens are captured by immature DC in peripheral tissues and processed to form MHC-peptide complexes. As a consequence of antigen deposition and inflammation, DCs begin to mature, expressing molecules that will lead to binding and stimulation of both B and T cells within lymphoid tissues. After activation, T and B cells leave the lymphoid organ. B cells move to the lining of the intestine, the bone marrow, and other parts of the lymphoid tissue, such as the medulla of lymph node becoming antibody-secreting plasma cells. T cells migrate to the original site of antigen deposition, recognizing changes in the inflamed blood vessels and responding vigorously to cells that are presenting antigen. This limits the immune response to the site of microbial infection.

The effectiveness of immune response depends on information about identity and pathogenicity of its potential target, and about the character of the pathogen. The availability of such information provides selective orientation of the response against the pathogen avoiding at the same time potentially dangerous reaction to non-pathological, harmless/self antigens. It also provides the selection of those effector mechanisms that are most effective against the particular pathogen and optimal for the action within a specific tissue. Migrating activated DCs are the key element in providing all these information to naïve T cells (Kadowaki, 2007) (Figure 4).

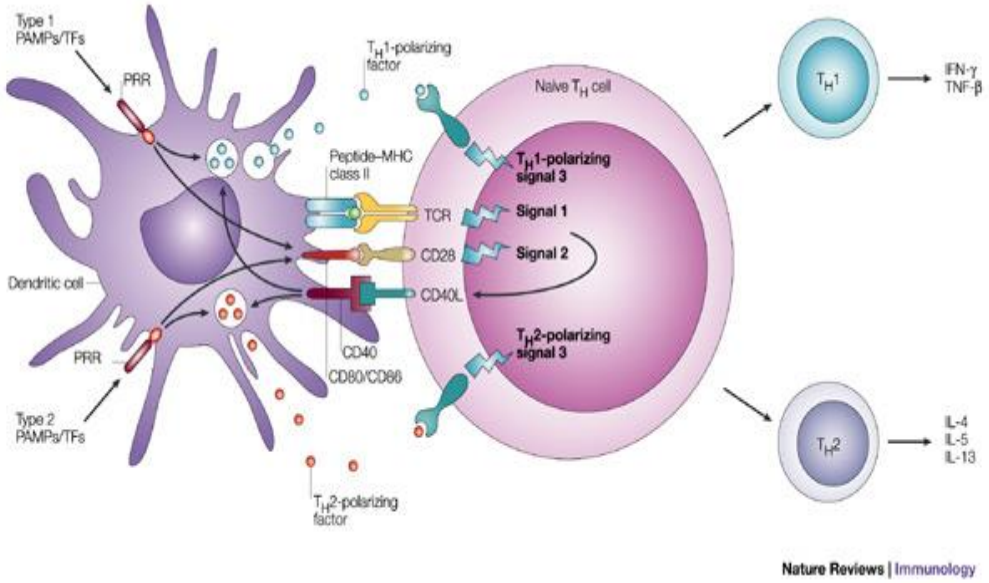

Figure 4. **Polarization of T cell response.** Signal 1 is the antigen-specific signal that is mediated through T-cell receptor (TCR) triggering by MHC class-II-associated peptides processed from pathogens after internalization through specialized pattern recognition receptors (PRRs). Signal 2 is the co-stimulatory signal, mainly mediated by triggering of CD28 by CD80 and CD86 that are expressed by DCs after ligation of PRRs, such as TLRs that are specialized to sense infection through recognition of pathogen-associated molecular patterns (PAMPs) or inflammatory tissue factors (TFs). Signal 3 is the polarizing signal that is mediated by various soluble or membrane-bound factors, such as interleukin-12 (IL-12) and CC-chemokine ligand 2 (CCL2), that promote the development of Th1 or Th2 cells, respectively. The nature of signal 3 depends on the activation of particular PRRs by PAMPs or TFs. Type 1 and type 2 PAMPs can be defined as those that selectively prime DCs for the production of high levels of TH1-cell-polarizing or TH2-cell-polarizing factors. Whereas, the profile of T-cell-polarizing factors is primed by recognition of PAMPs, optimal expression of this profile often requires feedback stimulation by CD40 ligand (CD40L) expressed by T cells after activation by signals 1 and 2 (Kapsenberg, 2003).

When an activated mature DC carrying MHC-antigen complex on their surface arrives in the closest lymph node, it encounters differentiated naïve T helper (Th) cells expressing T cell receptor (TCR) and CD3. TCR-CD3 complex binds strongly to the antigen-MHC complex present on the surface of DC. CD4, a co-receptor of the TCR complex, also binds to a different section of the MHC molecule. These interactions bring these proteins closer together, allowing the intracellular kinases present on the TCR, CD3 and CD4 proteins to activate each other via phosphorylation. This triggers the major biochemical pathways in the cytosol of the Th cell. This signal is known as *SIGNAL 1* of T cell activation, as it is the first and primary pro-activation signal in a Th cell and it provides the information about the molecular identity of the pathogen.

Having received the first TCR-MHC signal, the naïve T cell must activate a second independent biochemical pathway, known as *SIGNAL 2*. It involves an interaction between CD28 on the Th cell and the proteins CD86 and CD80 on the DC. This verification step is governing the magnitude of the response and is protective in the sense that it ensures that a T cell is responding only to a foreign antigen. In this way, if an antigen was not immunogenic enough to trigger the up-regulation of co-stimulatory molecules on DC, but still gets presented to T cell, in the lack of this second signal, T cell will not become reactive, but instead anergic. This anergic cell will not respond to any antigen in the future, even if both signals are present later on. Therefore, co-stimulation plays an important role in decreasing the risk of T cell auto-immunity against harmless/self antigens. Once the two signal activation is complete the Th cell then starts to proliferate by releasing a potent T cell growth factor called interleukin 2 (IL-2) which acts upon itself in an autocrine fashion (Granucci et al., 2003).

Mature DCs not only carry antigenic and costimulatory signals, but also are well equipped to transmit an additional *SIGNAL 3* thereby contributing to the initial bias of naive Th cells towards Th-cell subsets characterized with distinct cytokine-secretion profiles intimately associated to their effector functions (Murphy and Stockinger, 2010; Fietta and Delsante, 2009). The best defined role in Th-cell differentiation is played by soluble factors secreted greatly, but not exclusively by DCs. One of the most explored factor within this group is IL-12 (Gee et al., 2009; Del Vecchio et al., 2007; Watford et al., 2003), which currently appears to be the most crucial APC product, driving the development of naive precursors into Th1 cells. Th1 cells mediate cellular immunity against intracellular bacteria and viruses by secreting cytokines such as IFN-γ and tumor necrosis factor-α (TNF-α). The lack of IL-12 or its receptor IL12R is associated with impaired Th1 responses and recurrent infections with salmonella and mycobacteria stressing its role in the defense against pathogens located in intracellular vesicles

(Altare et al., 1998). Additionally to Th1 cells, IL-12 provides essential signals for the activation and survival also to NK, cytotoxic CD8 and B cells. It is important to mention that IL-12 production by DC strongly depends on the stimulus type and conditions of stimulation of DC. While IFN-γ and IL-4 are potent enhancers of bioactive IL-12 production, many other factors such as IL-10, corticosteroids and PGE_2 downregulate IL-12 production by DC (Bogdan et al., 1991);(Elenkov, 2004; Son et al., 2006). The factors that suppress Th1 response usually drive the polarization towards Th2. Th2 cells produce IL-4, IL-5 and IL-13 which encourage antibody production, particularly IgE responses and also enhance eosinophil proliferation and functions, thus regulating humoral immunity, allergic responses (Araujo et al., 2010) and immunity against extracellular parasites (Moreau and Chauvin, 2010).

An interesting possibility is that the type of Th-cell responses can also be differentially influenced by DCs of different lineages, such as lymphoid and myeloid DCs. *In mice,* Th1 response is induced by $CD8^+$ subpopulation and Th2 response by $CD8^-$ subpopulation of DCs. These subpopulations were considered ontogenetically different which led to the characterization of lymphoid DCs as Th1 and myeloid DCs as Th2 inducers. However, it has been shown that CD8 is irrelevant as a marker of lymphoid DCs (Manz et al., 2001) so the role of DC lineage in Th polarization has been relativized. *In humans,* monocyte-derived $CD11c^+$ DCs polarize naive T cells predominantly towards a Th1 profile. The induced pattern of Th1 cytokine secretion is dependent on the DC production of IL-12 and IL-12 is indeed secreted mostly by $CD11c^+$ subset. Reorientation to a distinct cytokine profile is not restricted to CD4 T cells and also applies to CD8 T lymphocytes and NK T cells (Kadowaki et al., 2001).

The characterization of another novel T helper subtype, T helper 17 cells (Th17) has cast further doubt on the basic Th1/Th2 model (Stockinger et al., 2007). These IL-17 producing cells were initially described as a pathogenic population implicated in autoimmunity but are now thought to have their own distinct effector and regulatory functions (van de Veerdonk et al., 2009).

There are also other types of T cells that can influence the expression and activation of helper T cells, such as natural regulatory T cells which typically serve to modulate and deactivate the immune response. Within mucosal surfaces DCs are present within or adjacent to the lining epithelia as well as in the mucosal-associated lymphoid tissues. Here, mucosal DC are able to induce regulatory T cells that prevent immune reactivity to harmless environmental antigens (Hubert et al., 2007). Regulatory T cells produce the cytokine transforming growth factor-beta (TGF-β) and IL-10. Both cytokines are inhibitory to helper T cells; TGF-β suppresses the activity of most of the immune system. There is evidence to suggest

that TGF-β may not suppress activated Th2 cells as effectively as it might suppress naive cells, but it is not typically considered a Th2 cytokine.

DCs are of high importance also for the B cell responses, directly enhancing proliferation of CD40-activated naïve and memory B cells and their differentiation into secreting plasma cells (Ma and Clark, 2009).

On the interaction with DCs rely also NK cells for the induction of their cytotoxic activity (Walzer et al., 2005; Moretta et al., 2006).

Dendritic cells are also involved in the induction of immune tolerance. There are two types of tolerogenic process: central and peripheral. Central tolerance (negative selection of T cells) represents acquisition of autotolerance in the central lymphoid organs as a result of encounter and interaction between immature autoreactive thymocytes with self-antigens. Negative selection is interceded by different cells of thymus microenvironment (cortical and medular epithelial thymic cells) of which thymic DCs have the most selective potential based on their pronounced potential of internalization and processing of antigens as well as costimulatory potential. Peripheral tolerance is the mechanism by which mature T cells that recognize self-antigens in peripheral tissues become incapable of subsequently responding to these antigens. These mechanisms are responsible for T cell tolerance to tissue-specific self antigens that are not abundant in the thymus. Anergy of these autoreactive T cells can be induced by antigen presentation by tissue DCs that are normally in the resting state and express few or no costimulators. In this way T cells survive but remain incapable of responding to the antigen even if it is later presented by competent DC.

