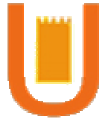

UNIVERSITÀ DEGLI STUDI DI ROMA "TOR VERGATA"

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Doctoral thesis in Immunology and Applied Biotechnology

XXII CYCLE

Adaptive immunity in a human chronic inflammatory disorder: phenotypic and functional characterization of T lymphocytes in a cohort of Chronic Granulomatous Disease patients.

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To my family

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ABBREVIATIONS

APC: antigen presenting cell
BAL: bronchoalveolar lavage
BCG: bacillus Calmette–Guérin
CGD: chronic granulomatous disease
CIA: collagen-induced arthritis
CMV: cytomegalovirus
CTMX: trimethoprim-sulphamethoxazole
DC: dendritic cell
DHR: dihydrorhodamine 123
DOK8: cytokinesis 8
EAE: experimental allergic encephalitis
FITC: fluorescein isothiocyanate
GVHD: graft versus host disease
HIES: hyper IgE syndrome
H_2O_2 : hydrogen peroxide
IBD: inflammatory bowel disease
IDO: indoleamine 2.3-dioxygenase
IFN-γ: interferon γ
IL-1β: interleukin 1β
IL-4: interleukin 4
IL-6: interleukin 6
IL-10: interleukin 10
IL-17A: interleukin 17A
IL-17F: interleukin 17F
IL-23: interleukin 23
IL-12: interleukin 12
MPO: myeloperoxidase
MS: multiple sclerosis
NADPH: nicotinamide adenine dinucleotide phosphate
NBT: nitroblue tetrazolium test
NCF1: neutrophil cytosolic factor 1
NOX: NADPH oxidase
$OH^{\bullet}$: hydroxyl radical
$O_2^{\bullet -}$: superoxide anion

PBMC:	peripheral blood mononuclear cells
PCR:	polymerase chain reaction
PE:	R-phycoerythrin
PHA:	phytohaemaglutinin
RA:	rheumatoid arthritis
RORγt:	retinoic acid-related orphan receptor γt
ROS:	reactive oxygen species
RT-PCR:	real-time PCR
SD:	standard deviation
SH3:	src homology 3 domains
SI:	stimulation index
SOD:	superoxide dismutase
STAT3:	signal transducer and activator of transcription 3
TGF-β:	transforming growth factor-β
TYK2:	tyrosine kinase 2

ABSTRACT

Chronic granulomatous disease (CGD) is a rare primary immunodeficiency syndrome characterized by mutations in one of the four genes that encode the subunits of NADPH oxidase, a enzyme principally involved in the ROS mediated pathogen killing. The patients are characterized by life-threatening and recurrent infections mainly by catalase-positive microorganisms, due to the inability of their phagocytes to express a respiratory burst against invading bacteria (e.g. *Staphylococcus aureus*) and fungi (e.g. *Aspergillus fumigatus* and *Candida albicans*). Patients, also suffer from a variety of sterile inflammatory conditions as acute and/or chronic inflammation with fibrosis containing non-caseous granulomas in the intestinal tract, liver, lymph nodes, urogenital tract, skin, and brain, most probably caused by an intrinsic dysregulation of the inflammatory mechanisms that can involve other cell types than the known defective phagocytes.

Although in literature there are few immunological studies about cell function in CGD, a recent work on CGD animal model, has highlighted that in acute fungal infection, a dysregulation of IL-17-secreting cells, coupled with a decrease IFN-γ-producing effector cells and of IL-10-producing regulatory cells, could have a pathogenic role. Moreover, a decreased frequency of a specific T lymphocytes compartment, called Th17, for their characteristic IL-17A production, was associated with increased susceptibility to

fungal end bacterial infection in several human chronic disease as hyper IgE syndrome (HIES) and chronic mucocutaneous candidiasis.

In this study, we have investigated the potential involvement of T lymphocyte deregulation in CGD manifestations, by comparison of proliferation, mRNA expression, cytokine production and T lymphocyte phenotype upon fungal and bacterial antigen stimulation, of peripheral blood mononuclear cells (PBMC) between a cohort of CGD patients and of healthy controls.

Despite a very large variability in functional responses found in both patient and controls, we showed a statistical significant increased of proliferation upon *C. albicans* lysate and of IL-10 production upon *A. fumigatus* in CGD patients, coupled with a slight increase of IFN-γ and IL-17A upon fungal stimulation.

Furthermore, upon *A. fumigatus* in the CDG cohort was found a statistically significant increased of IL-17A production, excluded one patient with chronic inflammation, and increased IL-17A mRNA expression, in agreement with an involvement of IL-17A$^+$ cells in CGD pathogenesis, as shown by the CGD animal model of invasive aspergillosis.

We also observed that, in agreement with a protective role of IFN-γ, CGD patients that experienced severe aspergillosis expressed low IFN-γ production compared with other patients, but similar to controls, while patient that not experienced severe aspergillosis had increased IFN-γ production compared to controls, demonstrating that

a compensatory IFN-γ increased was need to protect patients with defect of ROS-mediated killing.

Concerning the responses to *C. albicans* yeast and hyphae stimulation, we observed similar amount of IFN-γ and increased of IL-17A and IL-10 levels, compared to controls, showing that probably different pathological mechanism lead to increased *Candida albicans* infections in CGD with respect to HIES, because it would not be dependent on a decrease of IL17A response to fungal antigens.

Moreover, in comparison to CGD patients, HIES patients expressed lower value of IFN-γ for all antigens, but differently to STAT3-mutated HIES patient, that not express IL-17A upon all kinds of stimulation, in STAT3-wt HIES patient IL-17A production upon both *C. albicans* yeast and hyphae was similar to CGD and increased compared to age-matched healthy controls. Thus the reduced frequencies of *C. albicans* infection in CGD compared to STAT3-wt HIES, may be explained through the main protective level of IFN-γ. Moreover we believe that *in vitro* immunological test can help us to exclude a STAT3 mutation, in patients who have a positive score for HIES, before performing a molecular analysis of the gene.

Key words: *Aspergillus fumigatus*, *Candida albicans*, chronic granulomatous disease (CGD), hyper IgE syndrome (HIES), IFN-γ, IL-17A, NADPH oxidase, reactive oxygen species (ROS), Th17 cells, T lymphocytes.

RIASSUNTO

La malattia granulomatosa cronica è una rara immunodeficienza primitiva, dovuta a mutazioni di uno dei quattro geni che codificano per le componenti catalitiche e regolatorie della NADPH ossidasi, un enzima coinvolto nella produzione dei ROS, necessari per mediare l'uccisione del patogeno fagocitato da neutrofili, monociti e macrofagi. I pazienti sono quindi caratterizzati dall'avere un'aumentata suscettibilità ad infezioni dovute a microrganismi catalasi-positivi come batteri (es. *Staphylococcus aureus*) e funghi (es. *Aspergillus fumigatus* e *Candida albicans*). Inoltre, i pazienti soffrono di infiammazioni acute e croniche, con fibrosi e formazione di lesioni granulomatose in vari organi quali fegato, pelle, tratto digerente e urogenitale, linfonodi e nei casi più gravi cervello. Questi danni cronici, non direttamente riconducibili ad infezioni microbiche, sono probabilmente causati da alterazioni delle risposte immunitarie che coinvolgono anche altre cellule diverse dai fagociti.

Ci sono pochi studi immunologici sui linfociti nei pazienti affetti da CGD, ma un recente lavoro condotto sul modello murino di CGD con aspergillosi invasiva, ha mostrato che nella fase acuta dell'infezione, i linfociti produttori di IL-17A vanno incontro ad una espansione incontrollata, con una ridotto numero di cellule effettrici IFN-γ $^{+}$e di cellule regolatorie IL-10^{+}. Inoltre la diminuita frequenza di linfociti Th17, produttrici di IL-17 è stata associata con l'aumento

di infezioni batteriche e di *Candida* in due malattie croniche come la sindrome da iper IgE (HIES) e la candidiasi mucocutanea cronica.

In questo studio abbiamo investigato il potenziale coinvolgimento dei linfociti T nella patogenesi della CGD, comparando: test di linfoproliferazione, quantificazione dell'espressione del mRNA e della produzione delle citochine, e l'analisi del fenotipo linfocitario, tra pazienti e controlli sani.

Nonostante la grande variabilità nella risposta tra tutti i soggetti analizzati, i pazienti hanno mostrato un significativo aumento di proliferazione indotta dal lisato di *C. albicans* e un significativo aumento nella produzione di IL-10 con stimolazione da parte d *A. fumigatus*, ma anche un lieve aumento di produzione di IFN-γ e IL-17A dopo stimolazione con antigeni fungini, rispetto alla popolazione di controllo.

L' aumentata produzione di IL-17A diventava significativa quando dalla corte dei pazienti si escludeva un paziente particolare, che aveva un'infiammazione cronica intestinale. Anche l'espressione del mRNA per l'IL-17A è stata trovata aumentata nei PBMC non stimolati dei pazienti, rispetto ai controlli, indicando un possibile coinvolgimento di questa citochina nella patogenesi della CGD come mostrato nel modello animale.

Inoltre, in accordo con il suo ruolo protettivo nell'infezione da *A. fumigatus*, la produzione di IFN-γ indotta dal fungo, era ridotta nei pazienti che avevano subito un'aspergillosi, rispetto agli altri CGD,

ma simile rispetto ai controlli, mentre i pazienti che non avevano avuto aspergillosi, producevano livelli più elevati d'IFN-γ rispetto ai controlli, indicando che un aumentato livello d'IFN-γ è probabilmente richiesto per compensare la ridotta degradazione del fungo presente nei pazienti con CGD.

Rispetto ai controlli, i PBMC dei pazienti stimolati con lieviti e ife di *C. albicans*, producevano livelli simili d'IFN-γ, ma aumentati livelli di IL-17A e IL-10, indicando che l'aumentata frequenza alle infezioni di *Candida* nei CGD forse richiede un meccanismo diverso rispetto alla HIES , che non dipende dalla diminuita frequenza di linfociti Th17.

I PBMC dei due pazienti con HIES, rispetto a quelli dei CGD, producevano livelli più bassi d'IFN-γ dopo stimolo con tutti gli antigeni. Per quanto riguarda l'IL-17A, diversamente dal paziente HIES con mutazione di STAT3 che non produceva affatto, il paziente HIES senza mutazione, dopo stimolazione con lieviti e ife di *C. albicans* produceva livelli di IL-17A simili a quelli trovati per i pazienti CGD e maggiori rispetto a quelli dei controlli. Quindi è plausibile che la ridotta frequenza d'infezioni da *Candida* nei pazienti CGD, rispetto ai pazienti HIES, sia dovuta alla maggior protezione mediata da più alti livelli d'IFN-γ. Inoltre, poiché i pazienti HIES con mutazione STAT3 non producono IL-17A, noi pensiamo che l'utilizzo di test immunologici *in vitro*, possa servire a

meglio indirizzare le future indagini molecolari nei pazienti con sospetta HIES.

Key words: *Aspergillus fumigatus*, *Candida albicans,* cellule Th17, IFN-γ, IL-17A, linfociti T helper, malattia granulomatosa cronica (CGD), NADPH ossidasi, sindrome da Iper IgE (HIES), specie reattive dell'ossigeno (ROS).

INTRODUCTION

THE PHAGOCYTE NADPH OXIDASE

Story, tissues expression and features of NOX-family's members

From 1933, when the respiratory burst was discovered in neutrophils, to 1999, the only molecular complex involved in oxygen consumption (other the mitochondrial oxidative chain) was the phagocyte reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex in which a trans-membrane catalytic core (cytochrome b_{558}) was formed by two subunits called $gp91^{phox}$ and $p22^{phox}$ (from phagocyte oxidase). In 1999, the first of the homologues of $gp91^{phox}$ (NOX 1, from NADPH oxidase) was discovered by searching homology in the public database *(Suh Y. A. et al. 1999)*, and successively cloned from colon epithelial cells. Over the next few years, other three homologues of $gp91^{phox}$ (NOX 3, NOX 4, NOX 5) were cloned in a variety of non-phagocytic cells indicating that phagocyte NADPH oxidase belongs to a family of enzymes named NOX *(Lambeth J. D. 2002)*. $Gp91^{phox}$ was renamed NOX 2 and two other longer homologues, DUOX 1 and DUOX 2 (form dual oxidase) were also identified and enclosed in the NOX family, for a total seven members; NOX 1-5, DUOX 1-2 (fig. 1a and b).

The tissue distribution of this enzymes is very spread *(Krause K. H. 2004)*; while NOX 2 is the true phagocyte NADPH oxidase present in neutrophil, granulocytes, monocyte/macrophages and eosinophils, with microbicidal action, NOX 1 is expressed in the colon, and perhaps in the uterus and prostate, and has an inducible expression in vascular smooth muscle. In the colon it would be able to exert microbicidal function while in the vascular system, its participation in blood pressure regulation appears probable. NOX 3 is expressed in the fetal kidney and in the inner ear, where it seems important for equilibrium-perception through otoconia formation. NOX 4 appears to be the most widely expressed among the various NOX isoforms, it is abundantly expressed in the kidney cortex, but it is also found in endothelial cells, smooth muscle cells, ovary, testis, osteoclasts, fibroblasts and astrocytes. Its function remains elusive, the most popular hypothesis claims a role in the oxygen sensing in the kidney cortex, but a role in regulation of cell proliferation has also been postulated. NOX 5 is essentially found in lymphoid tissues, mammary glands, spleen, cerebrum, sperm and testis, where it could have a function in different stages of spermatogenesis as regulator of transcription factors. DUOX 1 and DUOX 2 are both expressed in the thyroid gland but also in respiratory epithelial (DUOX 1) and salivary and rectal gland epithelia (DUOX 2). In mammals both the enzymes are involved in thyroid hormone synthesis, into thyroid different tissues their functions remain poorly understood.

NOX-family enzymes are present in most of eukaryotic and in all mammalian animals. As showed in figure 1b, all enzymes are composed of six trans-membrane α-helices containing two hemes, in the N-terminal region, and NADPH-binding and FAD-binding domains in the C-terminal cytoplasmic region. NOX 5 and both DUOX enzymes also contains four and two $Ca2^{+}$ binding EF-hand motifs, respectively, with activatory (NOX 5) and inhibitory (DUOXs) $Ca2^{+}$ depending functions. Different from the previous, NOX1-4 need to increase their membrane stability by binding $p22^{phox}$, another trans-membrane protein containing two α-helices.

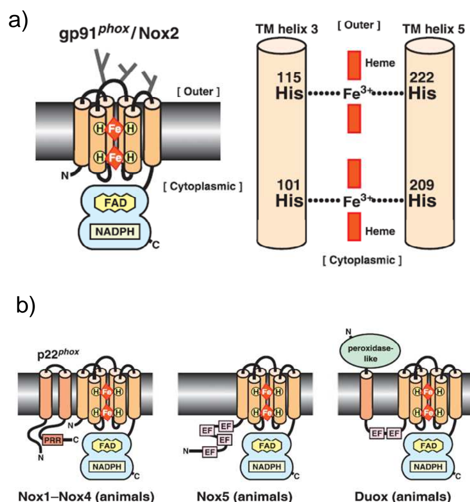


Figure 1. Structure of NOX proteins. Model for the structure of $gp91^{phox}$, cylinders represent six a-helices a), models for structures of various subtypes of Nox-family enzymes. EF: Ca^{2+}-binding EF-hand motif (from *Hideki Sumimoto 2008*).

Phagocyte NADPH oxidase: organization

In the best characterized neutrophil NADPH oxidase, the gp91^{phox}/p22^{phox} complex, called flavocytochrome b_{558}, form a complete apparatus that transports electrons from cytosolic NADPH , *via* FAD and two hemes, to molecular oxygen to the other site of membrane, in the extracellular or intraphagosomal space. Besides flavocytochrome b_{558}, complete NADPH oxidase comprises the small G-protein Rap1A, and the cytosolic regulatory components: p47^{phox}, p67^{phox}, p40^{phox}, the small G-proteins Rac2 and Cdc42, and the newly identified p29 peroxiredoxin, described and characterized in the neutrophils mainly from studies on Chronic granulomatous disease (CGD) *(Lomax K. J. et al. 1989; Dinauer M. C. et al. 1990; Leto T. L. et al. 1990).*

In resting neutrophils, 15% of the flavocytochrome b_{558} is located in the plasma membrane and the remaining 85% within the membrane of the specific granules, gelatinase granules and secretory vesicles (fig 2a) *(Ginsel L. A. et al. 1990).* When the phagocytic cell recognizes microbes, the flavocytochrome b_{558} and Rap1A are translocated in the plasma membrane to perform NADPH oxidase in the future phagolysosome *(Huang J. et al. 1995).* Upon oxidase activation, the cytosolic subunits p47^{phox}, p67^{phox} and p40^{phox} undergoing phosphorylation with conformational changes, translocate to the plasma membrane, and bind with the

flavocytochrome b_{558} complex *(Clark R. A. et al. 1990)*. Additionally, also the small GTPase proteins Rac2 and Cdc42 are involved in the assembly and activation of the NADPH oxidase *(Gabig T. G. et al. 1995)* (fig. 2b).

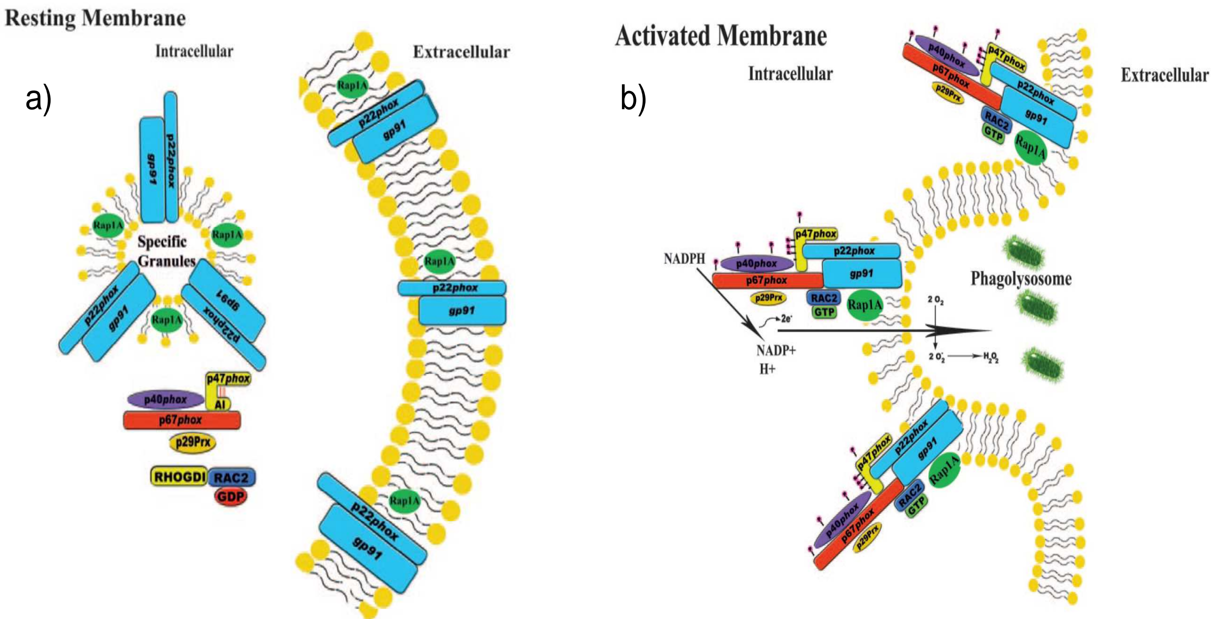


Figure 2. Phagocyte NADPH oxidase. Representative picture of a NADPH oxidase components in resting a) and in activates neutrophils (from *Sheppard FR, et al. 2005*)

NADPH oxidase: catalytic activity regulation

P47^{phox}, or NCF1 (neutrophil cytosolic factor 1), is the subunit responsible for transporting the whole cytosolic regulatory complex (p47^{phox}-p67^{phox} -p40^{phox}) to the docking site during NADPH oxidase activation. Thus, it is considered as the organizer subunit because it coordinates the interaction of the different NADPH oxidase subunits allowing the formation of an active complex. In resting non phosphorylated state, p47^{phox} has a constrained conformation due to the tight intramolecular interaction between its src homology 3 (SH3) domains and its auto-inhibitory region (AIR) *(Yuzawa S. et al. 2004)*. The phosphorylation of p47^{phox}, in its carboxy-terminal tail, relaxes this intramolecular interaction (SH3/AIR), allowing the binding of its cryptic SH3 domains to the proline-rich region of p22^{phox} in the membrane *(Shiose A. and Sumimoto, H. 2000)*. P47^{phox} also has a phox homology (PX) domain which binds to phosphatidylinositol 3.4 biphosphate and phosphatidic acid *(Kanai F. et al. 2001; Karathanassis D. et al. 2002; Stahelin R. V. et al. 2003)*. This binding is important for membrane stability and localization of the complex (p47^{phox}-p67^{phox}-p40^{phox}) and may help to assemble the NADPH oxidase complex at precise sites of the ingested pathogens *(Zhan Y. et al. 2002)*.

P67^{phox} contains a catalytic NADPH binding site for electron transfer to FAD in the cytochrome b_{558} complex *(Smith R. M. et al. 1996)*, therefore it was considered the NADPH oxidase activator.

The p67^{phox} subunit is phosphorylated during activation *(Benna J. E. et al. 1997)*, although to a lesser degree than p47^{phox}. At the membrane, it binds to the cytochrome b_{558} *(Dang P. M. et al. 2001a)* and regulates its activity via the activation domain (AD) *(Nisimoto Y. et al. 1999)* and by interaction with Rac2.

Rac2 is a small G-protein, required for NADPH oxidase activation *(Quinn M. T. et al. 1993)*, that upon activation, rapidly converts from a GDP- to GTP-bound state, dissociates from its inhibitor, and migrates to the membrane *(Abo A. et al. 1994)*.

