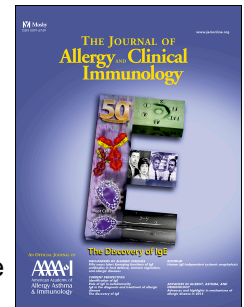


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mTOR inhibition counterbalances the inflammatory status of immune cells in Chronic Granulomatous Disease

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Abstract

Background

Chronic granulomatous disease (CGD) is primary immunodeficiency caused by defective production of reactive oxygen species in phagocytic cells that results in life-threatening infections and severe inflammatory manifestations. The treatment of inflammatory manifestations remains challenging, as it can be associated with an increased risk of infections. Previous studies have shown that CGD phagocytes display a defect in autophagy and a reactive oxygen species-independent activation of the inflammasome.

Objective

Since the intersections between autophagy and inflammasome have been observed in various diseases and microbial infections, we investigated the possible benefit of restoring the autophagy defect through rapamycin – a potent autophagy inducer -in the setting of CGD.

Methods

We studied fifteen patients diagnosed with CGD and followed in our institution. All patients were free of any active infection at the time of the study.

Results

We show that CGD patients present a consistent inflammatory phenotype defined by (i) increased 'non-classical' and 'intermediate' monocytes (ii) a pro-inflammatory state of mononuclear phagocytes with increased IL-1 β and TNF- α content (iii) a TH17 bias of CD4⁺ T-cells (iv) and an increase in IL-17A secreting neutrophils. We document the reversion of CGD inflammatory status by the mTOR inhibitor rapamycin on the different immune cell subsets. We also provide evidence for the enhancement of rapamycin inhibitory effect on IL-1 β secretion by the IL-1 receptor antagonist anakinra in CGD phagocytes.

Conclusion

Altogether these data open new therapeutic approaches for CGD inflammatory manifestations.

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55 **Clinical Implications**

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57 This study suggests that CGD patients with steroid refractory/dependent inflammatory manifestation
58 could benefit from a rapamycin treatment.

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60 **Capsule summary**

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62 This study provides evidence that rapamycin - a potent mTOR inhibitor and autophagy inducer -
63 counterbalances the inflammatory status of immune cells in patients with chronic granulomatous
64 disease, opening the field for its use for the treatment of CGD inflammatory manifestations.
65

66 **Key words.**

67
68 Chronic Granulomatous Disease, Inflammatory Manifestations, mTOR inhibition, Autophagy,
69 Inflammasome, Interleukin 17A, Interleukin 1 β .
70

71

72 **Abbreviations/Acronyms**

73

74 AKT Protein kinase B
75 CGD Chronic Granulomatous Disease
76 DMSO Dimethyl Sulfoxide
77 HC Healthy Control
78 IL Interleukin
79 LC3 Microtubule-associated protein 1A/1B-light chain 3
80 MDM Monocyte-derived macrophages
81 mTOR mammalian Target Of rapamycin
82 NADPH Nicotinamide Adenine Dinucleotide Phosphate
83 PI3K Phosphoinositide 3-kinase
84 PMA Phosphomolybdic acid
85 ROR γ t RAR-related Orphan Receptor γ
86 TNF- α Tumor Necrosis Factor α
87 TH T Helper
88
89