Role of DC in pathological conditions and their clinical application

Given their central role in the control of immunity, DCs are implicated in a plethora of pathologies - infectious diseases, tumors, allergies and autoimmunity. Some of these conditions are major health problems throughout the world, such as acquired immunodeficiency syndrome (AIDS) and tuberculosis.

DCs are shown to be very important in the control of HIV infection. The confirmation of their infectivity with HIV-1 virus comes from the studies showing that both plasmacytoid and myeloid DC populations isolated from HIV patients contain proviral DNA (Donaghy et al., 2003). Apparently, on their surface DC express molecules that are engaged by HIV to enter the host cell among which the most noted is DC-SIGN (C-lectine grabbing non-integrin) (Steinman, 2000). Others are langerin present in Langerhans cells and mannose receptor (Turville et al., 2002). Once internalized, the virus gets to lysosomal compartments where it

maintains the infectivity (Kwon et al., 2002). Using DCs as viral reservoirs the HIV gets transported to lymph nodes and transmitted to T lymphocytes during antigen presentation (Geijtenbeek et al., 2000). It has also been shown that the virus replicates in greater extent in immature DCs since the mature ones inhibit translation of the virus once the proviral cDNA is integrated in the cell genome (Patterson et al., 2001; Bakri et al., 2001).

The ability of HIV infected DC to up-regulate the co-stimulatory molecules is maintained but it seems that the production of major pro- and anti-inflammatory cytokines is dysregulated. Proinflammatory IL-12 is indispensable for IFNγ production by NK and T cells directing in that way the immune response towards Th1. It has been shown that only non infected DC of HIV patients produce IL-12 (Marshall et al., 1999). Production of IL-10 is, however, increased in HIV infected DC (Carbonneil et al., 2004). Numerous studies show the reduction in number of DC in peripheral blood of HIV patients that correlates directly with viral load. AIDS remains one of the most important deadly infections today, due to the lack of preventing vaccine and limited access to medical care in developing countries. Thus, understanding the molecular mechanisms of DC involvement in the course of the disease could be one step forward in improving the current available therapy based on life long retroviral treatment.

Another worldwide distributed infection is tuberculosis, with eight million cases each year. DCs play a crucial role in the pathogenesis of *Mycobacterium tuberculosis* (Mtb) infection as well, being a bridge between innate resistance and adaptive immune response against intracellular pathogens. The production of IL-12 in response to Mtb is a key event of protective immunity. Mycobacterial infection of DCs results in the up-regulation of the regulatory cytokines such as IL-12 and TNFα. The IL-12 production potentiates the development of IFNγ and TNFα producing T cells, both potent activators of bacterial killing by infected macrophages (Cooper et al., 1997). This contributes to the development of the inflammatory process, through the recruitment of leukocytes leading the granuloma formation and the containment of bacterial dissemination (Hernandez-Pando et al., 1997). Interaction of DCs with Mtb also results in direct cell maturation. In fact, Mycobacteria-infected DCs have a decreased endocytic activity (Henderson et al., 1997) and they up-regulate the expression of co-stimulatory molecules such as CD80, CD86, the intercellular adhesion molecules (ICAM-1) and the signalling molecules CD40 (Thurnher et al., 1997). Mtb is able to survive in DCs that present antigen to CD4 T cells in association with MHC molecules and this process is essential to establish persistence or latent infection. In years, several studies have shown contrasting results about the regulation of MHC molecule expression. It has been reported that Mtb infection up-regulates the expression of MHC molecules in

DCs (Demangel et al., 1999), but more recent evidence show a fine mechanism of escape by Mtb to evade antigen presentation down modulating MHC molecules in a TLR dependent mechanism (Harding and Boom, 2010). This reduction of antigen presentation might reflect a general mechanism of negative feedback regulation that prevents excessive T cell mediated inflammation and that Mtb has subverted to create a niche for survival in infected APCs and evade the recognition by CD4 T cells. These several highlighted features of DC relationship in intracellular infections put them in focus as targets for possible future therapies.

In oncology, DC involvement has been well documented. In particular, DCs are currently being studied as adjuvants for vaccines or as a direct target for remedy by inducing specific immunity against cancer. In fact, DCs loaded with tumor lysates, tumor antigen-derived peptides, MHC class I restricted peptides, or whole protein have all been shown to generate anti-cancer immune responses, including in some cases the ability to induce complete regression of existing tumor (Young and Inaba, 1996). Thus, there is a great desire to test these strategies and use tumor-antigen bearing DCs as a vaccine in humans. Human clinical trials are ongoing in many institutions to use new protocols relying on DCs to induce immunity to antigens against breast cancer, lung cancer, melanoma, prostate and renal cell cancers.

At this point, it is worth noting that DCs come out as a significant element in a variety of pathological conditions. Nevertheless, in non-pathogenic conditions such as chronic autoimmune diseases, they are often the source of cytokines responsible for their initiation and progression. The effector cells activated by DCs in autoimmune diseases like Crohns disease are mainly Th1 and Th17 cells, which secrete cytokines (TNFα, IL2, IL6 and IFNγ) now targeted for treatment of these diseases as well as in conditions such as psoriasis (Gottlieb, 1988) and rheumatoid arthritis (Cavanagh et al., 2005).

Dendritic cells and sepsis

Sepsis is a serious medical condition that is characterized by a whole-body inflammatory state (called a systemic inflammatory response syndrome or SIRS) initiated by infection that resulted in a disrupted immune response. Inflammatory dysregulation consequently affects multiple organs via effects on endothelial, epithelial and immune cell types thus leading to irreversible damage. The poor outcome in sepsis is considered to be a consequence of an overactive systemic inflammatory response elicited by the invading micro-organisms (Ruokonen et al., 1991). The endotoxic shock is followed by a mechanism set to end the excessive inflammation (known as compensatory anti-inflammatory response - CARS)

counteracting SIRS by releasing of anti-inflammatory molecules (Cavaillon et al., 2003). Imbalance and overreaction of either response can result in host damage caused by excessive inflammation or immune dysfunction reflected in the balance between the production of pro- and anti-inflammatory cytokines and this is critical for the outcome of sepsis. IL-12 and its counterpart IL-10 play a major role during sepsis since the increase in IL-10 amount increases survival (Walley et al., 1996) whereas administration of IL-12 increases sepsis-induced mortality (Echtenacher et al., 2001).

It has been hypothesized that DCs play a fundamental role in sepsis-mediated immune suppression. Much of the knowledge we have today about the role of DCs in the inflammatory response comes from studies done on mice. There are two methods mostly used in mice induce the symptoms similar to those found in septic patients. Cecal ligation and puncture (CLP) serves as a model for polymicrobial sepsis whereas bacterial LPS is used in mice models for endotoxemia. These two models induce different sepsis responses (Echtenacher et al., 2001; Remick and Ward, 2005) but both of them represent good models to explore how DCs behave to microbial products and presumably microbial invasion.

Any defects in the cascade of DC antigen capture, processing and presentation result in the impairment of the body's ability to clear invading microorganisms such as that seen with sepsis. It has been shown that targeted depletion of DCs dramatically increases mortality to sepsis, while adoptive transfer with wild type bone marrow-derived DCs restores survival in this model of polymicrobial sepsis (Scumpia et al., 2005). Several studies were published on the role of DCs during sepsis response in human patients and mice CLP model. It has been reported that the patients who died from sepsis had lost DCs from the spleens (Hotchkiss et al., 2002). The depletion of DCs was further described in spleen (Tinsley et al., 2003; Ding et al., 2004), lymph nodes (Efron et al., 2004), lung (Benjamim et al., 2003) and peritoneum (Ding et al., 2004) in mice CLP model for polymicrobial sepsis. Besides DC depletion, their functional properties are also affected by environmental factors such as pro- and anti-inflammatory cytokines. DCs are the major producers of IL-12, a proinflammatory cytokine which modulate host immune response (Cella et al., 1996). It was described that splenic DCs from septic mice showed reduced ability for IL-12 production while the peritoneal DCs produced significantly higher amounts of IL-12 than control mice (Ding et al., 2004). Lung DCs isolated from septic mice when stimulated with LPS showed reduced production of IL-12 and TNFα and increased production of IL-10 (Benjamim et al., 2003; Benjamim et al., 2005) suggesting a change in DCs cytokine profile towards the Th2-type, probably in order to down-modulate the proinflammatory response.

Although scarce in number and truly effector cells of immunity, DC's unique ability to link innate and adaptive immunity sets them in focus for manipulation of their functional properties in order to cure many diseases including sepsis. Interestingly, recent findings of our group have implicated a particular enzyme of a large transglutaminase family of proteins - transglutaminase type 2 (TG2) as an important factor in the mechanism through which the sepsis is developed acting on multiple levels (Falasca et al., 2008). Also, use of TG2 inhibitors is demonstrated to be useful in reversing some of the inflammatory processes (Sohn et al., 2003).

Transglutaminase type 2 (TG2)

TG2 is a multifunctional enzyme belonging to thiol- and Ca2+-dependent acyl transferase family of transglutaminases (TGases) involved in a variety of biological functions (Figure 5). TGases act as catalyzer of a covalent bond formation between the γ-carboxamide groups of peptide-bound glutamine residues and various primary amines, including the ε-amino group of lysine in certain proteins (Greenberg et al., 1991).

Three functionally characterized families of TGases have been identified: papain-like TGases (factor fXIIIA, human TG2 and TG3); protein disulphide isomerase like TGases and bacterial TGases (E.coli cytotoxic factor and B. bronchoseptica neurotoxin) with predominantly deamidating activity. All transglutaminase enzymes are encoded by a family of closely related genes which have a high degree of sequence similarity and similar gene organization with remarkable conservation of intron distribution (Grenard et al., 2001). The human TG2 gene has been localized to chromosome 20q12 (Gentile et al., 1994) and has a full length of 687 aminoacids with predicted molecular mass of approximately 80kDa. It is a highly conserved gene with 90% similarity to bovine, mouse and guinea pig TG2 genes.

In mammalian tissues, TG2 is the most ubiquitously distributed enzyme of all TGase family belonging enzymes so far described (Thomazy and Fesus, 1989). It is also the only one among them, which, in addition to protein-protein crosslinking, incorporation of amines and glutamine deamidation has an additional, G-protein signaling activity (Murthy et al., 2002; Mhaouty-Kodja, 2004). TG2 is expressed extracellularly – on the cell surface associated with cell matrix (Gaudry et al., 1999), intracellularly and in a cytosolic form.

