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"Mycoplasma and Tumors: Synergistic Effect on Human Monocyte-Derived Dendritic Cells Immunosuppressing Activity"

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ABSTRACT

The contribution of microenvironment on DC differentiation and function is critical for the outcome of the entire immune response. Here we study the combined effect of tumor-derived factors (TDFs) and mycoplasma, a microbe often detected in several human neoplasia, on monocyte derived DC development. DC generated in the presence of mycoplasma-infected tumor cell lines-conditioned medium express high amounts of CD83, CD86, CD80, markers typical of terminal differentiated cells, namely mature DC. Upon the exposure to TLRs ligands, including bacterial lypopolysaccaride (LPS) and polyriboinosinic polyribocytidylic acid (Poly I:C), these cells lost the ability to secrete pro-inflammatory cytokines (TNFα, IL-12) through the classical p38/MAPK pathway and induce apoptosis of memory/effector T cells. Neutralization of the well-known TDFs (IL-6, TGF-β, EGF, VEGF, IL-10, IDO) does not prevent these immunosuppressive effects. On the contrary, treatment with mycoplasma antibiotic drug (Ciprofloxacin) completely reverts the effects of TDFs. We propose that tumors would act as a mycoplasma bio-reactor, promoting the persistence of the infection in local site to drive the immune response toward the immunosuppressive pathway. In this context, evaluation of mycoplasma infection and its pharmacological treatment would be considered as a mandatory immunotherapeutic strategy to improve the response against tumor cells.

BACKGROUND

Dendritic Cells (DC) are a heterogeneous population of bone-marrow derived cells that play a pivotal role in controlling both innate and adaptative immune response (Steinman 1991). Beside precursors (pre-DC), dendritic cell subsets contain conventional dendritic cells (cDC) and plasmacytoid pre-dendritic cells (pDC). Both cDC and pDC are bone marrow derived cells. Although the common functions of DC are antigen-processing and T-lymphocyte activation, they differ in surface markers, migratory patterns, and cytokine output (Wu L. 2004, Shortman K et al. 2007).

Plasmacytoid dendritic cells (pDC) are present at low level in peripheral blood, express TLR7 a TLR 9 and have the hallmark to produce high amount of type I IFN after viral challenging (Colonna et al. 2004, Lanzavecchia et al. 2001, Spits et al. 2000, Dzioneck et al. 2000, Cella et al.1999, Edwards et al. 2003).
Conventional DC are characterized *in vivo* by expression of CD11c, CD33 and absence of CD45RA, CD123 and lineages markers (Kadowaki et al. 2001, Sieling et al. 2002). Myeloid DC expresses a wide range of TLRs (TLR1, 2,3,4,5,6,8,10) and secrete a variety of cytokines but not type I IFN upon activation (Steinman RM 2006). They are distributed in blood, peripheral tissues and lymphoid organs and show an unique ability to activate and polarize naive T-cells (Banchereau et al. 1998). In peripheral tissues, cDC exist in two functional and phenotypically distinct states, immature (iDC) and mature (mDC). iDC are characterized by a high rate of endocytosis and low antigen-presenting capability. This asset let them to be a powerful microenvironment sensor highly active to capture extracellular antigens during fluid phase pinocytosis and macropinocytosis. Although, the terminal differentiation program of iDC includes a decrease in the "capturing" activity, it generates mDC: specialized antigen presenting cells with the unique ability to activate naïve T cells in lymphonode T-cells areas (Maldonado-Lopez et al. 2001). mDC express on their surface high levels of a variety of molecules involved in the activation process of T cell, including major histocompatibility complex (MHC) class I and class II molecules, adhesion molecules, B7-family members (CD80, CD86, PD-L2/B7-DC, ICOSL), TNF family members (CD137/4-1BBL, CD 134/OX-40L, CD70). The activation program also involved dramatic changes in the profile of chemokines receptors: among them, CCR5 and CCR7, play a key role to re-locate mDC at the appropriate paracortical area of secondary lymphoid tissue, namely the "traffic zone", a strategic place to meet and engage trafficking naïve T cell (Ebert LM 2005.).

A number of studies revealed terminal differentiation as a very plastic process that can be oriented by a variety of microenvironment factors and can generate mature DC showing a wide range of biological properties ranging from inflammatory to tolerogenic phenotype (Lanzavecchia et al. 2001). Pathogens

derivatives offer good examples of DC plasticity modulators: Toxoplasma gondii, bacteria, viruses and mycoplasma drive DC toward a strong pro-inlammatory phenotype. Again, prolonged exposure to LPS or Toxoplasma gondii lead to a paralysis of IL-12 production (Karp et al. 1998, Reis 1999). Beside pathogen derivatives, cytokines present in the microenvironment at the moment of stimulation can also affect the production IL-12. IFN-gamma product by activated lymphocytes enhances Il-12 production, while IL-10 and TGF-β display opposite effects (Snijders et al. 1998, Hochrein 2000, De Smedt et al. 1997). DC exposed to IL-1, TNFα, colera toxin fail to produce IL-12 and generate DC favoring a Th2 response (Reis et al. 1999, Braun et al. 1999, Gagliardi et al. 2000, Rescigno et al. 2000, Kalinsky et al. 1999). Finally, a variety of agents elevating the intracellular levels of cAMP, including prostaglandins, vasoactive intestinal peptide, extracellular ATP, can drive DC toward a regulatory phenotype (Galgani M 2004). Kinetic of DC activation is an additional relevant factor regulating the secretion of Il-12: DC are able to produce IL-12 till 16 hours from the challenge, while at later time points became refractory to further stimulation (Langenkamp et al. 2000).

DC can engulf apoptotic or necrotic tumor cells, process the tumor-associated antigens and present them to CD4+ and CD8+ T cells. Dying cells have been postulated to engage several mechanisms to signal the innate immune system: (i) 'find me', (ii) 'eat me' and (iii) 'stay away' (Dhodapkar et al. 2007). 'Find me' signals are soluble factors, such as lysophosphatidylcholine, able to induce migration of phagocytes to apoptotic cells (AC) (Lauber et al. 2003). 'Eat me' signals are usually membrane bound and serve as markers for phagocytes to recognize and ingest ACs. These include alterations in cell surface phospholipid composition with exposure of phosphatidylserine (PS), alterations in cell surface charge or expression of specific molecules (Fadok et al. 1998). A number of receptors expressed on immature DC such as $\alpha_v\beta_5$ integrin, complement receptors and CD36 are thought to be involved in apoptotic cells uptake (Fadok et al. 2001, Albert et al. 1998). Furthermore, 'eat me' signals might be indirect, that is, mediated by serum or phagocyte-derived proteins, which can opsonize ACs, and thereby promoting their capture (Verbovetski et al. 2002, Mevorach et al. 1998, Hanayama et al. 2002, Anderson et al. 2003). Finally, the 'Stay away' (or 'do not eat me') signals may be critical to prevent the uptake of activated but live cells and include CD47 or CD31 (Brown et al. 2002). Although the adaptive immune response should be able to eradicate tumors, this option represents a rare event. The inability of tumor associated antigen (TAAs) to elicit an effective immune response is not a passive process since tolerizating factors play an active role in the tumor microenvironment (Curiel et al. 2004, Curiel et al. 2003, Gabrilovich et al. 1996, Munn et al. 2004a, Zou et al. 2001).

In the early 1990s, there was the surprising observations that most antigens expressed by tumor cells were not necessarily neo-antigens uniquely present in

cancer cells but, maybe most important for an immunological point of view, tissue-differentiation antigens also expressed in normal cells (Boon at al. 2006, Rosenberg 1999). These unexpected findings supported the concept that tumors are able to escape the immune system surveillance in subtlest ways, because malignant cells are very difficult to dissect from normal cells (Sotomayor et al. 1996). Several experimental evidences supported this hypothesis. Bogen and Levitsky independently demonstrated that antigen-specific CD4+ T cells were indeed rendered tolerant during tumor growth (Bogen 1996, Staveley-O'Carroll et al. 1998). Following the initial report of this phenomenon, termed as tumor-induced anergy, several studies showed that this state of T cell unresponsiveness also occurs during the growth of hematologic or solid tumors expressing model or true tumor antigens during the progression of spontaneously arising tumors and, most importantly, during the progression of human cancers (Cuenca et al. 2003, Morgan et al. 1998, Overwijk et al. 2003, Lee et al. 1999, Willimsky et al. 2005, Noonan et al. 2005). After the understanding of the tumor-induced tolerance, the first question that arises from was about the role of antigen presenting cells in the instauration of this phenomenon. Utilizing parent-into-F1 bone marrow chimeras, researchers demonstrated that tumor antigen processing and presentation by APCs (not direct presentation by tumor cells) is the dominant mechanism underlying the development of tumor antigen–specific CD4+ T cell tolerance. This critical role of APCs was operative not only in mice challenged with tumor cells that have intrinsic antigen-presentation capabilities (B cell lymphoma), but also in mice challenged with solid tumors that are ill-equipped to present cognate antigen to CD4+ T cells. These studies therefore demonstrated that the intrinsic APC capacity of tumor cells has little influence over T cell priming versus tolerance, a critical decision that is regulated at the level of bone marrow–derived APCs. It has been reported that TAA-priming might happen not only in the draining lymph nodes, but also in the tumor microenvironment to some degree, where naïve T cells and DC can be found. The first tumors in which DC has been found are renal cell carcinomas, head and neck cancer (Thurnher et al. 1996, Tas et al. 1993). It has been found that within the tumor, primarily iDC not mDC are present, while mDC were detected only in the marginal zones (Troy et al. 1998, Troy et al. 1998, Sandel et al. 2005). Furthermore, labeling experiments revealed that most of the intratumoral DC remain inside the tumor instead to migrate out (Fejoo et al. 2005). From this point of view the tumor is a false lymphoid organ, and T-cell priming in the tumor microenvironment is compromised by the fact that APC present in the tumor are either dysfunctional or induce T cell tolerance. One important raison why the tumor microenvironment have the ability to influence the DC function, reside in his ability to produce different factor with immunosuppressing activity on DC. Below, we provide a short description of the most well known Tumor Derived Factors.