Introduction

Chronic granulomatous disease (CGD) is a primary immunodeficiency of innate immunity caused by defects in phagocyte nicotinamide adenine dinucleotide phosphate (NADPH) oxidase subunits. Loss-of-function mutations in the NADPH oxidase components (i.e. gp91phox, p22phox, p67phox, or p47phox) abrogate oxidase activity and compromise host immunity against certain bacteria and fungi¹. As patients' life expectancy increases thanks to progress in diagnosis and management, the burden of inflammatory manifestations occurring independently of infectious agents has become more evident. CGD patients develop granulomatous disorders, notably colitis, as a consequence of dysregulated inflammatory response². The treatment of such manifestations remains challenging, as it can be associated with an increased risk of infections³. Previous studies have shown that NADPH complex deficient phagocytes display a defect in autophagy and a reactive oxygen species-independent activation of the inflammasome^{4,5}. As IL-1 β blockade decreases the activation of inflammasome, and restores autophagy in CGD mice *in vivo*, and in human cells *in vitro*, clinicians have tempted to treat CGD patients with the IL-1 β receptor antagonist (anakinra) with mixed results^{6,7}. Rapamycin, is a macrolide naturally produced by the bacterium *Streptomyces hygroscopicus* initially developed as an antifungal agent⁸. However, rapamycin - a potent mTOR inhibitor - has mainly been used in clinical care for its immunosuppressive properties⁹. Through mTORC1 inhibition, rapamycin has also been shown to act as an autophagy inducer¹⁰. Since the intersections between autophagy and inflammasome have been observed in various diseases and microbial infections, we investigated the possible benefit of restoring the autophagy defect through rapamycin in the setting of CGD¹¹. For this purpose, we investigated the biological inflammatory status of a French cohort of CGD patients and performed an *in vitro* study of the effect of rapamycin treatment on CGD immune cells.

Methods

Patients

We studied fifteen patients diagnosed with chronic granulomatous disease followed in our institution. Characteristics of the patients are detailed in Table I. Informed consent for participation in the study was obtained from the patient's parent(s) or from the patient, in accordance with French regulatory requirements, the approval of the Institutional Review Board of the Necker-Enfants Malades Hospital and the Declaration of Helsinki. Patient's median age was 8 years (1-22). No patients presented active

bacterial or fungal infection at the time of the study. Eight patients presented with inflammatory manifestations (colitis n=7, and granulomatous cystitis n=1). Contribution of each CGD patient to the biological study is detailed in Table E1, in this article's Online Repository at www.jacionline.org.

Cohort of Healthy Controls

The median age of the healthy controls (HC) was 24 years (2-56). The control cohort comprised 5 children (2, 4, 7, 10 and 14 years old). We did not observe any significant correlation between age of the HC and the different biological parameters evaluated. Contribution of each HC to the biological study is detailed in Table E2, in this article's Online Repository at www.jacionline.org.

Monocytes, CD4+ T-cells, Neutrophils and Monocytes derived Macrophages assay

All assays, including cell isolation, flow cytometry analysis, cell stimulation analysis, cytokine secretion assay, macrophage differentiation, caspase-1 activation assay, immunofluorescence detection of Microtubule-associated protein 1A/1B-light chain 3 (LC3) were performed according to standard protocols. Detailed methods are provided in the Methods section in this article's Online Repository at www.jacionline.org.

Statistical analysis.

Statistical analysis was performed with Graphpad Prism software (version 6.0; GraphPad, La Jolla, CA). Man-Whitney T-test was used for comparison of unpaired values, whereas Wilcoxon paired T-test was used for comparison of paired values. Correlation between two variables was assessed using the non-parametric Spearman correlation.

Results

Circulating monocytes in CGD patients display an inflammatory profile

We first investigated the circulating monocytes from patients with CGD followed at the Necker-Enfants Malades Hospital. Patient's median age was 8 years (1-22). No patients presented active bacterial or fungal infection at the time of the study. Eight patients presented with inflammatory manifestations (colitis n=7, and granulomatous cystitis n=1). Characteristics of the patients are detailed in Table I. Absolute count of circulating monocytes was higher in CGD patients versus healthy controls (HC) (median count per μL : 750 versus 400 respectively, $p=0.006$). The analysis of monocytes subsets - defined as $\text{CD14}^+\text{CD16}^-$ 'classical', $\text{CD14}^+\text{CD16}^+$ 'intermediate' and $\text{CD14}^{\text{low}}\text{CD16}^+$ 'non-classical' monocytes - showed a significant increase in absolute number of circulating non-classical and