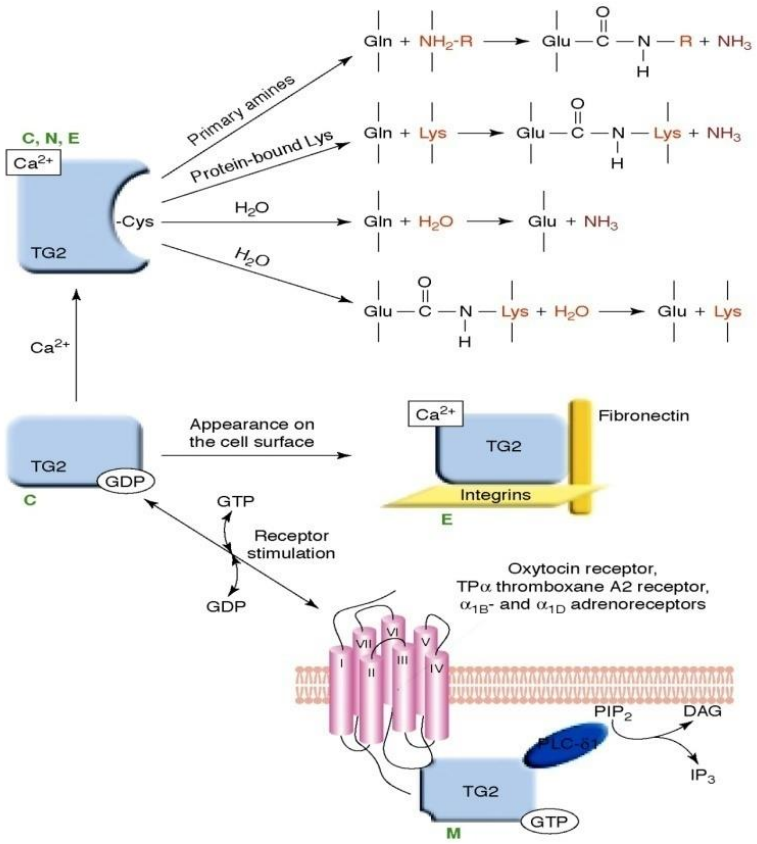

Figure 5. **Catalytic activities of TG2.** TG2 catalyzes Ca2+-dependent acyl-transfer reaction between γ-carboxamide group of a specific protein-bound glutamine and either the ε-amino group of a distinct protein-bound lysine residue or primary amines such as polyamines and histamine. Water can replace amine donor substrates, leading to deamidation of the recognized glutamines. TG2, similar to factor XIIIa, has Ca^{2+}-dependent isopeptidase activity and, at least under test tube conditions, can hydrolyse γε isopeptides. TG2 can be exposed on the external leaflet of the plasma membrane. The presence of TG2 outside the cell has been proposed to depend on its interaction with fibronectin and integrins. TG2 binds and thereby activates phospholipase C following stimulation of several kinds of cell surface receptors; its endogenous GTPase activity ensures proper regulation of transmembrane signalling through these receptors. Functions of TG2 are performed in the cytosol (C), the nucleus (N), at the cell membrane (M) and in the extracellular space (E). Except for its isopeptidase activity, all other functions have been shown to occur in intact cells and/or tissues. (Fesus and Piacentini, 2002).

TG2 enzyme's characteristics - wide tissue distribution, together with high ontological conservation and the fact that it's multiple activities are of major importance for the cell function homeostasis makes it a very intriguing potential player in a variety of cellular processes as well as a possible target enzyme in treatment of number of pathological conditions.

Molecular structure, activity and TG2 involvement in cellular processes

Molecular structure of TG2 is similar to the other TGs. The enzyme consists of four distinct domains (Figure 6): N-terminal β-sandwich, bearing fibronectin and integrin binding sites; a catalytic core, bearing the catalytic triad for the acyl-transfer reaction and a putative BH3 domain; and finally, the two C-terminal β-barrel domains. A unique guanidine nucleotide-binding site, which has not been found in any TG proteins, is located in a cleft between the catalytic core and the first β-barrel. The binding of GTP, but not that of Ca^{2+}, proves to be important for the stability of the enzyme's conformation, suggesting the molecular mechanism by which GTP inhibits TG2 activity. Accordingly, the GTP-TG2 adopt a conformation which decreases the accessibility of the protein matrix to the solvent, thus rendering the accessibility of the active site more difficult (Di Venere et al., 2000).

The 3D organization of the Cys277, His335 and Asp358 in the catalytic triad is similar to that of thiol proteinases, such as papain (Pedersen et al., 1994; Yee et al., 1994), and confers high reactivity to Cys277, which might form thioesters with peptidyl-glutamine moieties of the protein substrates. The high reactivity of Cys277 has been employed to develop a wide range of active-site-directed irreversible inhibitors of the enzyme. In the absence of Ca^{2+}, TG2 assumes the basic latent conformation and the reactivity of Cys277 is decreased either by hydrogen-bonding with the phenolic hydroxy group of Tyr516 or by formation of a disulphide with a neighbouring cysteine residue, namely Cys336 (Noguchi et al., 2001). Further, the pro-apoptotic and pro-survival activities of TG2 are confined in discrete domains along the primary sequence of the protein, suggesting a modular and independent phylogenetic evolution.

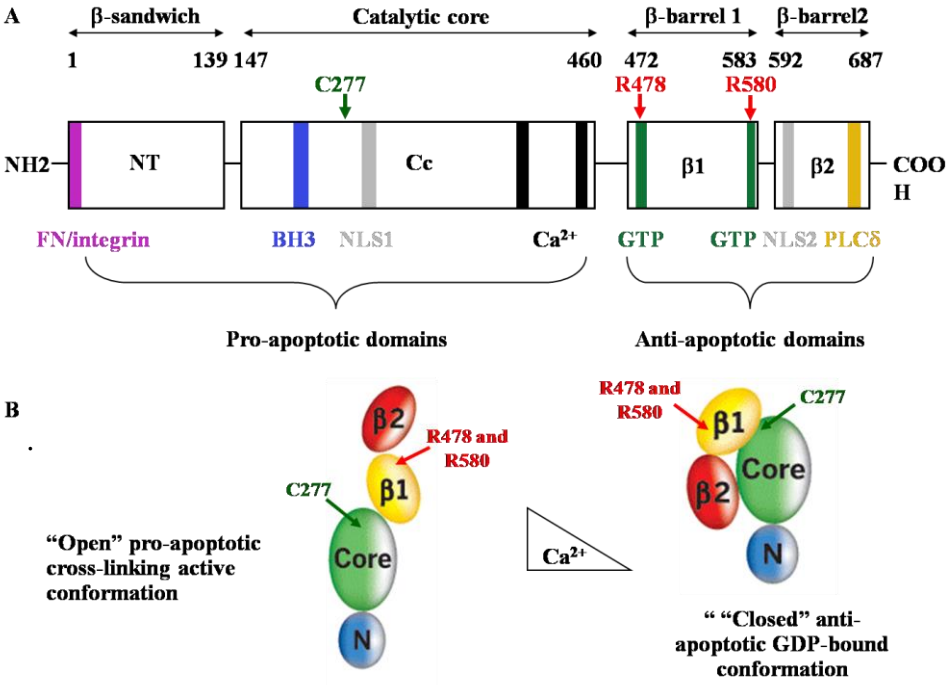

Figure 6. **TG2 structure and domains.** Panel A. Graphic representation of the four domains of TG2: FN/Integrin binding motif; BH3 domain; C277, active site's cysteine; NLS1 and 2, Nuclear Localisation Signals; Ca2+ binding motif; GTP binding motif and relative binding impairing mutations (R478/580); PLC binding motif. Panel B. Ca^{2+} concentration may control the shift between the "open" pro-apoptotic (high [Ca^{2+}]) and the "closed" anti-apoptotic (low [Ca^{2+}]) conformations of TG2.

When expressed at membrane locations TG2 interacts with other macromolecules such as phospholipase Cδ (Hwang et al., 1995). This interaction depends on the C-terminal domain of TG2 and is crucial to the role of the enzyme as a GTP-binding effector protein in the transduction of extracellular α_1-adrenergic signals, coupled with phosphatidylinositide metabolism. Further characterization of this signalling complex has revealed the association of TG2 with a 50 kDa protein – Ca^{2+}-binding protein calreticulin, which, when exposed on the cell surface serves as a determinant immunogenic cell death (Obeid et al., 2007).

TG2 also displays a strong binding affinity for heparin and heparan sulphates, but the functional role of this effect and identification of heparin-binding regions has not yet been fully explored, although this might be relevant for interaction of

TGases with extracellular-matrix protein components and membrane proteins such as integrins (Zemskov et al., 2006).

The interaction of TG2 with fibronectin involves the N-terminal region (Gaudry et al., 1999) and is believed to play a role in localizing TGase in regions of tissue damage (Nardacci et al., 2003). This is particularly important for transformed cells and might be relevant for the involvement of TG2 in the stabilization of the extracellular matrix, since loss of TG2 by proteolytic cleavage would facilitate increased cell migration and invasion in the metastatic process (Chhabra et al., 2009; Kotsakis and Griffin, 2007; Mangala and Mehta, 2005).

Interestingly, TG2 can also get translocated into the nucleus under certain conditions (Lesort et al., 2000) where it can function either as a G protein(Singh et al., 1995) or as a transamidase activated by nuclear Ca^{2+}-signals to crosslink histones (Ballestar et al., 2001) and retinoblastoma (Rb) (Oliverio et al., 1997) proteins. This suggests that TG2 could have a direct role in chromatin modifications and/or gene expression regulation.

When activated by Ca^{2+} TG2 interacts and modifies major components of the cytoskeleton such as β-tubulin and microtubule-binding proteins (Piredda et al., 1997) including tau, which can be crosslinked by the enzyme (Murthy et al., 1998). In this way, TG2 may contribute to the formation of neurofibrillary tangles in Alzheimer's disease where it has already been demonstrated that its activity is elevated (Wilhelmus et al., 2009).

TG2 has been widely related to programmed cell death as it is selectively expressed during apoptosis (Piacentini et al., 2005). First, its overexpression prepares cells for dying by sensitizing them for apoptosis. TG2 interacts with mitochondria and shifts them into higher polarized state and altered redox status. During the late phase of apoptosis the massive increase of cytosolic Ca^{2+} determines the switch of TG2 to its cross-linking configuration in all cellular compartments leading to extensive polymerization of intracellular proteins (including cytoskeleton proteins, which can lead to formation of detergent-insoluble structures. These protein scaffolds stabilize the structure of the dying cell before its clearance by phagocytosis, limiting the release of harmful intracellular components and consequently inflammatory or autoimmune responses (Piredda et al., 1997).

TG2 involvement in pathological conditions

This topic attracts a lot of scientific interest given that some of the broad spectrum of different activities of TG2 could be potentially targeted in treatment of

chronic diseases, in particular inflammatory diseases (wound healing, tissue repair and fibrosis), autoimmune conditions, chronic degenerative diseases (arthritis, atherosclerosis and neurodegenerative pathologies) as well as tumors.