P67^{phox} also binds P40^{phox} that it is weakly phosphorylated during activation *(Bouin A. P. et al. 1998)*. P40^{phox} is not required for NADPH oxidase activation in a cell-free system but it has a positive stimulatory effect on enzyme activation in vivo *(Kuribayashi F. et al. 2002; Ellson C. D. et al. 2006)*. Activation of the NADPH oxidase system occurs by at least three signalling triggers that involve protein kinases, lipid-metabolizing enzymes and nucleotide-exchange proteins that activate the GTPase Rac. In agreement with the above selective phosphorylation of p47^{phox}, it seems that *in vivo*, depending on the nature of the agonist encountered by neutrophils, a combination of kinases could participate to the phosphorylation of p47^{phox} and to NADPH oxidase activation. Thus PKCζ *(Dang P. M. et al. 2001b)*, PKCβ *(Dekker L. V. et al. 2000)*, PKCδ *(Cheng N. et al. 2007)*, PAK *(Martyn K. D. et al. 2005)*, ERK1/2 *(Dewas C. et al. 2000)* and AKT *(Chen Q. et al. 2003)* were shown to play a

stimulatory role in formylated peptides (fMLF) or PMA-induced NADPH oxidase activation.

Besides the microbial antigens also the cytokines are important to NADPH activation, and proinflammatory cytokines such as GM-CSF and TNFα, which do not activate NADPH oxidase but prime its activation in response to a secondary stimulus such as fMLF *(El-Benna J. et al. 2008)*, induce partial phosphorylation of p47phox on Ser345 by ERK1/2 or p38MAPK and promote NADPH oxidase assembly *(Dewas C. et al. 2003; Dang P. M. et al. 2006).*

To increase membrane binding also lipid modifications are required and phosphatidylinositol 3-kinase (PI3K) and phospholipase D, that produce 3-phosphorylated phosphatidylintositols (PtdInsP) and phosphatidic acid, respectively, providly lipids to which the $p47^{phox}$ and $p40^{phox}$ PX domains bind *(Kanai F. et al. 2001; Zhan Y. et al. 2002)* .

ROS microbicidal activity

In the phagolysosome, in the granule or in the plasma membrane the activity of NADPH oxidase leads to a reduction of molecular oxygen to superoxide anion ($O_2^{\cdot -}$), that in turn provides the source for formation of all toxic oxygen derivatives called " reactive oxygen species" (ROS) *(Rada B. et al. 2008)*, as hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot}$), hypohalous acids as hypochlorous acid

(HOCl), and electronically excited state of molecular oxygen as singlet oxygen ($^1O_2$).

Superoxide anion itself is only weakly reactive and reacts with a few macromolecules, and its direct toxicity is still controversial *(Sawyer D. T. and Valentine, J. S. 1981)*. Superoxide is unstable, cannot reach very far from the site of its production, and is not able to cross lipid bilayers.

Hydrogen peroxide is formed by the spontaneous or superoxide dismutase (SOD)-catalyzed dismutation of superoxide. Two superoxide anions will produce one hydrogen peroxide molecule by incorporating two hydrogen ions and releasing one molecule of oxygen. Hydrogen peroxide is a well-known oxidizing agent capable of reacting with a wide range of macromolecules, it is relatively stable and membrane permeable, so it can diffuse away from the site of its formation.

Although hydrogen peroxide is reactive, its toxicity can be increased dramatically by forming further derivatives when reacts with myeloperoxidase (MPO), an enzyme expressed only in polymorphonuclear leukocytes, monocytes, and certain types of macrophages. MPO resides in the primary granules of neutrophils and is released into the phagosome upon engulfment of bacteria. MPO catalyzes the oxidation of different substrates by hydrogen peroxide. The most common substrates are halides: chloride,

bromide, fluoride, or iodide, that undergo oxidation by hydrogen peroxide into hypohalous acids.

Due to the high concentrations of chloride in the phagosome, hypochlorous acid is formed in the highest amounts. Hypochlorous acid is a strong oxidizing agent, it attacks a broad range of biologically relevant compounds, but the preferred targets are: thiols, thioesters, amines, phenols, unsaturated bonds; it is membrane permeable *(Hampton M. B. et al. 1998).*

Although formation of $OH^{\cdot}$, a very reactive radical occurs in vitro from hydrogen peroxide catalyzed by iron, its contribution to microbial killing remains doubtful since the iron released into the phagosome by either the bacteria or the neutrophils is bound to lactoferrin *(Britigan B. E. et al. 1989).*

Singlet oxygen is a highly reactive and short-lived radical attacking a wide range of biomolecules. Although singlet oxygen has been suggested to be present in neutrophils' phagosome, the contribution of singlet oxygen to killing in neutrophil phagosome is still difficult to interpret due to imperfect specificity of singlet oxygen scavengers *(Clifford D. P. and Repine, J. E. 1982).*

The anti-microbial function of ROS is well explained in human, where mutations on $gp91^{phox}$, $p22^{phox}$, $p47^{phox}$, $p67^{phox}$ and Rac, that impair NADPH oxidase activity, lead to CGD, characterized by increase susceptibility to catalase positive microbial infection and hyperinflammation. But elimination of potentially pathogenic

microorganisms is a complex process that requires directed migration, phagocytosis, production of toxic oxygen (and nitrogen) metabolites, and release of antibacterial proteins and enzymes stored in the different granule types. Thus, elimination of microorganisms can not be ascribed to any single function or protein; it should rather be regarded as a joint action of several equally important mechanisms.

CHRONIC GRANULOMATOUS DISEASE (CGD)

Genetics of CGD

Chronic granulomatous disease (CGD) is a rare primary immunodeficiency syndrome (estimated incidence of 1/200000 to 1/250000) characterized by mutations in four genes that encode subunits of NADPH oxidase (gp91^{phox}, p22^{phox}, p47^{phox}, p67^{phox}) *(Segal B. H. et al. 2000).*

Dependently from the locus of the mutated gene, the inheritance can be distinguished in recessive x-linked and autosomal recessive.

The X-linked recessive transmission of CGD, characterized by mutations in the CYBB gene (on the short arm of chromosome X) encoding gp91^{phox}, is the most frequent form of CGD (X91-CGD) (approximately 65-70% of cases). This type of CGD is due to a heterogeneous group of mutations gathered in two database (the

Human Gene Mutation Database (HGMD) *(Stenson P. D. et al. 2003)* and the Immunodeficiency database (ID) *(Piirila H. et al. 2006)* that includes: single-nucleotide substitutions (missense or nonsense mutations including splicing or not) account for 58% of the defects; small deletions, insertions, and insertion–deletions account for 26% and large deletions and insertions for 14%. These mutations can strike all domains of $gp91^{phox}$, and they can lead to a lack of protein expression ($X91^0$ phenotype) in the most of cases (55%), or to an incomplete loss of protein and partial dysfunction ($X91^-$ phenotype) *(Heyworth P. G. et al. 2001)* in less than 5% of cases, and finally the mutations, principally located in the COOH terminus tail of protein, can lead to a normal expression of mutated gp91phox ($X91^+$ phenotype) for the another 5% of cases *(Stasia M. J. 2007)*.

The second most common form of CGD is autosomal recessive (AR-CGD), accounting for approximately 30-40% of the cases. This type of CGD is caused by mutations in one of the three genes CYBA, NCF1, and NCF2 (presents on chromosomes 16, 7, and 1) that encoding, respectively, $p22^{phox}$, $p47^{phox}$, and $p67^{phox}$ *(Stenson P. D. et al. 2003; Piirila H. et al. 2006)*. The most frequent form of AR-CGD is caused by mutations on $p47^{phox}$ present in about the 30% of patients, while both mutations on $p22^{phox}$ and $p67^{phox}$ are extremely rare (frequency <5% for both). Moreover, independently from the type of mutation, the phenotype of AR-CGD is always characterized

by the loss of the expression of the protein (with A22^0, A47^0 and A67^0 phenotype).

Clinical features: etiology of infections and acute inflammation

Given that the disease is caused by the defect of the NADPH oxidase, mainly present in the phagocytes, in which is principally involved in the ROS mediated pathogen killing, the patients with CGD are characterized by life threatening and recurrent infections with mainly catalase-positive microorganisms, due to the inability of phagocytes to mount a respiratory burst against invading bacteria and fungi.

The comparison of the studies on different cohorts of patient from USA *(Winkelstein J. A. et al. 2000)*, United Kingdom *(Jones L. B. et al. 2008)*, Italy *(Martire B. et al. 2008)*, Israel *(Wolach B. et al. 2008)* and Spain *(Soler-Palacin P. et al. 2007)*, has shown that in CGD the most common complications from infection are respectively pneumonia (79% of total infections), suppurative adenitis (53%), subcutaneous abscess (42%), liver abscess (27%), osteomyelitis (25%), sepsis (18%), but also dermatitis, enteritis, otitis, perianal abscess and urinary tract infection can be present.

Since in many cases, the search of pathogens is not performed and often, despite a proper procedure (such as bronchoalveolar lavage, blood culture, needle aspiration or biopsy), only negative

cultures were obtained, the identification of microorganisms has been possible only for 33% of the infections (range 9.6-49%).

The pathogens responsible for the majority of infections in CGD are *Staphylococcus* spp. (20-34% of the total isolated). They are the first agents of suppurative adenitis (it was isolated from 26 to 100% of this infection), subcutaneous abscess (27-50%), liver abscess (50-87%) and suppurative dermatitis (100%); less frequently were in: lung abscess (8-33%), osteomyelitis (6-50%), pneumonia (12%) and bacteremia (9-37%).

The second most common pathogens in CGD infections are *Aspergillus* spp. (14-34% of the total isolated). They are the principal cause of pneumonia (found from 33 to 45% in this infection), osteomyelitis (20-80%), and of the rare cases of brain abscess (58-100%). Moreover, they are also found in lung abscess (17-23%), subcutaneous abscess (5%) and liver abscess (3%).

Candida spp. are the third agents that cause infection in the patients (14% of the total isolated). These fungi are the first cause of meningitis (found in the 20% of this infection) and fungemia (11-40%), but they are also found in suppurative adenitis (7%), pneumonia (2-20%), subcutaneous abscess (4%), liver abscess (2%).

Other microorganisms frequently involved were Gram negative, such as *Serratia marcescens* and *Burkolderia cepacia*, identified in 10% and from 2 to 4% of the total isolated, respectively.

Serratia spp. are the first cause of osteomyelitis (present in the 29% of this infections), the second important pathogens for subcutaneous abscess (15%) and suppurative adenitis (9%). These kinds of bacterial can be isolated also from liver abscess (5%), pneumonia (5%) and sepsis (6-20%).

Burkolderia cepacia is the third microbe more frequently isolated from pneumonia (8-10%), and lung abscess (7%). Further it is also the second pathogen in the sepsis (3-12%) and it was also isolated from meningitis (7-50%).

Other important etiological pathogens, in CGD, are *Streptococcus* spp. (6.8% of the total isolated microbes), that can be consider the third most frequent pathogens isolated from liver abscess (5-6%), and important also in sepsis (11%) and lung abscess.

Other potential pathogens isolated from cultures of patients are: *Klebsiella* spp., *Nocardia* spp., *Pseudomonas* spp., *Acinetobacter* spp., *Enterobacteriaceace* spp., *Paecilomyces* spp., *E.coli* and other, but their frequency was found very low.

Clinical features: chronic inflammation and autoimmunity

Patients with CGD, beside having recurrent infections, also suffer from a variety of inflammatory conditions, classified as "complications not obviously caused by infection" *(Johnston R. B., Jr. 2001)*. The origin of these complication, most probably, is caused

by intrinsic dysregulation of inflammatory mechanisms, because often these lesions are sterile, respond to immunomodulators, such as steroids, but not to antibiotics, and in animal models of CGD, they can be induced by sterile fungal cell wall preparations *(Schappi M. et al. 2008)*.

The most commonly described feature is an acute and/or chronic inflammation with fibrosis containing non-caseous granulomas in the intestinal tract, liver, lymph nodes, urogenital tract, skin and brain.

Gastrointestinal inflammatory manifestation are reported as the most common hyperinflammatory symptom in CGD patients *(Martire B. et al. 2008)*, with a prevalence of 33% *(Marciano B. E. et al. 2004)*. Gastrointestinal inflammatory lesions may affect any part of the gastrointestinal tract with growth failure, anaemia, failure to thrive, abdominal pain, diarrhoea, with or without blood, nausea, vomiting, constipation, and weight loss *(Schappi M. G. et al. 2001; Marciano B. E. et al. 2004)*. The gastrointestinal inflammatory manifestation can be presents how focal obstructive lesions as well as diffuse inflammation. Focal obstructive lesions are observed principally in distal stomach where they lead to gastric outlet obstruction, but can also be present in the oesophagus and in the duodenum. Diffuse inflammation is observed in the oesophagus, small bowel, and in the colon, where it is associated with colitis, enteritis and inflammatory bowel disease (IBD), with oral ulcers,

esophagitis, gastric outlet obstruction, villous atrophy, fistulas, and perirectal abscesses.

The genitourinary (GU) tract is the other viscera frequently involved in inflammatory-associated manifestations in CGD patients (incidence around 40%) *(Walther M. M. et al. 1992)*. The most frequently reported lesions are urinary obstruction due to granuloma, and cystitis without apparent infection, but also immune complex glomerulonephitis, renal granulomas, granulomatous orchitis, and penescrotal granulomas have been reported. These lesions can sometimes lead to hydronephrosis *(Forbes G. S. et al. 1976)* and even renal insufficiency *(Casale A. J. et al. 1989)*, with symptoms as suprapubic pain, dysuria, urinary retention and haematuria.

Another site of inflammatory involvement in CGD patients is the eye *(Kim S. J. et al. 2003)*, with chorioretinitis as most frequent lesion (incidence 30%). This lesion affects visual activity only when it is extensive and otherwise can be asymptomatic *(Goldblatt D. et al. 1999)*. Moreover also rare cases of oculomucocutaneus syndrome *(Kelleher D. et al. 1986)*, chronic uveitis *(Matsuura T. et al. 2006)*, ulcerative keratitis *(Leroux K. et al. 2004)***,** have been reported.

In addiction to higher susceptibility to infection and hyperinflammation, CGD patients are also predisposed to a variety of autoimmune diseases. Thus, discoid and systemic lupus erythematosus *(Winkelstein J. A. et al. 2000; Martire B. et al. 2008)* is reported in up to 3.8% of CGD patients *(Foster C. B. et al. 1998)***,**

as well as cutaneous lesions similar to discoid lupus that are the most common phenotype in X-CGD carriers (26%) and kindreds. There are also case reports of other autoimmune diseases such as juvenile rheumatoid arthritis, erythema nodosus, autoimmune thrombocytopenia *(Trelinski J. et al. 2005)*, idiopathic thrombocytopenic purpura, rheumatoid arthritis, eosinophilic cystitis, IgA nephropathy, sarcoidosis, and celiac disease with pulmonary hemosiderosis *(De Ravin S. S. et al. 2008)*.

Diagnosis

The laboratory diagnosis of CGD is based on the demonstration of absence of phagocyte oxidizing activity with tests which detect superoxide production by $Fe3^{+}$ cytochrome C reduction *(Weisbart R. H. et al. 1986)* or by Nitroblue Tetrazolium test (NBT) *(Baehner R. L. and Nathan, D. G. 1968)*, or by dihydrorhodamine 123 (DHR) flow cytometric assay, which reveals absent or markedly reduced oxidase activity in stimulated neutrophils *(Vowells S. J. et al. 1995)*.

The qualitative dihydrorhodamine 123 (DHR) flow cytometric assay is today's the most accurate diagnostic test for CGD, although the qualitative (microscopial) and less discriminant is still in clinical use. The flow cytometry assay allows more sensitive and reproducible detection of affected patients and carriers, and can indicate the nature of the underlying molecular defect *(Crockard A.*

D. et al. 1997). Western blot analysis of neutrophil lysates, which reveals the level of protein expression, helps in the identification of CGD subtype. Although functional tests are important in the diagnosis of CGD, identification of molecular defects, by sequencing remains an essential tool for counseling family members at risk for being CGD carriers and for prenatal diagnosis.

The X-linked CGD is generally the most common and also presents the most serious disorders (as gastrointestinal symptoms and lesions of the urogenital tract) than the autosomal recessive (AR) form of disease. Thus, while X-linked CGD is diagnosed in childhood, very often (76% of patients) before the age of 5 years, some patients (more women that men), mainly those with AR CGD, can be diagnosed in adult life (after the age of 20 years) *(Winkelstein J. A. et al. 2000; Wolach B. et al. 2008).*

Therapy

To date, the only definitive treatment is haematopoietic stem cell transplantation from a healthy compatible donor. However, despite recent advance in transplant conditioning regimens, the immune suppressive treatment required to avoid graft versus host disease (GVHD) increases the risk of infections *(Martire B. et al. 2008).* Furthermore it is still difficult to find compatible donors and transplant for CGD remains a high risk procedure.

Currently, the treatment is basically preventive and is based on rigid infectious prophylaxis provided by lifelong co-administration of an antibacterial and an antifungal agent. The usefulness of Trimethoprim-Sulphamethoxazole (CTMX) as antimicrobial prophylaxis in patients with CGD has been well established since the early 1990s, when a significant reduction was achieved in bacterial infections in these patients *(Margolis D. M. et al. 1990)*. But to avoid also fungal infection, a long life administration of Intraconazole, is required. The use of this drug is in agreement with an European study in which Intraconazole has shown excellent tolerance and a reduced rate of *Aspergillus* infections *(Mouy R. et al. 1994)*.

Despite controversial study, also the Interferon-γ is now recommended as life long therapy for infection prophylaxis in CGD patients, but the cost of long-term prophylactic is high and the need to be injected intramuscularly, making the compliance to this treatment rather poor**.** Consequently Interferon-γ prophylaxis is offered only in selected CGD cases, by most European physicians, while it is universally prescribed in the USA.

Moreover steroid treatment is required in cases of inflammation complications such as gastrointestinal inflammatory obstruction, colitis, severe eczema, urogenital tract inflammation, or lupus-like rashes and also in the clinical management of some severe infection.

Outcome and causes of death

The antibacterial and antifungal prophylactic protocols, administered early at time of diagnosis, has significantly increase the survival of CGD patients. Thus, if CGD was originally named "fatal granulomatous disease of childhood" presenting, in the 1970s, the 45% of patient dead before the age of 7 years *(Johnston R. B., Jr. and Baehner, R. L. 1971)*, the mortality rate, in the recent studies with American and Italian cohort, was reduced to 17.6% and 13% respectively *(Winkelstein J. A. et al. 2000; Martire B. et al. 2008)*, with a median age at death of 14 years. However, despite the therapy, the survival curve show that at 25 years from diagnosis, the mortality still remains severe (about 54%).

Pneumonia and/or sepsis due to *Aspergillus* and *Burkholderia* are the most common causes of death in CGD patients *(Winkelstein J. A. et al. 2000)*, bat also pneumonia caused by *Candida* spp. and complications after transplantation *(Martire B. et al. 2008)*, severe Pneumococcal and *Candida* sepsis, or disseminate Aspergillosis *(Wolach B. et al. 2008)*, and *Pseudomonas*, *Staphylococcus* or *Nocardia* septicaemia *(Jones L. B. et al. 2008)* are reported.

Immunological studies in humans

Since the NADPH oxidase is mainly express in the phagocytic cells, CGD is considered caused mainly by the oxidative defect of neutrophils and monocytes, there are many works characterizing these cellular compartments in CGD patients.

Previous studies have suggested that the lack of a functional NADPH-oxidase confers an excessive response to phagocytes, with reduced spontaneous apoptosis in neutrophils and down regulation of anti-inflammatory mediators during phagocytosis in macrophages *(Brown J. R. et al. 2003)*.

Another study has linked the dysregulation of PMNs of CGD patients with reduced quantities of reactive oxygen species (ROS). In this study differential gene transcription was measured with oligonucleotide microarrays analysis after phagocytosis in human PMNs, and it has shown that the expression of 206 genes was changed in CGD compared to healthy donors. Importantly, in CGD patients, genes encoding mediators of inflammation and host defence (TLR5, IL-8, CXCL1, FcγR1, FcαR, calgranulins A and B) were up-regulated constitutively, while some anti-inflammatory genes involved in TGF-β signalling, that play a prominent role in suppressing inflammatory responses of phagocytic cells, were down regulated *(Kobayashi S. D. et al. 2004)*.

The differential expression of important receptors for pathogens recognition it has also been shown in a work in which the PMNs from patients with CGD had lower expression levels of TLR5, TLR9, CD11b, CD18, CD35, and CXCR1 compared with those from healthy controls, and the expression of TLR5 and CD18 correlated inversely with the frequency of lymphadenitis and pneumonia in *(Hartl D. et al. 2008).*

A recent study indicated that peripheral blood mononuclear cells (PBMC) of CGD patients, stimulated with TLR agonists such as lypopolysaccharide (LPS) or peptidoglycan (PGN), were hyper-responsive in terms of IL-6 and TNF-α production *(Bylund J. et al. 2007).*

This work is in agreement with another study on monocytes of CGD patients, in which the expression levels of major inflammatory mediators, including IL-1β, IL-6, IL-8, IL-10, IL-12β, TNF-α, IFN-γ and CCL8, were significantly up-regulated in CGD resting and TLR activated monocytes compared to control cells. While genes that encode anti-inflammatory mediators, such as the cytokines TGFβ2, IL-13, IL-18-binding protein (IL-18BP) and the TGF-β receptor (TGFBR2) did not display elevated expression in CGD patients compared to controls *(Brown K. L. et al. 2008).*

All these data indicate that phagocytes from CGD patients have a specific cellular dysfunction, rather than a more general enhancement of gene transcription and this is in agreement with the

hypothesis that the defect of NADPH oxidase can lead to a specific pro-inflammatory phenotype in phagocytic cells that in turn could contribute to the chronic inflammations found in CGD patients. However, if there is consensus on the role of the NADPH oxidase in increasing the apoptosis of neutrophils, on the induction of pro-inflammatory phenotype in monocytes the data are still conflicting. Indeed, a recent paper has shown that purified monocytes of CGD patients stimulated via TLRs would produce more IL-10 and less pro-inflammatory cytokines, as IFN-γ, IL-6 and IL-13, compared with monocytes of healthy donors *(Rahman F. Z. et al. 2009)*.