intermediate monocytes in CGD patients versus HC (mean 'non-classical' and 'intermediate' monocytes count of 18.5 ± 9.7 and 13.6 ± 6.5 per μL for CGD patients versus 6.7 ± 3.8 and 4.4 ± 2.4 respectively for HC, $p < 0.0001$ and $p = 0.0001$) (Fig 1A and B). This is reminiscent of other clinical inflammatory diseases in humans^{12,13}. Moreover intracytoplasmic staining for the proinflammatory cytokines IL-1 β , IL-6, IL-10 and Tumor Necrosis Factor alpha (TNF- α) revealed an increased content of IL-1 β and TNF- α – evaluated by the geometric mean of fluorescence intensity (GMFI) of the specific staining versus the isotype control – in monocytes of CGD patients versus HC. This increase was observed in all monocytes subsets (Fig 1 C, D and E)

TH17 orientation of CD4⁺ T cells in patients with CGD

We then investigated the cytokines profile of CD4⁺ T-cells, looking for an imbalance in T-helper polarization. Interestingly, intracytoplasmic staining of non-stimulated CD4⁺ T cells showed an increase of the IL-17A and TNF- α content of total CD4⁺ T-cell as evaluated by the GMFI of IL-17A and TNF- α staining versus isotype control ($p = 0.002$ and 0.02 respectively) (Fig E1, in this article's Online Repository at www.jacionline.org). Then, we analyzed the frequency of T-helper 1 (TH1), TH2 and TH17 cells among CD4⁺ T-cells of CGD patients by intracytoplasmic staining of IFN- γ , IL-4 and IL-17A of purified CD4⁺ T-cells after stimulation with Phosphomolybdic acid (PMA) and calcium ionomycin (Fig 2A). The frequency of IL-17A secreting CD4⁺ T cells was significantly higher in CGD patients ($n = 10$) versus HC ($n = 8$) (mean percentage of IL-17⁺ CD4⁺ T-cells of 3.6 for CGD versus 1.1% for HC, $p = 0.003$). We did not observe an increase in the proportion of CD4⁺ T-cells producing concomitantly IFN- γ ⁺ and IL-17⁺ in CGD patients as compared to HC (data not shown). Interestingly, the increased frequency of IL-17A secreting CD4⁺ T-cells was correlated with increased age of patients (Spearman $\rho = 0.73$, $p = 0.02$). However, we did not find any association between the frequency of IL-17A secreting CD4⁺ T-cells and the existence of inflammatory manifestation nor history of fungal infection. In parallel, we also noted an increased proportion of TNF- α secreting CD4⁺ T-cells after PMA/ionomycin stimulation in CGD patients versus HC (mean percentage of TNF- α ⁺ CD4⁺ T-cells of 50 for CGD versus 23% for HC, $p = 0.001$). We did not observe any difference in the proportion of IFN- γ and IL-4 secreting CD4⁺ T-cells. These data were confirmed by cytokine analysis of supernatant collected after 48 h of polyclonal stimulation (anti-CD3/CD28) as we noted a higher secretion of IL-17A (mean level of 769 ± 260 pg/mL versus 314 ± 72 pg/mL $p = 0.001$), TNF- α (mean level of 4132 ± 440 pg/mL versus 3511 ± 195 pg/mL $p = 0.005$) and IL-6 (mean level of 2298 ± 410 pg/mL versus 1052 ± 410 pg/mL $p = 0.005$)

by CGD CD4⁺ T-cells (=10) than HC CD4⁺ T-cells (n=6) (Fig 2B). It is noteworthy that CD4⁺ T-cells from CGD patients secreted less IL-10 than CD4⁺ T-cells from HC after stimulation (mean level of 2290±909 pg/mL versus 696±198 pg/mL, $p = 0.004$), leading to a higher IL-6/IL-10 pro-inflammatory ratio (Fig 2B). Altogether, these data confirm the skewing of the TH profile towards TH17 in CGD patients.