VEGF. The first identified factor with immunomodulatory effects on DC was vascular endothelial growth factor (VEGF) (Ellis et al. 1996, Toi, et al. 1996, Carmeliet et al. 2000, Kryczek et al. 2005, Gabrilovich et al. 1998). Its physiological role is linked to neo-vascularization and hematopoiesis during embryogenesis. However VEGF is produced by most tumors and its plasma amount increase in cancer patients correlate with an unfavorable prognosis. Furthermore, expression of VEGF inversely correlated with DC number in tumor tissues and in the peripheral blood (Saito et al. 1998, Almand et al. 2000). The initial findings above the immunosuppressing role of VEGF on DC function, derive from *in vitro* experiments that demonstrate the ability of VEGF neutralization to revert the negative effects of tumor conditioned media. These evidences was supported by *in vivo* experiments on tumors bearing mice treated with anti-VEGF antibodies, that achieved a better DC differentiation as well as number rescue (Gabrilovich et al. 1999, Ishida et al. 1998).

M-CSF and IL-6. Macrophage colony-stimulating factor (M-CSF) and IL-6 are produced by a large number of tumors and have also been reported to be involved in the tumor-mediated regulation of DC differentiation (Gabrilovich et al. 1996, Menetrier-Caux et al. 1998). Renal carcinoma cell lines were shown to release soluble factors that inhibit the differentiation of CD34+ progenitor cells into DC and instead trigger their differentiation towards monocytic cells. Both neutralizing IL-6- and M-CSF-specific antibodies abolished the impact of renal cell carcinoma conditioned medium on DC and the combination of IL-6 and M-CSF displayed a similar effect on inhibition of DC differentiation (Menetrier-Caux et al. 2001). IL-6 plays an important role in abnormal DC differentiation in multiple myeloma (Ratta et al. 2002). Furthermore, sera from patients with multiple myeloma inhibited the generation of DC, which could be reverted by anti-VEGF and/or anti-IL-6 antibodies (Hayashi et al. 2003). In another recent study, IL-6 was found to suppress DC maturation *in vivo* and play a major role in maintaining immature DC (Park et al. 2004). The suppressive role of IL-6 could be attributed to activation of the transcription factor STAT3.

IL-10. IL-10 plays an important role in DC defects in cancer. DC derived from transgenic mice with IL-10 over-expression have suppressed ability to stimulate allogeneic T-cell and CTL responses as well as IL-12 production (Sharma et al. 1999). IL-10 might contribute to the conversion of iDC into tolerogenic APCs by decreasing the expression of co-stimulatory molecules (Steinbrink et al. 1997). Treatment of human DC with IL-10 was found to induce suppression of antigen-specific proliferation of CD4+ and CD8+ T cells via cell-cell contact (Steinbrink et al. 2002). Furthermore, the blockade of differentiation of monocytes to DC could be attributed to IL-10, which drives the differentiation process towards a macrophage cell type rather than DC (Allavena et al. 1998, Buelens et al. 1997). IL-10 also inhibits the function of Langerhans cells,

monocyte derived DC, or CD34+ progenitors (Beissert et al. 1995, Enk et al. 1993, Peguet-Navarro et al. 1994, Caux et al. 1994, Steinbrink et al. 1997). A mouse tumor model revealed that tumor derived IL-10 was responsible for DC dysfunction *in vivo*. DC function was not affected in IL-10 deficient tumor bearing mice (Yang et al. 2003). Even though different tumor cells might produce and release IL-10, the majority of IL-10 is probably produced by tumor-associated macrophages (TAM) with some contribution from tumor-infiltrating lymphocytes (Seo et al. 2001, Sica et al. 2000).

TGF-β (transforming growth factor-β). Cytokines of the TGF-β family are essential factors in embryonic development and tissue repair. This family includes three types of TGF-β (β1, β2 and β3), inhibins and activins, as well as various bone morphogenetic proteins (BMPs) and mullerian inhibiting substance. Activin βA and TGF-β1 share functions in inflammatory reactions including tissue repair and suppression of immune response (Munz et al. 1999, Rosendahl et al. 2001). Both cytokines share SMAD2/3 and SMAD4 as intracellular signaling targets of their receptors (Itoh et al. 2000). In an adoptive transfer model TGF-β revealed its capability of inducing suppressive regulatory T cells (Treg) by its ability to generate DC that promote tolerance in a manner dependent on MHC class II molecules (Alard et al. 2004). Specifically, generation of Treg cells was attributed to immature DC, and TGF-β prevents the maturation of DC by maintaining a low expression of co-stimulatory molecules (Geissmann et al. 1999, Roncarolo et al. 2001)

Indoleamine-2,3-deoxigenase (IDO). This is a heme-containing enzyme that catalyzes the oxidative breakdown of the essential amino acid tryptophan via the kynurenine pathway (Mellor et al. 2000). Munn and colleagues (1998) provided the first evidence showing that IDO may play a role in the establishment of immune privilege; they demonstrated that IDO preserves the feto-placental unit from T cell attack. IDO expression has been documented in murine as well as in human DC. IDO catalyzes the oxidative catabolism of tryptophan, an aminoacid essential for T cell proliferation and differentiation, IDO+ DC reduce the access of lymphocytes to free Trp blocking in such a way cell cycle progression. T cells are inhibited in their clonal expansion and subsequently are inducted to undergo apoptosis. Uyttenhove (2003) found that immunogenic tumors engineered to over-express IDO growth more aggressively: this effect correlated with a decreased accumulation of activated T cells at the tumor site. Importantly, *in vivo* administration of the IDO inhibitor 1-methyltryptophan resulted in reduced tumor mass and stimulation of anti-tumor CTL responses. Although the precise mechanisms that regulate IDO expression still remain to be ascertained, Muller and colleagues (2005) recently showed that IDO is under the genetic control of the tumor suppression gene *Bin1*, which is attenuated in many human tumors. IDO+ DC can be generated *in vitro* from human monocytes, but has been found *in vivo*,

in patients affect by breast tumor and in draining lymph nodes in patients with melanoma and patients with lung, colon, breast, pancreatic cancer.

EGF. The epidermal growth factor receptor (EGFR; HER1/erbB-1) has recently been identified as a target for cancer therapy in multiple human tumors (Bellone et al. 2007, Arteaga 2002 Baselga 2000, Raymond et al 2000, Baselga 2001, O'Dwyer and Benson 2002, Mendelsohn and Baselga 2000). On endogenous ligand binding, EGFR activation occurs, with receptor homo or heterodimerization and autophosphorylation of the intracellular tyrosine kinase domain (Schlessinger 2002, Sako et al. 2000). Subsequently, a complex network of signal transduction pathways is induced, which plays a key role in regulating cell proliferation, differentiation, motility, invasion and angiogenesis (Schlessinger 2002, Sako et al. 2000, Schlessinger 2000, Olayioye et al.1999, Kim and Muller 1999). EGFR is expressed in a variety of human malignancies and its high level of expression has been previously correlated with poor patient prognosis and resistance to treatment in many tumor entities including cervical carcinoma (Kim and Muller 1999, Nicholson et al 2001, Mendelsohn and Fan 1997, Corvo et al 2001, Kersemaekers et al. 1999, Kim et al. 2004).

Mycoplasma. Mycoplasmas are distinguished phenotypically from other bacteria for the minute size and the total lack of a cell wall (Shmuel et al.1998), a property taxonomically used to classify mycoplasmas as classe Mollicutes (from latin Mollis, soft; Cutis, Skin). Mycoplasmas are widely distributed in nature as parasites of humans, mammals, reptiles, fish, arthropods and plants, but the list of host that harbor mycoplasma is destined to increase. The major difficulty for mycoplasma research is the difficulty to growth *in vitro*. This is due to the requirement of exogenous supplies. Mycoplasmas apparently lost almost all the genes involved in the biosynthesis of amino acids, fatty acids, cofactors, and vitamins and therefore depend on the host microenvironment to supply the full spectrum of biochemical precursors required for the biosynthesis of macromolecules. Competition for these biosynthetic precursors by mycoplasmas may disrupt host cell integrity and alter host cell function. Many animal mycoplasmas depend on adhesion to host tissues for colonization and infection. Adherence is one of the most important virulence factors for mycoplasma. M. pneumoniae is the model for the study of adhesin and its receptors. A surface 169 kDa protein designated P1 and a 30kDa called P30 are providing polarity to the cytoadherence event (Inamine et al. 1988, Dallo et al. 1990). Both proteins elicit a strong immunological response in convalescent-phase sera from humans and experimentally infected hamsters, and anti-P1 or anti-P30 monoclonal antibodies block this cytadherence (Balish et al. 2002, Razin and Jacobs 1992, Krause 1996 Su et al. 1989). Currently theories propose that mycoplasmas remain attached to the surface of epithelial cells, although some mycoplasma have evolved