Although, to date, the mechanism underlying such abnormal response of the immune system has not yet been elucidated, it may involve other cell types than the known defective phagocytes, but unfortunately there are few studies that attempted to characterize the lymphocytes of CGD patients.

In a study of 2002, fifty-three patients with CGD and forty-two age-matched controls were studied by flow cytometry to identify different T cell subpopulations *(Heltzer M. et al. 2002)*. When CD3, CD4 and CD8 positive T cell numbers were compared between patients and controls, patients with CGD older than 3 years, had diminished T cell numbers (CD3, CD4 and CD8), and the difference increased with age. These results are in agreement with those previously found in the work by Hasumi *(Hasui M. et al. 1993)*, where 17 patients with CGD were examined and memory T cells

($CD4^+$ $CD29^+$ cells) and suppressor T cells ($CD8^+$ $CD11b^+$ cells) were significantly decreased compared to healthy donors.

Also a profound reduction in memory B lymphocytes ($CD19^+$ $CD27^+$), independent of age, genotype and clinical status of the patients, and correlating with the defective NADPH oxidase, was found in a cohort of 50 CGD patients in comparison to thirty healthy subjects, suggesting a role for

NADPH in the process of memory B cell formation *(Bleesing J. J. et al. 2006)*.

The study of Heltzer *(Heltzer M. et al. 2002)* also analyzed lymphocyte proliferation upon stimulation with PHA, and it has shown that, T lymphocytes form CGD patients had a reduced proliferative responses than controls, but the difference was not statistically significant.

A decreased PBMC proliferation of CGD patients, upon PHA stimulation, has also been shown in a more recent work performed only on three patients (one $A47^0$ and two $A67^0$) and six controls *(Salmen S. et al. 2007)*, but no differences were observed in lymphocyte number or phenotypes between CGD and control. Interestingly, in this work, a diminished percentage of CD40L expression after PMA stimulation was observed in T lymphocytes from CGD patients.

The cytokine induction in lymphocyte after PMA plus ionomycin stimulation, was analyzed in another study in which six patients with

CGD (five X-linked and one $p47^{phox-/-}$) were used as controls for Hyper IgE syndrome. Thus, although there was not difference in the IL-4 induction, the intracellular expression of IFN-γ, in $CD4^+$ and $CD8^+$ T lymphocytes from peripheral blood of CGD patients, was found higher than healthy control, suggesting a greater Th1/Th2 ratio in the patients *(Ohga S. et al. 2003)*.

Other then the classical Th1 and Th2 effector T lymphocyte subsets, also the new recently characterized Th17 lymphocyte compartment could be involved in the CGD increased susceptibility to fungal infections and chronic damage, as showed in the recent works with both fungal infection *(Zelante T. et al. 2007)* and CGD animal models *(Romani L. et al. 2008)*. Up to date, there are not studies on CGD patients but, the potential role of Th17 cells in the pathogenesis of human CGD is supported by: the in vitro ability of *Candida albicans* hyphae to drive naïve T $CD4^+$ lymphocytes toward IL-17 producing cells; the presence of *Candida* specific Th17 memory cells, showing molecular markers of skin and mucosal homing, in peripheral blood of healthy donors *(Acosta-Rodriguez E. V. et al. 2007b)*; the reduced number of $IL17^+$ cell in PBMC of patient with disease characterized by increased fungal and bacterial infections as Chronic Mucocutaneous Candidiasis (CMC) and Hyper-Immunoglobulin E Syndrome (HIES) *(Eyerich K. et al. 2008; Ma C. S. et al. 2008)* and the presence of highly differentiated Th17

lymphocytes in some chronically inflamed human tissues *(Pene J. et al. 2008)*.

TH17 CELLS

Discovery of Th17 cell and role in extracellular and intracellular pathogen infections

The T helper 1 (Th1) cell and Th2 cell paradigm, first proposed by Mosmann and Coffman, *(Mosmann T. R. and Coffman, R. L. 1989)*, in which antigens-presenting cells (APC) drive the differentiation of naïve CD4+ lymphocytes to either interferon-γ (IFN-γ)-producing Th1 cells or IL-4-producing Th2 cells, has been used to explain how hosts elicit different adaptive immune responses to eradicate the infection of various pathogens. Uncontrolled and persistent effector T cell responses, however, can drive the onset of various complications as shown in animal model where uncontrolled Th2 cell responses led to atopic diseases, such as asthma *(Cohn L. et al. 2004)*, while the abnormal Th1 cell responses mediated at least in part, many other autoimmune diseases, including psoriasis and inflammatory bowel disease (IBD) *(Bouma G. and Strober, W. 2003)*. It has also become clear, however, that many complex pathological situations cannot be simply explained by the Th1 cell and Th2 cell paradigm. But, the recent discovery that other kind of

cells, such as Treg and Th17, can differentiate from naïve T lymphocyte and to take part to immune responses, can contribute to explain the pathogenesis of several diseases.

The discovery of the involvement of Th17 lymphocytes (producer of IL-17A) in human pathology, came after the discovery of IL-23, a cytokine produced by APC, that consists of a unique p19 subunit and shares a common p40 subunit with IL-12 *(Oppmann B. et al. 2000)*. Subsequently, it was demonstrated that IL-23p19$^{-/-}$ but not IL-12p35$^{-/-}$ mice are resistant to experimental allergic encephalitis (EAE), a model for human multiple sclerosis and collagen-induced arthritis (CIA) *(Cua D. J. et al. 2003; Murphy C. A. et al. 2003)*. These findings suggested that, in contrast to the previous concept, not IL-12/Th1 but the IL-23/Th17 axis is crucial in a variety of autoimmunity manifestations as subsequent studies found for rheumatoid arthritis (RA) *(Lubberts E. et al. 2004; Hsu H. C. et al. 2008)*, multiple sclerosis (MS) *(Tzartos J. S. et al. 2008)*, psoriasis *(Wilson N. J. et al. 2007)*, and inflammatory bowel disease as Crohn's disease *(Fujino S. et al. 2003)*.

IL-17A is disulfide-linked homodimeric glycoprotein, consisting of 155 amino acids *(Yao Z. et al. 1995)*, exerting part of its actions as a homodimer with a molecular weight around 35kD. IL-17A and IL-17F can either exist as IL-17A homodimers and IL- 17F homodimers or as IL-17A-IL-17F heterodimers *(Liang S. C. et al. 2007)*.

Besides being produced by Th17 cells, both IL-17A and IL-17F are also produced by a variety of cell of innate immunity γδ T cells, NKT cells, NK cells, neutrophils, and eosinophils *(Starnes T. et al. 2001; Ferretti S. et al. 2003; Lockhart E. et al. 2006).*

Despite the ability of Th17 cells to mediate inflammation in numerous models of auto-immune conditions, Th17 cells are committed to confer immunity to extracellular, bacterial and fungi. Indeed, increased susceptibility to infection by either *Klebsiella pneumoniae* or *Citrobacter rodentium* in IL-17A receptor-deficient and IL-23p19-deficient animals indicates the importance of the IL-23/IL-17 axis in immunity to extracellular bacteria *(Happel K. I. et al. 2005; Mangan P. R. et al. 2006)*, moreover IL-17A receptor-deficient mice are also highly susceptible to systemic infection by *C. albicans*, and neutrophils are not recruited into the sites of infection in these mice *(Huang W. et al. 2004).*

Although Th17 cells play a less direct role in protection against pathogens that primarily reside within the infected cells, several studies also indicate that Th17 cells contribute to protection against intracellular infections by *Mycobacteria* spp., *Listeria* spp. *(Miyamoto M. et al. 2003)*, and *Salmonella* spp. *(Schulz S. M. et al. 2008).* As shown in a vaccination model for protective antigen of *Mycobacterium tuberculosis*, IL-17-roducing $CD4^+$ T cells populate the lung, producing chemokines that recruit IFN-γ-producing antigen-specific Th1 cells to the site of infection *(Khader S. A. et al.*

2007). Furthermore, IL-17 is induced during both the innate and the adaptive immune responses against *Mycobacterium bovis* bacille Calmette–Guérin (BCG) infection and is required for proper formation of granulomas during mycobacterial infection *(Umemura M. et al. 2007)*.

Development of Th17 in mouse and human

Mouse In 2006, three independent studies found that a combination of the immunoregulatory cytokine transforming growth factor-β (TGF-β) and the pro-inflammatory and pleiotropic cytokine IL-6 is required to induce IL-17 in naive T cells *(Bettelli E. et al. 2006; Mangan P. R. et al. 2006; Veldhoen M. et al. 2006)*.

The complete Th17 differentiation required expression of the retinoic acid-related orphan receptor γt (RORγt) *(Ivanov, II et al. 2006)* and RORα *(Yang X. O. et al. 2008)*, both dependent on STAT3 pathway and preferentially activated by IL-6 IL-21, and IL-23 *(Kimura A. et al. 2007; Yang X. O. et al. 2007)*. However, IL-6 alone cannot induce RORγγt and TGF-β is absolutely required both for the initial induction of IL-17 in naive $CD4^+$ T cells and for the induction of IL-23R, which makes differentiating Th17 cells responsive to IL-23 and therefore further promotes their maturation (Fig. 3).

Because TGF-β is a critical differentiation factor also for the generation of regulatory T cells (Treg) *(Chen W. et al. 2003)*, that

express the transcription factor forkhead box P3 (Foxp3) *(Hori S. et al. 2003)*, this finding demonstrate a dichotomy in the generation of Th17 and Treg, and that IL-6 plays a pivotal role in dictating whether in mouse, an immune response is dominated by Foxp3^{+} Treg or Th17 cells.

Because Th17 cells are a major source of IL-21 that both suppressed the TGF-β-induced expression of Foxp3 and, together with TGF-β, induces IL-17 in naive T cells *(Korn T. et al. 2007)*, an autocrine amplification loop was proposed by which Th17 cells enhance their own differentiation and precursor frequency *(Nurieva R. et al. 2007; Zhou L. et al. 2007)* (Fig. 3).

Human. In 2007, several studies claimed that TGF-β was dispensable for the differentiation of human Th17 cells and the combinations of IL-1β plus IL-6 *(Acosta-Rodriguez E. V. et al. 2007a)* or IL-1β plus IL-23 *(Wilson N. J. et al. 2007)* was proposed as the differentiation factors for human Th17 cells.

Subsequently, three new reports come out proving that TGF-β is essential also for the differentiation of human Th17 cells from naive T cells *(Manel N. et al. 2008; Volpe E. et al. 2008; Yang L. et al. 2008)*, as well. TGF-β is required to induce RORC (the human homolog of RORγt), but its expression and function are inhibited by excess TGF-β. Only when additional cytokines such as IL-6 plus IL-23 or IL- 21 are present, RORC is relieved from inhibition, and then naïve T cells can begin transcribing IL-17 *(Manel N. et al. 2008)*.

Thus, at a molecular level, the differentiation conditions of mouse and human Th17 cells do not appear to be different (fig. 3).

Differently, more recent studies showed that human Th17 cells originated exclusively from a $CD161^+CD4^+$ T cell precursor using umbilical cord blood (UCB) or single positive $CD4^+CD8^+$ thymocytes in the presence of IL-1β and IL-23. Naïve $CD161^+CD4^+$ T cells were found to express both RORC2 and IL-23 receptor constitutively, indicating that RORC2 expression did not depend upon TGF-β *(Annunziato F. et al. 2008; Cosmi L. et al. 2008; Santarlasci V. et al. 2009)*. TGF-β did not have a direct critical role on the differentiation of human IL-17-secreting T cells, but rather acted indirectly to favour IL-17 induction by inhibiting selectively the expansion of IFN-γ-secreting T cells. This would result in a greater degree of positive selection of an already existing RORC2-expressing T cell population and relative expansion of an IL-17 positive population induced by IL-1β and IL-23.

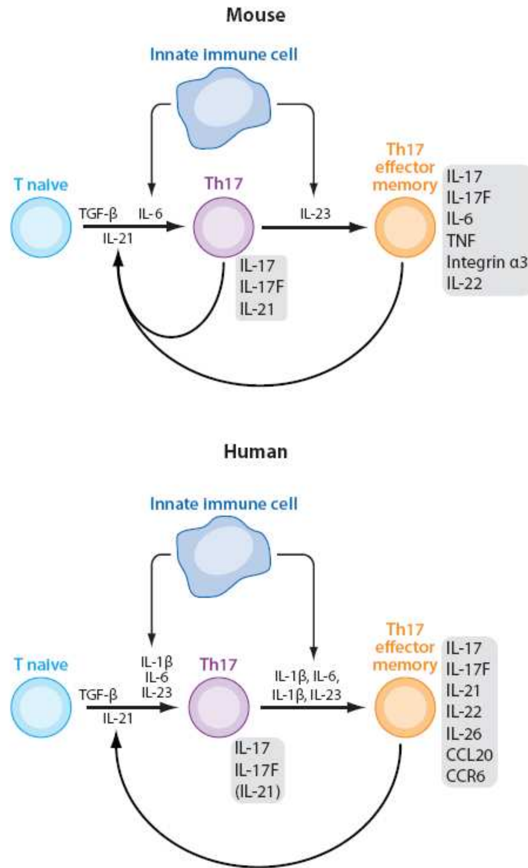


Figure 3. Differentiation of Th17 cells in mice and humans. Factors required to induce the development of Th17 cells in mice starting from naive T cells (light blue). IL-21 feeds back on developing Th17 cells, amplifying their frequency, as do IFN-γ and IL-4 in the differentiation of Th1 and Th2 cells, respectively. IL-23 might be required to induce further effector molecules in committed Th17 cells (purple) to establish their terminally differentiated effector phenotype (orange). Differently, in humans, IL-1β and IL-23 seem more important in respect to IL-6 and TGF-β to lead to the commitment and to the differentiation of Th17 from naïve T lymphocytes (Modified from *Thomas Korn T. et al. 2009*).

Th17 in hyper IgE syndrome (HIES)

Hyper-immunoglobulin E (IgE) syndromes (HIES) is a very rare primary immunodeficiency, characterized by clinical triad of high serum level of IgE (> 2000 IU/ml), recurring staphylococcal skin abscesses and pneumonia with pneumatocele formation. Fungal infections, including mucocutaneous candidiasis and pulmonary aspergillosis, are also common in HIES. Eczema usually begins during the neonatal period, before the onset of atopic dermatitis. Patients with HIES suffer from atopic dermatitis associated with extremely high serum IgE levels and eosinophilia, but are usually free from other allergic manifestations, such as allergic rhinitis, asthma, urticaria, and anaphylaxis *(Grimbacher B. et al. 2005)*.

Most case are sporadic, but both autosomal dominant form of HIES (AR-HIES) and autosomal recessive form (AD-HIES) have been described.

The most frequent AD-HIES (more than 90% of cases), is caused by a mutation of the STAT3 gene with patients showing low number or absent Th17 cells and a lower production of IL17A, and IFN-γ *(Ma C. S. et al. 2008; Milner J. D. et al. 2008; Renner E. D. et al. 2008)* and increased susceptibility to extracellular fungal infections.

The AR-HIES are clinically distinct, more severe, and may be associated with autoimmunity and vasculitis. Along with severe atopic dermatitis and recurrent skin infections, these patients have

serious cutaneous viral infections by *Molluscum contagiosum*, *Herpes simplex* virus, and *Varicella zoster*. Although pneumonias may occur, these individuals do not form pneumatoceles and have no connective tissue abnormalities. Two of this patient had mutation on tyrosine kinase 2 (*TYK2)* gene involved in signal transduction for various cytokines such us IL-12 and IFN-α *(Minegishi Y. et al. 2006)* and also IL-23 *(Shaw M. H. et al. 2003)*. Although the involvement of Th17 was not explored by the authors, the failure to produce IL-17A cannot be excluded.

More recently, mutations on cytokinesis 8 (*DOK8*) gene, encoding a protein implicated in the regulation of the actin cythosckeleton, were found responsible for many, although not all, cases AR-HIES *(Engelhardt K. R. et al. 2009)*, indicating a possible involvement of other pathway than IL-23/IL-17 in pathogenesis of HIES.

CGD ANIMAL MODEL OF INVASIVE ASPERGILLOSIS

CGD animal models

The first mouse model of CGD was a knock-out mouse generated in the laboratory of Mary Dinauer by targeting the gene encoding $gp91^{phox}$, creating a model for human X-linked CGD *(Pollock J. D. et al. 1995)*. Contemporarily, a second model was generated by Jackson

et al., through disruption of the $p47^{phox}$ gene *(Jackson S. H. et al. 1995)*. More recently, also $Rac2^{-/-}$, $p40^{phox-/-}$ *(Roberts A. W. et al. 1999; Ellson C. D. et al. 2006)* and $p22^{phox-/-}$ mouse *(Nakano Y. et al. 2008)*were generated as different models for AR-CGD.

Although all these mouse models lack phagocyte superoxide production and manifest many characteristics of CGD, as increased susceptibility to infection, for $Rac2^{-/-}$ and $p40^{phox-/-}$ there are not described mutations that lead to CGD in humans. Furthermore, although Rac2 deficiency results in a lack of host clearance of Chlamydia, which probably leads to chronic joint inflammation *(Zhang X. et al. 2005)*, since Rac2, other to NADPH oxidase activation, has functions involved in the organization of the cytoskeleton, some alterations observed in Rac2-deficient mice, as defects in F-actin polymerization, chemotaxis, and exocytosis of primary granules, cannot be unequivocally attributed to a CGD phenotype *(Abdel-Latif D. et al. 2004)*.

$Gp91^{phox-/-}$ and $p47^{phox-/-}$ CGD mouse models recapitulate the severe infections found in patients with spontaneous phenotype for $gp91^{phox\ -}$ by fungal and bacterial pathogens such as *Aspergillus, Candida, Staphylococcus* or *Pseudomonas* species.

Although a high percentage of patients suffer from gut disease, no such spontaneous phenotype has been described in $gp91^{phox}$ deficient mice, but other signs of hyperinflammation and of chronic damages of different tissues.

Thus, intradermal injection of heat-inactivated *A. fumigatus* cell wall causes severe hyperinflammation in gp91$^{phox-/-}$ CGD mice *(Morgenstern D. E. et al. 1997)*, with the maximal inflammation at 72 h persisting up to 4 weeks. More recently, branched fungal β-glucan [β-(1-3)(1-6)-glucan] was described as the cell wall components that cause persistent severe inflammation characterized by lesions with central necrosis *(Schappi M. et al. 2008)*. However, no sterile cell wall preparations from bacteria (*S. aureus, P. aeruginosa, E. coli*) caused prolonged and severe skin inflammation in CGD models.

In gp91^{phox} and p47^{phox} knock-out mice, compared to wild-type, there is a more severe arthritis induced by zymosan and poly-L-lysine coupled lysozyme, and granulomatous synovitis and increased matrix and connective tissue destruction as well as enhanced expression of inflammatory mediators *(van de Loo F. A. et al. 2003)*. There are many studies on joint inflammation in this models, thus the evidence that decreased ROS generation plays a role in arthritis development is strong.

With respect to the lungs, acidophilic macrophage pneumonia is a non-infectious condition normally found in aging mice and in 100% of both gp91^{phox} and p47^{phox} knock-out mice 2.5 months old *(Bingel S. A. 2002; Harbord M. et al. 2002)*. Although the cause of this lung inflammation is poorly understood and could be related to a problem of degradation of phagocytosed material, the injection of heat-

inactivated *A. fumigatus* wall, causes neutrophil infiltrations five times larger that in wt mice, with microabscesses and granuloma formation that persists up to 6 weeks *(Morgenstern D. E. et al. 1997)*.

Altered IL-17$^+$ lymphocyte homeostasis in CGD mouse model of invasive aspergillosis

Recent studies in a mouse model mimicking CGD (p47$^{phox-/-}$) indicate that a key player in inducing inflammation and tissue damages could be IL-17. CGD mice infected with *A. fumigatus* conidia, showed hyperinflammation in the lung and increased frequency in IL-17A-producing T cells in combination with a reduction of both, Th1 lymphocyte (producing IFN-γ) responsible for normal anti-fungal activity as well as Treg lymphocyte (Foxp3$^+$ regulatory cells) controlling inflammation *(Romani L. et al. 2008)*.

The involvement of IL-17 in the CGD mouse model is in agreements with the data found by Zelante and colleagues, in the first study demonstrating that IL-23/IL-17 pathway promotes inflammation and susceptibility to fungal infection through negative regulation of Th1-mediated immune resistance and by subverting the inflammatory program of neutrophils *(Zelante T. et al. 2007)*.

In this study both IL-23$^{-/-}$ mice, intragastrically infected with *C. albicans* and intranasally with *A. fumigatus* conidia, exhibited

reduced pathogen burden compared to IL-12$^{-/-}$ infected mice, suggesting a superior activity of IL-12 over IL-23 in opposing fungal infectivity. Moreover, if double negative IL-12/IL-23 mice showed minimal inflammation one week after infection, IL-12$^{-/-}$ mice had increased IL-23 production, a higher number of IL-17 producing T cells and severe pyogranulomatous inflammation, while IL-23$^{-/-}$ mice exhibited increased IL-12 production, a higher number of IFN-γ producing T cells and a mild mononuclear inflammation. Furthermore, in wild type mice, antibody-neutralization of IL-17 or IL-23, that correlated with an increased frequency of IFN-γ^{+} Th1 cells and a decreased frequency of Th17 cells in the draining lymph nodes, greatly reduces fungal burden and corrects inflammation. Thus, all this data clearly show that during mouse fungal infection IL-23 and IL-12 are mutually regulated and demonstrate that the IL-23/IL-17 pathway confers susceptibility to fungal infections by inhibition of protective Th1 immunity.

This study also examined the effects of IL-23 on neutrophils and showed that neutrophils express IL-23 receptor (IL-23R) and then are directly responsive to IL-23. Thus, neutrophils from wild type mice exposed to IL-17 or IL-23 showed impaired *Candida* and *Aspergillus* fungal killing activity, also in the presence of IFN-γ that normally increase their microbicidial potential. In addiction, it has been shown that both IL-23 and IL-17, not only promoted expression of matrix metalloproteinase 9 and myeloperoxidase that could

increase the tissue damages, but also reduced the apoptotic program of wild type neutrophils and completely antagonized the IFN-γ induction of indoleamine 2.3-dioxygenase (IDO), an important enzyme that suppresses T cell responses and promotes tolerance.