Increased circulating IL-17A neutrophils in CGD patients

We next decided to evaluate the circulating neutrophil compartment from CGD patients. As human peripheral blood neutrophils are able to produce IL-17A after stimulation with IL-6 and IL-23 in a ROR γ t dependent manner, we investigated the presence of IL-17A secreting neutrophils in CGD patients by intracytoplasmic staining¹⁴. As shown in Fig 3A, CGD patients display a higher proportion and absolute number of IL-17A secreting neutrophils in blood compared to HC, (mean of 0.4±0.3 versus 0.08±0.06%; and mean of 18±11 versus 3±2 per μ L respectively, $p = 0.002$). Of note, the increased frequency of IL-17A secreting neutrophils was also correlated with the age of the patient (Spearman $\rho = 0.67$, $p = 0.039$) but was not associated with the existence of inflammatory manifestation or history of fungal infection. Moreover, total neutrophils of CGD patients tend to display a higher, though not significant, expression of the transcription factor ROR γ t than total neutrophils from HC as evidenced by intracytoplasmic staining (Fig 3B).

Effect of rapamycin on the inflammatory status of monocytes-derived macrophages (MDM) from CGD patients

Previous studies have shown that macrophages from CGD patients presents with a defect in autophagy. This defect in autophagy is associated with inflammasome activation and increased IL-1 β release⁷. De Luca and al have also shown that IL-1 blockade with the receptor antagonist anakinra, in addition to inflammasome inhibition, restored autophagy in CGD mice *in vivo*. As autophagy and inflammasome are mutually regulated, we wondered if a direct activation of autophagy could also inhibit inflammation activation in NADPH oxidase defectives mononuclear phagocytes. Thus, we decided to evaluate the inflammatory profile of CGD mononuclear phagocytes and the effect of rapamycin, a potent autophagy inducer, on this profile¹⁰.

For this purpose, we performed macrophage differentiation of CD14⁺ enriched monocytes from CGD patient and HC in serum free medium (X-VIVO 20) supplemented with 100 ng/mL GM-CSF - condition

known to drive monocyte differentiation toward M1 macrophages^{15,16}. This differentiation was performed in presence of dimethyl sulfoxide (DMSO) or 10nM rapamycin. Supernatants collected at day+6 after monocyte differentiation towards M1 macrophage from CGD and HC were then analyzed for cytokines production. During their differentiation toward MDM, CGD monocytes produced higher level of IL-1 β (34 $\pm$ 19 pg/mL versus 14 $\pm$ 5 pg/mL, p = 0.001), IL-6 (305 $\pm$ 175 pg/mL versus 4 $\pm$ 3 pg/mL, p = 0.0002) and TNF- α (1442 $\pm$ 307 pg/mL versus 560 $\pm$ 148 pg/mL, p = 0.0002) than HC monocytes (Fig 4A and Fig E2A, in this article's Online Repository at www.jacionline.org). These results are in agreement with previous studies, and confirmed a pre-activation state of CGD macrophage as compared to HC.

To further characterize the inflammatory profile from CGD patients' macrophages, we evaluated their cytokine secretion after 24h of lipopolysaccharides (LPS) stimulation. LPS-stimulated MDM from CGD patients produced higher levels of IL-1 β than LPS-stimulated MDM from HC (66 $\pm$ 30 pg/mL versus 32 $\pm$ 4 pg/mL, p = 0.001). Moreover, LPS-stimulated MDM from CGD patients secreted less IL-10 than MDM from HC (3296 $\pm$ 491 pg/mL versus 2242 $\pm$ 809 pg/mL, p = 0.03) leading to a higher IL-6/IL-10 pro-inflammatory ratio. We did not observe any difference in IL-6, IL-12 p70, IL-23 and TNF- α secretion after LPS stimulation between CGD patients and HC (Fig 4B and Fig E2B, in this article's Online Repository at www.jacionline.org).