mechanisms for entering host cells that are not naturally phagocytic. The intracellular localization is a privileged niche, well protected from immune system and from the action of many antibiotics. Mycoplasmas known to be surface parasites as M. penetrans, *fermentans*, *genitalium* and *gallisepticum* under certain circumnstances can reside within non-phagocytic cells. The lack of a rigid cell wall allows direct and intimate contact of the mycoplasma membrane with the cytoplasmic membrane of the host cell, and under appropriate conditions, such contact may lead to cell fusion. Mycoplasma *fermentans* is known as one of the most fusogenic mycoplasma competent to target a variety of cells. It has been shown that the polar lipid fraction of this organism is able to enhance the fusion of small, unilamellar phosphatidylcholine-cholesterol (1:1 molar ratio) vesicles with Molt-3 lymphocytes in a dose-dependent manner, suggesting that a lipid component acts as a fusogen. During the fusion process, mycoplasma components are delivered into the host cell and affect the normal cell functions. A whole array of potent hydrolytic enzymes has been identified in mycoplasmas. Most remarkable are the mycoplasmal nucleases that may degrade host cell DNA (Paddenberg et al. 1998). It has recently been shown that M. *fermentans* contains a potent phosphoprotein phosphatase. The delivery of an active phosphoprotein phosphatase into the eukaryotic cell upon fusion may interfere with the normal signal transduction cascade of the host cell. In addition to delivery of the mycoplasmal cell content into the host cell, fusion also allows insertion of mycoplasmal membrane components into the membrane of the eukaryotic host cell. This could alter receptor recognition sites as well as affect the induction and expression of cytokines and alter the cross-talk between the various cells in an infected tissue. It is documented that Mycoplasma can exert different effects on DC differentiation and immunomodulatory activity, through mechanisms not yet completely investigated, but involving the ability of mycoplasma PAMPs to bind TLRs. (Link C et al. 2004, Mariolina et al. 2000, Quah BJ and O'neill HC 2007). The involvement of mycoplasmas in cancer progression is now well documented. Prolonged mycoplasma infection is responsible for irreversible malignant transformation, including the ability to form tumors *in vivo* and high soft agar cloning efficiency *in vitro* (Shaw-Huey et al. 1999). In this context it has been also proposed that the mycoplasma protein p37 would promote invasiveness and promote genomic instability on tumor cells (Schmidhauser C et al. 1990; Shien T et al. 1995). Furthermore, the presence of mycoplasma in tumor cell lines positively correlates with aberrant expression of oncogenes and tumor suppressor genes (Zhang S et al 2006). Several studies have shown that mycoplasma infection correlates positively with a large spectrum of human cancers and negatively with the prognosis (Chan PJ et al. 1996, Huang S et al. 2001, Pehlivan M et al 2005). Finally, PCR and immunohistochemistry analysis showed mycoplasma in several

human cancer including gastric carcinoma, renal cell carcinoma, ovarian, lung, breast, esophageal and glioma cancers.

AIM OF THE STUDY

In the last decade, a number of reports have investigated the immunomodulatory role of cancer identifying a variety of tumor-derived factors capable to interfere with the immune response and more specifically with the differentiation programs of Dendritic Cells. Although these studies contributed to improve our knowledge on this field, only few of them have take in account the possibility that a frequently detected intracellular parasite like mycoplasma plays a major role in the immunomodulatory process. Mycoplasma is the most frequent parasite of tumor cell lines and has been detected in several human tumor lesions including gastric, ovarian and breast carcinoma. This microbe has been proposed to be involved in the oncogenetic mechanisms and, more recently, an increasing number of evidences have shown its powerful immunomodulatory properties. Our study has been aimed to define the role of mycoplasma infection in the immunomodulatory thyroid tumor. Specifically, we have analyzed the role of mycoplasma infection in the immunoregulatory effects exerted by a large number of thyroid tumor cell lines on the activation program of monocyted-derived DC. Our results shade new light about the role of mycoplasma infection in the outcome of the anti-tumoral immune response and would open novel perspective for immunotherapeutical intervention in human cancer.

MATERIALS AND METHODS

Media and Reagents

DC were generated in RPMI 1640 (Invitrogen Life Technologies), supplemented with 2 mM L-glutamine, 50 ng/ml streptomycin, 50 units/ml penicillin, and 10% heat-inactivated fetal calf serum (Hyclone Laboratories, Logan, UT), 50 ng/ml GM-CSF (Schering-Plough, Kenilworth, NJ) and 1000U/ml IL-4 (ImmunoTools, Germany). Phenotype was evaluated by cytometry. LPS was from Sigma (Sigma, Milano, Italia).

Cell lines

Human thyroid cancer cell lines conditioned medium from (TPC-1, FB2, NIM, BHP10-3, BHP17-10, BHP14-9, BC-PAP, BHP2-7, BHP5-16, Cal-62, 850-5c, Fro, ARO) and from normal thyroid cells, were provided from Dr RM Melillo. Cell culture method has been described previously (Cerutti et al.,1996; Ohta et al., 2001; Basolo et al., 2002). Briefly, they were maintained in DMEM supplemented with 10% foetal bovine serum, 1% penicillin–streptomicin, and 1% glutamine.

Mycoplasma detection

The mycoplasma infection was tested by microbiological assay at Section of Microbiology of Department of Clinical Pathology, University of Naples Federico II and verified by PCR amplification of mycoplasma genome (MycoProbe® Mycoplasma Detection Kit, R&D System)

To eradicate mycoplasma from cell culture, Ciprofloxacin was added to medium for 7-21 days. Spent medium of each cell line culture was replaced with fresh medium containing one of the various antibiotics during the treatment period. Cultures were always thoroughly mixed in order to ensure optimal distribution and access of the reagents to the mycoplasma cells commonly attached to the eukaryotic cell membrane. It is particularly important to break up clumps and clusters because these may represent sanctuaries to which the antibiotics do not have access. At the end of the treatment periods of 7–21 d, the cells were washed twice and left in fresh complete medium without antibiotics. Cells were then grown antibiotic free for at least 2 wk in order to enrich any residual mycoplasmas up to detectable levels or to get rid of residual mycoplasmal DNA. Ciprofloxacin (Ciprobay 100) is from Bayer (Leverkusen, Germany). The original ready-to-use solution of 100 mg/ml of the quinolone enrofloxacin (Baytril from Bayer) was diluted 1:100 with RPMI1640 medium.

In vitro Generation and Culture of Human DC

DC were generated from peripheral blood mononuclear cells, as described (Sallusto, F., and Lanzavecchia, A. (1994) J. Exp. Med. 179, 1109–1118), with

some modification. Briefly, peripheral blood mononuclear cells were obtained from 30 ml of leukocyte-enriched buffy coat from healthy donors by centrifugation with F Lymphoprep gradient (Axis-Shield PoC AS, Oslo, Norway), and the light density fraction was recovered. Monocytes were purified by positive selection using anti-CD14 conjugated magnetic microbeads (Miltenyi Biotec, Bologna, Italy). $CD14^+$ cells were cultured at a concentration of 1 x 10^6 cells/ml in RPMI 1640 supplemented with 10% fetal calf serum or, and 2 mM glutamine (complete medium) , 50 ng/ml streptomycin, 50 units/ml penicillin, containing 50 ng/ml granulocytes monocytes-colony stimulating factor (Schering-Plough, Kenilworth, NJ) and 1000U/ml IL-4 (Immunotools, Friesoythe, Germany). Cells were cultured for 4–5 days to obtain a population of iDC. For preparation of mDC, iDC were cultured for 24–48 h in the presence of 1 mg/ml LPS (Sigma).

Neutralization of cytokines and enzymes

For neutralization of cytokines in culture, goat anti-human IL-6 from Sigma-Aldrich was used (cat no AF-206-NA), anti Il-10 was from BD Pharmingen (cat.no 559076), anti TGF-β was from R&D system (cat.no MAB1835). For IDO neutralization 1-Methyl-DL-Triptophan from Sigma Aldrich was used.
Bevacizumab and Cetuximab were kindly provided by Dr. Giampaolo Tortora.

Lymphocytes proliferation and dead cells evaluation

PBL were isolated from peripheral blood of healthy donors by Lymphoprep (Nyegaard, Oslo, Norway) density gradient centrifugation. PBL was obtained as plastic non-adherent fraction. The adherence was performed in RPMI serum-free, at 37°C for 1 hour.

CD4 and CD8 cells were separated by negative selection using anti-Ig-coated magnetic beads (Dynabeads, Dynal; Oslo, Norway). The CD4+CD45RA cells were separated by negatively selected CD4 T cells and anti-Ig-coated magnetic beads (Dynabeads, Dynal; Oslo, Norway). All protocols were performed following the manufacturer's instruction. Purity of the lymphoid population was tested by flow cytometry.

Lymphocytes ($1x10^6$/ml) were cultured in 24-well, flat-bottomed plates (Falcon) with anti-CD3/CD28 antibody covered beads (Dynabeads, Dynal; Oslo, Norway) (0.5 beads/cell). To analyze the proliferation, lymphocytes were labeled with 5,6-carboxyfluorescein-diacetate-succinimidyl ester (CFSE; Molecular Probes, Eugene, OR) and than stimulated with anti-CD3/CD28 beads (Lyons, A. B. (1999). Proliferating cells can be tracked by flow cytometry, based on the sequential loss of fluorescence intensity. Dead cells were identified by using the propidium iodide staining and the Annexin V^{FITC} Apoptosis Detection kit; Beckton Dickinson, according to the manufacturer's directions.