Wound healing requires the involvement of several distinct TGases, which co-operate with each other to reconstitute tissue integrity damaged by traumatic or other pathological injuries. TG2 is probably involved in the angiogenic phase of wound repair as well as in its interaction with and stabilization of the extracellular matrix, possibly through its role as an independent cell-adhesion protein or as an integrin co-receptor. TG2 is also involved in tissue fibrosis and scarring which has been demonstrated in studies on severe chronic inflammations found in liver cirrhosis and fibrosis (Elli et al., 2009), alcoholic hepatopathy (Tatsukawa and Kojima, 2010) and type C hepatitis (Grenard et al., 2001; Mirza et al., 1997b). Also, TG2 involvement has been confirmed in renal and lung fibrosis (Liu et al., 2005; Richards et al., 1991). In addition, its role in the pathogenesis of the chronic inflammatory diseases of the joints, including rheumatoid arthritis and osteoarthritis, has been reported (Johnson et al., 2001). It is hypothesized that TG2 is involved in the activation of pro-inflammatory cytokines such as TGFβ (Rosenthal et al., 2000). Activated cytokines such as TGFβ can stimulate pyrophosphate release in diseased joints, leading to mineralization and progression of diseases such as arthritis. Also, TGFβ together with IL-6 and TNFα can in turn lead to further induction and expression of TG2, leading to an effective, but vicious, autocrine loop.

Several autoimmune diseases are characterized by the production of autoantibodies reactive against TG2. Data in this perspective have been collected mostly for coeliac disease (Arentz-Hansen et al., 2000), Type 1 (insulin-dependent) diabetes (Lobner et al., 1999), thyroid diseases, and, more recently, systemic lupus erythematosus and Sjörgen syndrome (Villalta et al., 2002). In all these instances evaluation of immunoreactivity against TG2 can be a valuable help for diagnostic purposes and in predictions of the progression of the disease.

Quite different are the mechanisms whereby TG2 is involved in the pathogenesis of several chronic neurodegenerative diseases, which are characterized by the accumulation of highly cross-linked insoluble protein materials. These include senile dementia of the Alzheimer’s disease (AD) and the polyglutamine (polyQ) tail diseases, such as Huntington's disease (HD), rubropallidal atrophy and spinocerebellar palsy. In AD brain, the elevated TG2 activity is manifested by polymerization of a number of proteins, including β-amyloid precursor protein (Rasmussen et al., 1994) and the microtubule-associated tau protein and formation of neurofibrillary tangles (as previously mentioned). In contrast, the polyQ diseases are primarily characterized by the synthesis of proteins

with abnormal tail extensions that represent the sites of TGase-mediated protein cross-linking (Cooper et al., 2002). Recent studies have demonstrated that administration of the TG2 inhibitor cystamine to transgenic mice expressing exon 1 of huntingtin containing an expanded polyglutamine repeat was found to alter the course of the disease in a favorable way, providing further evidence for the involvement of TG2 in this disease (Karpuj et al., 2002).

An additional field of active research on the importance of TG2 in human pathology is that of neoplastic diseases. It has been shown that tumor cells, when observed *in vitro*, generally have a lower TG2 content than normal ones. The decline of TGase activity in tumours is potentially a bad prognostic biomarker (Birckbichler et al., 2000) and is possibly related to tumor metastatic potential, dictating the ability of the cells to cross basal membranes and to invade the bloodstream (Knight et al., 1990).

The important role played by TG2 in different pathologies was confirmed in knockout mice carrying homozygous deletion of TG2 gene. Under normal physiological conditions these mice are born with Mendelian frequency, viable, and are of normal size and weight. Moreover, no significant alterations were observed in developmental apoptosis, the structure of extracellular matrix nor in the heart function (Nanda et al., 2001) where G-protein activity is thought to be important. The possible explanation for this lack of major changes in phenotype could be the compensation of the TG2 functions by other types of TGases.

However, several particular changes of phenotype in $TG2^{-/-}$ mice have been reported, especially under certain stressful and pathological conditions. For example, normal thymocyte apoptotic turnover of immature T-cells is not affected by the lack of TG2, but in dexametasone-induced cell death $TG2^{-/-}$ thymocytes have increased susceptibility. Also, primary fibroblasts of $TG2^{-/-}$ mice show decreased adherence when plated in normal or fibronectin-coated plates (Nanda et al. 2001). Further, $TG2^{-/-}$ mice have impaired glucose-stimulated insulin secretion and a reduced glucose tolerance with a phenotype resembling that of MODY (maturity onset diabetes of young) patients (Bernassola et al., 2002). In the cases of experimentally induced Parkinson disease (by MPTP which inhibits mitochondrial complex I in nigrostriatal dopaminergic neurons) $TG2^{-/-}$ mice have lower extent of nigrostriatal damage and higher overall survival (Battaglia et al., 2007). In another neurodegenerative condition, Huntington disease, $TG2^{-/-}$ mice show differences in the phenotype compared to wild type animals. In the animal model transgenic for huntingtin carrying 116 repeats which exibit the HD after 16 weeks upon birth, TG2 ablation leads to an increased number of misfolded ubiquitinated protein aggregates. The $TG2^{-/-}$ HD animals also show better survival and improved motor performances (Mastroberardino et al., 2002).

Apoptosis clearance is another process that gets deregulated when TG2 is ablated. This phenomenon has been shown in the thymus as well as liver of $TG2^{-/-}$ mice (Szondy et al., 2003) and it is accompanied by inflammatory reactions. This has as a consequence increased incidence of developing autoantibodies, splenomegaly and glomerulonephritis in these animals.

TG2 implication in a variety of cellular processes underlying many pathological conditions sets the need for understanding molecular mechanisms through which TG2 performs its actions especially since numerous TG2 inhibitors are being developed that can be very useful for the specifically designed therapies in these conditions.

TG2 and sepsis

TG2 is expressed at low levels in many different tissues but is inappropriately activated in a variety of pathological conditions, including neurodegenerative diseases, atherosclerosis, inflammatory diseases, autoimmune diseases, and fibrosis (Kim et al., 2002). In many inflammatory diseases, including celiac disease, Crohn's disease, and sporadic inclusion-body myositis, increased TGase activity is closely associated with inflammation (Bruce et al., 1985; D'Argenio et al., 1995). To date many reports show that expression of TG2 is increased in inflammatory diseases and big efforts were made during the last several decades to unveil the physiological roles of TG2 in the inflammatory process in various cell types, however, the mechanism of its action is not yet clear.

It is known that increased TG2 activity triggers NF-kB activation without I-kBα kinase signaling. TG2 induces the polymerization of I-kBα rather than stimulating I-kBα kinase. This polymerization of I-kBα results in the direct activation of NF-kB in various cell lines. TG inhibition reverses NF-kB activation. Interestingly, a central event in the induction of cytokines and inflammatory mediators is the activation of NF-kB (Liu and Malik, 2006). NF-kB activity is markedly increased in every organ, both in animal and in human sepsis models which is associated with a higher rate of mortality and worse clinical outcome. Taking into account that TG2 expression can be induced directly by NF-kB activation (TG2 promoter contains a NFkB-binding motif as shown by Mirza et al. 1997) the absence of TG2 could be an advantage during endotoxic shock, because this deficiency appears associated with an activation of NF-kB that is transient, thus allowing the restoration of the immunological equilibrium.

It has been was demonstrated that the ablation of TG2 confers resistance to LPS-induced septic shock in mice (Falasca et al., 2008). TG2 knockout mice

display enhanced survival (90%) to LPS challenge. The absence of TG2 is associated with a profound reduction of the inflammatory response and attenuated organ damage. Production of proinflammatory mediators is elicited in $TG2^{-/-}$ mice by LPS treatment, indicating that the capacity to respond to endotoxic stimulus is still present in these animals, but as opposed to the WT, $TG2^{-/-}$ mice have the capacity to restore the initial equilibrium. IL-6 and IFNγ were released at significantly high amounts in the early phase after LPS injection both in WT and $TG2^{-/-}$ mice. These molecules are responsible for the development of fever and cachexia (Leon et al., 1998), the symptoms of endotoxic shock observed initially also in TG2 animals. However, it seems that the levels of IL-6 and IFNγ appear to discriminate between survivors and non-survivors: WT mice have significantly higher, and long lasting, proinflammatory cytokine levels compared to $TG2^{-/-}$ (Falasca et al., 2008).

Also, in $TG2^{-/-}$ mice under unstimulated conditions there is a different pattern of distribution of the various NF-kB subunits, p65/p50/p52 on the target gene promoter. In particular, in nuclear extracts of $TG2^{-/-}$ macrophages, the p65 subunit appeared constitutively. An increase of p65 subunit in the nucleus, and the formation of polymerized I-kBα in the cytoplasm, are induced by LPS in macrophages from WT animals but no changes are present in the absence of TG2 (Falasca et al., 2008). Taking into account that TG2 expression can be induced directly by NF-kB activation, because the TG2 promoter contains a NF-kB-binding motif (Mirza et al., 1997a) the absence of TG2 could be an advantage during endotoxic shock, because this deficiency appears associated with an activation of NF-kB that is transient, thus allowing the restoration of the immunological equilibrium, whereas normally the increased expression of TG2 induced by LPS causes a continuous activation cycle in the inflammation process.

It is also relevant to mention that LPS stimulation of different types of cells (eg macrophages) increases TG2 expression (Akimov and Belkin, 2001). Considering that up to date most therapies against sepsis are still not effective and the identification of novel therapeutic strategies is needed, as well as the fact that in the last years numerous TG2 inhibitors are being developed for purpose of treating numerous pathological conditions in which TG2 is involved, TG2 inhibition could be taken into account for development of future therapies also for sepsis.

Aim

Sepsis is defined as a systemic inflammatory response and it is associated with immune suppression. Scientific investigations have demonstrated the important role played by DCs in regulation of sepsis, since their depletion is pronounced in various lymphoid and non-lymphoid tissues, in patients as well as in septic mice (Scumpia et al., 2005; Hiramatsu et al., 1997). Moreover, DC depletion is associated with an increase in mortality. Recently, results obtained by our group demonstrated that TG2, a multifunctional enzyme involved in many important biological processes, is a very important factor in the induction of sepsis (Falasca et al., 2008). TG2 expression has been reported to be induced by LPS in several tissues and organs and it has been shown that the ablation of TG2 confers resistance to LPS induced septic shock in mice. $TG2^{-/-}$ mice have the capacity to restore the initial equilibrium of circulating cytokines and pro-inflammatory mediators.

Aim of this thesis was to evaluate whether TG2 expressed by DC exert a role on their function during septic shock. Furthermore, the mechanisms by which TG2 regulates the response of DCs to septic shock were analyzed.

To this purpose two mammalian model systems were used: human monocyte-derived DC in which the cross/linking activity of TG2 was blocked using a specific inhibitor and a murine model where TG2 gene was ablated ($TG2^{-/-}$).