Addition of 10 nM rapamycin during CD14⁺ monocyte differentiation toward macrophage did not seem to alter macrophage phenotype - evaluated by the expression of CD11b, CD68, HLA-DR and CD86 – whether monocytes originated from CGD patient or HC (Fig E2E, in this article's Online Repository at www.jacionline.org). As expected, addition of rapamycin in the culture medium decreased the level of phosphorylation on serin 235 and 236 of the S6 ribosomal protein (Fig E2C, in this article's Online Repository at www.jacionline.org) and induced autophagy in treated MDM as shown by evaluation of LC3 foci induction by immunofluorescence (Fig E3, in this article's Online Repository at www.jacionline.org).

To investigate if rapamycin could counterbalance the pre-activation state of CGD macrophage, we analyzed cytokine secretion in supernatant collected at day+6 after monocyte differentiation toward macrophage from CGD and HC. If addition of rapamycin did not change the level of IL-1 β and IL-6 secretion by MDM from CGD patients, it significantly reduced their TNF- α secretion (556 $\pm$ 225 pg/mL for treated versus 1442 $\pm$ 307 pg/mL for untreated MDM, p = 0.0002) (Fig 4A). These results were

confirmed by intracytoplasmic staining for IL-1 β , IL-6 and TNF- α (Fig E2C and E2D in this article's Online Repository at www.jacionline.org).

To determine the effect of rapamycin on macrophage activation, we stimulated treated and untreated (DMSO) MDM macrophage from CGD patients and HC with 100 ng/mL LPS for 24h and collected supernatant for cytokines analysis. After LPS stimulation, MDM from CGD patients treated with rapamycin produced lower levels of IL-1 β (36 $\pm$ 22 pg/mL versus 66 $\pm$ 30 pg/mL, p = 0.006), IL-6 (1451 $\pm$ 818 pg/mL versus 2899 $\pm$ 988 pg/mL, p = 0.002) IL-23 (1260 $\pm$ 399 pg/mL versus 92 $\pm$ 80 pg/mL, p = 0.001) and TNF- α (1053 $\pm$ 763 pg/mL versus 2881 $\pm$ 1135 pg/mL, p = 0.002) (Fig 4B and Fig E2B, in this article's Online Repository at www.jacionline.org). If IL-10 production by LPS stimulated MDM was also significantly reduced (p = 0.002) by the rapamycin treatment, IL-6/IL-10 pro-inflammatory ratio was significantly lowered by the addition of the mTOR inhibitor (data not shown, p = 0.002). The same changes in cytokine production were observed for LPS-stimulated MDM from HC.

To assess the effect of rapamycin on inflammasome activation we stimulated MDM from CGD patient with LPS and ATP and evaluated caspase 1 activity by a fluorescent inhibitor of active caspase-1 (FLICA) (Fig 4C and 4D). As anticipated, rapamycin decreased inflammasome activation in CGD MDM.

Altogether, these data show that rapamycin is not only able to inhibit IL-1 β secretion by MDM through inflammasome inhibition but significantly reduces the inflammatory profile of NADPH deficient cells.

Counterbalance of the TH17 profile of CGD CD4⁺T cells by rapamycin

ROR γ t serves as the master regulator for TH17 cells and enhanced expression of ROR γ t in naive CD4⁺ T-cells induces genes essential for TH17 differentiation^{17,18}. Studies have shown that the suppression of the PI3K-Akt-mTORC1 axis - that accelerates nuclear translocation of ROR γ t to the nucleus - impairs Th17 differentiation *in vitro* and *in vivo* in a S6K1/2-dependent fashion^{19,20}. We wondered whether rapamycin could reverse the TH17 cytokine secretion profile observed in CGD CD4⁺ T-cells independently of its action on the production of pro-TH17 cytokines by phagocytic cells. Purified total CD4⁺ T-cells obtained from CGD patients (n = 6), were stimulated with anti-CD3/CD28 for 48 H with or without (DMSO) 10 nM rapamycin. Supernatants were then collected for cytokine analysis by cytometric bead assay (MacsPLEX). CGD patient's CD4⁺ T-cells treated with rapamycin produced lower levels of IL-6 (810 $\pm$ 290 pg/mL for rapamycin treated cells versus 1475 $\pm$ 423 pg/mL for

untreated cells, $p=0.03$), IL-17A (292 ± 121 pg/mL versus 896 ± 241 pg/mL, $p=0.03$), and TNF- α (1766 ± 364 pg/mL versus 4696 ± 421 pg/mL, $p=0.03$), with a reduction of the IL-6/IL-10 pro-inflammatory ratio (Fig 4E).