Flow Cytometry

We used the following monoclonal antibodies conjugated to FITC or phycoerythrin for direct staining: PE-anti-CD86, PE-anti-CD1a, FITC-anti-CD83, FITC-anti-CD14 from BD Biosciences. The cells were also stained with the corresponding FITC- or phycoerythrin-conjugated isotype-matched control antibody from BD Biosciences. All incubations were in the presence of 10 µg/ml human IgG to prevent binding through the Fc portion of antibodies. For intracellular cytokine detection, Brefeldin A (5 µg/ml; Sigma) was added to the culture medium. Cells were then fixed and permeabilized by using a cytokine staining kit following the manufacturer's instructions (Caltag Laboratories, Burlingame, CA). Intracellular TNFα, IL-10, IL12 were detected by using PE-conjugated antibodies (BD Biosciences) and analyzed by FACSCalibur flow cytometer and Cellquest software (BD Biosciences).

Immunoenzymatic Detection of Cytokines

TNFα, IL-12, and IL-10 were measured by an ELISA developed in our laboratory using cytokine-specific capture and detection antibodies (BD Pharmingen) according to the manufacturer's instructions (monoclonal antibody 11 for detection of TNFα; antibody JES3-12G8 for detection of IL-10, and antibody C11.5 for detection of IL-12). Standard curves were generated by using corresponding human recombinant cytokines (BD Pharmingen). The concentration of cytokines in the cell supernatants was determined by extrapolation from the appropriate standard curve.

Western Blot

Total cell lysates were obtained in 50 mM HEPES (pH 7.5), 250 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 10 mM sodium fluoride, 1 mM sodium orthovanadate, and 2 µg/ml aprotinin, leupeptin, and pepstatin. 50 µg of total proteins from each lysate was subjected to SDS-PAGE under reducing conditions. After electrophoresis proteins were transferred on a nitrocellulose filter membrane (Protan, Schleicher & Schuell) by using a Trans-Blot Cell (Bio-Rad) and transfer buffer containing 25 mM Tris, 192 mM glycine, 20% methanol. Membranes were placed in 5% nonfat milk in phosphate-buffered saline, 0.5% Tween 20 (PBST) at 4 °C for 2 h to block the nonspecific binding sites. For detection of phospho-p38, phospho-ERK-1/2, tubulin, phospho-CREB, phospho-JNK, phospho-STAT-3, IKB specific antibodies from Santa Cruz Biotechnology (Santa Cruz, CA) were used. Filters were incubated with specific antibodies before being washed three times in TBST and then incubated with a peroxidase-conjugated secondary antibody (Amersham Biosciences). After further washing with PBST, peroxidase activity was detected by using the ECL system (Amersham Biosciences).

RESULTS AND DISCUSSION

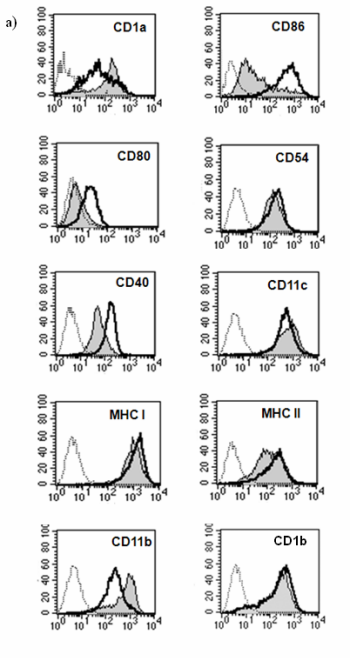
Thyroid TDFs interfere with differentiation program of monocyte-derived DC.

18 human thyroid tumor cell lines (15 tumoral and 3 untrasformed) were growth at 80% of confluence in regular cell medium before being washed and cultured for additional 24 hours in fresh medium. After 18 hours, tumor-

Tab.1 Mycoplasma detection in thyroid cell lines

Cell lines	Hystological type and oncogene	Mycoplasma levels	Mycoplasma specie
TPC-1	PTC; RET/PTC1	100.000U/ml	*Fermentans*
FB-2	PTC; RET/PTC1	100.000U/ml	*Fermentans*
FBA-2	PTC; RET/PTC1	<10.000U/ml	*Fermentans*
NIM-1	PTC; RET/PTC1	100.000U/ml	*Fermentans*
BHP 10-3	PTC; RET/PTC1	<10.000U/ml	*Fermentans*
BHP 2-7	PTC; RET/PTC1	<10.000U/ml	*Fermentans*
BHP 14-9	PTC; Braf V600E	<10.000U/ml	*Fermentans*
BHP 5-16	PTC; Braf V600E	<10.000U/ml	*Fermentans*
BC-PAP	PTC; Braf V600E	<10.000U/ml	*Fermentans*
NPA87	PTC; Braf V600E	100.000U/ml	*Fermentans*
CAL-62	ATC; N-ras Q61K	100.000U/ml	*Fermentans*
850-5C	ATC; Braf V600E	100.000U/ml	*Fermentans*
FRO	ATC; Braf V600E	100.000U/ml	*Fermentans*
ARO81	ATC; Braf V600E	100.000U/ml	*Fermentans*
FB-1	ATC; Braf V600E	100.000U/ml	*Fermentans*
P5-2N	Normal thyroid; unknown	Undetectable	
P5-3N	Normal thyroid; unknown	Undetectable	
P5-4N	Normal thyroid; unknown	Undetectable	

conditioned media were collected and tested for the presence of Mycoplasma infection and for their ability to affect differentiation of monocytes toward DC lineage. The results of the coltural biological assay documented the presence of *mycoplasma fermentans* in all conditioned medium derived from thyroid tumor cell lines with the exception of that derived from normal thyroid cell lines (tab. 1). To evaluate the ability of TDFs to interfere with differentiation program of dendritic cells, freshly isolated CD14+ monocytes were cultured in optimal



amount of GM-CSF and IL-4 for 7 days in the presence or absence of TDFs. At this time point, the expression of typical differentiation markers of DC was evaluate by cytometry. We found that the exposure of monocytes to TDFs strongly interfered with generation of cells displaying the classical phenotype of immature DC (Fig. 1a). On the contrary, a detectable increase of CD83, CD86, CD80, CD40, MHC II was detected (Fig.1a). Notably, the most prominent effects were exerted by cell lines with a level of mycoplasma infection >100.000 U/ml, while BHP 5-10, BHP 10-3, BHP 14-9, BHP 2-7 and BC-PAP (mycoplasma infection level <10.000 U/ml) did not interfere with the generation of typical iDC (Fig.1b).

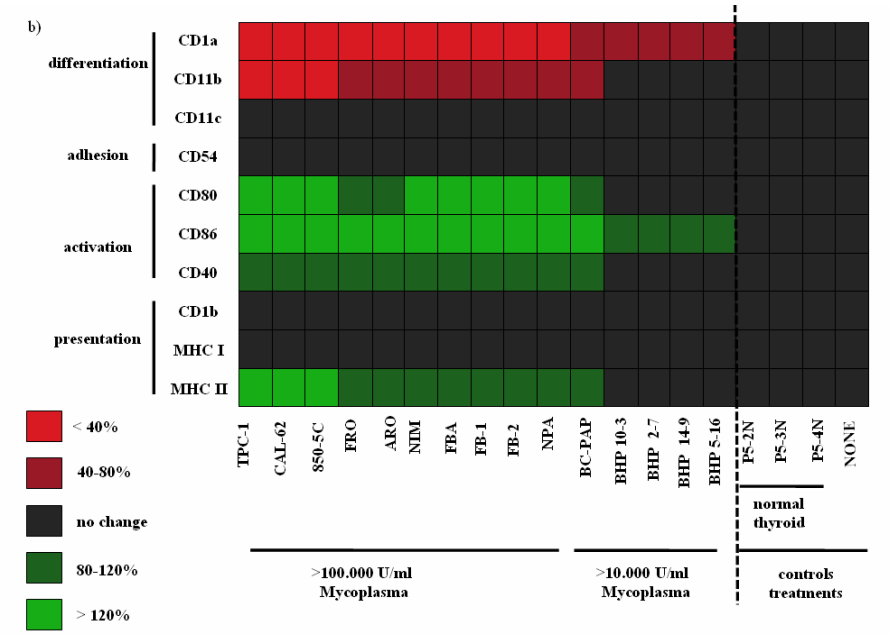


Fig.1 Effects of TDFs on DC immunophenotype

(a) FACS profiles of DC generated from monocytes cultured for 7 days with GM-CSF and IL-4 in the presence or absence of 10% TPC conditioned medium. Results are representative of five independent experiments. (*Gray, filled histograms: untreated; black, open: TPC; dotted, open: isotype control*). Panel b reports the results expressed as the percentage of untreated values measured in DC cultured in regular medium (100%=Mean Fluorescence Units of DC generated in the absence of TDF).

TDFs interfere with terminal differentiation of DC induced by TLRs ligands4.