Materials and methods

Mice

Male mice 6-8 weeks old were used in all experiments. Animals were maintained in the pathogen-free animal facility of the University of Rome "Tor Vergata" under the guidelines and ethically approved protocols. Wild-type (WT) C57BL/6 mice and knockout $TG2^{-/-}$ mice on pure C57BL/6 background were obtained from the laboratory of Gennaro Melino.

mAbs and reagents

The following monoclonal antibodies (mAbs) were purchased from BD Biosciences (USA) for flow cytometry experiments: FITC-conjugated anti-mouse CD86 mAb, PE-conjugated anti-mouse TLR4/MD-2 mAb, PE-conjugated anti-mouse MHC class II mAb (I-Ab), APC-conjugated anti-mouse CD11c mAb and rat anti-mouse FcR mAb (2.4G2), ApoAlert Annexin V-FITC Apoptosis Kit; FITC-conjugated anti-human CD1a mAb, FITC-conjugated anti-human CD80 mAb, PE-conjugated anti-human CD11c mAb, PE-conjugated anti-human CD86 mAb, PerCP-conjugated anti-human MHC class II mAb (HLA-DR), APC-conjugated anti-human MHC class I mAb (HLA-A,B,C), APC-conjugated anti-human CD14 mAb and purified anti-human CCR7 mAb.
Human recombinant IL-4 and GM-CSF were purchased from Peprotech (USA). LPS was purchased from Sigma Aldrich (USA).
Mouse anti-human monoclonal antibody TG2 (Clones CUB 7402 + TG100) was purchased from NeoMarkers (CA, USA); mouse anti-human GAPDH mAb (6C5) was purchased from Calbiochem (Darmstadt, Germany). CD14 microbeads for human monocytes isolation were purchased from Miltenyi Biotec (Germany).

DC differentiation and cultures

Human PBMCs were isolated from healthy adult donors by density gradient centrifugation using Lympholite-H (Cederlane, Canada). Monocytes were positively separated by anti-CD14 magnetic beads (MACS, Miltenyi Biotec, Germany) according to the manufacturer's specifications. Monocyte derived DCs

(MoDCs) were obtained by culturing monocytes at 37°C in 5% CO_2 at a density of $1x10^6$ cells/ml in the RPMI1640 medium with 10% FCS, 10mM Hepes, 2mM L-Glutamine, 2mM penicillin and 50µg/ml streptomycin, 50ng/ml of human recombinant GM-CSF and IL-4 for 5 days. MoDCs were matured for 24h by the addition of LPS (200ng/ml). TG2 cross-linking activity was inhibited by addition of different concentrations (10, 50 and 100µM) of KCC009 inhibitor (Choi et al., 2005) on day 0 of culture.
Murine bone marrow cells were extracted from femurs and tibias as previously described (Lutz et al., 1999). Cells were cultured at 37^0C in 5% CO_2 at a density of $2x10^5$ cells/ml in the RPMI1640 medium with 10% FCS, 2mM L-Glutamine, 2mM penicillin and 50µg/ml streptomycin. Mouse recombinant GM-CSF and IL-4 (40ng/ml) were added on days 0, 4 and 8. On day 10 LPS (200ng/ml) was added for 24h to induce maturation of DC.

In vivo LPS treatment of murine DCs

WT and $TG2^{-/-}$ mice were injected intraperitonealy with 40mg/kg LPS solubilized in sterile PBS. Control animals were injected with the same volume of PBS. 18-20h after, the animals were sacrificed and total spleenocytes were isolated. DC phenotype was analyzed by flow cytometry on gated CD11c positive cells.

Flow Cytometry

Surface immunophenotyping of mouse cells was performed by pre-incubation with rat anti-mouse FcR mAb 2.4G2 for 15 minutes, then washed and stained with fluorochrome-conjugated mAbs for 15 minutes at 4°C. After washing, cells were fixed in 1% paraformaldehyde (PFA) and acquired using a FACScalibur cytometer (Becton Dickinson Italia S.p.A.).
Human cells were stained with fluorochrome-conjugated mAbs for 15 minutes at 4°C, then washed and fixed in 1% PFA. Acquisition was performed using a FACScalibur cytometer. Multiparameter data acquisition and analysis were performed with CellQuest software (BD Immunocytometry Systems).

Naive T cell polarization assay

The ability of MoDC to stimulate and polarize naïve T cells was evaluated. Allogeneic naïve $CD4^+$ T cells were isolated from PBMCs using CD4 naïve T cell isolation kit (Miltenui Biotec, Germany) according to the manufacturer's specifications. After extensive washing MoDCs (2×10^5) were cultured with allogeneic T cells (8×10^5) in 24-well plates. After 9 days culture supernatants were collected and stored at -80°C.

Cytokine assays

Supernatants of MoDCs (1×10^6/ml) derived in different conditions, as well as the supernatants from MoDC-T cell co-cultures, were collected and stored at -80°C. IL-10 (limit of sensitivity < 5pg), IL-12 (p70 subunit) (limit of sensitivity < 5pg) and IFNγ (limit of sensitivity < 5pg) levels were determined by ELISA kits (Pierce Endogen, USA) according to the manufacturer's specifications. Results are expressed as pg/ml and reported as means.
Supernatants of murine bone marrow derived DC (BMDC) and splenocytes treated overnight with LPS (200ng/ml) were collected and stored at -80°C. IL-10 (limit of sensitivity < 30pg), IL-12 (p70 subunit) (limit of sensitivity < 15pg) and IFNγ (limit of sensitivity < 0.7pg) levels were determined by ELISA kits (BD Biosciences, USA) according to the manufacturer's specifications. Results are expressed as pg/ml and reported as means.

Western blotting

MoDCs were pelleted and lysed using ice-cold Cell lytic buffer (SIGMA). Protein extracts were heated for 10 minutes at 97°C in Laemmli buffer, and loaded on 10% SDS polyacrylamide gel. The gel was run in Tris-Glycine buffer at 100V and transferred to nitrocellulose membrane (Whatman, Germany). The membranes were incubated with 5% milk in PBS-Tween to prevent nonspecific binding of the mAbs. The membranes were then incubated with anti-TG2 and anti-GAPDH primary Abs overnight at 4°C. After several washes with PBS-Tween membranes were incubated with the appropriate secondary Abs for 1h. Following a final wash step, protein signal was detected by chemiluminescence ECL plus system (Amersham and HealthCare).

Statistical analysis

Statistical analysis was conducted using a non parametric, unpaired test. $p<0.05$ values were considered statistically significant. GraphPad Prism version 5.00 for Windows, (GraphPad Software, San Diego California USA) was used to perform the analysis.

Results

TG2 expression during DC differentiation

It has been reported that the expression of TG2 increases drastically during monocyte differentiation into macrophages (Akimov and Belkin, 2001). To determine whether TG2 is included in the differentiation of DCs, we first analyzed its protein levels during human monocyte differentiation into MoDC. To this aim blood-derived human monocytes were grown in the presence of recombinant GM-CSF and IL-4 for 5 days, followed by another day in the presence of LPS; time course analysis of TG2 protein levels was carried out by Western blot analysis. The data reported in Figure 1 show that monocytes express very low amount of TG2, but the enzyme levels keep increasing significantly during MoDC differentiation. Interestingly, we observed that mature DCs express higher TG2 levels compared to the immature ones, thus suggesting a possible role of TG2 in MoDC differentiation process and functions.

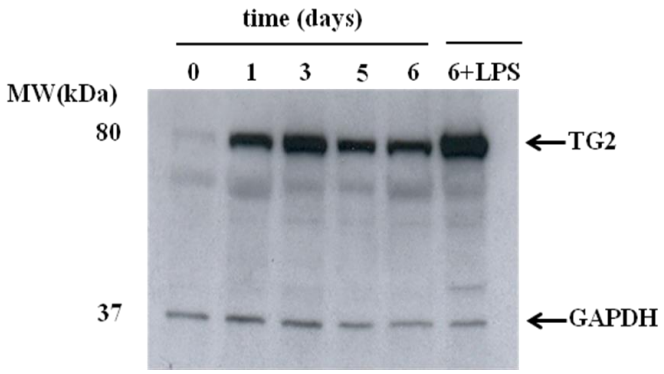

Figure 1**. Western blot analysis of the TG2 expression in differentiating MoDCs.** Human DCs were generated from monocytes for 5 days in culture with GM-CSF, and IL-4 and LPS treatment was performed on day 5 for 24h. Cells were collected on day 0 (monocytes), 1, 3, 5 and 6 (control and LPS treated), lysed and total protein extract was analyzed by Western blot for TG2 expression. Anti-GAPDH mAb was used as control of total amount of collected protein

Effect of TG2 inhibition on human MoDC differentiation

After observing the increase of TG2 expression during human MoDC differentiation, our next step was to examine the effect of the inhibition of TG2 in differentiated immature MoDC. To this aim we used a specific TG2 cross-linking activity inhibitor, KCC009.

First, MoDCs were differentiated in the medium with GM-CSF and IL-4. The inhibitor KCC009 (100µM) was added at day 0. After 5 days of culture immature MoDC were collected and analyzed for the phenotype. We observed no significant changes in the phenotype of control and KCC009 treated cells (panel A of Figure 2). In fact, TG2-inhibited MoDCs displayed similar levels of HLA class I, class II, and costimulatory molecules compared to untreated controls. To further substantiate these observations we treated already differentiated immature MoDC with the TG2 inhibitor for 24h and analyzed their phenotype. Again, we did not observe significant differences in the phenotype of control and KCC009 treated cells (panel B of Figure 2) suggesting that, at least cross-linking activity of TG2 is not involved in DC differentiation.

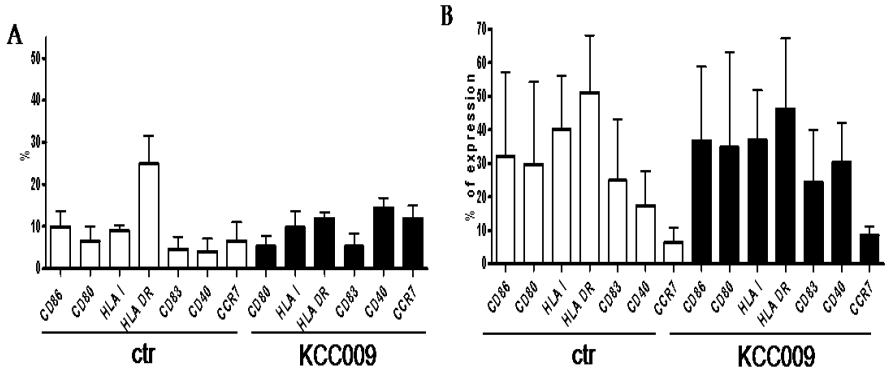

Figure 2. **Phenotypic analysis of immature differentiated MoDC treated with the KCC009 inhibitor.** Human MoDC were cultured from monocyte for 5 days in medium with GM-CSF and IL-4 after this period cells were collected and their phenotype was analyzed. Panel A shows the phenotype of immature MoDC treated with the KCC009 inhibitor (100µM) on day 0 of culture. Panel B shows the phenotype of immature MoDC treated with KCC009 for 24h after their differentiation.

To test whether TG2 has toxic effects DC viability test was performed by Tripan blue exclusion. The number of live cells was high (>99%) both in the control cells as well as in presence of KCC009, indicating that KCC009 had no effect on DC viability.