Combination therapy of rapamycin and anakinra

We then wondered if anakinra, a competitive inhibitor of the binding of IL-1 to its receptor, could enhance the anti-inflammatory action of rapamycin. Whole blood from CGD patients ($n=8$) was incubated 24 hours with rapamycin or DMSO and increasing dose of anakinra (i.e. 0, 40 and 400 ng/mL) before stimulation with LPS²¹. Secretion of IL-1 β and TNF- α by CGD monocytes was evaluated by intracytoplasmic staining (Fig 4F and G). Secretion of IL-1 β and TNF- α by monocytes was significantly reduced by rapamycin (mean percentage of IL-1 β + monocytes of 28.1 for rapamycin treated samples versus 44.7 for untreated samples (DMSO), $p=0.008$ and mean percentage of TNF- α + monocytes of 15.2 for rapamycin treated samples versus 34.8 for untreated samples, $p=0.008$). Addition of anakinra at low concentration (i.e. 40 ng/mL) to rapamycin significantly enhanced the inhibition of IL-1 β secretion (mean percentage of IL-1 β + monocytes of 28.1 for rapamycin treated samples versus 18.6 for samples treated with rapamycin and low-dose anakinra, $p=0.015$). We did not observe a benefit to the increase in dose of anakinra. Of note, the combination of low-dose anakinra plus rapamycin reduced more efficiently IL-1 β secretion than low-dose anakinra alone (mean percentage of IL-1 β + monocytes of 27.3 for samples treated with low-dose anakinra versus 18.6 for samples treated with rapamycin and low-dose anakinra, $p=0.015$). Anakinra had no effect on TNF- α + secretion by monocytes after LPS stimulation. Its combination with rapamycin did not enhance the inhibitory effect of rapamycin on TNF- α + secretion.

Discussion

The progress in management of CGD has underscored the burden of inflammatory/granulomatous manifestations in CGD patients. Indeed, CGD patients develop granulomatous disorders involving diverse organs with a high prevalence - up to 69% depending on the reported series -². We report herein one of the largest series analyzing the inflammatory phenotype of patients presenting with CGD. Independently of any active chronic infection, CGD patients present a biological inflammatory state involving all immune cells subsets. It is noteworthy that even in patients free of evident inflammatory manifestations, we observed a consistent inflammatory phenotype characterized by: (i)

increased 'non-classical' and 'intermediate' monocytes (ii) a pro-inflammatory state of mononuclear phagocytes with increased IL-1 β and TNF- α content (iii) a TH17 bias of CD4⁺ T-cells and (iv) an increase in IL-17A secreting neutrophils.

The increase of 'non-classical' and 'intermediate' monocytes is a common feature observed in diverse inflammatory conditions^{12,13}. Our data on monocytes and MDM are in line with previous reports underlying the important role of IL-1 β and TNF- α in CGD pathophysiology of inflammatory manifestations^{4,5,7,22}.

We report a TH17 bias of CD4⁺ T-cells in CGD patients, a feature that has already been described by others²³. This bias could be related to the production of the key Th17-polarizing cytokines, IL-1 β , IL-6 and IL-23 and TGF- β by mononuclear phagocytes as reported in our study and by others²⁴. Even if activation of the IL17-IL23 axis has been implicated in the pathogenesis of inflammatory conditions such as multiple sclerosis, inflammatory bowel disease or Behçet disease, the contribution of a TH17 imbalance in the genesis of CGD inflammatory lesions still remains unclear despite some data obtained in NADPH-deficient mice^{25–28}. Interestingly, we identified the presence of a subset of IL-17A secreting neutrophils in CGD patients. This finding is reminiscent of the neutrophil population described by Taylor et al that produces IL-17A in a ROR γ t dependent manner and uses it in an autocrine manner to enhance ROS production and antifungal activity¹⁴.