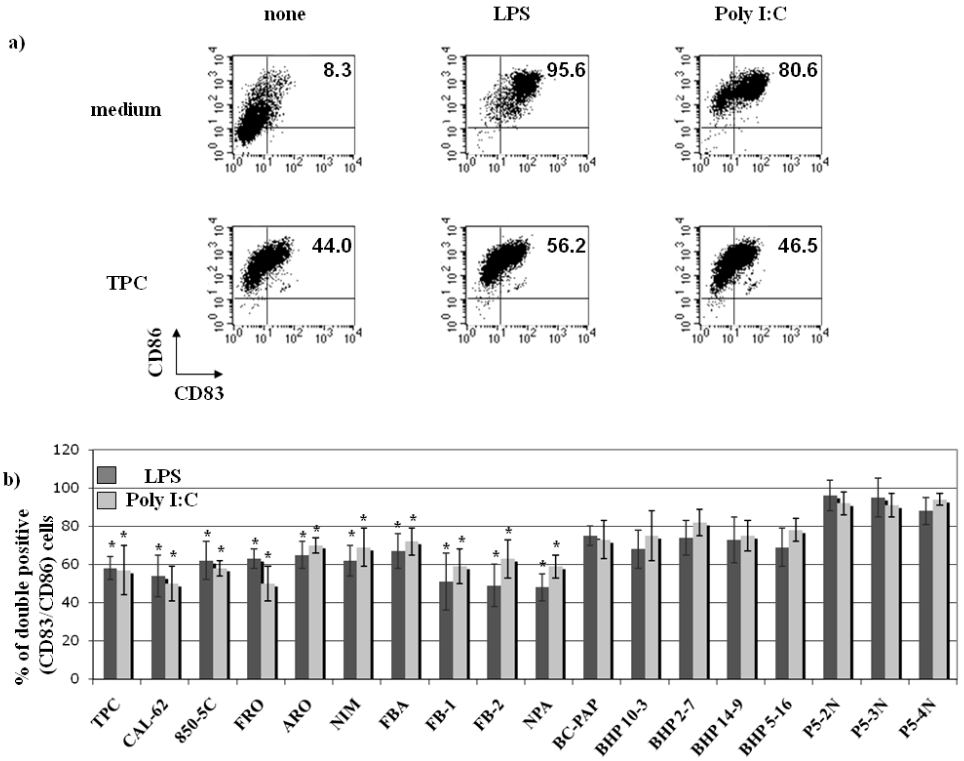


Fig.2 Effects of TDFs phenotypical maturation and TLRs signaling.
a) iDC generated in presence of TPC cell line conditioned medium were collected, washed and exposed to LPS (1μg/ml) or Poly I:C (1ooμg/ml). After 24 hours, cells were analyzed by double-staining cytofluorimetric assay for the expression of the indicated surface markers. The data shown are representative of three independent experiments.
b) Bars histogram represent the percentage of double positive CD83/CD86 DC, generated from 7 days culture of monocytes in the presence or absence of 10% conditioned medium of indicated cell lines and exposed to bacterial endotoxin (1μg/ml) or Poly I:C (1ooμg/ml). After 24 hours, cells were analyzed by double-staining cytofluorimetric assay for the expression of the indicated surface markers. The results are representative of three independent experiments.

iDC generated in the presence or absence of TDFs were washed and exposed for additional twenty-four hours to TLR4 and TLR3 ligands, namely Lipopolysaccaride (LPS) and Polyinosinic: polycytidylic acid (poly I:C) and immunophenotype were evaluated by cytometry. As expected, TLRs ligands induced a marked increase in double positive CD86/CD83 in DC growth in regular

medium supplemented with optimal amount of GM-CSF/IL-4. Otherwise, TDFs-DC expressed high levels of CD83 and CD86 even in the absence of TLRs ligands and addition of LPS or poly I:C did not induced any significant increase of double positive DC (Fig.2a). Notably, CD83 and CD86 levels positively correlate with the presence of mycoplasma in conditioned media (Fig.2b).

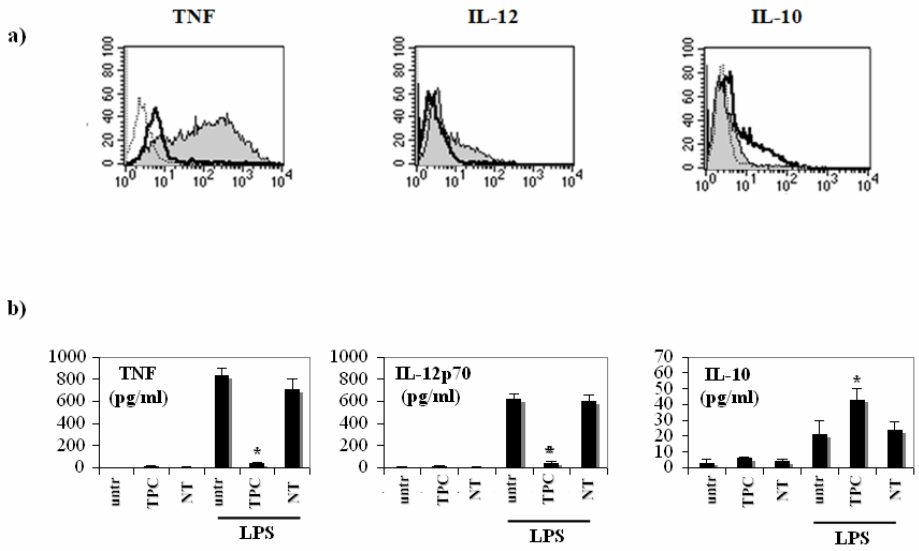


Fig.3. TDFs-exposed DC are tolerant to LPS.
a) iDC generated in presence of 10% of TPC-TDF were collected, washed and exposed for additional 24 hours to LPS (1μg/ml) in the presence or absence of 5μg/ml Brefeldine A (a and b panels, respectively). After 24 hours, cells were analyzed by intracellular staining (a) and levels of cytokines evaluated by ELISA in supernatants (b). Panel a reports a typical FACS profile of intracellular cytokines staining. In panel b, bar graphs report mean and standard deviation of 6 independent experiments.

To analyze the effects of TDFs on cytokines synthesis we evaluate the intracellular levels as well as the concentration in DC supernatants of TNFα, IL-12 and IL-10. As expected, the exposure to LPS induced the synthesis and secretion of all three analyzed cytokines (fig. 3). On the contrary and regardless to the presence of TLRs ligands in the culture media, TDFs-exposed DC failed to synthesize and release any cytokines (Fig3a).

DC generated in the presence of TDFs induce lymphocytes cell death.

To analyze the effect exerted by TDFs-generated DC on T cells, we co-cultured these cells with CSFE-labeled purified T cells DC and in the presence or absence of anti-CD3/CD28 conjugated beads. After 3 days, cells were stained with Propidium Iodide (PI) and analyzed by flow cytometry (Fig.4a). Results shown a significant increase in the percentage of death cells in lymphocytes cultured in the presence of TPC-TDFs DC . In addition, we found a positive correlation between the percentage of cell death and the activation stage of lymphocytes. A time course analysis of this phenomenon, demonstrate that after 7 days of culture, the percentage of live (PI negative)/CFSE positive lymphocytes activated with anti CD3/CD28 was less than 5% (Fig.4b). On the contrary, normal thyroid conditioned medium didn't induce cytotoxic DC (Fig.4b). Furthermore, we analyzed, with the same experimental model, the sensitivity to TDFs-DC induced cell death of the different lymphocytes subsets. Thus, CD4, CD8 and CD4CD45RA naïve T lymphocytes were separated from peripheral blood and stained with CFSE before being co-cultured with DC (ratio DC:PBL 1:25). These experiments revealed that CD4CD45 lymphocytes were resistant to the DC-induced death. Otherwise, no differences were observed in CD4 and CD8 subsets. (Fig.4c).