Effect of TG2 inhibition on human MoDC maturation and function

In order to determine the role of TG2 in the response of MoDC to LPS we differentiated MoDCs in the presence of increasing concentration of KCC009 for 5 days, followed by another day in the presence of the maturation stimulus, LPS. Then we tested whether the inhibition of TG2 plays a role in MoDC maturation by performing phenotype analysis of mature MoDC treated with TG2 inhibitor. We observed that after LPS treatment, TG2 inhibited MoDCs present a dose dependent down-modulation of CD80, CD86, HLAI and CCR7 (Figure 3A and B) suggesting that TG2 cross-linking activity could be involved in MoDC maturation process.

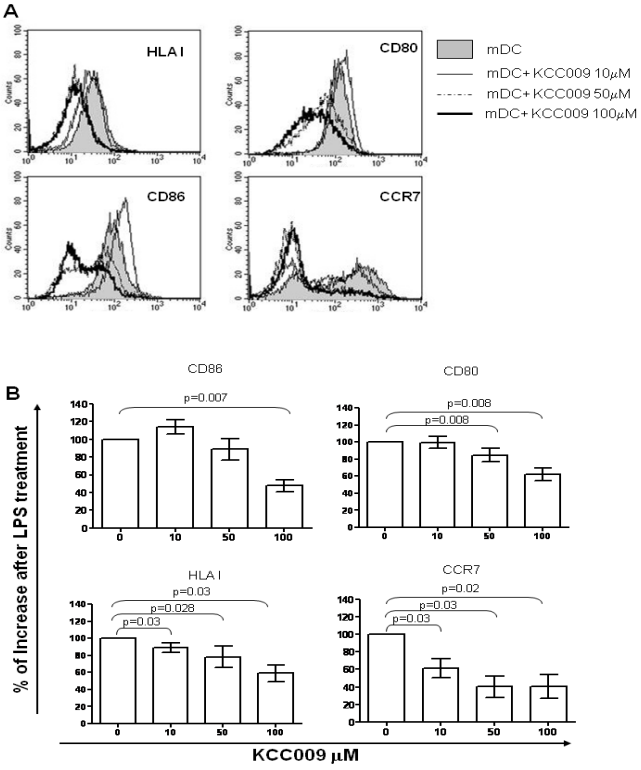

Figure 3. **Phenotypic analysis of MoDCs treated with TG2 inhibitor KCC009**. Human DCs were generated from monocytes after 5 days of culture with GM-CSF and IL-4 and in the presence of different concentrations of KCC009. Then cells were treated with LPS for 24h and DC phenotype was analyzed. Panel A shows the expression of the indicated molecules (as histograms) of a representative experiment. In panel B the effect of KCC009 on DC maturation obtained from all the experiments is represented. The results are shown as percentage of increase of molecule expression in the LPS treated DCs, upon treatment with different concentrations of KCC009.

Upon proper stimulation DC are able to secrete IL-10 and IL-12, which play a central role in the regulation of the immune response. We therefore evaluated whether the TG2 inhibition by KCC009 was able to prevent the release of IL-10 and IL-12 by MoDC after 24 hours upon LPS treatment. Indeed, the pre-treatment of MoDC with the TG2 inhibitor strongly impaired the secretion of both IL-10 (Fig.4A) and IL-12 (Fig.4B), indicating a regulatory role played by the enzyme on DC functions.

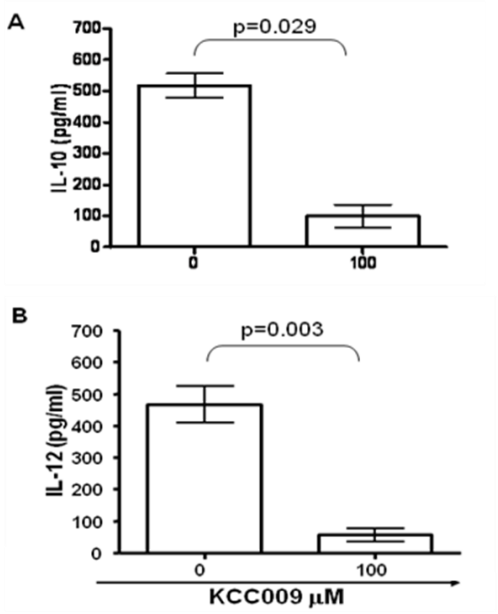

Figure 4. **IL-10 and IL-12p70 production by MoDCs.** Human DCs were generated from monocytes after 5 days of culture with GM-CSF and IL-4 and in the presence of 100 µM TG2 inhibitor KCC009. Cells were treated with LPS (200ng/ml) for further 24h, then supernatants were collected and samples stored at –80°C. The levels of IL-10 (panel A) and IL-12 (panel B) were tested by ELISA. Results are expressed as mean values (+ SEM) of 6 independent experiments.

KCC009 treated DCs are not able to polarize naïve CD4$^+$ T cells

MoDCs play a major role in directing the immune response, having a unique capacity to stimulate naïve T lymphocytes, driving them into distinct classes of effector cells (Th1 and Th2 and Treg). Considering that TG2 inhibition reduces significantly the production of regulatory cytokines by MoDCs, we wondered whether the treatment with KCC009 alters their capacity to polarize the immune response. To address this issue we examined the nature of primary allogeneic CD4 naïve T cell response induced by KCC009 treated MoDC, after 9 days of co-culture. T cell derived IFNγ was measured by ELISA. As expected, T cells co-cultured with allogeneic mature MoDCs produced high amount of IFNγ. In

contrast, T cells co-cultured with allogeneic MoDCs differentiated in presence of KCC009 showed a significant decrease in the IFNγ release (Figure 5), indicating that the TG2 inhibition also impaired the MoDC capacity to polarize Th1 immune response. However, when IL-4 secretion by T cells in response to control and KCC009 treated DCs was tested, we did not find detectable levels of IL-4, suggesting a complete impairment of DC-induced T cell polarization.

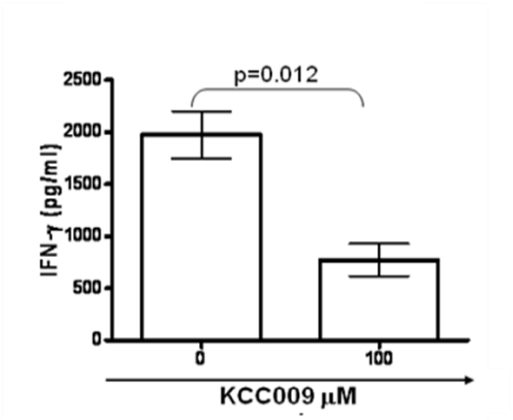

Figure 5. **IFN-γ accumulation in the supernatants of naïve CD4 T cells cultured with allogeneic MoDCs**. Naïve CD4 T cells ($1x10^6$) were cultured with heterologous MoDCs differentiated in the presence of KCC009 (100μM) and the supernatants were collected after 9 days and stored at –80°C. The levels of IFNγ profuced by T cells were tested by ELISA. Data are expressed as mean values (+ SEM) of cytokine levels of 4 independent experiments.

Effect of TG2 ablation on mouse DC functions.

The above described results indicate an important role for TG2 in the maturation and functions of human DCs. Furthermore, our previous work demonstrated that TG2 ablation leads to partial resistance to experimental sepsis reflecting a reduction of tissue injury and a better homeostasis of the proinflammatory mediators (Falasca et al., 2008). These findings raised the question whether the TG2-ablated DC population could play a role in this phenomenon.

To this aim we performed the characterization of $TG2^{-/-}$ DCs upon in vivo stimulation with LPS. We injected LPS in the peritoneal cavity of WT and $TG2^{-/-}$ mice, and analyzed splenic DC phenotype after 18h. Our results showed that in the steady state $TG2^{-/-}$ DCs express higher levels of MHC class II molecule on their surface compared to WT DCs (Fig.6A and B). Upon LPS stimulation, DCs from $TG2^{-/-}$ mice have lower ability to up-regulate MHC class II (Fig.6A and B) and the co-stimulatory molecule CD86 compared to WT (Fig.6C and D), suggesting that, as in human, mouse TG2 is involved in the DC response to LPS.

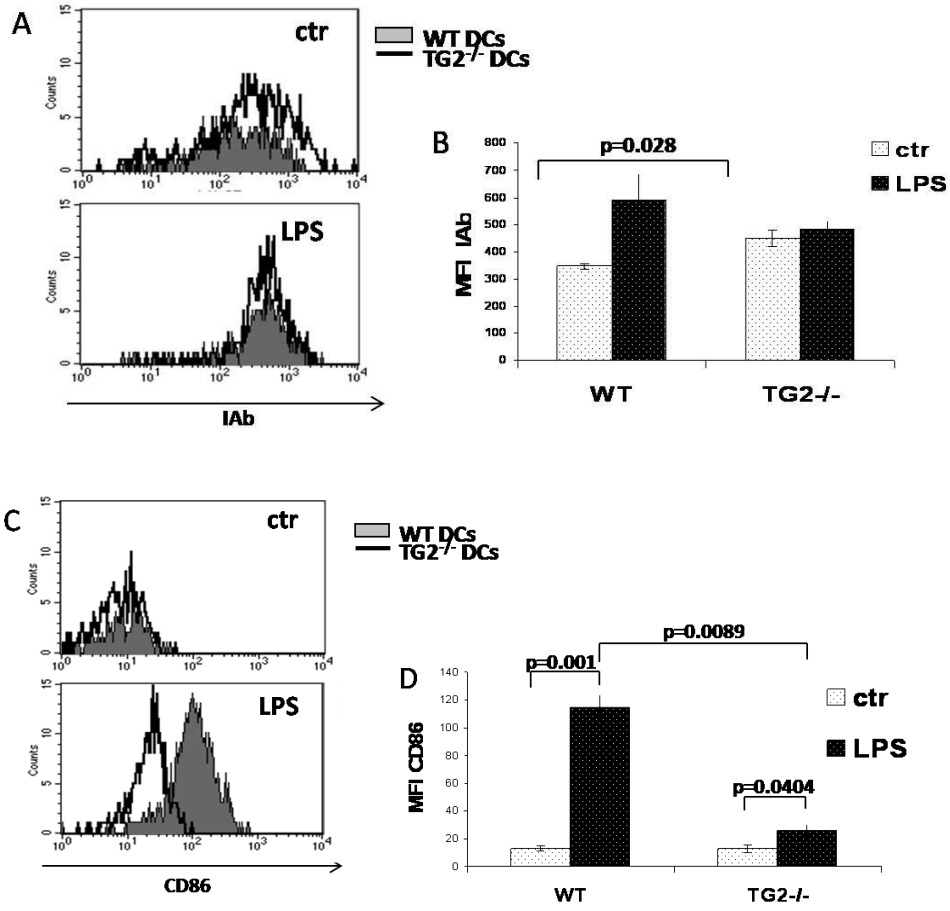

Figure 6**. Expression of MHC class II and CD86 in spleen DC of WT and TG2$^{-/-}$ septic mice.** WT and TG2$^{-/-}$ C57BL/6 mice were injected with LPS (40mg/kg) intraperitonealy. After 18-20 hours, total cell population of spleen was isolated and phenotype analysis of DCs was performed for expression of MHC class II (I-Ab). Panel A shows two representative dot histograms (ctr and LPS) of the expression of I-Ab on gated CD11c positive splenic cells (WT filled histogram; TG2$^{-/-}$ empty histogram). Panel B represents the mean values (expressed as mean fluorescence intensity, MFI) for the I-Ab expression (ctr white columns; LPS black columns) in WT (3 mice) and TG$^{-/-}$ (3 mice). Panel C shows two representative dot histograms (ctr and LPS) of the expression of CD86 from gated CD11c positive splenic cells (WT filled histogram; TG2$^{-/-}$ empty histogram). Panel D represents the mean values (expressed as mean fluorescence intensity, MFI) for the CD86 expression (ctr white columns; LPS black columns) in WT (3 mice) and TG$^{-/-}$ (3 mice).