The immuno-pathological features of IL-$12^{-/-}$ mouse infected by *Aspergillus*, are in agreement with the more recent CGD mouse model of acute *Aspergillosis (Romani L. et al. 2008)*, in which knock-out of $p47^{phox}$ leads to a defect of NADPH oxidase activity. When this mouse was intratracheally infected with *A. fumigatus* conidia, an increased susceptibility to lethal acute pulmonary infection was observed. All the CGD mice died within 8 days compared with control mice that exceeded 60 days of survival.

In CGD mouse, the histopathology of lung performed after 4 days from infection, revealed abundant pyogranulomatous lesions with central neutrophilic infiltrates and exuberant neutrophilic response with increased number of eosinophils and lymphocytes were also shown from bronchoalveolar lavage (BAL) fluid. Furthermore neutrophils from CGD mouse, as well as the IL-$12^{-/-}$ mouse model, had a higher expression of metalloproteinase 9 and myeloperoxidase than control mouse, and also showed a defective IDO activity.

The ELISA test performed on lung homogenate and on sorted $CD3^{+}$ T lymphocytes, showed a increasing number of IL-17 and IL-23 producing cells along all time of infection with a decreased IFN-γ, IL-12, IL-10 and TGF-β secreting cells, showing an altered balance

between antifungal effectors Th1 cells, regulatory Treg cells and IL-17 secreting lymphocytes. Although the altered equilibrium was also found in thoracic lymph node of CGD mouse, where the increased Th17 lymphocytes was identified by detecting expression of gene *Rorc* in the $CD4^+$ sorted cells, in lungs the major source of pro-inflammatory IL-17 were γδ-T lymphocytes.

During the first phase of inflammation, γδ-T cells control innate responses, including recruitment and infiltration of neutrophils to inflamed tissues, through their production of IL-17 *(Lockhart E. et al. 2006; Shibata K. et al. 2007)*. In the lung of CGD mouse γδ-T cells had at least two phenotype: Vγ1 γδ-T IL-17 producing cells and Vγ4 γδ-T cells mostly producing IL-10.

Importantly, Romani and colleagues found that wild type uninfected mouse, treated with inhibitor of IDO, recapitulate the features of *Aspergillus* infected CGD mouse with the increased number of Vγ1 IL-17 producing γδ-T cells, increased susceptibility to infection as well as the decreased survival *(Romani L. et al. 2008)*.

The indoleamine 2.3-dioxygenase (IDO) is an important enzyme that mediates conversion of the essential amino acid L-tryptophan (L-Trp) to first (L-kynurenine) of metabolic products collectively known as kynurenines (fig. 4). IDO as other enzymes along kynurenine pathway is normally induced in dendritic cells and phagocytes upon inflammation (depends on IFN-γ or TGF-β stimulation) and acts as immunoregulatory factor that contributes to

generate tolerance in same physiopathologic conditions such as allograft rejection, protection against autoimmunity, maternal tolerance in pregnancy, allergy and inflammatory pathology. The mechanisms through IDO induces tolerance are based both on L-Trp starvation that can inhibit cell proliferation, but also on Kynurenines induction of: down regulation of T cell receptor ζ-chain, apoptosis of Th1, inhibition of NK cell proliferation and induction of T regulatory Treg cells from naïve T lymphocytes *(Belladonna M. L. et al. 2009)*.

Although *in vitro* studies indicated that there are not differences in IDO expression and in IDO IFN-γ induction in neutrophils of CGD compared with control wild type mouse, the production of L-kynurenine was dramatically reduced in CGD mouse both in lung homogenates and *in vitro* IFN-γ stimulated neutrophils, indicating a possible involvement of IFN-γ independent altered IDO regulation in the pathogenesis CGD.

The link between mutated NADPH oxidase and altered activation of IDO in CGD mouse was subtended also by a recent study that account a possible function of superoxide anion like a cofactor and substrate precursor in IDO dependent L-Trp degradation *(Macchiarulo A. et al. 2007)*. This study showed that superoxide anion binding lead to conformation changes and more reactive catalytic site in IDO, that in turn could more efficiently catalyze the L-Trp degradation and L-kynurenine production.

In their animal model of CGD, Romani and colleagues showed that kynurenines could induce a selective apoptosis of Vγ1 IL-17 producing γδ-T cells but not of regulatory Vγ4 producing IL-10 γδ-T lymphocytes. Moreover the *in vivo* administration of L-kynurenine associated with IFN-γ, that induced kynurenine pathway enzymes, restoring the correct balance between IL-17 producing cells, Th1 and Treg was able to correct the inflammatory injury and lead to complete cure of CGD mice *(Romani L. et al. 2008).*

Thus, this important study revealing that, in mouse, alteration in NADPH oxidase function lead to decreased IDO tollerogenic activity and an increase of IL-17 producing cells, raises the possibility of a potential involvement of Th17 lymphocytes in the pathogenesis of same chronic clinical manifestation of CGD patients.

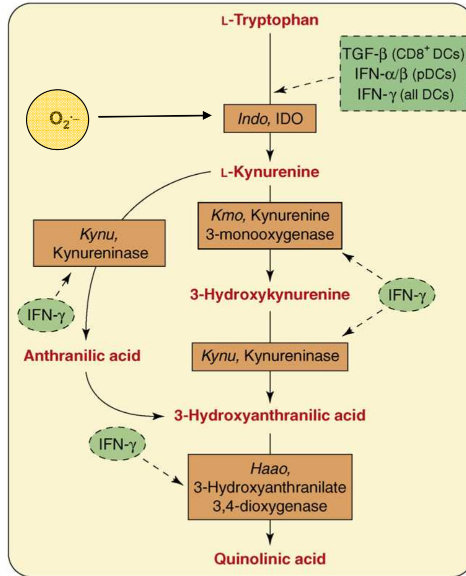


Figure 4. Kynurenine pathway. Tryptophan catabolism in mammalian cells, in which IDO catalyzes the initial and rate-limiting step under transcriptional regulation by IFN-γ. The superoxide anion ($O_2^{\cdot-}$) acts as cofactor and substrate precursor that increase IDO activity. Indicated in the figure are the enzymes and the corresponding genes (modified from *Belladonna ML, et al., Trends Mol Med, 2009*).

AIMS OF THE STUDY

Chronic granulomatous disease is a rare primary immunodeficiency syndrome, due to mutations in genes that codify proteins of the NADPH oxidase complex, and characterized by an increased susceptibility to fungal and bacterial infection. Other important features manifested by CGD patients and animal model of CGD, are chronic inflammations and granulomas in various tissues not completely explained by chronic infection. Indeed, in the last decade, parallel to the decreased mortality, an increase in chronic manifestations of exaggerated inflammation has emerged as a major clinical problem. To date, the mechanism underlying such abnormal response of the immune system has not yet been elucidated in humans, but it may involve other cell types than the known defective phagocytes.

Recently a decreased frequency of a specific T lymphocytes compartment, called Th17 for their characteristic IL-17A production, was associated with increased susceptibility to fungal end bacterial infection in several human chronic disease as hiper IgE syndrome (HIES) and chronic mucocutaneous candidiasis (CMC).

Although in literature there are few immunological studies about cell function in CGD, a recent work on a CGD animal model, has highlighted that in acute fungal infection, a dysregulation of IL-17 cell, coupled with a decrease of FN-γ-producing effectors cells and of IL-10-producing regulatory cell, could have a pathogenic role.

Primary aim of the study, therefore, was to investigate the potential involvement of T lymphocyte deregulation in CGD manifestations, by comparison of the immunological responses of peripheral blood mononuclear cells between a cohort of patients and of healthy controls.

To this aim we performed:

i) proliferation tests

ii) cytokine detections

iii) quantifications of cytokine mRNA expression

iv) cytofluorimetric analysis of T cell subsets in resting PBMC and upon mitogens and antigens stimulation.

The second aim of the thesis was to correlate, the clinical characteristic of the patients with the immunological responses through:

i) stratification of the cohort for specific pathogen infections

ii) comparison of patients with specific clinical and genetic characteristics with other CGD patients, with patients with HIES and with healthy subjects.

RESULTS

MOLECULAR AND CLINICAL CHARACTERIZATION OF THE COHORT OF CGD PATIENTS

The cohort of CGD patients studied was characterized in our laboratory as a part of longer CGD cohort, described in a previously published work *(Di Matteo G. et al. 2009)*, in witch the kinds and sites of gene mutations, the kinds and domains of the amino-acid changes, and the CGD phenotype, were evaluated by sequencing of PCR products and western-blot analysis (tab. 1).

The patients displayed very heterogeneous kinds of mutations; 5 patients showed a single nucleotide mutation, that led to a single amino acid change (nonsense mutation in P. M. and P. G.), to a stop codon introduction (missense mutation in B. S. and B. A.) and to a silent mutation with exon 2 and 3 skipping (splice site mutation in M. F. M.) previously described by Ishibashi *(Ishibashi F. et al. 2001)*. Moreover, patient C. A. had a double non-contiguous single nucleotide deletion that led to a frameshift mutation, while other two patient showed a single but very large deletion (> 27 KB) that overlap all the 13 exons of $gp91^{phox}$ (C. L. and D. A. G.) with no translation product.

All patients except one, did not show a $gp91^{phox}$ bands in western-blot analysis and then were classified $X91^0$, while P. M. was $X91^-$ due the decreased intensity of $gp91^{phox}$ bands.

For one patient (A. A. K.), the genetic mutation assessment are still on going, but differently from the other CGD patients, showed gp91^{phox} band but not p22^{phox} and was classified A22^{0}.

As expected, several sites and domains of gp91^{phox} were interested by the mutations, with alteration of FAD-binding site (B. S. and P. M) and NADPH-binding site (P. G.) (tab. 1).

Although the homogeneity of the current therapy (all patients are under anti-bacterial and anti-fungal prophylaxis with Cotrimozazole and Intraconazole), the clinical history is very heterogeneous and at least 3 patients (P. M.; C. L. and C. A.), experimented *Aspergillus* infection with severe lung aspergillosis. Although can not be excluded that more that one of our patients undergoing on *C. albicans* and *S. aureus* infections, only one patient (B. S.) had a documented severe *C. albicans* infection (that led to osteomyelitis) and a *S. aureus* sepsis.

Other patogens that infected CGD patients were *Mycobacterium tuberculosis* (with severe meningitis in B. A.) and *Serratia* and the respiratory syncytial virus (RSV) that led to pyodermitis and bronchiolitis, respectively.

Finally, all patients (except A22^{0} patient that showed a residual activity) showed a decreased oxidative activity upon PHA stimulation (evaluated with DHR-test), both in frequency of oxidated granulocytes and in MFI, when compared with healthy subject.

Table 1. Molecular characterization of CGD patients.

Patients	Genonic DNA change	Exon/Intron location	Amino-acid change	CGD type (w/b analysis)	Mutated domain
MFM	252 G > A	Exon 3	none	X91°	
BS	1018 G > T	Exon 9	336 Glu > Stop	X91°	FADBD [a]
PM	1088 G > C	Exon 9	359 Gly > Ala	$X91^-$	FADBD
CA	1299 del T + 1302 del C	Exon 10	Frameshift C428	X91°	
PG	1369 T > A	Exon 11	453 Trp > Arg	X91°	NADPHBD [b]
CL	Del Exons 1-13 (>27 KB)	Exons 1-13		X91°	
DAG	Del Exons 1-13 (>27 KB)	Exons 1-13		X91°	
BA	83 G > A		28 Trp >Stop	X91°	
AAK	nd	nd	nd	A22°	nd

The mutations found in CGD patients by sequencing of PCR products, were characterized for genomic nucleotide changes, site of mutation and alteration in translational amino-acid. Moreover the resulting phenotype, as classified by western-blot analysis, was also shown.

[a] FADBD: Flavin adenine dinucleotide binding domain

[b] NADPHBD: Nicotinamide adenine dinucleotide phosphate binding domain

Table 2. Clinical features of CGD patients.

Patients	Date of birth	Age at diagnosis (years)	CGD TYPE	DHR test: % (MFI)	Severe infections	Minor infections	Non infectious complications	Profilaxis
BA	13/01/206	3	$X91^0$		Liver abscess, meningitis (*Mycobacterium tuberculosis*)			Cotrimoxazole, Itraconazole
BS	04/10/1979	10	$X91^0$		Sepsis (*Staphylococcus*), Osteomyelitis (*C. albicans*)	Salmonellosis, pyodermitis, anal fissures, epididimitis	Inflammatory bowel disease (IBD) and chronic uveitis	Cotrimoxazole, Itraconazole
PM	19/12/1996	1	$X91^-$		Lung aspergillosis, suppurative lymphadenitis	Pneumonia		Cotrimoxazole, Itraconazole
PG	15/07/1998	6	$X91^0$	7 (24)		Lymphadenitis, aphthous stomatitis		Cotrimoxazole, Itraconazole
CL	18/08/1998	2	$X91^0$		Lung aspergillosis, lung abscess	Recurrent lymphadenitis, pneumonia pyodermitis	Celiac disease	Cotrimoxazole, Itraconazole
DAG	03/08/1991	12	$X91^0$	7 (110)	Lung abscess, lymphadenitis, pyodermitis (*Serratia*)	Pneumonia		Cotrimoxazole, Itraconazole
MFM	24/10/2007	1 month	$X91^0$	27 (50)		Bronchiolitis (respiratory syncytial virus RSV)		Cotrimoxazole, Itraconazole
CA	13/12/2004	2	$X91^0$	29 (82)	Lung aspergillosis	Pneumonia, lymphadenitis	Granulomatous cystitis	Cotrimoxazole, Itraconazole
AAK	16/01/2004	4	$A22^0$	60 (350)	BCG infection, liver abscess, lymphadenitis	Salmonellosis, enteritis		Cotrimoxazole, Itraconazole

The CGD cohort of patients was characterized by severe and minor infection and non infectious complications. When documented, also the etiological agents are shown. In DHR-test the residual oxidative activity is shown as percentage (reference CTRL values 67-100 %) and mean fluorescence intensity (MFI)(reference CTRL values 500-1300) of granulocytes, upon PHA stimulation.

PROLIFERATION TEST UPON MITOGEN AND ANTIGEN STIMULATION

To verify differences of proliferative responses, PBMC from CGD patients and healthy control were stimulated with mitogens (PHA and OKT3/antiCD28), with bacterial and fungal antigens (TT, CMV, *C. albicans* lysate) and with heat-inactivated preparations of *A. fumigatus* hyphae, *C. albicans* yeast, *C. albicans* hyphae and *S. aureus*. For these last stimulations was used the best cell/antigen ratio, previously identified in a dose-response tests (see materials and methods).

There were not significant differences of proliferation between CGD *versus* CTRL upon mitogen stimulations (SI mean ± SD: 85.8 ± 41.8 *vs* 88.6 ± 57.0 with PHA and 74.8 ± 58.5 *vs* 80.4 ± 49.1 with OKT3/antiCD28 stimulation) (fig. 5a).

Stimulation with TT did not show differences between CGD and CTRL (6.1 ± 3.6 *vs* 5.0 ± 3.0), differently PBMC from CGD showed a higher SI compared to CTRL (7.2 ± 5.8 *vs* 4.0 ± 3.4) after stimulation with CMV (fig. 5b). Whereas, upon stimulation with C. *albicans* total lysate C. *albicans* lysate), a statistically significant difference was found between CGD *vs* CTRL (mean 9.2 ± 9.1; median 6.5; 1.4-18.1 *vs* 1.2 ± 0.5; 1.4; 0.7-1.5; U=5.50; *P*=0.0319), condition upon witch CTRL did not proliferate (SI_{max}=2.1 with SI cut-off = 2) (fig.5b).

After stimulation with the heat-inactivate preparations, no statistically significant differences were observed for all antigens tested (fig. 5c), with similar proliferation between CGD and CTRL upon *A. fumigatus* hyphae and *S. aureus* stimulations (10.9 ± 5.4 *vs* 11.0 ± 8.9) and (11.9 ± 7.1 *vs* 14.3 ± 12.5) respectively, and with small reduction of proliferation upon *C. albicans* yeast (17.1 ± 11.5 *vs* 22.6 ± 14.1) and increase upon *C. albicans* hyphae stimulations (17.8 ± 15.8 *vs* 13.6 ± 9.5) in CGD patients (fig. 5c).

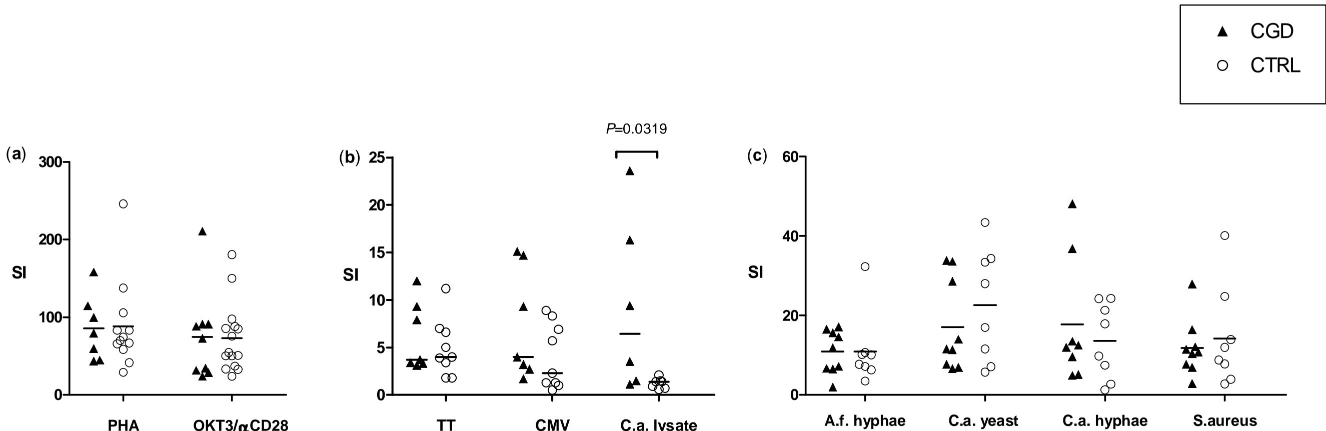


Figure 5. PBMC proliferation upon mitogen and antigen stimulation. Proliferation, determined by ^{3}H timidine incorporation was evaluated in upon 72h PHA (CGD patients n=7; CTRL n=12) and OKT3/antiCD28 (CGD patients n=9; CTRL n=15) stimulations (**a**); 120h upon TT (CGD patients n=7; CTRL n=9), CMV (CGD patients n=7; CTRL n=9) and *C. albicans* lysate stimulations (CGD patients n=6; CTRL n=7) (**b**); and upon 168h upon *A. fumigatus* hyphae (CGD patients n=9; CTRL n=8), *C. albicans* yeast (CGD patients n=9; CTRL n=8), *C. albicans* hyphae (CGD patients n=8; CTRL n=8) and *S. aureus* stimulations (CGD patients n=9; CTRL n=8) (**c**). Bars indicate stimulation index mean value. For *C. albicans* lysate stimulation difference between patients and controls was evaluated by Mann-Whitney non-parametric test ($U = 5.5$; $P = 0.032$).

CYTOKINE PRODUCTION UPON MITOGEN AND ANTIGEN STIMULATION

To verify differences in cytokine production between CGD patients and healthy controls, after mitogens (PHA and OKT3/antiCD28), and antigens *in vitro* stimulation (TT, CMV, *C. albicans* lysate, *A. fumigatus*. hyphae, *C. albicans*. yeast, *C. albicans*. hyphae, and *S. aureus*), the concentration of the cytokine was evaluated by ELISA in the supernatants of stimulated and unstimulated PBMC.

IFN-γ. All mitogen stimulations did not lead to differences in IFN-γ production between CGD and CTRL (with PHA: 12122 ± 17765 pg/ml *vs* 17277 ± 20826 pg/ml; with OKT3/αCD28: 34146 ± 34248 pg/ml *vs* 36714 ± 42405 pg/ml) (fig. 6a).

No statistically significant differences were observed upon 5 days antigen stimulation, with a lower IFN-γ production by CGD *vs* CTRL with TT (241 ± 278 pg/ml *vs* 432 ± 593 pg/ml) and CMV stimulations (357 ± 415 pg/ml *vs* 609 ± 1029 pg/ml), and a mean increase with *C. albicans* lysate stimulation (1336 ± 2495 pg/ml *vs* 47 ± 89 pg/ml) (fig. 6b).

A increased production of IFN-γ in CGD patients was observed also after *A. fumigatus* (688 ± 644 pg/ml *vs* 338 ± 423 pg/ml), and *C. albicans* hyphae stimulation (7519 ± 8842 pg/ml *vs* 2232 ± 2611 pg/ml), while very little differences were found after *C. albicans*

yeast (11561 ± 10098 pg/ml *vs* 11784 ± 8930 pg/ml) and *S. aureus* stimulation (3307 ± 3550 pg/ml *vs* 5447 ± 6501 pg/ml) (fig. 6c).

IL-17A. No differences were found with mitogens stimulation between CGD *vs* CTRL (PHA: 346 ± 320 pg/ml *vs* 455 ± 529 pg/ml; OKT3/αCD28: 279 ± 353 pg/ml *vs* 332 ± 364 pg/ml) (fig. 7a), and with TT stimulation (123 ± 187 pg/ml *vs* 129 ± 205 pg/ml) (fig. 7b)

Small differences were observed with CMV and *C. albicans* lysate (7 ± 12 pg/ml vs 28 ± 50 pg/ml; 87 ± 84 pg/ml vs 45 ± 62 pg/ml) (fig. 7b), with CMV stimulus unable to induce IL-17A production in CGD.

The bigger difference was found with *A. fumigatus* stimulation that induced a 3 fold increase of IL-17A production in CGD compared to CTRL (523 ± 635 pg/ml *vs* 167 ± 160 pg/ml) (fig. 7c). A two fold differences were observed also for *C. albicans* yeast and hyphae (913 ± 1294 pg/ml *vs* 1848 ± 3382 pg/ml and 790 ± 560 pg/ml *vs* 438 ± 504 pg/ml) and for *S. aureus* (177 ± 151 pg/ml *vs* 376 ± 565 pg/ml), respectively (fig. 7c).