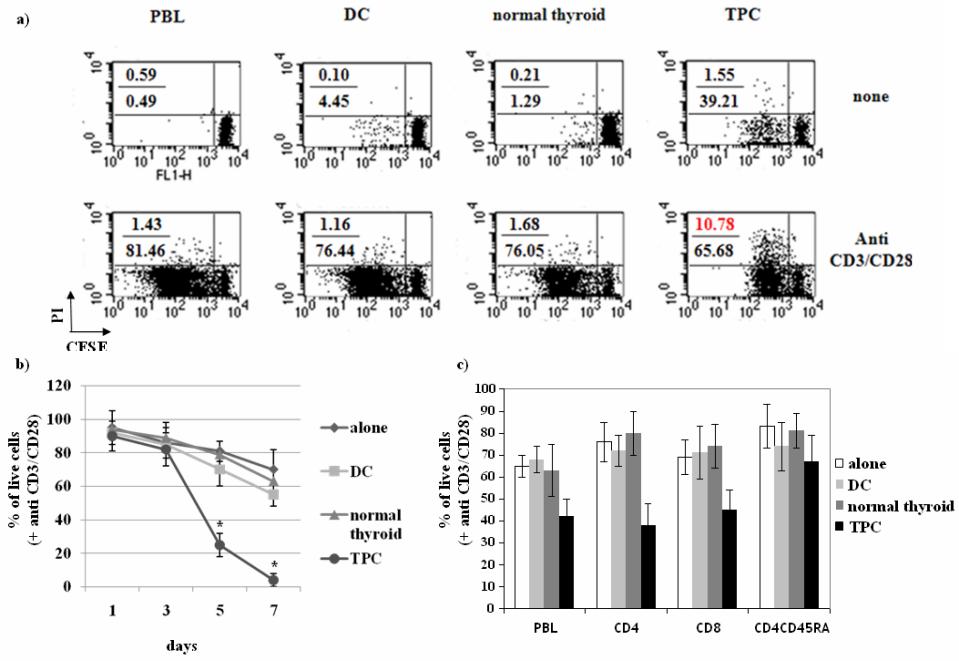
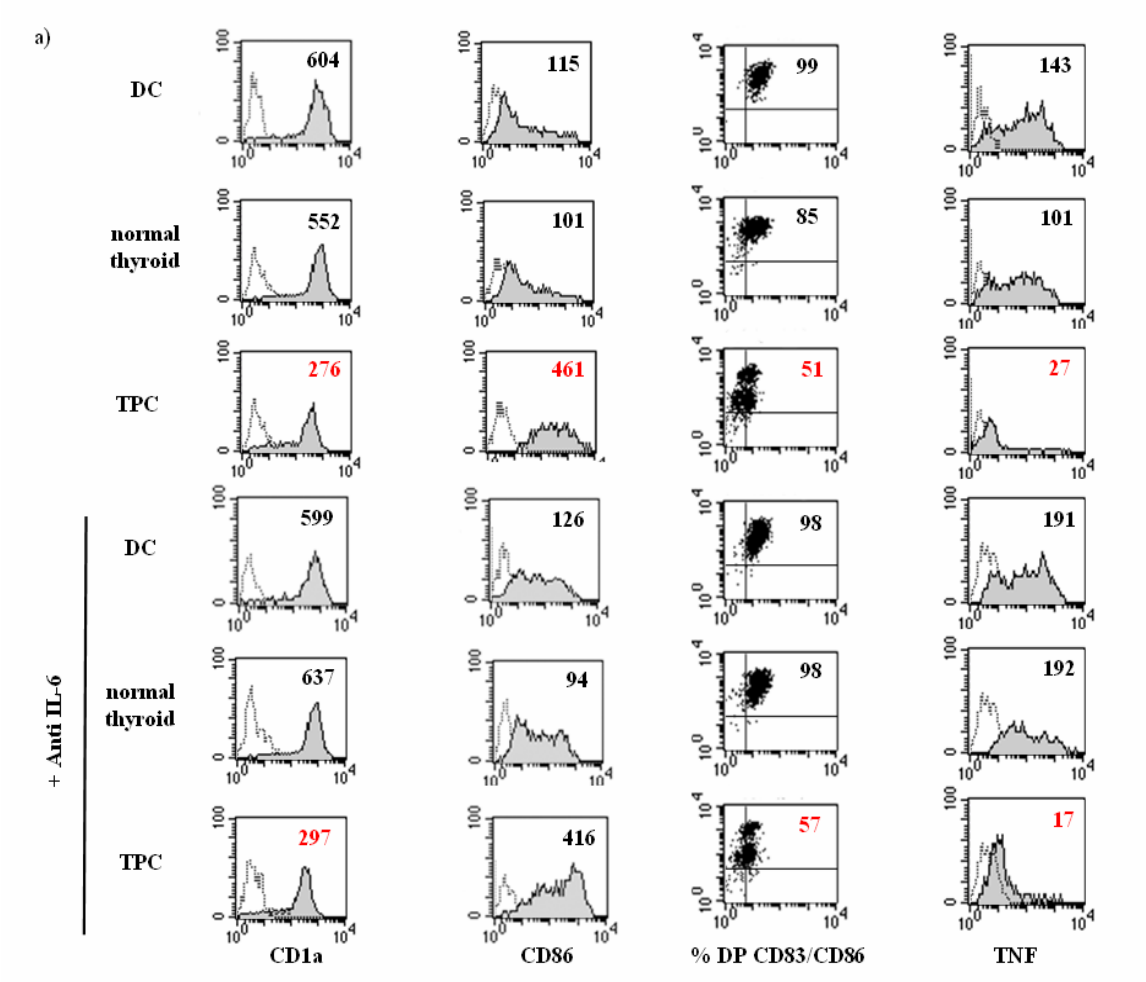


Fig.4 TDFs induce DC with cytotoxic activity against activated T lymphocytes
a) iDC generated in presence of TPC cell line conditioned medium were collected, washed co-cultured with CFSE labeled Peripheral Blood Lymphocytes (PBL) in a 1:25 ratio, in the presence or absence of anti-CD23/CD28-coated beads (0.5 beads/cell). After 3 days, cells were analyze for CFSE dilution and stained with Propidium Iodide (PI). A typical dot plot analysis has been shown in panel a. Panel b reports mean and SD of viable T-cell calculated on 5 independent experiments.
c) Histograms report mean and SD of the percentage of viable T cells calculated for five independent experiments.

Neutralization of IL-6, IL-10, TGF-β, VEGF, EGF, IDO do not revert the effects induced by TDFs on DC.

To verify the contribute of well known TDFs on DC differentiation, we added neutralizing antibodies to TDFs before being added to monocytes culture. IDO activity was blocked by using the anti-metabolite drug 1-methyl-DL-tryptophan. None of the above mentioned treatments were able to interfere with the immunomodulatory activity of TDFs (fig. 5).



c)

		CD1a (MFI)	CD86 (MFI)	DP CD83/CD86 (% of gated)	TNF (MFI)
	anti IL-10	210	396	55	11
	anti TGF-β	193	401	54	14
	anti VEGF (BEVACIZUMAB)	240	435	52	23
	anti EGFR (CETUXIMAB)	187	412	46	12
	1-Me-Trp	231	387	54	8
	TPC	193	422	49	13
control treatments	normal thyroid	638	169	97	93
control treatments	none	598	214	94	85

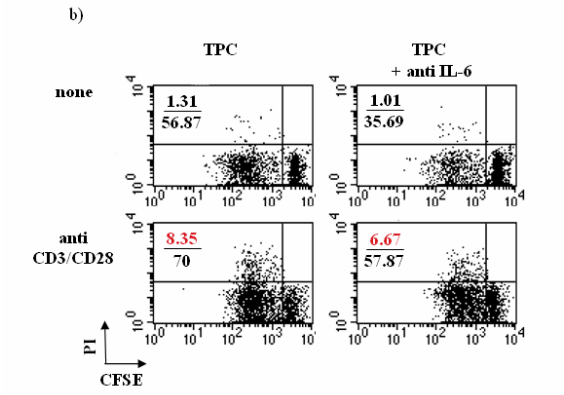


Fig.5 Neutralization of IL-6, IL-10, TGF-β, VEGF, EGF and IDO activity does not interfere with the immnuomodulatory activity of TDFs.
iDC were generated in the presence or absence of 10% TPC cell line or normal thyroid conditioned medium in the presence or absence of 5μg/ml of the reported neutralizing antibodies . After 7 days culture cells were collected, washed and exposed to LPS for 24 hours to induce maturation. BFA (5μg/ml) was added to cells to detect TNFα production. iDC were analyzed for expression of CD1a and CD86. mDC were analyzed by double staining for CD83/CD86, and intracellular staining for TNFα production. All molecules were detected by cytofluorimetric assay. Data shown are representative of three similar experiments. b) Typical dot plot analysis of T cells cultured for 3 days with anti-CD3/CD28 beads and in the presence of DC generated in the presence of 10% of TPC conditioned medium in the presence or absence of anti-IL-6. PBL alone and with anti CD3/CD28 was used as control (not shown). Data shown are representative of three independent experiments. c) Tabel c report the results of the neutralization of TDFs in the presence of the indicated molecule. Data shown are representative of three independent experiments.