It was reported in literature that during septic shock a severe depletion of DCs occurs (Hiramatsu et al., 1997; Scumpia et al., 2005). Thus, we wondered whether the ablation of TG2 could restore DC number. To this aim we tested the number of splenic DCs before and after in vivo LPS treatment. We found that when untreated, $TG2^{-/-}$ mice have lower number of CD11c positive cells. However, this difference showed no statistical significance. After LPS treatment the number of splenic DCs (CD11c positive cells) decreased (approximately 35%) in WT mice, as expected (Ding et al. 2004). In contrast, in $TG2^{-/-}$ mice the percentage of splenic DCs did not change (Figure 7), confirming that TG2 plays a role in regulating DC response to LPS.

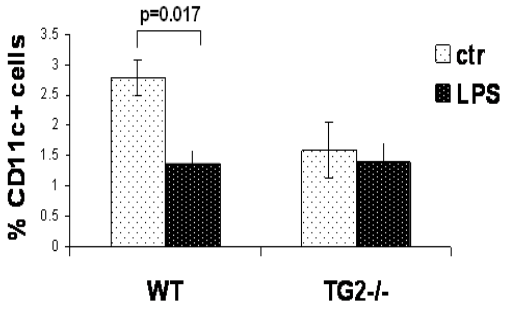

Figure 7. **Effect of TG2 ablation on the number of spleen DC in response to in vivo LPS injection.** WT and $TG2^{-/-}$ C57BL/6 were injected with LPS (40mg/kg) intraperitonealy. After 18-20 hours, total cell population of spleen was isolated and phenotype analysis of DCs was performed for expression of CD11c. The graph represents the percentage of splenic CD11c positive cell population from WT (3 mice) and $TG^{-/-}$ (3 mice) (ctr white columns; LPS black columns).

Since TG2 is involved in the regulation of cell death by apoptosis (Fesus and Piacentini, 2002), we asked whether the depletion of splenic DCs upon LPS treatment is a consequence of cell death or perhaps due to migration of DCs to other sites of inflammation. To this aim we analyzed the apoptosis levels (annexin V expression) in DC population resident in spleen after in vivo LPS treatment. Our results show that the level of apoptosis in splenic DCs from $TG2^{-/-}$ mice is much lower both before and after endotoxin challenge compared to WT (Figure 8A and B). These data suggest that in $TG2^{-/-}$ mice the decrease in DC depletion could be due to a general resistance to apoptosis.

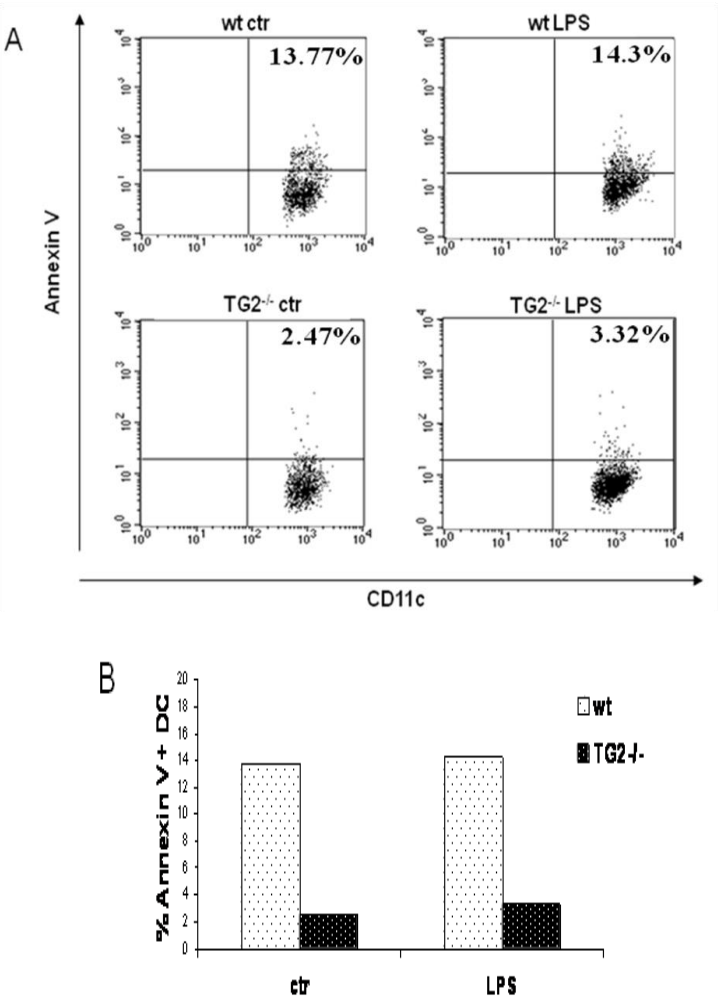

Figure 8. **In vivo effect of LPS on apoptosis on spleen DCs from TG2$^{-/-}$ and WT mice.** Annexin V expression was tested by flow cytometry. In panel A two representative dot plot graphs for the number of cells positive for annexin V are shown. Panel B shows the percentage of annexin V positive splenic DC from WT and TG2$^{-/-}$ ctr (white columns) and LPS injected (black columns) mice from the same representative experiment.

In order to characterize apoptosis dynamics after exposure to LPS we performed an *in vitro* annexin V apoptotic assay on CD11c gated cells isolated from spleens of WT and TG2$^{-/-}$ mice at different time points after LPS treatment. Figure 9 shows

that the level of apoptosis in splenic DCs from TG2$^{-/-}$ mice is lower both before and after endotoxin challenge compared to WT.

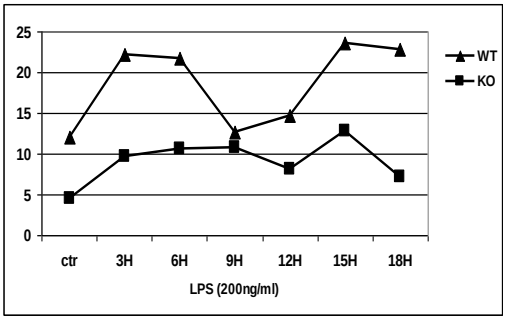

Figure 9. **Effect of TG2 ablation on the apoptosis of splenic DCs in response to in vitro LPS treatment .** Splenic DCs were isolated from WT and TG2$^{-/-}$ C57BL/6 mice and put in culture with LPS (200ng/ml). After 3, 6, 9, 12, 15 and 18 hours, cells were collected and annexinV expression was measured on gated CD11c positive cells.

To understand whether the TG2 ablation had a direct effect on DCs or the microenvironment could have had an influence, we used the in vitro generated DC model. We isolated bone morrow cells from WT and TG2$^{-/-}$ C57BL/6 mice and cultured them in the presence of IL-4 and GM-CSF for 10 days. After this time LPS was added for 18h and cells were collected at different timepoints for annexinV apoptotic assay analysis. Results shown in Figure 10 confirm that DC from TG2-/- mice were more resistant to LPS induced apoptosis.

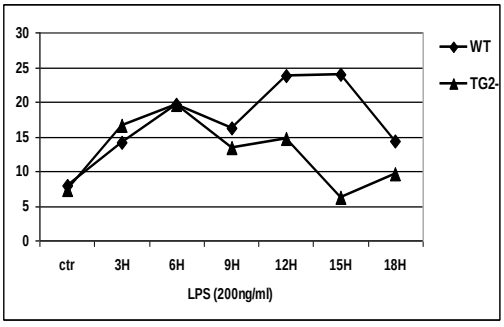

Figure 10. **Effect of TG2 ablation on the apoptosis of in vitro differentiated DC in response to LPS treatment.** Bone marrow cells from WT and TG2$^{-/-}$ C57BL/6 mice were cultured for 10 days in mediun with GM-CSF and IL-4 and on day 10 treated with LPS (200ng/ml). After 3, 6, 9, 12, 15 and 18 hours, cells were collected and annexinV expression was measured on gated CD11c positive

Altogether, these data indicate that TG2 regulates DC response to LPS affecting their phenotype and apoptosis.

Cytokine profiles of mouse TG2$^{-/-}$ dendritic cells

Our findings demonstrate that TG2 plays a role in a response of DCs to endotoxic shock by affecting their phenotype and apoptosis. To better characterize the effect of TG2 ablation in murine DC model system, we asked whether also DC functions were affected. To address this issue we performed ELISA cytokine analysis on in vitro bone marrow derived DC system. Bone marrow precursor cells were isolated from femurs and tibia of wt and TG2$^{-/-}$ mice and put in culture with recombinant mouse GM-CSF and IL-4 for 10 days. After this time bone marrow derived DCs were treated with LPS for 24h and their supernatants were collected and stored at -80^{o}C. Cytokine analysis of the supernatants showed that wt and TG2$^{-/-}$ BMDCs produce similar levels of IL-12 and IFNγ. However, TG2$^{-/-}$ BMDCs produce significantly higher levels of IL-10. When stimulated with LPS both wt and TG2-/- BMDCs respond with several fold increase in the production of IL-12p70 and IFNγ, although not statistically significant differences were detected between the two groups analyzed (Figure 11). The relative increase in IL-10 was much lower in TG2$^{-/-}$, without statistical significance, however.

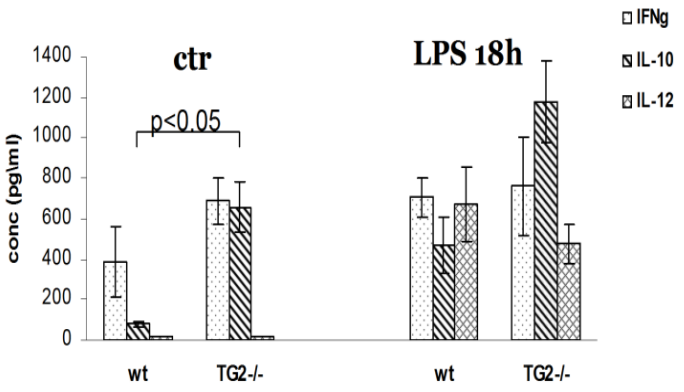

Figure 11. Cytokine production in response to LPS treatment in TG2$^{-/-}$ and WT mice. Bone marrow cells from WT and TG2$^{-/-}$ C57BL/6 mice were cultured for 10 days in medium with GM-CSF and IL-4 and on day 10 treated with LPS (200ng/ml). Supernatants were collected and IL-10, IL-12p70 and IFNγ levels were measured by ELISA test. Results shown are from 3 independent experiments. Panel A shows concentrations of secreted cytokines by splenocytes of WT and TG2-/- control and LPS injected mice. Panel B shows concentrations of secreted cytokines by BMDC of WT and TG2$^{-/-}$ mice treated with LPS overnight.