IL-10A. All mitogen stimulations induced a decreased production of IL-10 in CGD compared with CTRL (PHA: 262 ± 289 pg/ml *vs* 311 ± 169 pg/ml; OKT3/αCD28: 92 ± 104 pg/ml *vs* 169 ± 181 pg/ml) (fig. 8a).

IL-10 production upon TT, CMV and *C. albicans* lysate was very small for CGD and CTRL (TT: 5 ± 10 pg/ml *vs* 29 ±35 pg/ml; CMV: 30 ±72 pg/ml *vs* 15 ± 20 pg/ml; *C. albicans* lysate: 1 ± 2 pg/ml *vs* 0 ±

1 pg/ml) (fig. 8b) and only one of CGD patients tested was able to produce IL-10 after TT and CMV stimulation.

Interestingly, *A. fumigatus*, induced a statistically significant increase in the IL-10 production in CGD compared to CTRL (mean: 237 ± 243 pg/ml; median: 167 pg/ml; 92-309 pg/ml *vs* 35 ± 33 pg/ml; 35 pg/ml; 0-50 pg/ml; U=5.00; *P*=0.0033) (fig. 8c). Both *C. albicans* yeast and hyphae also induced an increased production of IL-10 in CGD patient compared with control (132 ± 116 pg/ml *vs* 53 ± 52 pg/ml; 45 ± 57 pg/ml *vs* 2 ± 4 pg/ml). Whereas no differences were found upon *S. aureus* stimulation (35 ± 35 pg/ml *vs* 23 ± 24 pg/ml)(fig.8c).

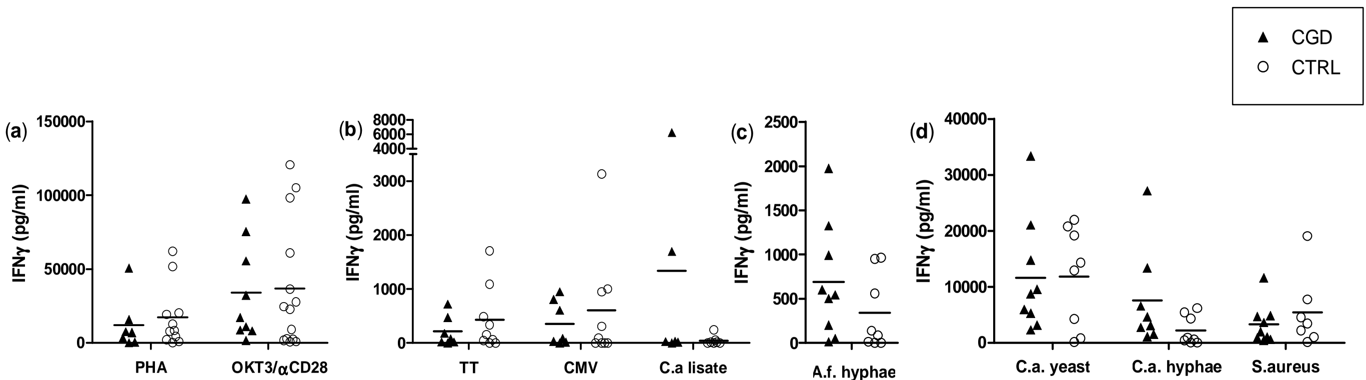


Figure 6. IFN-γ production upon mitogen and antigen stimulation. IFN-γ production by PBMC determined by ELISA, was evaluated in cell colture supernatants upon 72h PHA (CGD patients n=7; CTRL n=11) and OKT3/antiCD28 (CGD patients n=9; CTRL n=14) stimulations (**a**); 120h upon TT (CGD patients n=7; CTRL n=9), CMV (CGD patients n=7; CTRL n=9) and *C. albicans* lysate stimulations (CGD patients n=6; CTRL n=7) (**b**); and upon 168h *A. fumigatus* hyphae (CGD patients n=9; CTRL n=8), *C. albicans* yeast (CGD patients n=9; CTRL n=8), *C. albicans* hyphae (CGD patients n=8; CTRL n=8) and *S. aureus* stimulations (CGD patients n=9; CTRL n=7) (**c**). Bars indicate cytokine concentration mean value.

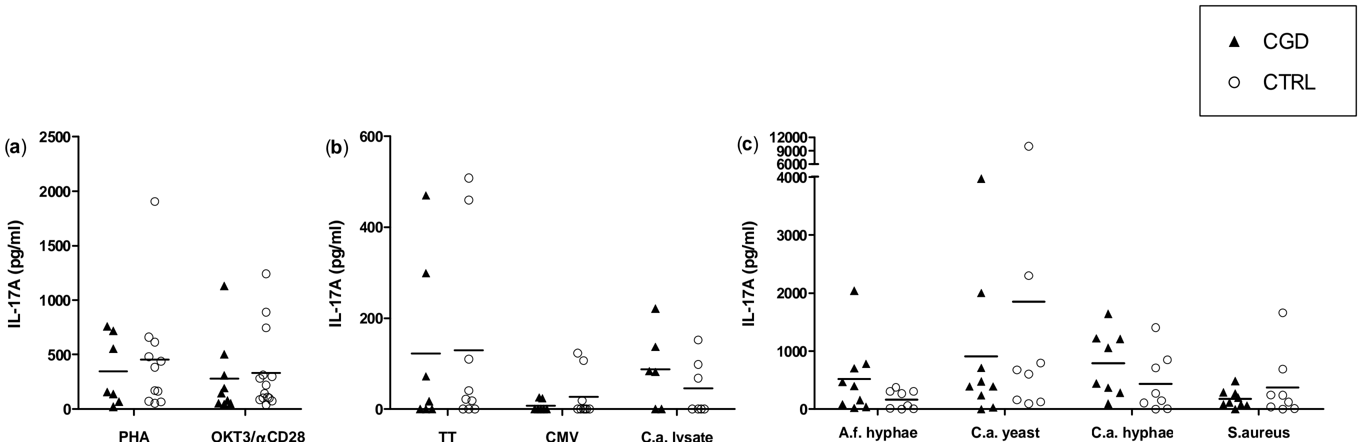


Figure 7. IL-17A production upon mitogen and antigen stimulation. IL-17A production by PBMC determined by ELISA, was evaluated in cell colture supernatants upon 72h PHA (CGD patients n=7; CTRL n=11) and OKT3/antiCD28 (CGD patients n=9; CTRL n=14) stimulations (**a**); 120h upon TT (CGD patients n=7; CTRL n=9), CMV (CGD patients n=7; CTRL n=9) and *C. albicans* lysate stimulations (CGD patients n=6; CTRL n=7) (**b**); and upon 168h *A. fumigatus* hyphae (CGD patients n=9; CTRL n=8), *C. albicans* yeast (CGD patients n=9; CTRL n=8), *C. albicans* hyphae (CGD patients n=8; CTRL n=8) and *S. aureus* stimulations (CGD patients n=9; CTRL n=8) (**c**). Bars indicate cytokine concentration mean value.

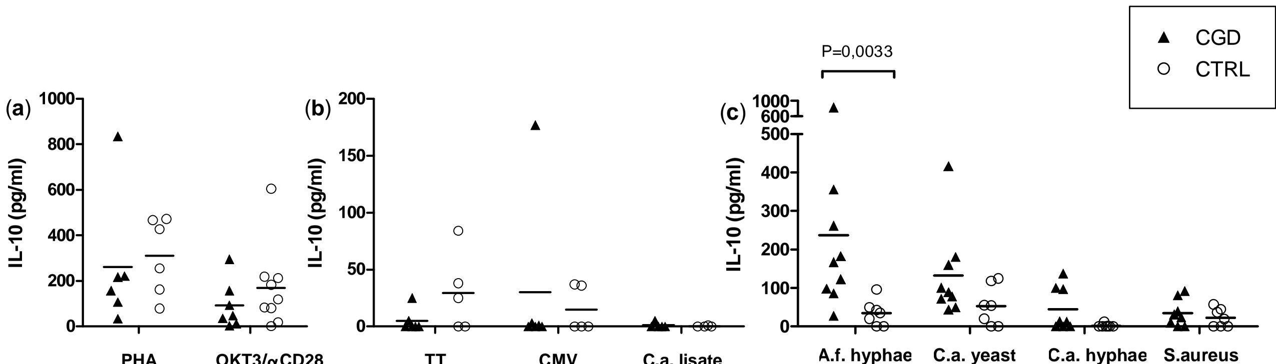


Figure 8. IL-10 production upon mitogen and antigen stimulation. IL-10 production by PBMC determined by ELISA, was evaluated in cell colture supernatants upon 72h PHA (CGD patients n=6; CTRL n=6) and OKT3/antiCD28 (CGD patients n=7; CTRL n=9) stimulations (**a**); 120h upon TT (CGD patients n=6; CTRL n=5), CMV (CGD patients n=6; CTRL n=5) and *C. albicans* lysate stimulations (CGD patients n=5; CTRL n=4) (**b**); and upon 168h *A. fumigatus* hypahe (CGD patients n=9; CTRL n=7), *C. albicans* yeast (CGD patients n=9; CTRL n=7), *C. albicans* hyphae (CGD patients n=8; CTRL n=7) and *S. aureus* stimulations (CGD patients n=8; CTRL n=7) (**c**). Bars indicate cytokine concentration mean value. For *A. fumigatus*. hyphae stimulation, difference between patients and controls was evaluated by Mann-Whitney non-parametric test ($U = 5.0$; $P = 0.0033$).

IFN-γ, IL-17A AND IL-10 mRNA BASAL EXPRESSION IN PBMC

To investigate whether our patients display a different basal IFN-γ, IL-17A and IL-10 production, quantitative real-time PCR was performed on the cDNA from unstimulated PBMC of 7 CGD, 7 age-matched healthy controls, two HIES patients and one CGD patient who had previously undergone bone marrow transplantation.

Although the β-actin relative expression of both cytokines showed low values, as expected in unstimulated PBMC, there were no significant differences in relative expression of INF-γ mRNA among CGD, CTRL, and HIES patients (mean: 401 ± 541, 487 ± 791 and 404 ± 42; median: 173, 153 and; 404, respectively). The expression of INF-γ by the only transplanted CGD was smaller than the mean values of the other groups (174 ± 54), but comparable with several single CGD and CTRL values (fig. 9a).

For the basal relative expression of IL-17A mRNA, CGD patients showed values higher than CTRL (298 ± 379; 79 *vs* 113 ± 129; 59; *P*= ns)(fig. 9b). As expected, the HIES patients had an absent basal expression of IL-17A (5 ± 5), while, interestingly, the transplanted CGD patient (fig. 9b, sample 9) showed a higher expression (1139 ± 238) than other subjects analyzed and resembles the mRNA expression of his brother who is carrying the same mutation (fig. 9b, sample 6).

IL-10 basal mRNA expression was increased in CGD patient compared to CTRL and HIES (mean: 120816 ± 172463, 24515 ± 38563 and 29089 ± 39279; median: 56107; 4443, and 29089, respectively), with high level also in the transplanted CGD patient (95890 ± 22235 vs 24515 ± 38563).

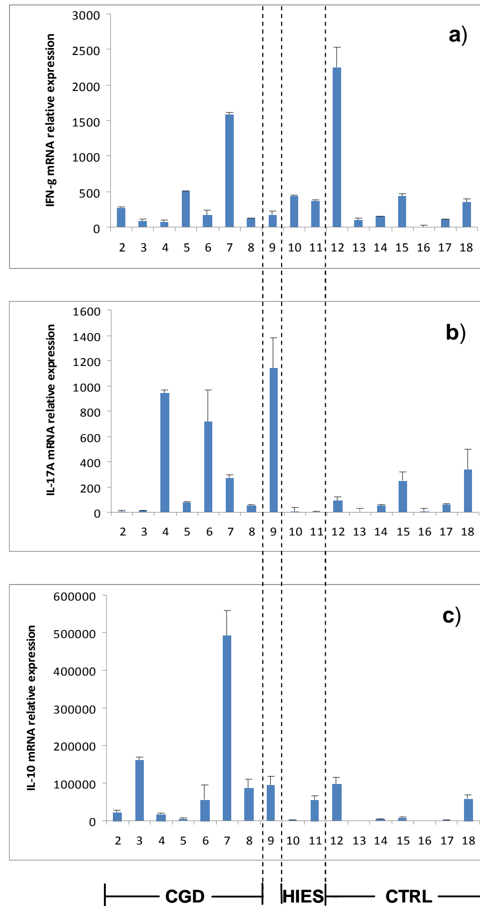


Figure 9. IFN-γ, IL-17A and IL-10 mRNA basal expression. Real-time was performed in unstimulated, thawed PBMC from n=7 CGD patients (line 2-8: PM; BS; CA; BA; PG; AAK; MFM), n=7 healthy control (line 12-18), one transplanted CGD patient (line 9: PA) and n=2 HIES patients (STAT3 mutated line 10, PA; STAT3 wt line11, LB). After the cDNA production the β-actin relative expression was evaluated for IFN-γ mRNA **a**), IL-17A mRNA **b**) and IL-10 mRNA **c**) Values shown are relative expression levels of duplicate samples (means and SD).

PHENOTYPIC ANALYSIS OF CD3$^+$ CELLS FROM UNSTIMULATED AND STIMULATED PBMC

To evaluate the differences of IFN-γ (Th1 lymphocytes) and IL-17A (Th17 lymphocytes) secreting cells between CGD patient and CTRL, the frequency of IFN-γ$^+$ cells; IL-17A$^+$ cells and IFN-γ$^+$ IL-17A$^+$ cells were estimated in the CD3$^+$ cell compartment by cytofluorimetric analysis performed on fresh PBMC without antigen stimulations (fig. 10, T0)(tab. 3), and after 7 days of stimulations upon *A. fumigatus* hyphae, *C. albicans* yeast and hyphae and *S. aureus* (fig.10, T1) (tab. 4 and 5). The fold inductions, (FI = frequency at T1 / frequency at T0) were also calculated for each cell compartment (tab. 4 and 5).

As expected, in unstimulated PBMC of 4 CTRL analyzed, the mean frequency of IL-17A$^+$ (0.5 ± 0.3 % of CD3+ cells) and IFN-γ$^+$ IL-17A$^+$ cells (0.3 ± 0.2 % of CD3+) was smaller compared with that found in IFN-γ$^+$ cells (13.7 ± 7.6 %).

Up to now we analyzed PBMC of 2 CGD patients (A22^0 and X91^0), one transplanted CGD (patient P. A.) and one HIES patient (STAT3 wt). Probably due to the large variability in CTRL, we did not find any differences in unstimulated PBMC of patients compared to CTRL (tab. 3).

After 7 days upon *C. albicans* yeast and hyphae stimulation, the PBMC of CTRLs showed an increased frequency (compared to that

at T0) for all $CD3^+$ compartments, with a major increased of IFN-γ^+ IL-$17A^+$ cells (FI for yeast: 6.6 ± 3.8; hyphae 5.7 ± 7.4). No relevant changes were observed with other stimuli (tab.4 and 5).

Compared to the CTRL values, CGD $A22^0$ patient had similar fold inductions upon *A. fumigatus* hyphae, *C. albicans* yeast, and *S. aureus*, but upon *C. albicans* hyphae, the frequency of IL-$17A^+$ cells and IFN-γ^+ IL-$17A^+$ cells was found markedly increased (FI: 10.2 *vs* 2.8 ± 1.4 and 13.0 *vs* 5.7 ± 7.4) (tab.5).

For the CGD $X91^0$ patient, although no differences were observed in induction of IFN-γ^+ cells for all antigens tested, a impairment of both IL-$17A^+$ and IFN-γ^+ IL-$17A^+$ cells was detected upon *C. albicans* yeast (0.5 *vs* 2.0 ± 0.5 and 3.1 *vs* 6.6 ± 3.8), and of IL-$17A^+$ cells induction upon *C. albicans* hyphae (0.9 *vs* 2.8 ± 1.4).

His bone-marrow transplanted sibling, showed a decreased values for all $CD3^+$ cell compartments after 7 days upon *C. albicans* yeast stimulation, and a slight increase of IFN-γ^+ IL-$17A^+$ cells induction upon *C. albicans* hyphae.

In HIES PBMC, *C. albicans* yeast and hyphae stimulations led to a impaired induction of IFN-γ^+ and IFN-γ^+ IL-$17A^+$ cells compared to CTRL, on the other hand, *C. albicans* hyphae stimulation allowed a small increase of IL-$17A^+$ cells compartment (4.8 *vs* 2.8 ± 1.4) (tab.4 and 5).

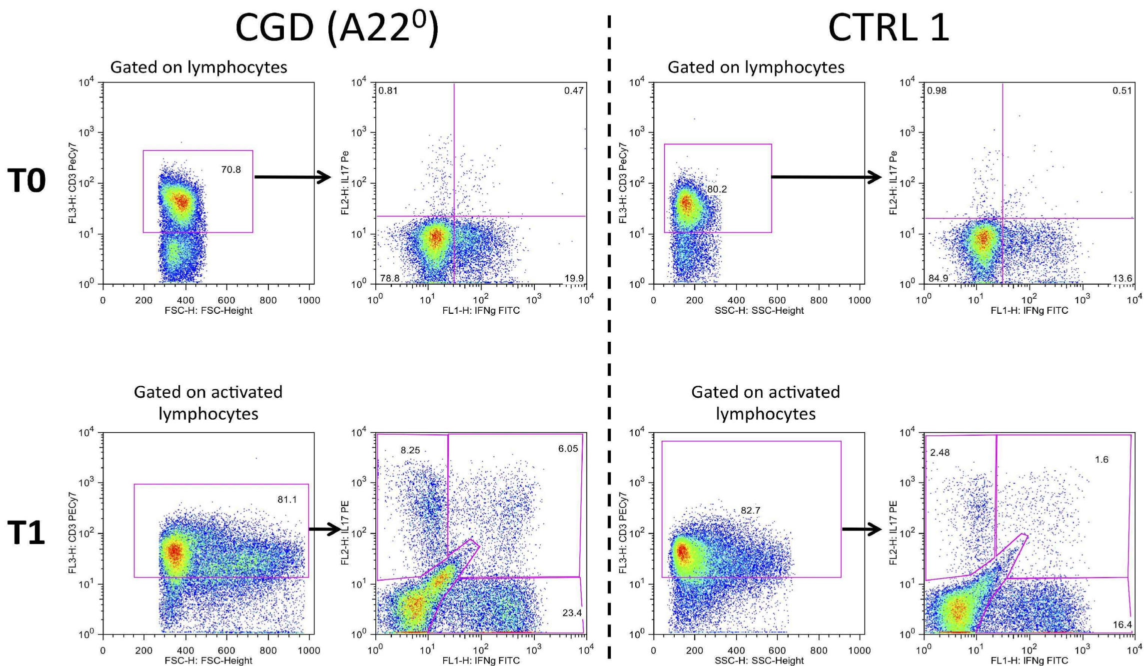


Figure 10. IL-17A and IFN-γ intracellular staining of unstimulated and stimulated PBMC. After membrane staining with anti-CD3-PeCy7, a intracellular staining for IL-17A-Pe and IFN-γ FITC was performed in unstimulated (T0) and 7 days antigen-stimulated PBMCs (T1) from a CGD ($A22^0$) patient and from a healthy subject (CTRL 1). For all experiments the frequency of IFN-γ^+, IL-17A^+ and IFN-γ^+ IL-17A^+ cells, was evaluated within the $CD3^+$ cells gate as shown in this representative experiment.

Table 3. Phenotype of unstimulated PBMC.

	T0		
	$CD3^+$ IFN-γ^+ %	$CD3^+$ IL-17A$^+$ %	$CD3^+$ IFN-γ^+ IL-17A$^+$ %
CTRL 1	13.6	0.98	0.51
CTRL 2	3.18	0.31	0.09
CTRL 3	17.6	0.24	0.08
CTRL 4	20.5	0.63	0.47
CTRL (mean ± sd)	13.7 ± 7.6	0.5 ± 0.3	0.3 ± 0.2
CGD (A22^0)	19.9	0.81	0.47
CGD (X91^0)	10.4	0.22	0.13
CGD (transplanted)	20.5	0.77	0.31
HIES (STAT3 wt)	16.2	0.21	0.1

After membrane staining with anti-CD3 Ab, a intracellular staining for IL-17A and IFN-γ was performed in unstimulated PBMC from 4 healthy controls, 2 CGD patient (with A22^0 and X91^0 phenotype), one CGD transplanted patient and one HIES patient. For controls subjects the means and standard deviations are also shown for each cell compartment.

Table 4. Phenotype of *A. fumigatus* hyphae and *C. albicans* yeast stimulated PBMC.

	T1 (*A. fumigatus* hyphae)			T1 (*C. albicans* yeast)		
	$CD3^+$ IFN-γ^+ % (**FI**)	$CD3^+$ IL-$17A^+$ % (**FI**)	$CD3^+$ IFN-γ^+ IL-$17A^+$ % (**FI**)	$CD3^+$ IFN-γ^+ % (**FI**)	$CD3^+$ IL-$17A^+$ % (**FI**)	$CD3^+$ IFN-γ^+ IL-$17A^+$ % (**FI**)
CTRL 1	12.8 (**0.9**)	1.5 (**1.5**)	0.8 (**1.6**)	21.2 (**1.6**)	1.6 (**1.6**)	3.1 (**6.1**)
CTRL 2				25.2 (**7.9**)	0.7 (**2.3**)	1.0 (**11**)
CTRL 3	15.2 (**0.9**)	0.3 (**1.3**)	0.1 (**1.3**)	18.9 (**1.1**)	0.6 (**2.5**)	0.6 (**7.5**)
CTRL 4	12.6 (**0.6**)	0.2 (**0.3**)	0.1 (**0.2**)	15.6 (**0.8**)	0.9 (**1.4**)	0.8 (**1.7**)
CTRL (FI: mean ± sd)	0.8 ± 0.2	1.0 ± 0.6	1.0 ± 0.7	2.9 ± 3.4	2.0 ± 0.5	6.6 ± 3.8
CGD ($A22^0$)	16.9 (**0.8**)	1.2 (**1.5**)	0.8 (**1.7**)	26.0 (**1.3**)	2.4 (**3.0**)	4.1 (**8.7**)
CGD ($X91^0$)				20.3 (**2.0**)	0.1 (**0.5**)	0.4 (**3.1**)
CGD (transplanted)				8.2 (**0.4**)	0.4 (**0.5**)	0.5 (**1.6**)
HIES (STAT3 wt)				9.3 (**0.6**)	0.6 (**2.9**)	0.2 (**2.0**)

After 7 days upon *A. fumigatus* hyphae and *C. albicans* yeast stimulation, membrane staining for CD3, and a intracellular staining for IL-17A and IFN-γ are performed on PBMC from 4 healthy controls, 2 CGD patient (with $A22^0$ and $X91^0$ phenotype), one CGD transplanted and one HIES (*STAT3* wt) patient. The frequency and the fold induction (FI = T1 % / T0 %) are shown. For controls subjects the means and standard deviations are also shown for each cell compartment.