Ciprofloxacin treatment completely reverts TDFs immunomodulatory activity

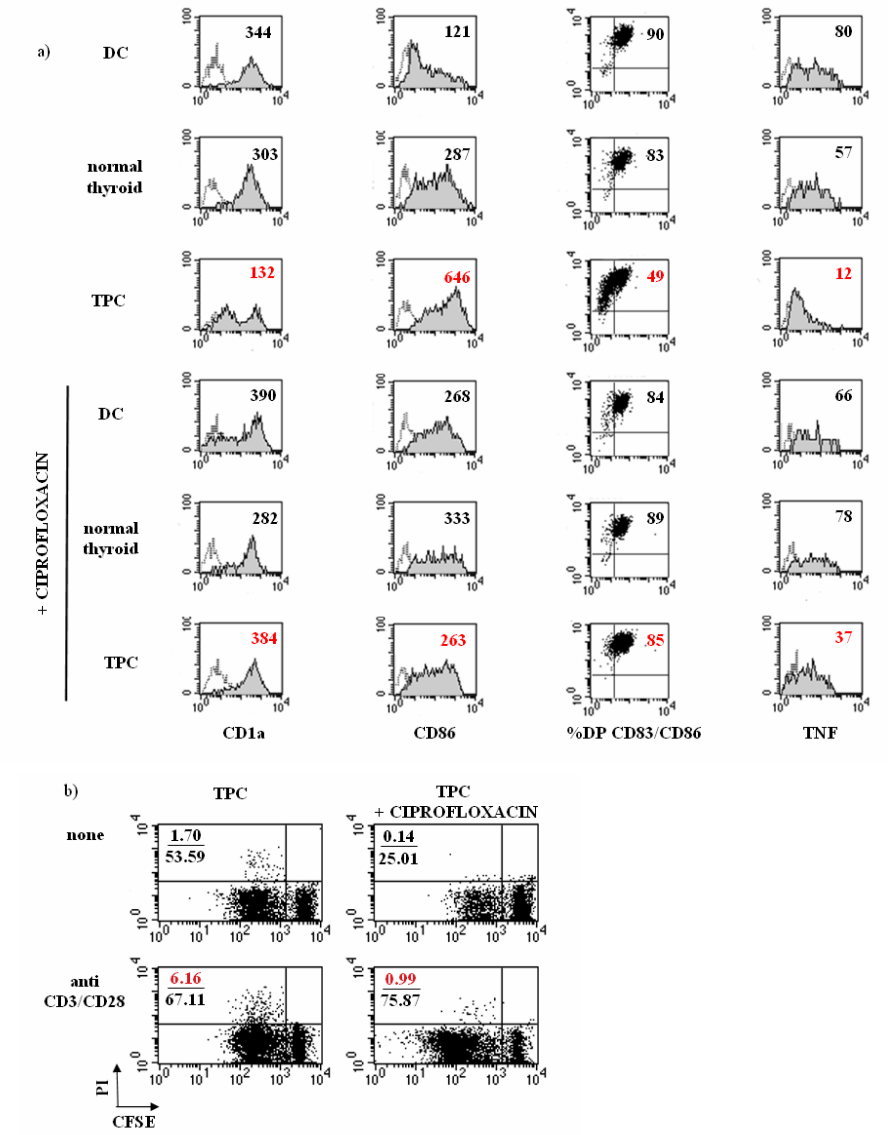


Fig.6 *Mycoplasma eradication abrogates the immunomodulatory activity of TDFs.* Typical immunophenotype of DC generated in the presence of the reported conditioned medium. b) Typical dot plot analysis of T cells cultured for 3 days with anti-CD3/CD28 beads and in the presence of DC generated in the presence of 10% of Ciprofloxacin-treated or untreated TPC conditioned medium.

TPC cell line was treated with antibiotic drug Ciprofloxacin for 15 days, and after a washout time were growth at 80% of confluence in regular cell medium before being washed and cultured for additional 24 hours in fresh medium. After 18 hours, tumor-conditioned media were collected and tested for the presence of Mycoplasma infection and for the ability to affect differentiation of monocytes toward DC lineage. The biological assay confirmed the eradication of mycoplasma from TPC. CD14+ monocytes were cultured in the presence of Treated-treated conditioned TPC medium, in the presence of DC lineage differentiating cytokines. After 7 days culture, iDC were analyzed for their immunophenotype (Fig.6a) and were stimulated with LPS to induce terminal differentiation. After 24 hours, expression of activation markers, production of pro-inflammatory cytokines and ability to induce death of CD3/CD28 stimulated PBL were analyzed by flow cytometry (Fig.6b). iDC generated in the presence of treated TPC conditioned medium displayed an immunophenotype comparable to the normal thyroid and untreated DC, with restoration of CD1a expression, low CD86 basal level and undetectable CD83 (Fig.6a). Up-regulation of CD83 and CD86 after a 24 hours bacterial LPS stimulation is up to 90% and the production of TNFα was perfectly restored (Fig.6a). Co-culture of DC with CFSE-stained PBL shown PI positively lower than 1% after 3 days culture (Fig.6b).

Thyroid TDFs induce loss of function of TLR4 signaling

DC obtained culturing for 7 days monocytes with GM-CSF and IL-4 in presence or absence of TPC tumor conditioned medium were washed and exposed to LPS. The expression of proteins related to TLR-4 signaling pathway, were analyzed by western blot at the time point indicated in Fig.7. LPS binding to TLR-4 induce a complex event of intracellular signaling, leading to the activation of MAPK cascade and NF-Kb activation. We found that JNK and p38 phosphorylation is widely impaired, while accumulation of the phosphorylated form of ERK and CREB were not affected by in TDFs-exposed DC (Fig.7). On the other hand, TDFs-exposed DC loss the ability to decrease IkB levels upon stimulation with LPS (Fig.7). Moreover, we investigate the role of STAT-3. Furthermore, phospho-STAT-3 was undetectable in unstimulated DC, while unstimulated TDFs-exposed DC show higher p-STAT3 levels compared to DC generated in the presence of normal thyroid supernatants of just in regular medium supplemented with the differentiating cytokines cocktail.

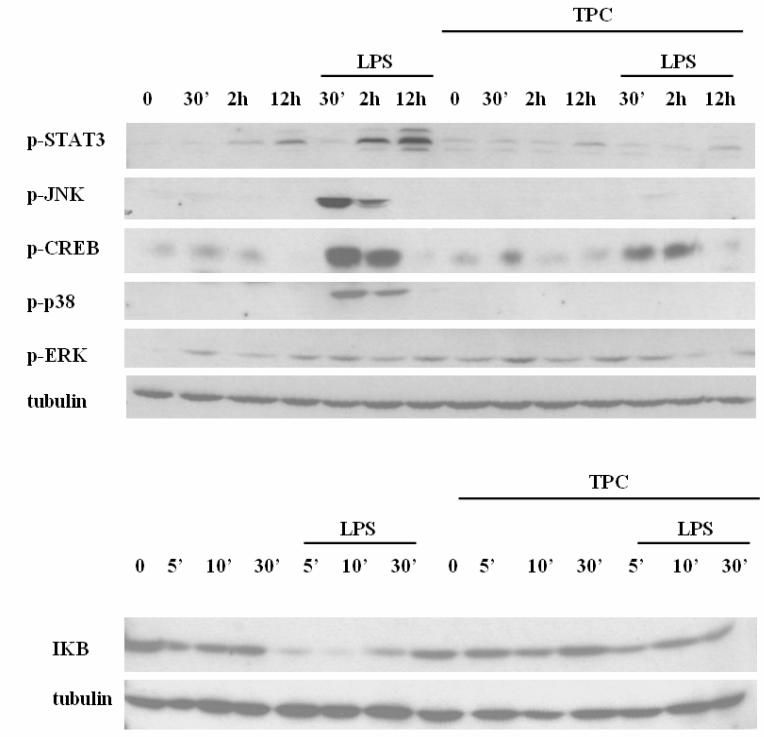


Fig.7 Thyroid TDFs induce loss of function of TLR4
Typical immunoblot of DC generated in the presence or absence of TPC-TDFs and left in regular medium for the reported time in the presence or absence of LPS. The results are representative of four independent experiments.

The capability of human tumor cells to interfere with the differentiation program of DC has been shown in a variety of experimental models, either *in vivo* or *in vitro.* Notably, a large fraction of these studies documented the ability of cancer cells to dissociate the terminal differentiation program by generating the unconventional $CD1a^{low}$ $CD86^{high}$ DC phenotype that fail to respond appropriately to TLRs ligands with secretion of pro-inflammatory cytokines (Roth P 2000). In the present study, we confirm and extend these data to thyroid cell lines recapitulating almost all alterations previously described by other authors, including the defect in TLRs signaling and alterations in phospho-STAT-3 accumulation, a hallmark of DC generated in the presence of TDFs (Nefedova Y 2005, Nefedova Y 2004, Wang T 2004). However, our study takes into account, beside TDFs, an additional factor that has been poorly investigated by most of the previous studies: the presence of mycoplasma in cancer cells. Unexpectedly, and in agreement with a few other reports, this parasite seems to play a crucial role in the mechanism leading to the generation of unconventional immunosuppressive DC. The biological significance of this finding is crucial to understand the exact contribute of TDFs and mycoplasma in the orchestration of the immune response against tumors.

Mycoplasmas infection *per se* is able to induce alterations of dendritic cells differentiation and functions. Several reports documented the ability of DC to sense mycoplasma infection, displaying up-regulation of maturation markers CD83 and CD86 (Salio M 2000, Link C 2004) and, more specifically, the capability of M. *fermentans* to activate cells of the monocytes/macrophages lineage (Muhlradt PF 1997). In addition, there are evidences that suggest that M.*fermentans* could act as accessory factors in the activation of AIDS (Blanchard A 1994, Lo SC 1990). The ability of Mycoplasma to interfere with monocyte toward DC differentiation is related, at least in part, to proteins that act as TLR ligands, as the lypoprotein M161Ag of M.*fermentans* that can bind and activate TLR2 (Nishiguchi M 2001). Actually, Mycoplasma is considered as maturation inducer factor for human monocyte-derived DC (Salio M 2000, Nishiguchi M 2001) as well as for murine DC (Link C 2004). At the same extent of previous reports, we find that DC exposed to mycoplasma-infected thyroid tumors conditioned medium exhibit a CD83+CD86high phenotype which is considered characteristic of terminal differentiation. In accord to previous observations (Salio M 2000), we also show that they fail to further up-regulate CD83 and CD86 upon LPS exposure. Moreover, it is reported that M.*fermentans* is a strong inducer of pro-inflammatory cytokines release by monocytes and DC (Link C 2004, Weigt H 2003).