TLR4 expression after LPS treatment is responsible for the attenuated response in TG2$^{-/-}$ mice

We found that DC from TG2$^{-/-}$ mice respond to LPS treatment in a lesser extent, so we wondered whether the impaired response could be due to a defective expression of one of the main LPS receptors TLR4. Interestingly, WT and TG2$^{-/-}$ express similar levels of TLR4 under physiological conditions; by contrast, upon LPS injection, DC from TG2$^{-/-}$ mice showed a lower number of TLR4 positive cells compared to WT (Figure 12) suggesting an impaired TLR4 signalling.

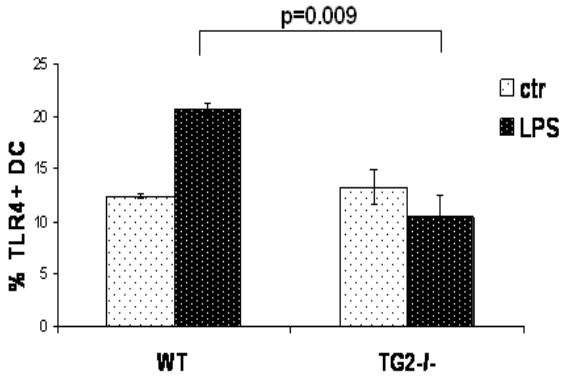

Figure 12. In vivo effect of LPS on TLR4 expression on splenic DCs from TG2$^{-/-}$ and WT mice. TLR4 expression was tested by flow cytometry. The graph shows the mean percentage of TLR4 positive splenic DCs in WT and TG2$^{-/-}$ control mice (grey columns) and LPS injected (black columns), from 3 independent experiments.

Discussion

Dendritic cells are antigen presenting cells with a unique ability to initiate primary immune responses. They are strategically distributed in the peripheral tissues where they screen the microenvironment for the intruding microorganisms and foreign antigens. When activated by the presence of these agents, DCs mature and migrate to peripheral lymphoid organs where they pass the detailed information about the character and location of the antigen to T helper cells which will further orchestrate different effector cells of immune system in order to eliminate the potential danger. From this brief overview of DC function it is already clear how important their role is in the regulation of immune response. Therefore DCs are of great interest as targets in therapeutic strategies that use the immune system as a tool to combat different microbial invaders or pathological conditions. On the other hand, in conditions of excess immune response that can cause the tissue damage it might be useful to manipulate DC properties in order to diminish the extent of immune reactions.

One of the conditions in which the over-pronounced inflammatory response leads to severe damage of multiple tissues and organs causing death in majority of cases is septic shock. The role of DCs in septic shock has been highlighted by the fact that they are profoundly depleted in both septic mice and patients (Hiramatsu et al., 1997; Hotchkiss et al., 2002; Scumpia et al., 2005). However, due to the complexity of interactions of DCs with other cells and microbes, the mechanisms triggering activation, maturation, migration and tolerogenic properties of DCs are not yet clearly understood. Recent data published by our group indicated TG2 as a possible "player" in the molecular and cellular mechanisms leading to improper regulation of inflammatory response involved in sepsis (Falasca et al., 2008). TG2 is known to function in various cell locations and to play important roles in many processes including cell death, cell movement, adhesion and proliferation (Fesus and Piacentini, 2002). The defective expression of TG2 activity is associated with diverse disorders; for example Huntington's, Alzheimer's and Parkinson's disease are related to transamidation, and celiac disease to deamidation.

It has been already demonstrated that TG2 expression increases during monocyte trans-endothelial migration and differentiation into macrophages (Thomas-Ecker et al., 2007). Thus, in the current study we explored the potential role of TG2 in differentiation and function of human DCs, since we found that TG2 increased as the DC differentiation progressed. Moreover, when stimulated with LPS, human DCs further up-regulated the levels of TG2, again suggesting a role in the DC functions. Our results showed that by inhibition of TG2 activity by KCC009, a small-molecule TG2 inhibitor (Choi et al., 2005), monocyte

differentiation to phenotypically immature DCs was unaffected. However, KCC009 did influence the ability of these DCs to mature when stimulated with LPS. This was reflected in the DC phenotype as well as in the cytokine production, indicating that the enzyme might have a role in the DC's function.

It is well known that DCs are able to polarize naive T cells toward IFNγ producing Th1 or IL-4, or IL-10 producing Th2 cells, depending on the DCs subtypes and micro environmental conditions (Diebold, 2008). Our results showed a defect also in this DC function when they were treated with the TG2 inhibitor; in fact, DCs were unable to produce a proper Th1 response in co-culture with allogeneic T lymphocytes. On the basis of these findings, we might suggest that cross-linking activity of TG2 is involved in the final maturation and function of DCs, thus contributing to the development of sepsis pathogenesis. One of the substrates for TG2 cross-linking activity is CD38 protein. Human CD38 is a 45-kDa transmembrane protein that acts as a bifunctional ectoenzyme, catalyzing the synthesis of cyclic ADP-ribose (cADPR) from NAD^+ and the hydrolysis of cADPR to ADP-ribose. CD38 is down-modulated during differentiation into immature DCs and expressed again upon maturation (Fedele et al., 2004). CD38 mediates signalling in mature DCs very likely acting as a receptor and its activation leads to CD83 expression and IL-12 secretion (Fedele et al., 2004). CD38 has been shown to act as a substrate for the TG2-catalyzed cross-linking reaction resulting in the the formation of p190 multimers (Umar et al., 1996). The purified p190 showed more cyclase activity than CD38, conversely, p190 was less active than CD38 in hydrolyzing cADPR to ADPR. These findings indicate that post-translational modification of CD38 may represent a mechanism for regulating two catalytic activities of this bifunctional enzyme and could explain the difference in IL-12 production we observed in DCs lacking TG2. However, this hypothesis needs more investigation.

Our previous data showed that the TG2 expression increases during endotoxemia in mice, and that a better homeostasis of the proinflammatory mediators was observed in $TG2^{-/-}$ mice, suggesting that TG2 could be responsible for vicious inflammatory cycle (Falasca et al., 2008). In order to further substantiate this hypothesis, we studied the role of TG2 in DC functions in the same mouse model.

When we analyzed splenic DC in septic $TG2^{-/-}$ mice for their phenotype we observed that upon LPS stimulation DC have the lower ability to up-regulate MHC class II and co-stimulatory molecule CD86 in comparison to DC from WT mice. This confirmed our conclusions that TG2 is implicated in phenotypic changes of DC upon LPS treatment.

In mice, many studies have been performed in order to investigate the change in expression of DCs surface costimulatory molecules during sepsis, which is a

hallmark for DC activation and maturation. Ding et al. 2004 reported no change in CD40, CD80 and CD86 expression by splenic DCs 24h after sepsis induction. Similarly, no significant difference was observed in the percentage of mature DCs in the lymph nodes of septic mice compared to the controls (Efron et al., 2004). The splenic DCs from septic mice with no significant change in their expression of costimulatory molecules was found to have similar antigen presenting capacity as that of DCs from control mice.

We also found an inappropriate regulation of the TLR4 and reduced apoptosis upon LPS stimulation. Interestingly, we found that LPS induced a splenic DC depletion not detected in $TG2^{-/-}$ mice, in which the number of DCs upon the endotoxin stimulation was preserved. One explanation can be the reduced susceptibility of $TG2^{-/-}$ DCs to undergo apoptosis upon LPS challenge. In agreement, DC survival was demonstrated to be pivotal in the resistance to lethal endotoxic shock (Falasca et al., 2008).

These findings might be also explained by the absence of TG2 normally localized on DC's plasma membrane (Raki et al., 2007). In fact, it is well known that TG2 can be externalized and, by interacting with the extracellular matrix, can regulate the adhesive properties of various cells (Zemskov et al., 2006). In addition, it is tempting to hypothesize that the ectoTG2 could also affect the assembly of the MHC class I. In fact, upon synthesis, the MHC class I heavy chain binds to the membrane-associated endoplasmic reticulum chaperone, calnexin. Upon dissociation from calnexin, the heavy chain binds to β2 microglobulin and is then incorporated into the peptide-loading complex (Fesus et al., 1981). The other constituents of the complex are the two subunits of the transporter associated with antigen processing (TAP1 and TAP2), the transmembrane glycoprotein tapasin, the soluble chaperone calreticulin, and the thiol oxidoreductase ERp57. Interestingly, TG2 is known to interact with calreticulin (Feng et al., 1999) and to crosslink β2 microglobulin (Fesus et al., 1981), thus it seems plausible that hypothesize that the enzyme could play a role in the proper assembly of the MHC class I complex.

Altogether these data indicate that TG2 ablation markedly affects DC maturation and functions, thereby determining a decreased capacity of these cells to produce a pro-inflammatory response upon endotoxic shock stimulation. An important reason for DC unresponsiveness might relay in the expression of the LPS receptor (Nagai et al., 2002). DCs and other cells react to bacterial motifs, such as LPS, via Toll like receptor 4, and consecutively up-regulate other members of Toll like family (TRL2 and TLR9) (Nagai et al., 2002; Powers et al., 2006). The TLR4-MD-2 complex resides on the plasma membrane and LPS induces its clustering, a mechanism critical for TLR4 signalling. When we tested $TG2^{-/-}$ DCs for the expression of TLR receptors, indeed we found that, while in DCs from LPS-treated WT mice the expected up-regulation of TLR4/MD-2 complex expression is

observed, in the DCs from TG2 deficient animals the receptor is down-regulated. Such a phenomenon has already been reported in unresponsive animals upon LPS stimulation (Fan and Cook, 2004), confirming that the defect in TLR4 receptor expression detected in DCs obtained from $TG2^{-/-}$ mice could also be responsible for the impaired responsiveness of these cells to sepsis.

Taken together, our results indicate a pleiotropic TG2 role in the function, maturation and survival of DCs, which might explain the impaired response to LPS stimulation we reported in $TG2^{-/-}$ mice. Future studies should clarify by which mechanism(s) TG2 participates in the regulation of DC function in response to bacterial compounds. To achieve this result the identification and characterization of the enzyme's substrate protein profile in APCs is absolutely required. Finally, these findings highlight TG2 inhibition as a new target for the treatment of inflammatory processes associated with sepsis.

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