Table 5. Phenotype of *C. albicans* hyphae and *S. aureus* stimulated PBMC.

	T1 (*C. albicans hyphae*)			T1 (*S. aureus*)		
	$CD3^+$ IFN-γ^+ % (**FI**)	$CD3^+$ IL-$17A^+$ % (**FI**)	$CD3^+$ IFN-γ^+ IL-$17A^+$ % (**FI**)	$CD3^+$ IFN-γ^+ % (**FI**)	$CD3^+$ IL-$17A^+$ % (**FI**)	$CD3^+$ IFN-γ^+ IL-$17A^+$ % (**FI**)
CTRL 1	16.4 (**1.2**)	2.5 (**2.6**)	1.6 (**3.1**)	12.5 (**0.9**)	0.6 (**0.6**)	1.2 (**2.4**)
CTRL 2	21.5 (**6.8**)	1.4 (**4.5**)	1.5 (**16.7**)			
CTRL 3	13.7 (**0.8**)	0.7 (**2.9**)	0.2 (**2.5**)	13.8 (**0.8**)	0.1 (**0.4**)	0.2 (**2.5**)
CTRL 4	16.2 (**0.8**)	0.7 (**1.1**)	0.3 (**0.6**)	13.4 (**0.7**)	0.6 (**1.0**)	0.5 (**1.1**)
CTRL (FI: mean ± sd)	2.4 ± 2.9	2.8 ± 1.4	5.7 ± 7.4	0.8 ± 0.1	0.7 ± 0.3	2.0 ± 0.7
CGD ($A22^0$)	23.4 (**1.2**)	8.3 (**10.2**)	6.1 (**13.0**)	22.5 (**1.1**)	0.6 (**0.7**)	1.3 (**2.8**)
CGD ($X91^0$)	8.2 (**0.8**)	0.2 (**0.9**)	0.5 (**3.8**)	13.8 (**1.3**)	0.1 (**0.5**)	0.3 (**2.3**)
CGD (transplanted)	19.0 (**0.9**)	1.6 (**2.1**)	2.6 (**8.4**)			
HIES (STAT3 wt)	8.6 (**0.5**)	1.0 (**4.8**)	0.2 (**2.0**)	7.8 (**0.5**)	0.1 (**0.5**)	0.1 (**1.0**)

After 7 days upon *C. albicans* hyphae and *S. aureus* stimulation, membrane staining for CD3, and a intracellular staining for IL-17A and IFN-γ are performed on PBMC from 4 healthy controls, 2 CGD patient (with $A22^0$ and $X91^0$ phenotype), one CGD transplanted and one HIES (STAT3 wt) patient. The frequency and the fold induction (FI = T1 % / T0 %) are shown. For controls subjects the means and standard deviations are also shown for each cell compartment.

CORRELATION BETWEEN CLINICAL, GENETICAL AND IMMUNOLOGICAL FEATURES

To better understand if differences of proliferative responses and cytokine production, could explain the heterogeneity of clinical features among CGD patients, we have attempted to compare the immunological data between stratified populations of patients, and when a patient showed a particularly interesting genetic, pathologic or immunological feature, we have also performed a single patient analysis.

Immunological response of PBMC from *A. fumigatus* infected and non infected CGD patients

To investigate the differences among CGD cohort, the patients have been stratified in two subgroups composed of: those that had manifested at least one demonstrated infection by *A. fumigatus* (CGD *A. f.* infected), and those who did not (CGD *A. f.* non infected), the proliferations and the cytokine production upon mitogens and *A. fumigatus* hyphae of the two subgroups and control subjects were compared.

Proliferation. Although the CGD *A. f.* infected patients had a higher proliferation rate for all mitogen stimulations (SI with PHA: 111 ± 49; with OKT3/αCD28: 124 ± 75), compared with CGD *A. f.*

non infected (67 ± 28; 50 ± 31), and controls (88 ± 57; 80 ± 50) (fig. 11a), no differences were observed in proliferation upon *A.fumigatus* hyphae stimulation among the two groups of patients and controls (11 ± 4; 11 ± 6; 11 ± 9), respectively (fig. 11b).

IFN-γ. IFN-γ production upon PHA stimulation was lower for CGD *A. f.* infected *versus* CGD *A. f.* non infected and healthy controls (5484 ± 9008 pg/ml vs 17100 ± 22360 pg/ml and 17277 ± 20826 pg/ml), while there were no differences among the three groups upon OKT3/αCD28 stimulation (34561 ± 35596 pg/ml; 33939 ± 37009 pg/ml and 36714 ± 42405 pg/ml) (fig. 12a).

Upon *A. fumigatus* hyphae stimulation, the group of CGD *A. f.* non infected showed a higher production of IFN-γ (907 ± 684 pg/ml) compared with other CGD patients and control that showed similar cytokine production (249 ± 231pg/ml and 338 ± 423 pg/ml, respectively) (fig. 12b).

IL-17A. The IL-17A production evaluated upon mitogens stimulation, showed a higher concentration for CGD *A. f.* infected for both PHA and OKT3/αCD28 stimulation (500 ± 415 pg/ml and 560 ± 542 pg/ml) compared with CGD *A. f.* non infected (230 ± 220 pg/ml and 138 ± 101 pg/ml) and control (455 ± 529 pg/ml and 332 ± 364 pg/ml) (fig. 13a).

Although the *A. fumigatus* hyphae induced a lower production of IL-17A in CGD *A. f.* infected *versus* CGD *A. f.* non infected patients (423 ± 348 pg/ml *vs* 573 ± 766 pg/ml), the IL-17A concentrations in

both CGD groups were increased compared to healthy controls (167 ± 160 pg/ml) (fig. 13b).

IL-10. Upon PHA stimulation IL-10 production was less in CGD *A. f.* infected (164 ± 81 pg/ml) than in CGD *A. f.* non infected patients (311 ± 358 pg/ml) and controls (311 ± 169 pg/ml). On the other hand, upon OKT3/αCD28 stimulation, CGD *A. f.* infected patients produced IL-10 in the same quantity as the control (152 ± 146 pg/ml *vs* 169 ± 181 pg/ml), but had a increased production compared to *A. f.* non infected patients (152 ± 146 pg/ml *vs* 47 ± 35 pg/ml) (fig. 14a).

When stimulated with *A. fumigatus* hyphae, the CGD *A. f.* infected produced a comparable concentration of IL-10 than *A. f.* non infected patients (208 ± 137 pg/ml *vs* 251 ± 293 pg/ml), but these last group showed a statistically significant increase compared to the healthy controls (mean 251 ± 293 pg/ml; median 145 pg/ml; 81-404 pg/ml *vs* 35 ± 33 pg/ml; 35 pg/ml; 0-50 pg/ml; U= 4.00; P=0.0140) (fig. 14b).

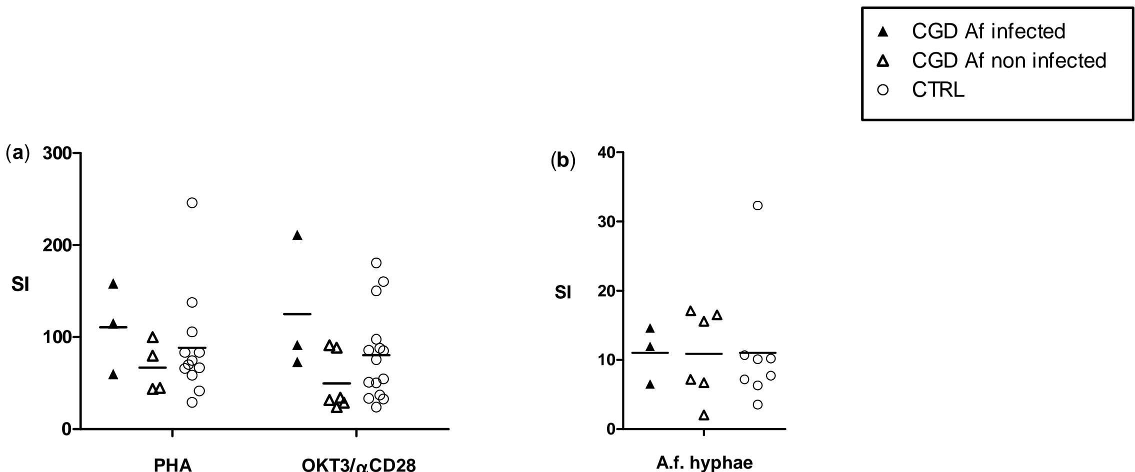


Figure 11. PBMC proliferation upon mitogen and *A. fumigatus* stimulation in *A. fumigatus* infected and non infected patients. Proliferation, determined by ^{3}H timidine incorporation was evaluated upon 72h PHA (CGD *A. f.* infected patients n=3; CGD *A. f.* non infected patients n=4; CTRL n=12) and OKT3/antiCD28 (CGD *A. f.* infected patients n=3; CGD *A. f.* non infected patients n=6; CTRL n=15) stimulations (**a**); and upon 168h *A. fumigatus* hyphae stimulation (CGD *A. f.* infected patients n=3; CGD *A. f.* non infected patients n=6; CTRL n=8) (**b**). Bars indicate stimulation index mean value.

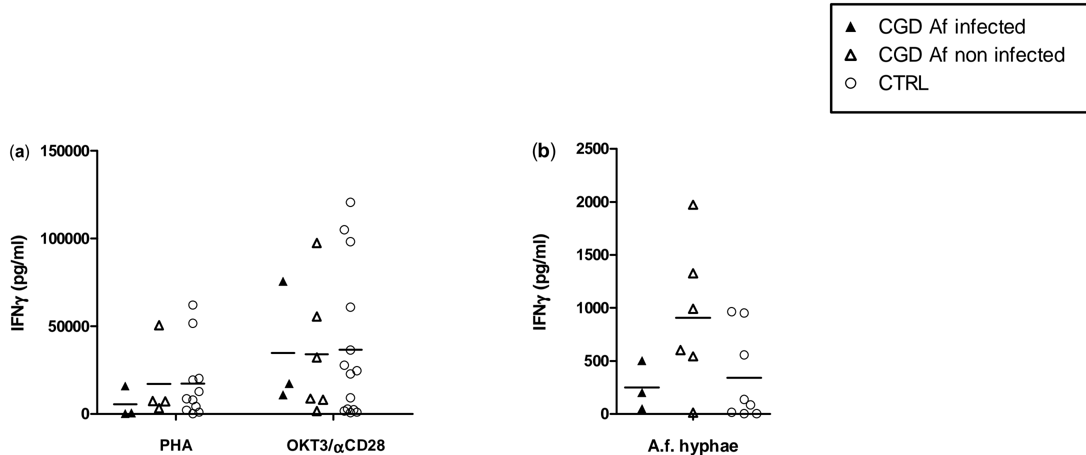


Figure 12. IFNγ production upon mitogen and *A. fumigatus* stimulation in *A. fumigatus* infected and non infected patients. IFNγ production by PBMC determined by ELISA, was evaluated in cell colture supernatants upon 72h PHA (CGD A. f. infected patients n=3; CGD *A. f.* non infected patients n=4; CTRL n=11) and OKT3/antiCD28 (*A. f.* infected patients n=3; CGD *A. f.* non infected patients n=6; CTRL n=14) stimulations (**a**); and upon 168h *A. fumigatus* hyphae stimulation (*A. f.* infected patients n=3; CGD *A. f.* non infected patients n=6; CTRL n=8) (**b**). Bars indicate cytokine concentration mean value.

Figure 13. IL-17A production upon mitogen and *A. fumigatus* stimulation in *A. fumigatus* infected and non infected patients. IL-17A production by PBMC determined by ELISA, was evaluated in cell colture supernatants upon 72h PHA (CGD *A. f.* infected patients n=3; CGD *A. f.* non infected patients n=4; CTRL n=11) and OKT3/antiCD28 (*A. f.* infected patients n=3; CGD *A. f.* non infected patients n=6; CTRL n=14) stimulations (**a**); and upon 168h *A. fumigatus* hyphae stimulation (*A. f.* infected patients n=3; CGD *A. f.* non infected patients n=6; CTRL n=8) (**b**). Bars indicate cytokine concentration mean value.

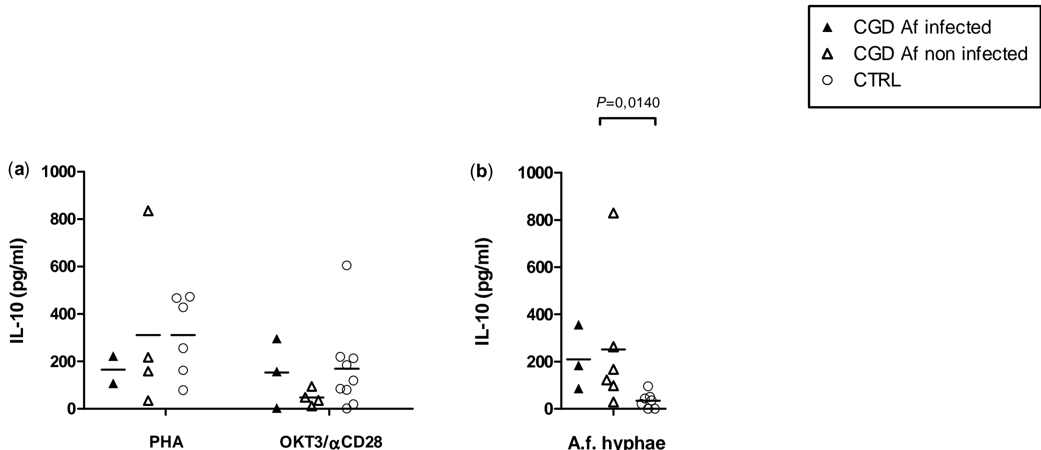


Figure 14. IL-10 production upon mitogen and *A. fumigatus* stimulation in *A. fumigatus* infected and non infected patients. IL-10 production, by PBMC determined by ELISA, was evaluated in cell colture supernatants upon 72h PHA (CGD *A. f.* infected patients n=2; CGD *A. f.* non infected patients n=4; CTRL n= 6) and OKT3/antiCD28 (*A. f.* infected patients n=3; CGD *A. f.* non infected patients n=4; CTRL n=9) stimulations (**a**); and upon 168h *A. fumigatus* hyphae stimulation (*A. f.* infected patients n=3; CGD *A. f.* non infected patients n=6; CTRL n=7) (**b**). Bars indicate cytokine concentration mean value. For *A. f.* hyphae stimulation, difference between *A. f.* non infected patients and controls was evaluated by Mann-Whitney non-parametric test ($U = 54.0$; $P = 0.014$).

Immunological characteristics of a A22^0 CGD patient

A.A.K. a 6 years old male child, was the only patient included in the CGD cohort, characterized by absence of the NADPH oxidase subunit p22^{phox} (A22^0 phenotype) in the western-blotting analysis. Although p22^{phox} mutation has not been yet identified, this patient conserved a gp91^{phox} and showed a residual oxidative activity by DHR test, compared to other CGD patients.

As expected from the positivity for anti-CMV IgG, upon CMV stimulation the proliferative response (SI: 15) was higher than other CGD and CTRL (6 ± 4 and 4 ± 3), with a comparable production of IFN-γ (607 pg/ml *vs* 315 ± 438 pg/ml; and 609 ± 1029 pg/ml), and of IL-17A (24 pg/ml *vs* 4 ± 10 pg/ml and 27 ± 50 pg/ml) and a greater production of IL-10 (177 pg/ml *vs* 1 ± 1 pg/ml and 15 ± 20 pg/ml).

Upon *C. albicans* lysate stimulation the PBMC of the patient, compared to other CGD, showed increased proliferation (SI: 16.3 vs 7.8 ± 9.4), increased IFN-γ and IL-17A production (IFN-γ: 1698 vs 1263 ± 2782; IL-17A: 221 vs 61 ± 60), but similarly to other GCD, did not produce of IL10.

Other marked differences were found upon *A. fumigatus* hyphae stimulation, with a very small proliferation and cytokine productions *versus* CGD and healthy controls (SI: 2 *vs* 12 ± 5 and 11 ± 9; IFN-γ: 13 pg/ml *vs* 772 ± 632 pg/ml and 338 ± 423 pg/ml; IL-17A: 41 pg/ml

vs 583 ± 650 pg/ml and 167 ± 160 pg/ml; IL-10: 28 pg/ml *vs* 263 ± 246 pg/ml and 35 ± 33 pg/ml).

Also upon *C. albicans* yeast and hyphae stimulation, A.A.K. revealed important differences in comparison to the other CGD, showing a very high proliferation level (yeast SI: 34 *vs* 15 ± 10; hyphae SI: 37 *vs* 15 ± 15), coupled to small amount of IFN-γ (for yeast: 2300 pg/ml *vs* 12719 ± 10137 pg/ml; for hyphae: 1540 pg/ml *vs* 8373 ± 9187 pg/ml) and IL-10 (for yeast: 78 pg/ml *vs* 139 ± 122 pg/ml; for hyphae: 13 pg/ml *vs* 49 ± 59 pg/ml), but to a increased levels of IL-17A (for yeast: 2000 pg/ml *vs* 777 ± 1312 pg/ml; for hyphae: 1640 pg/ml *vs* 669 ± 477 pg/ml) (A.A.K. was the most producer of IL-17A upon C. a. hyphae stimulation compared to the CGD and CTRL)

Upon *S. aureus* stimulation the proliferative response was similar to other CGD (SI: 12 *vs* 12 ± 7), with decreased IFN-γ (477 pg/ml *vs* 3660 ± 3622 pg/ml), decreased IL-17A (65 pg/ml *vs* 190 ± 155 pg/ml) and normal IL-10 production (39 pg/ml *vs* 34 ± 37 pg/ml).

Finally, due to differences in cytokines production, A.A.K. was found to have the smaller IFN-γ/IL-17A ratio upon *A. fumigatus* hyphae, *C. albicans* yeast and hyphae, compared with other CGD and healthy controls: 0.3 vs 9 ± 19 and 2 ± 1; 1.2 vs 75 ±107 and 16 ± 13; 0.9 vs 28 ± 50; 6 ± 6 respectively.

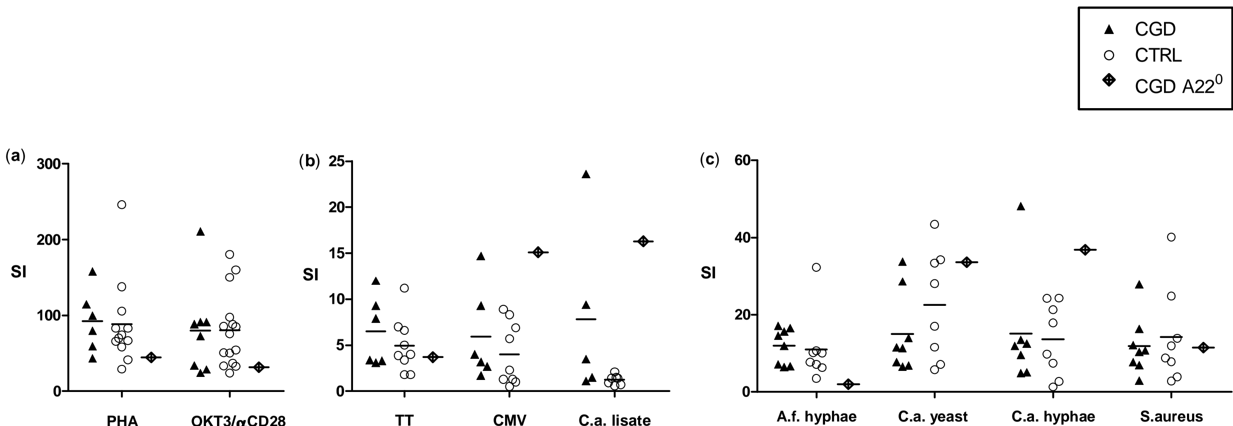
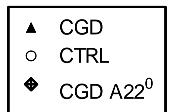


Figure 15. PBMC proliferation upon mitogen and antigen stimulation. Proliferation, determined by ^{3}H timidine incorporation was evaluated in upon 72h PHA (CGD patients n=7; CTRL n=12) and OKT3/antiCD28 (CGD patients n=9; CTRL n=15) stimulations (**a**); 120h upon TT (CGD patients n=7; CTRL n=9), CMV (CGD patients n=7; CTRL n=9) and *C. albicans* lysate stimulations (CGD patients n=6; CTRL n=7) (**b**); and upon 168h upon *A. fumigatus* hyphae (CGD patients n=9; CTRL n=8), *C. albicans* yeast (CGD patients n=9; CTRL n=8), *C. albicans* hyphae (CGD patients n=8; CTRL n=8) and *S. aureus* stimulations (CGD patients n=9; CTRL n=8) (**c**). Bars indicate stimulation index mean value.