The presence of mycoplasma in our experimental model is not conceived to investigate the role of this parasite per se, but as a symbiont of tumor. As a conditional pathogenic organism, mycoplasmas have been associated with a

variety of diseases, but the association with cancer remains still unclear. Persistent infection on mammalian host is capable to induce irreversible transformation of cell lines, increase tumor invasiveness and induce alteration in gene expression with features of classical carcinogens. In the initial phases of this interaction, there is a reversible stage during which removal of the mycoplasma cell culture infection leads to a restoration of eukaryotic cell function; there is no apparent chromosomal loss or translocation. After chronic infection, the transformation may become irreversible, and it is then associated with chromosomal alterations. With M. *fermentans* as the initial activator, cells develop altered morphology and unrestricted growth. This may require several weeks of interplay between cell and bacterium. When the transformation reaches the irreversible phase, removal of the mycoplasma does not have beneficial effects (Feng SH 1999, Zhang 2006, Tsai 1995). These reports suggest the hypothesis that mycoplasma could be considered an oncogenic factor for itself. Today, evidences linking cancer to mycoplasma infection are accumulating (Huang S 2001, Chan PJ 1996) and suggest the possibility of an association between the two, given also the data that support highest mycoplasma presence in the more advanced stadiums of cancers (Huang S 2001). Parasites as cofactors of human diseases are not a novel concept. One example concerns the well known relationship between Helicobacter pylori and gastric cancer (Cimolai N 1995).

Perhaps of lesser apparent importance, however, is the potential for microbes to act as cofactors, even weak ones, in disease causation (Pagano 1999). The study of cancer is complete with many examples where cofactors may work in concert to increase the risk for a given disease (Shirai 1993). In the course of either mycoplasma infections or other primary infections, a role for mycoplasmas as cofactors is recognized (Cimolai et al. 1995). Could this also be translated into the possibility that mycoplasmas may act as cofactors in malignancy? For example, certain human papillomaviruses are associated with the progression of pre-malignant changes that lead to cervical cancer. Could genital mycoplasmas modify the rate of progression when the virus is present (Kidder et al. 1998)?

Our study analyzes for the first time the interplay between mycoplasma and tumors from an immunological point of view, focusing on DC role in this symbiosis.

Conditioned medium of mycoplasma-infected thyroid tumor cells lines represent the microenvironment to which monocytes are exposed during the migration to the tumor site and the subsequent differentiation toward DC pathway.

According to previous reports, we obtained unconventional DC, characterized by early up-regulation of CD83, CD86, CD80, HLA-DR and low expression of CD1a (Roth P 2000). However, this maturation process was ultimately defective, resulting in DC that failed to produce TNFα and IL-12 after stimulation by LPS, failed to activate TLR-4 and TLR-2, and stimulate rapid apoptosis of

effector/memory T lymphocytes.

Recent studies have demonstrated an important role of the STAT-3 pathway in DC differentiation under physiological conditions and in cancers. Laouar et al reported, using conditional knockout mice, that STAT-3 is required for Flt-3 ligan-depend DC differentiation (Laouar 2003). At the same time Yu and Gabrilovich research groups demonstrated the hyperactivation of STAT-3 signaling is directly involved in the abnormal DC differentiation in cancer (Wang T 2004, Nefedova Y 2004). At the present state STAT-3 is recognized as negative regulator of DC activation and a hallmark of cancer induced DC defects. Consistent with these previous reports, we found that TDF of mycoplasma-infected thyroid cell lines induce a basal hyperactivation of STAT-3. We further extended these observations, investigating for the first time the role of this molecule upon LPS induced terminal differentiation. Our findings demonstrated that activation of STAT-3 strongly occurs after 24 hours from exposure to bacterial endotoxin, and that TDFs induce a dramatic loss in the ability of DC to phosphorilate this molecule.

Bacterial endotoxin induced maturation is tightly regulated at the level of the TLR-4 signaling pathway. On the basis of the inability of DC generated in the presence of mycoplasma infected thyroid cell lines conditioned medium to properly respond to LPS, we analyzed the integrity of the signal transduction pathway of TLR-4. We showed that, although normal expression of TLR4 mRNA (data not shown), the signaling through this receptor is disrupted, with little or no activation of classical MAPK/p38 pathway and failure to degrade the inhibitory subunity of NF-kB, IKBα. Therefore, it is demonstrated that DC can assume an endotoxin tolerogenic phenotype when they are exposed to maturation signals during their differentiation process. Resulting cells display high Il-10, low IL-12 secretory phenotype after a second exposure to the same maturation stimulus. (Jiang HR 2002, Rieser C 1998)

Previous reports shown that DC exposed to TDFs promote the development of early, but ultimately less potent, allostimulatory activity in monocyte-derived DC (Roth P 2000). Recent evidences described generalized suppression of CTL anamnestic response in mice bearing large tumor nodules, ascribing these anomalies to the activity of Myeloid Suppressor Cells (MSC) (Apolloni E 2000). We analyzed the ability of DC generated in the presence of mycoplasma infected thyroid tumor cell lines conditioned medium to activate allogeneic lymphocytes and induce activation-dependent cell death. We found that TDFs induce a potent cytotoxic activity in DC, leading to clonal deletion of CD3/CD28 activated effector/memory T lymphocytes. The mechanism by which DC induce lymphocyte death is not contact dependent, as clearly demonstrated by transwell experiments (data not shown).

To dissect the relative contribution of thyroid TDFs and mycoplasma to the defects

induced in DC differentiation and function, we tried to neutralize the most well know TDFs, that are shown to have a broad influence on DC differentiation, and, on the other hand, to eradicate mycoplasma infection from cell lines by ciprofloxacin treatment. Despite the big number of evidences that support the critical role of TDF on DC tumor-induced defect, neutralization of IL-6, IL-10, VEGF, EGFR, TGF-β, IDO was completely ineffective in the rescue of phenotype and function. At the contrary, conditioned medium obtained from cells treated with ciprofloxacin lead to complete restoration of DC classical features.

The finding that mycoplasma contribute exerts a dominant effect with the respect to TDFs, open new speculations regard the symbiosis between mycoplasma and tumors. We hypothesize that tumors act as a mycoplasma bioreactor.

Thyroid tumors are the most common malignancies of the endocrine system and include a broad variety of lesions with different biological and clinical behavior: benign adenomas and well differentiated (papillary and follicular), poorly differentiated and undifferentiated (anaplastic) carcinomas (Kroll et al., 2002). In 2002 (Batistatou et al. 2002) has been demonstrated that S100+ DC are present in thyroid tumors, with an inverse correlation with the respect to the prognosis and the onset of disease. Papillary Thyroid Carcinoma (PTC) is the most DC infiltrated tumors, while Poorly Differentiated Carcinoma (PDC) and Undifferentiated Carcinoma (UC) are poor of S100+ DC. More recently, an extensive characterization of DC infiltrating thyroid tumors shown that tumors with poor prognosis (PDC, UCs) were characterized by markedly reduced DC CD1a+infiltrates, strongly suggesting that the presence of fully differentiated DC exerts a protective role. In addition, they demonstrate that S100+ and CD1a+ DC localize preferentially in the thyroid nodule, not outside the tumor nodule. Thyroid tumors *in vivo* produce a number of DC chemoattractant molecules, such as RANTES, IP-10, MIP-1A, MIP-1B, MIP-3a. We can hypothesize that DC recruited in the mycoplasma infected tumor site are skewed to an immunosuppressing route by exposure to mycoplasma and mycoplasma-derived factors. Immunosuppressive rather than immunostimulatory DC induce death of activated T lymphocytes, inhibiting the subsequent clonal expansion and the immune response. Presence of a mycoplasma in tumors might be a function of the potential for opportunism with the bacterium entering systemically during the immunosuppression of the acute malignancy. Mycoplasma indoved in the tumor tissue could easily survive due to the abundance of metabolites derived from the accelerated growth of tumor cells, and at the same time might be efficacy protected from the activity of the immune sytem. At the same time, it can exploit the tumoral privileged niche to exert immunosuppressive functions, allowing new strategies for immune escape, based on DC alterations.

Despite the many publications that have discussed mycoplasmas in the

context of cancer, it is evident that this area has suffered from a lack of attention. In part, mycoplasmas have been seen by some as fastidious bacteria that are difficult to work with. Others in the context of biology laboratories may shun work with mycoplasmas because of the potential for mycoplasmas to contaminate cell cultures.

Our study shed new light about the synergy between malignancies and mycoplasma infection, underlying the potent immunosuppressive effect that this symbios could exert and suggesting that pharmacological treatment of mycoplasma infection would be considered as a mandatory immunotherapeutic strategy to improve the response against tumor cells.

CONCLUSIONS

Given the role of DC in the initiation and outcome of immune response, the effects that thyroid tumors conditioned medium exert on this cellular population spare on the entire ongoing of immune response. DC are not only enabled to initiate a correct immune response, but are also skewed to an immunosuppressing route. The immunosuppression is mediated by an aberrant cytokine pattern, and by target killing of activated T lymphocytes. From this point of view, tumors are mycoplasma incubators,that allow the growth and spread of this microorganism. Mycoplasma use tumors as “Troy Horse”, by the fact that can easily survive in the glucose rich and metabolically hyperactive tumor environment, which is also a privilegiate niche that can protect it from the immune system defence. By the way, tumors, that are themselves able to escape the immune surveillance, exploit mycoplasma immunosuppressing effect which improve this capacity in a synergic fashion.

We can ask if tumors develop from mycoplasma-dependent oncogenic activity, or if mycoplasma infection established on an already present neoplastic environment accelerate transformation progress. Most likely, the infection develops on the neoplastic environment owing of the low immune system activity. In this environment mycoplasma established a symbiontic relationship with tumor, which induce an increased escape of immune system activity and an improvement of neoplastic effect derived by mycoplasma.

So our study shows for the first time a synergic effect between mycoplasma and tumor on DC cells immunosuppressive activity. This result could be relevant for the *in vivo* progression of tumor and suggest that pharmacological treatment of mycoplasma infection could be a strategy to improve the immune response to tumor chemotherapy.

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