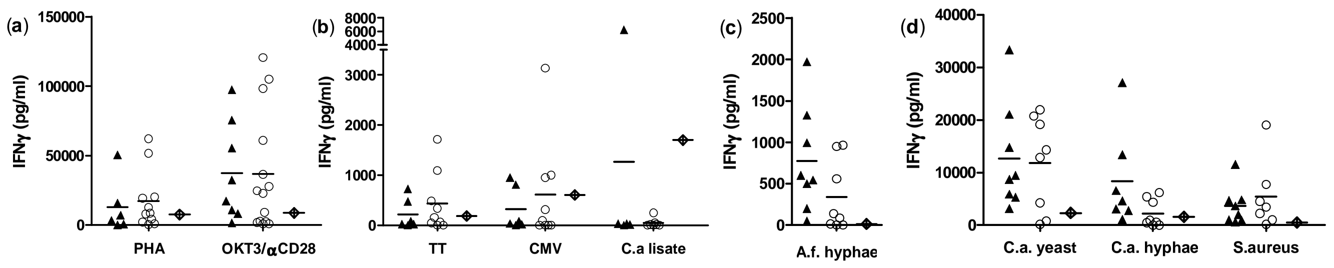


Figure 16. IFN-γ production upon mitogen and antigen stimulation. IFN-γ production by PBMC determined by ELISA, was evaluated in cell colture supernatants upon 72h PHA (CGD patients n=7; CTRL n=11) and OKT3/antiCD28 (CGD patients n=9; CTRL n=14) stimulations (**a**); 120h upon TT (CGD patients n=7; CTRL n=9), CMV (CGD patients n=7; CTRL n=9) and *C. albicans* lysate stimulations (CGD patients n=6; CTRL n=7) (**b**); and upon 168h *A. fumigatus* hyphae (CGD patients n=9; CTRL n=8), *C. albicans* yeast (CGD patients n=9; CTRL n=8), *C. albicans* hyphae (CGD patients n=8; CTRL n=8) and *S. aureus* stimulations (CGD patients n=9; CTRL n=7) (**c**). Bars indicate cytokine concentration mean value.

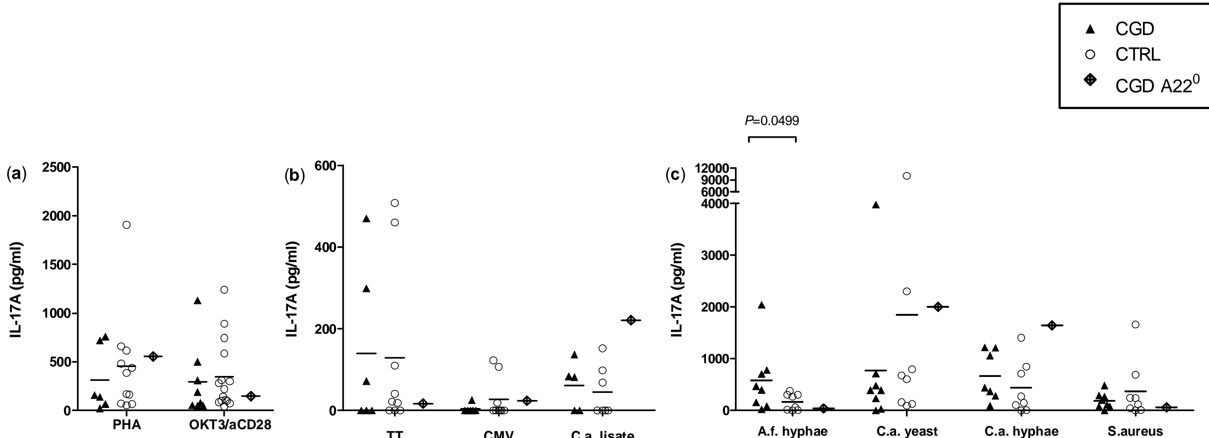


Figure 17. IL-17A production upon mitogen and antigen stimulation. IL-17A production by PBMC determined by ELISA, was evaluated in cell colture supernatants upon 72h PHA (CGD patients n=7; CTRL n=11) and OKT3/antiCD28 (CGD patients n=9; CTRL n=14) stimulations (**a**); 120h upon TT (CGD patients n=7; CTRL n=9), CMV (CGD patients n=7; CTRL n=9) and *C. albicans* lysate stimulations (CGD patients n=6; CTRL n=7) (**b**); and upon 168h *A. fumigatus* hyphae (CGD patients n=9; CTRL n=8), *C. albicans* yeast (CGD patients n=9; CTRL n=8), *C. albicans* hyphae (CGD patients n=8; CTRL n=8) and *S. aureus* stimulations (CGD patients n=9; CTRL n=8) (**c**). Bars indicate cytokine concentration mean value. For *A. fumigatus*. hyphae stimulation, difference between patients and controls was evaluated by Mann-Whitney non-parametric test ($U = 13.00$; $P = 0.0499$).

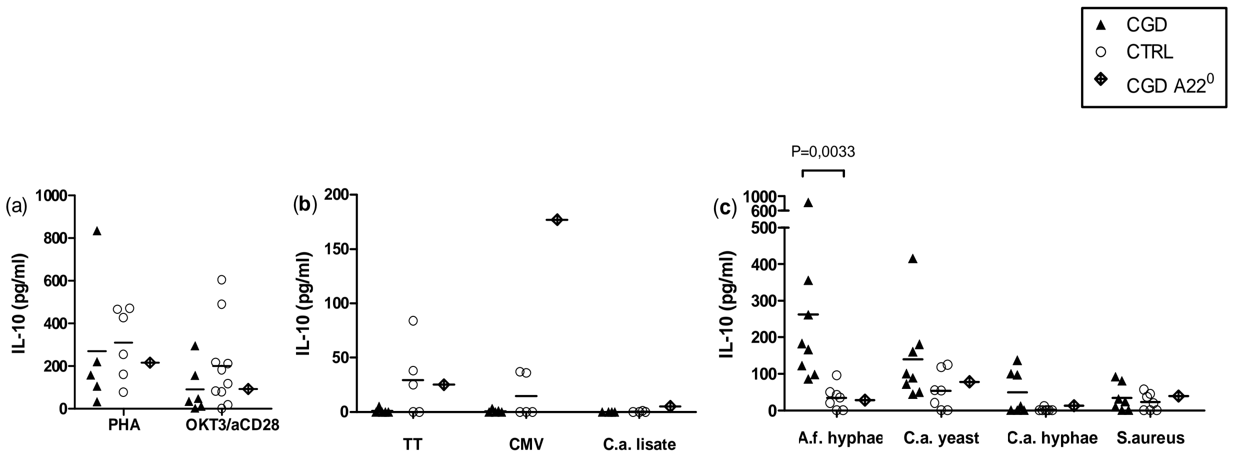


Figure 18. IL-10 production upon mitogen and antigen stimulation. IL-10 production by PBMC determined by ELISA, was evaluated in cell colture supernatants upon 72h PHA (CGD patients n=6; CTRL n=6) and OKT3/antiCD28 (CGD patients n=7; CTRL n=9) stimulations (**a**); 120h upon TT (CGD patients n=6; CTRL n=5), CMV (CGD patients n=6; CTRL n=5) and *C. albicans* lysate stimulations (CGD patients n=5; CTRL n=4) (**b**); and upon 168h *A. fumigatus* hypahe (CGD patients n=9; CTRL n=7), *C. albicans* yeast (CGD patients n=9; CTRL n=7), *C. albicans* hyphae (CGD patients n=8; CTRL n=7) and *S. aureus* stimulations (CGD patients n=8; CTRL n=7) (**c**). Bars indicate cytokine concentration mean value. For *A. fumigatus*. hyphae stimulation, difference between patients and controls was evaluated by Mann-Whitney non-parametric test ($U = 5.00$; $P = 0.0033$).

Immunological responses of a CGD patient affected by chronic inflammation

Another patient was B.S., a 30.5 years old a male, with gp91^{phox} deficiency (X91^{0}), who differed from other CGD patient for a documented acute *C. albicans* infection (osteomyelitis) at 10 years old, and is the only patient that also present a chronic inflammation (chronic enterocolitis).

Upon *A. fumigatus* stimulation, B.S. compared with CGD patients and CTRL had the best proliferative response (SI: 17 *vs* 10 ± 5 and 11 ± 9), with a very high production of IFN-γ (1324 pg/ml *vs* 608 ± 639 pg/ml and 338 ± 423 pg/ml), the lowest value of IL-17A (24 pg/ml *vs* 585 ± 648 pg/ml and 167 ± 160 pg/ml) and a value of IL-10 similar to other CGD, but increased respect CTRL (167 pg/ml *vs* 246 ± 258 pg/ml and 35 ± 33 pg/ml).

Upon *C. albicans* lysate, the patient displayed a marked proliferation (SI: 24 *vs* 6 ± 6 and 1 ± 1), with the greatest IFN-γ production (6240 pg/ml *vs* 355 ± 751 pg/ml and 47 ± 89 pg/ml), but did not produce IL-17A (0 pg/ml *vs* 105 ± 81 pg/ml and 46 ± 62 pg/ml) nor IL-10, similar to CGD and CTRL.

Moreover with *C. albicans* yeast and hyphae, B. S. showed respectively reduced (SI: 7 *vs* 18 ± 11 and 23 ± 14) and similar (SI: 14 *vs* 18 ± 17 and 14 ± 10) proliferation, with similar (9500 pg/ml *vs* 11819 ± 10763 pg/ml and 11784 ± 8930 pg/ml) and increased

(13400 pg/ml *vs* 6678 ± 9199 pg/ml and 2232 ± 2611 pg/ml) IFN-γ production; very small levels of IL-17A (yeast: 31 pg/ml *vs* 1023 ± 1337 pg/ml and 1848 ± 3382 pg/ml; hyphae: 95 pg/ml *vs* 890 ± 523 pg/ml and 438 ± 504 pg/ml) and increased production of IL-10 (yeast: 181 pg/ml *vs* 126 ± 122 pg/ml and 53 ± 52 pg/ml; hyphae: 100 pg/ml *vs* 37 ± 56 pg/ml and 2 ± 4 pg/ml).

Finally, upon *S. aureus*, despite PBMC proliferated similar to that of other patient (SI: 12 *vs* 12 ± 8 and 14 ± 13).they had as decrease of both IFN-γ (1012 pg/ml *vs* 3593 ± 3682 pg/ml and 5447 ± 6501 pg/ml) and IL-17A production (81 pg/ml *vs* 189 ± 157 pg/ml and 376 ± 565 pg/ml).

Summarizing, B. S. showed a tendency to produce high levels of IFN-γ coupled to a very small IL-17A production, and differs from other CGD patient to have bigger IFN-γ/IL-17A ratio for *C. albicans* lysate (6240 pg/ml *vs* 2 ±4 and 1 ± 2), yeast (307 *vs* 31 ± 36 and 16 ± 13), hyphae (141 *vs* 8 ± 7 and 6 ± 6), and for *A. fumigatus* hyphae (55 *vs* 2 ± 4 and 2 ± 1).

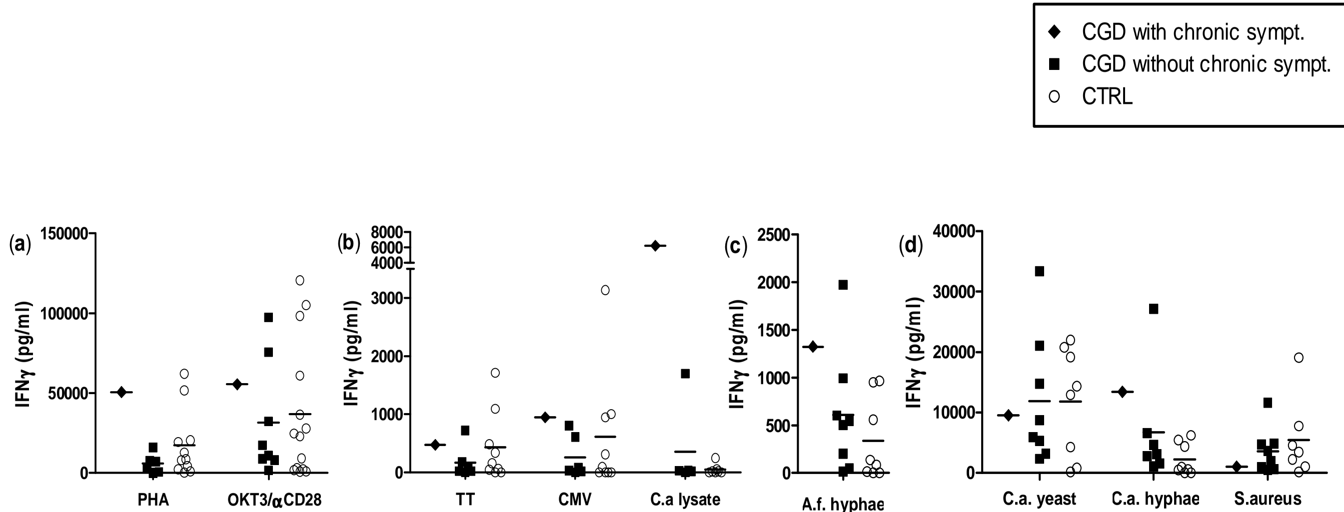


Figure 19. IFN-γ production upon mitogen and antigen stimulation. IFN-γ production by PBMC determined by ELISA, was evaluated in cell colture supernatants upon 72h PHA (CGD patients n=7; CTRL n=11) and OKT3/antiCD28 (CGD patients n=9; CTRL n=14) stimulations (**a**); 120h upon TT (CGD patients n=7; CTRL n=9), CMV (CGD patients n=7; CTRL n=9) and *C. albicans* lysate stimulations (CGD patients n=6; CTRL n=7) (**b**); and upon 168h *A. fumigatus* hyphae (CGD patients n=9; CTRL n=8), *C. albicans* yeast (CGD patients n=9; CTRL n=8), *C. albicans* hyphae (CGD patients n=8; CTRL n=8) and *S. aureus* stimulations (CGD patients n=9; CTRL n=7) (**c**). Bars indicate cytokine concentration mean value.

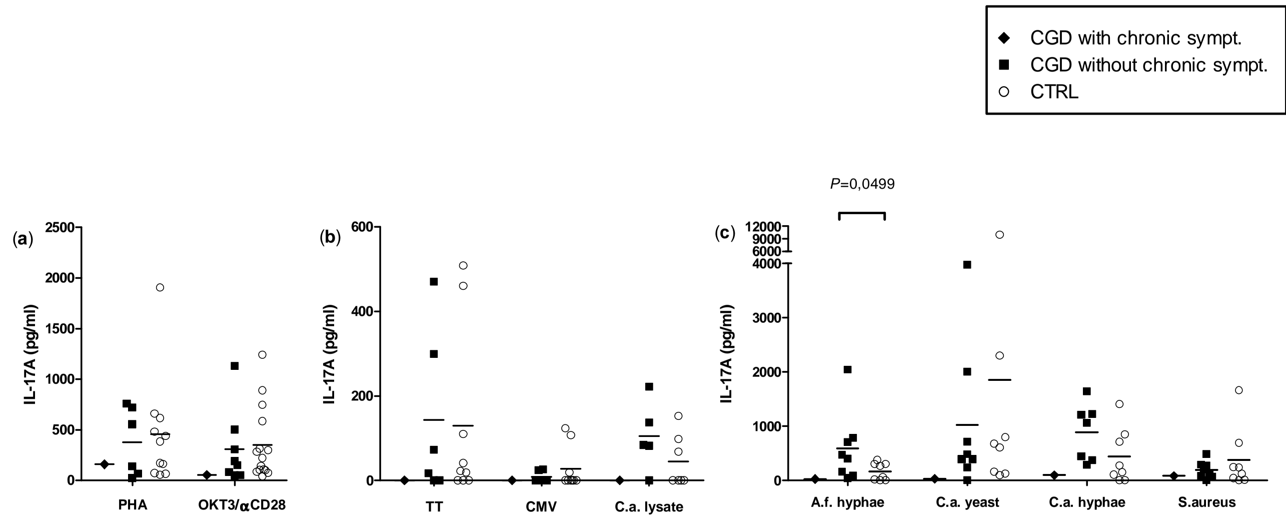


Figure 19. IL-17A production upon mitogen and antigen stimulation. IL-17A production by PBMC determined by ELISA, was evaluated in cell colture supernatants upon 72h PHA (CGD patients n=7; CTRL n=11) and OKT3/antiCD28 (CGD patients n=9; CTRL n=14) stimulations (**a**); 120h upon TT (CGD patients n=7; CTRL n=9), CMV (CGD patients n=7; CTRL n=9) and *C. albicans* lysate stimulations (CGD patients n=6; CTRL n=7) (**b**); and upon 168h *A. fumigatus* hyphae (CGD patients n=9; CTRL n=8), *C. albicans* yeast (CGD patients n=9; CTRL n=8), *C.*

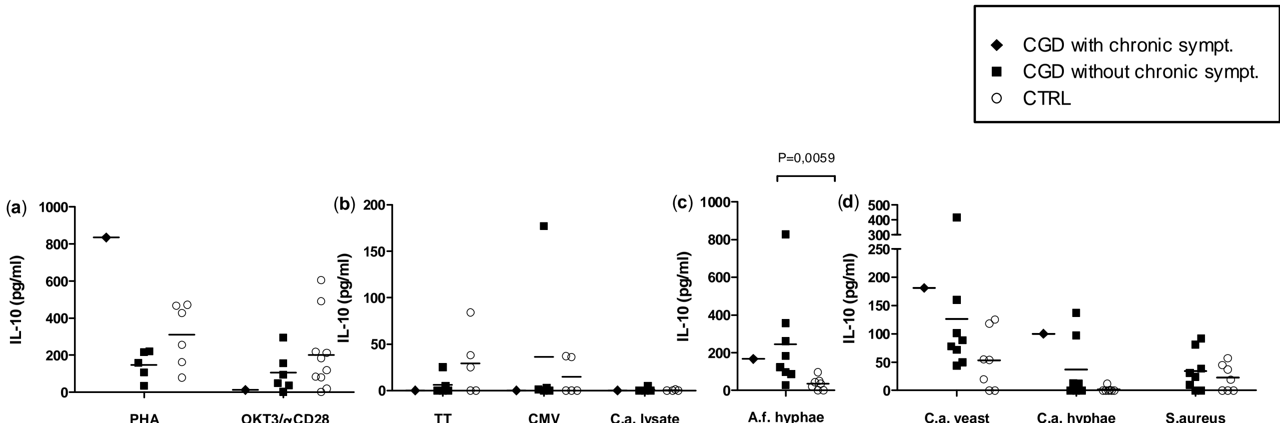


Figure 20. IL-10 production upon mitogen and antigen stimulation. IL-10 production by PBMC determined by ELISA, was evaluated in cell colture supernatants upon 72h PHA (CGD patients n=6; CTRL n=6) and OKT3/antiCD28 (CGD patients n=7; CTRL n=9) stimulations (**a**); 120h upon TT (CGD patients n=6; CTRL n=5), CMV (CGD patients n=6; CTRL n=5) and *C. albicans* lysate stimulations (CGD patients n=5; CTRL n=4) (**b**); and upon 168h *A. fumigatus* hypahe (CGD patients n=9; CTRL n=7), *C. albicans* yeast (CGD patients n=9; CTRL n=7), *C. albicans* hyphae (CGD patients n=8; CTRL n=7) and *S. aureus* stimulations (CGD patients n=8; CTRL n=7) (**c**). Bars indicate cytokine concentration mean value. For *A. fumigatus*. hyphae stimulation, difference between patients and controls was evaluated by Mann-Whitney non-parametric test (U = 5.00; P = 0.0059).

Two cases of Hyper-Immunoglobulin E Syndrome

Since HIES is characterized, like CGD, by abnormal susceptibility by infections of *C. albicans* and *S. aureus*, and by a reduced presence of IL-17$^+$ cells, we have also included two HIES patients in our cohort as control for CGD patients. One HIES patients (PG) was a 15 years old male, characterized by a mutation on *STAT3* gene (exon 21: 1909G>A; SH2 domain: V637M), while other patient (LB) was a 10 years old female, without *STAT3* mutation but that displayed a clinical features of HIES (score: 40).

Compared to the age-matched cohort of CTRL, SI upon OKT3/antiCD28 stimulation was reduced for PG (10 *vs* 71 ± 51) and increased for LB (256). The same pattern was found with TT stimulation (1; 6; *vs* 7 ± 5 for PG, LB and CTRL, respectively), while no difference were found upon CMV (3; 2; *vs* 1.2 ± 0.9) and *C. albicans lysate* (2; 3; 1.1 ± 0.7). HIES patients had comparable proliferation but reduced *versus* CTRL upon *A. fumigatus* hyphae (6; 5 *vs* 13.3 ± 10.7) and upon *S. aureus* (4; 5 *vs* 16.5 ± 13.4), but increased upon *C. albicans* hyphae stimulation (21; 20 *vs* 14.4 ± 9.7). Upon *C. albicans* yeast PG had the same SI that CTRL (24 *vs* 21.7 ± 14.2), whereas LB had a reduced proliferation (9.8).

ELISA performed with supernatants of PBMC coltures, showed that when cells were stimulated with OKT3/antiCD28 the IFN-γ production was reduced for PG (572 pg/ml) and comparable to

CTRL (32433 pg/ml ± 47384 pg/ml) for LB (28925 pg/ml), while upon CMV PG produced higher amount (867 pg/ml) than LB and CTRL (0 pg/ml; 31 ± 54.8 pg/ml).

For all other antigens, a tendency for HIES patient to producing smaller quantity of IFN-γ compared to CTRL was found: upon TT (0 pg/ml; 0 pg/ml *vs* 274 ± 249 pg/ml), *C. albicans* lysate (0 pg/ml; 0 pg/ml *vs* 8 ± 11 pg/ml), *A. fumigatus* hyphae (0 pg/ml; 29 pg/ml *vs* 497 ± 476 pg/ml), *C. albicans* yeast (4739 pg/ml; 1705 pg/ml *vs* 11807 ± 9168 pg/ml), *C. albicans* hyphae (38 pg/ml; 449 pg/ml *vs* 2615 ± 2930 pg/ml) and upon *S. aureus* (1023 pg/ml; 314 pg/ml *vs* 6281 ± 7727 pg/ml).

As expected for patient with *STAT3* mutation, PG did not produce IL-17A upon mitogen and antigen stimulations. For LB a reduced production of IL-17A was found *versus* CTRL upon OKT3/antiCD28 (120 pg/ml *vs* 442 ± 595 pg/ml), TT (27 pg/ml *vs* 175 ± 288 pg/ml) *C. albicans* lysate (19 pg/ml *vs* 76 ± 107 pg/ml) and *S. aureus* stimulation (42 pg/ml *vs* 414 ± 702 pg/ml); while we observed a comparable production upon *A. fumigatus* hyphae (141 pg/ml *vs* 193 ± 171 pg/ml) and *C. albicans* yeast (644 pg/ml *vs* 773 ± 889 pg/ml) and an increased production upon *C. albicans* hyphae (605 pg/ml *vs* 363 ± 387 pg/ml).

In comparison with CGD, both PG and LB showed a reduced proliferation for all antigens but interestingly high upon *C. albicans* yeast and hyphae (SI: 24; 9.8 *vs* 17 ± 11; and 21; 21 *vs* 18 ± 12

respectively), though, the IFN-γ production was even reduced with respect to CGD for all antigens and in particular for *C. albicans* yeast and hyphae was (4739 pg/ml; 1705 pg/ml *vs* 11561 ± 10098 pg/ml; and 38 pg/ml; 449 pg/ml *vs* 7519 ± 8842 pg/ml). Also IL-17A production was found always reduced in HIES patient compared to the CGD patient, except for the *STAT3*-mutation negative patient, who, upon *C. albicans* yeast and hyphae stimulation displayed a IL-17A production comparable to CGD (yeast: 644 pg/ml *vs* 913 ± 1294 pg/ml; hyphae: 605 pg/ml *vs* 790 ± 560 pg/ml).

DISCUSSION

The chronic granulomatous disease is a rare primary immunodeficiency syndrome in which a reduced activity of NADPH oxidase complex leads to decreased production of superoxide anion and other resulting ROS, with the consequence of an increased susceptibility to fungal and bacterial infections due to reduced clearance and killing of particular catalase-positive pathogens, such as *A. fumigatus*, *C. albicans* and *S. aureus*.

To date, there are not many studies that characterize the immunological features of CGD patients, specially for the response of lymphocytes to the specific antigens involved in severe infection.

With the aims to characterize the potential differences in lymphocytes functional responses, we have compared a cohort of CGD patients with age-matched healthy controls.

As expected, in our cohort the patients displayed very heterogeneous kinds of mutations (2 nonsense, 2 missense and 1 splice-site mutation, 2 single nucleotide deletion, and 2 extensive deletion) in the gene that codifies for NADPH oxidase subunit $gp91^{phox}$ with loss of ROS production; only one patient showed a $gp91^{phox}$ wild-type, but $A22^0$ phenotype and had a residual ROS production. Although all the patients are undergoing the same anti-bacterial and anti-fungal prophylaxis, the cohort is very heterogeneous also for clinical features, with 3 patients who experienced *Aspergillus* infection with severe lung aspergillosis, one

patient with documented *C. albicans* and *S. aureus* severe infections and chronic enterocolitis, and other patients affected by *Mycobacterium*, *Serratia* and RSV infections and severe lymphadenitis.

When we analyzed the total CGD cohort, after mitogen and antigen stimulations we observed statistically significant differences of proliferative response upon *C. albicans* lysate and of IL-10 cytokine production upon *A. fumigatus* hyphae stimulation, with patients having increased responses compared to the controls and an increase IL-10 mRNA expression has also been found in resting PBMC of CGD patients with respect to controls and hyper IgE syndrome (HIES) patients.

Normal subject inhale hundreds *A. fumigatus* spores (conidia) every day without signs or symptoms of disease, because in lung tissues, phagocytic cells mediate killing and clearance of spore before it germinates in invasive hyphae forms, while, in subject with compromised immunological system, conidia germinate toward hyphae and mycelia that invade lung tissues and in particular situation, other tissues through the circulation. Thus, if everyday conidia induce a immune response, it must also be controlled to avoid extensive tissues damages and probably, the IL-10 production is the natural mean to protect the host from exuberant innate and adaptive immune responses due to continuous antigens stimulation.

The increased IL-10 levels showed by our patients, was in agreement with other study performed in whole blood of CGD patients stimulated with *A. fumigatus* conidia, that demonstrated a statistical increase of IL-10 production with respect to controls *(Warris A. et al. 2003).* Although *in vivo* function of IL-10 during *A. fumigatus* infection remains undefined, several study in mouse and the in humans, showed that higher levels of IL-10 secretion were associated with poor prognosis and persistent invasive fungal infection *(Roilides E. et al. 2001; Hebart H. et al. 2002)*, moreover a polymorphism in IL-10 gene promoter, that leads to increased level of IL-10 production, was found to be a risk factor for invasive pulmonary aspergillosis *(Sainz J. et al. 2007).* Thus, all this data indicate that a high induction of IL-10 increases the susceptibility to the infection, and that may be a consequence of individual genetic predisposition, but do not exclude a possible pathogen-inducing escape mechanism from host immunity.

The $p47^{-/-}$ CGD mouse model for invasive aspergillosis *(Romani L. et al. 2008)*, showed a decrease of IFN-γ coupled with a increase of IL-17A but a decrease of IL-10 production. Differently, in our study we observe high amount of IL-10 irrespectively from severe infection, this finding would rule out the involvement of predisposing IL-10 promoter polymorphism. On the other hand, the increase of IL-10 could be due to a continuous antigen stimulation caused by a defect in ROS-mediated clearance.

Although our data did not confirm the trend of IFN-γ and IL-10 production found in the CGD mouse, with respect to IL-17A production, the patients displayed higher levels of IL-17A compared to controls after *A. fumigatus* stimulation. IL-17A production was further increased and became statistically significant when we excluded from the CGD cohort a patient that had a chronic inflammation and, differently from the other patients, had a tendency to produce a high amount of IFN-γ and a very low level of IL-17A. The increase of IL-17A production in PBMC of patients, was also confirmed from the quantification of IL-17A mRNA expression in unstimulated PBMC, but unfortunately we still have not characterized a consistent number of patients by cytofluorimetric analysis to identify the differences in Th17 lymphocytes frequency between patients and controls, to confirm an increase of this cell compartment in patients. Most probably, the discrepancy with the mouse model of fungal infection, could be due to: the status of acute inflammation in the lung of mouse compared with patients in non inflamed status, to the fact that cytokine analysis was performed on lung tissues, where the IL-17A$^+$ cells was most abundant, with respect to peripheral blood, or to differences in IL-17A$^+$ lymphocytes differentiation in mouse compared with human ***(de Jong E. et al. 2010)***. However, our results are in agreement with the hypothesis that the increased IL-17A production in CGD, could be a memory response formed during the previous acute infection experienced by

the patients, and lead us to consider that, as in the mouse, the IL-17A$^+$ cells could be a important cell compartment involved in this pathology.

We have also identified another important characteristic in the patients of our CGD cohort in relation to *A. fumigatus* infection; interestingly, after stratification for severe aspergillosis, the patients who experienced this severe inflammatory condition, showed a similar levels of IFN-γ compared to the healthy controls, but decreased compared to the other patients of the cohort that did not experience severe infection by *A. fumigatus* in their clinical history. The decreased levels of IFN-γ in CGD patient with severe aspergillosis compared with other CGD, were also displayed upon other fungal and microbial stimulations and most probably, this points out to an intrinsic feature of this group of patients independently from the kind of antigen stimulation. However, since the patients did not show a documented severe infection to other antigens than *A. fumigatus*, it may be possible that decreased IFN-γ level (coupled with other factors) was sufficient to protect the patients so far from *C. albicans* and *S. aureus* severe infection, indicating that *A. fumigatus* killing is more dependent on IFN-γ production, compared to other antigens.

Thus, if the role of IFN-γ is protective in human CGD as shown in animal models of fungal infection and in the CGD mouse model of aspergillosis, we are able to hypothesize that in the contest of CGD,

where the pathogen killing is decreased (due to reduced ROS production), the normal levels of IFN-γ are not sufficient to protect the patients from pathogens spreading and severe infection. Only the patients who express a increased levels of IFN-γ to compensate the decreased killing, are protected by severe *A. fumigatus* infection.

The stratifications not only helped us to show that there are at least two different populations of CGD patients (distinguished by levels of IFN-γ production), but also allowed another consideration respect to animals model of CGD aspergillosis: the patients who undergo severe aspergillosis, except for the increased levels of IL-10, are more similar to the animal model, for IFN-γ and IL-17A levels, than the other patients of our cohort.

Concerning the response to *C. albicans* stimulation, in CGD patients compared to controls, we observed similar amount of IFN-γ and IL-17A and increased levels IL-10 upon yeast, and an increase of all cytokines upon hyphae stimulation. The yeast and hyphae are the most powerful inductors of IFN-γ and IL-17A, respectively. Unfortunately, we could not obtain more information by stratification because only one patients of our cohort had a documented infection by *Candida*, and this patients also suffered of chronic bowel inflammation and chronic uveitis that might alter the specific responses to *Candida*. When we compared our data to what has been reported in other congenital immunodeficiencies as HIES and chronic mucocutaneous candidiasis (CMC), that like CGD, show

an increased susceptibility to recurrent bacterial and *Candida* infections, and both have a reduced frequency of IL-17A$^+$ cells *(Eyerich K. et al. 2008; Ma C. S. et al. 2008)* that is considered the potential cause of increased non-resolving *Candida* infections, we observed the CGD patients did not display the same cytokines profile. We can not perform yet a statistical analysis on Th17 cells number because the cytofluorimetric study is still on going, but in consideration of the slight increase of IL-17A levels found in our cytokine evaluation tests, we can conclude that in CGD the pathological mechanism that leads to increased *Candida* infection, most probably, is different than in HIES and CMC, and not dependent on defective IL17A response to microbial antigens.

In our cohort we have identified a particular CGD patient that differs from the others for conserved gp91^{phox} wt protein, absence of p22^{phox} protein and residual oxidative activity by DHR assay. Interestingly, he showed a tendency to produce less IFN-γ upon all stimulation (except for *C. albicans* lysate), but markedly increased levels of IL-17A upon all *C. albicans* stimulation. In this patients the increased IL-17A production was in agreement with an increased frequency of IL-17A$^+$ and IL-17A$^+$ IFN-γ$^+$ lymphocytes upon *C. albicans* yeast and hyphae stimulation. Since this patient has not experienced any documented severe fungal infection, we can attempt to theorize that the residual oxidative activity and the sustained IL-

17A production, cold compensate the low levels of IFN-γ to block fungal growth.

Here, we describe another CGD patient that showed the highest IFN-γ/IL-17A ratio upon all fungal stimulation due to high production of IFN-γ and very low production of IL-17A. This patient is affected by a chronic inflammation of bowel and eyes and by documented severe *Staphylococcus* and *C. albicans* infections in clinical history. For this patients, the low levels of IL-17A could be secondary to an counterregulatory effect of high levels of IFN-γ, sustained by the inflammatory condition.

We also analyzed two HIES patients as controls for the CGD cohort and we found a reduced production of IFN-γ compared to CGD for all antigens and, as expected, an absent IL-17A production for the patient with *STAT3* mutation upon all mitogens and antigens tested. In HIES *STAT3*-wt patient, the production of IL-17A was similar than in CGD and even increased compared to age-matched healthy controls, upon both *C. albicans* yeast and hyphae stimulation. The upregulation of Th17 in *STAT3*-wt HIES patient, upon both *C. albicans* yeast and hyphae stimulation was also confirmed by intracellular staining, and indicate that the signalling through the IL-6-STAT3 pathway was preserved, in spite of the low presence of Th17 cells in resting PBMC and absence of IL-17A mRNA in unstimulated PBMC. Thus, two consideration can be made on the basis of this data: first, in the CGD patients *C. albicans*

infection is less frequent compared to *STAT3*-wt HIES patients, most probably, through a more protective level of IFN-γ; second, not all non-autosomal dominant HIES patients have a decreased *C. albicans* specific Th17 cell compared to controls, when stimulated with antigen. Indeed, *STAT3*-wt HIES consists of an heterogeneous group of diseases depending on more that one genetic and/or immune defects in the mechanism devoted to the control fungal infections. The last consideration leads us to believe that, this *in vitro* immunological test can help to exclude a *STAT3* mutation in patients, who have a positive score for HIES, before performing a molecular analysis of the gene.

CONCLUSIONS

In this work we present the study of a cohort of CGD patients and compare this immunological response to that of a group of healthy controls. Although the cohort was extremely heterogeneous for clinical conditions and type of infections, and displayed a large variability of functional responses upon antigens stimulation, we can conclude that: a) upon fungal stimulation CGD patients express increased levels of IL-10 and slightly increased levels of IL-17A, b) CGD patients who experienced severe *A. fumigatus* infection were more similar, for cytokine production, to CGD animal model of aspergillosis than non-infected patients, c) it is possible to distinguish

at least two groups of patients, one that expresses high IFN-γ level and do not undergo *A. fumigatus* severe infection and one that expresses low IFN-γ level and experiences the infection. Thus, in agreements with the animal model, probably IFN-γ is protective for *A. fumigatus* infection also in CGD patient, d) upon *C. albicans* stimulation, CGD patients express increased value of IFN-γ and, differently from HIES, an increase of IL-17A e) the study of two HIES patients lead us to hypothesize that in comparison to CGD and controls, in some *STAT3*-wt HIES patient the occurrence of *Candida* infections could be due to a decreased IFN-γ instead of to a decrease of IL-17A production.

We conjecture that the extension of knowledge on *in vitro* T lymphocytes responses in CGD, could allow the identification of selected patients who might benefit from cytokine-modulating treatments.

Moreover, this studies also could be used for a more exhaustive evaluation of immune-function of CGD patients before haematopoietic stem cells transplantation.

MATERIALS AND METHODS

PATIENTS AND CONTROLS

For this study, ten CGD male patients, two HIES patients and sixteen age-matched healthy controls were enrolled.

CGD patients came from the Italian registry of Chronic Granulomatous Disease which is part of the Italian Primary Immunodeficiency Network of (IPINET). For all, diagnosis was based on abnormal granulocyte function tests evaluated by nitroblue tetrazolium test (NBT) and dihydrorhodamine 123 flow cytometric assay, and confirmed by molecular characterization of mutations and western-blot analysis for NADPH oxidase subunits. One of this patients underwent bone marrow transplant before the study had began.

HIES patients were enrolled in the University Department of Medicine of the Bambino Gesu' Childrens' Hospital. PG was a 15 years old male with *STAT3* gene mutation (exon 21: 1909G>A; SH2 domain: V637M) and LB was a 10 years old female without *STAT3*-mutatetion. Diagnosis was made on the basis of theclinical manifestations and laboratory analysis (HIES clinical score 64 and 40 respectively) and confirmed by molecular characterization of mutation only for one patients.

Controls were healthy age-matched individulas who were not affected by immune maediated diseases.

FUNGAL AND BACTERIAL PREPARATIONS

Conidia from *Aspergillus fumigatus* on Sabouraud-dextrose plate were harvest and filtered (with 40 µm nylon Cell Strainer, BD Biosciences, San Jose, CA, USA) in physiologic solution containing 0.2% (vol/vol) TWEEN-20, washed in phosphate-buffered saline (PBS), counted and suspended at 10^7/ml in RPMI and incubated in flask overnight at 28°C to obtain the switching toward hyphae. After switching, hyphae were separate from flask bottom by cell scraper, washed in PBS, heat-inactivated for 20 minutes at 120°C and stored at $0.5*10^7$ cells/ml to perform PBMC stimulation.

To obtain *Candida. albicans* yeast and hyphae preparation, yeast from Agar plate were grown in BHI-T medium overnight at 28°C, then washed with PBS, counted and separated in two aliquots. One of them, was switched in hyphae by incubation for 4h at 37°C in Germ-Tube medium; the switching was checked by microscope, then hyphae were washed in PBS. Subsequently, both yeast and hyphae, were heat-inactivated for 1h at 75°C in PBS, and stored at $7*10^7$ cells/ml to perform PBMC stimulation.

Staphylococcus aureus from Agar-blood plate, was expanded in BHI-T medium overnight at 37°C, wash in PBS, heat-inactivated for 1h at 100°C, and stored at $8*10^8$ cell/ml.

All microorganism preparations were analyzed for LPS contamination by the Limulus Amebocyte Lysate assay (Lonza,

Walkersville, MD U.S.A.) and were found to contain, at working dilution, less than 5 pg/ml of LPS.

PBMC PURIFICATION AND PROLIFERATION TEST

Peripheral blood mononuclear cells (PBMC) were isolated from peripheral blood of patients and healthy controls by density gradient centrifugation over Ficoll-paque PLUS (GE Healthcare Bio-Sciences, AB), washed twice in sterile PBS (Sigma, St. Louis, MO), and resuspended in complete medium: RPMI 1640 (Sigma) supplemented with 10% (vol/vol) heat-inactivated FBS, 200 U/ml Penicillin, 200 mg/ml streptomycin, 2 mM L-glutamine. The proliferation test upon mitogens and antigens stimulation was performed in triplicate on $2x10^5$ PBMC/well in 200 µl (total vol.) seeded in 96-well flat-bottom microplates as indicated: for mitogens stimulation OKT3 (3 µl/well) plus anti-CD28 (0.15 µg/ml, BD Pharmigen) and PHA (5 µg/ml, Sigma) were added in to wells for 72 h; for antigens stimulation Tetanus toxoid (TT) (0.5 µg/ml), CMV (0.5 µg/ml) and *C. albicans* total yeast lysate (*C. albicans* lysate) (0.4 µg/ml) (all from Nanogen, To, Italy) was added for 120 h, and for heat-inactivated preparations *A. fumigatus* hyphae (cells/antigens ratio = 5:1), *C. albicans* yeast (cells/antigens ratio = 5:1), *C. albicans* hyphae (cells/antigens ratio = 1:10) and *S. aureus* (cells/antigens ratio = 5:1) were added for 168 h. After incubation at 37°C in 5%

CO2 atmosphere, supernatants of unstimulated and stimulated cells were collected for future ELISA tests, and [^{3}H]-thymidine (0.5 μCi/well) (GE Healthcare, Amersham, Upsala, Sweden) was added in each well for 16 h, then harvested and analyzed by β-counter scintillator (Canberra Packard Instrument Company, Meriden, CT, U.S.A.). Stimulation Index (SI) was calculated as (CPM of stimulated/CPM of unstimulated correlated samples).

IN VITRO CYTOKINE SECRETION ASSAY

The *in vitro* cytokine production of unstimulated and mitogens and antigens stimulated PBMC was evaluated in supernatants collected in triplicates during proliferation test. IFN-γ and IL-17A concentration were evaluated by ELISA (Human IFNγ Sreeaning SeEndogen, Pierce Biotechnology Inc., Rockford, USA) and (READY-SET-GO! Human Interleukin-17 kit, BD Biosciences San Jose, CA, USA), while IL-10 concentrations was evaluated by Human Interleukin-10 (IL-10) ELISA kit (Endogen, Pierce Biotechnology Inc.). All tests were performed according to the manufacturer's protocols. The indicated cytokine concentrations upon stimulations were calculated as: (concentration of stimulated sample) – (concentration of unstimulated sample).

CYTOKINE mRNA QUANTIFICATION

IFN-γ and IL-17A RNA transcripts were analyzed by real-time PCR in unstimulated thawed PBMC of 7 CGD patients, 7 age-matched healthy controls, 2 HIES patients and one bone marrow transplanted CGD patient. After total RNA extraction using TRIzol according to the manufacturer's instructions (Invitrogen, Carlsbad, CA) and RNA quantification by spectrophotometer, 308 ng per sample were reverse-transcribed into cDNA, that was then amplified using the following conditions: denaturation 1 min at 95°C, annealing 30 s at (58°C for IFN-γ) 62°C for human IL-17A human IL-10 and human β-actin, followed by 30 s extension at 72°C. Primer sequences were as follows: IFN-γ, forward, 5′-TGG AGA CCA TCA AGG AAG AC-3′, reverse, 5′-GCG TTG GAC ATT CAA GTC AG-3′; IL-17A, forward, 5′-ACT ACA ACC GAT CCA CCT CAC-3′, reverse, 5′-ACT TTG CCT CCC AGA TCA CAG-3′; IL-10 forward, 5′-GGC ACC CAG TCT GAG AAC AG-3′; reverse, 5′-CTT GGC AAC CCA GGT AAC CC-3′; β-actin, forward, 5′-AAG ATG ACC CAG ATC ATG TTT GAG ACC-3′, reverse, 5′-AGC CAG GTC CAG ACG CAG GAT-3′. β-actin was used as a house-keeping gene. Gene expression was calculated using the ΔΔCt algorithm.

CYTOFLUORIMETRIC ANALYSIS

PBMCs from patients and healthy controls were stimulated for 5 hours with phorbol 12-myristate 13-acetate (25 ng/ml) and ionomycin (1 µg/ml) both Sigma-Aldrich, Munich, Germany) and, to block cytokine secretion, brefeldin-A (10 µg/ml, Sigma-Aldrich) was also added for the final 3 hours of stimulation. To evaluate IFN-γ and IL-17A intracellular accumulation in $CD3^{+}$ cells, PBMC were washed and stained with PeCy7-conjugated anti-human CD3 antibody (dil 1:50) (clone SK7; BD Biosciences San Jose, CA); fixed and then permeabilized with Fixation/Permeabilization buffer and Permeabilization buffer (both from eBioscience, San Diego, CA) according to the manufacturer's instructions, and stained with FITC-conjugated anti-human IFN-γ (dil 1:150) (Clone 4S.B3; BD Bioscience) and Pe-conjugated anti-human IL-17 (dil 1:100) (Clone eBio64CAP17; BD Bioscience). Cell samples were analyzed by FACScalibur flow cytometer (BD Biosciences) and flow cytometry data were analyzed by FlowJo software.

STATISTICAL ANALYSIS

All the data were analyzed upon evaluation of their distribution by Kolmogorov-Smirnov test. In case of Gaussian distribution the differences were evaluated by two-tailed parametric unpaired *t* test

with Weihch’s correction (for dissimilar variances), otherwise a two-tailed non-parametric Mann-Whitney test was performed. For all tests, *P* values less than 0.05 were considered significant. All statistical analysis were performed by Graphpad Prism 5.00 software.

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