It is noteworthy that the increase of 'non-classical' and 'intermediate' monocytes, and the higher frequency of IL-17A producing CD4⁺ T-cells and neutrophils reported did not seem to be associated with the presence of inflammatory manifestations. The apparent lack of association between inflammatory condition and these biological features could result from the anti-inflammatory therapy received by some patients, and/or the existence of subclinical inflammatory manifestations. Nevertheless, the significant correlation noted between IL-17A producing CD4⁺ T-cells and neutrophils and the age of the patient underline the importance of time in the occurrence of inflammatory complications in this disease².

Through the last decade, several groups have studied the mechanisms responsible for the inflammatory status observed in CGD showing that mononuclear phagocytes from CGD patients present with a defect in autophagy²⁹. This defect in autophagy results in activation of the inflammasome and increased IL-1 β release^{4,7}. As some experimental data have shown the potential

benefit of IL-1 blockade with the receptor antagonist anakinra in CGD, attempts have been made to treat CGD patients presenting with inflammatory manifestations with the IL-1 beta receptor antagonist (anakinra), with mixed results^{6,7}. Targeting the same pathway, we decided to evaluate the benefit of a rapamycin treatment on CGD inflammation. As rapamycin has been shown to act as an autophagy inducer through mTORC1 inhibition¹⁰, we reasoned that restoring the autophagy defect in CGD could probably contribute to reversion of the biological inflammation observed in CGD patients. We report that rapamycin is indeed able to significantly reduced basal TNF- α production and secretion from MDM. Through autophagy induction and caspase 1 inhibition, rapamycin inhibits efficiently IL-1 β secretion by LPS-primed MDM. This is in accordance with some recent data showing that blocking autophagy potentiated inflammasome activity while stimulating autophagy limited it³⁰. Moreover, rapamycin treatment of LPS-primed MDM significantly reduces their secretion of TNF- α other key Th17-polarizing cytokines –i.e. IL-6, IL-23 -. In accordance with data showing that suppression of the PI3K-Akt-mTORC1 axis impairs Th17 differentiation *in vitro* and *in vivo*, we show that rapamycin corrects the TH17 cytokine secretion profile observed in CGD CD4⁺ T-cells independently of its action on the production of pro-TH17 cytokines by mononuclear phagocytes cells^{19,20}. Finally, we show that inhibition of IL-1 receptor with the antagonist anakinra – even at low doses - enhances the inhibitory effect of rapamycin on IL-1 β secretion by phagocytes *in vitro*, suggesting that the combinatory therapy of rapamycin and anakinra could be beneficial for treatment of CGD patients with inflammatory complication that have no or partial response to anakinra.

Taken together, all these data suggest that rapamycin could be a useful therapy for managing inflammatory manifestations in CGD. Until recently, treatment of inflammatory/granulomatous manifestations has been mainly based on corticosteroids, but steady progress in the understanding of CGD pathophysiology have prompted the use of alternative therapies such as anti-TNF α monoclonal antibodies, thalidomide or anakinra^{4,6,7,31,32}. Compared to these therapies, rapamycin has the advantage of (i) inhibiting the constitutive inflammasome activation of CGD phagocytes through autophagy induction (ii) having additional immunomodulatory properties independently of caspase 1 inhibition – i.e. inhibition of secretion of TNF- α , IL-6, IL-23 by mononuclear phagocytes and inhibition of TNF- α , IL-6 and IL-17A by T-cells - , (iii) being ironically an antifungal agent despite its known immunosuppressive properties⁸. Finally, rapamycin could help correct the decrease in the functional activity of hematopoietic stem cells observed in patients with CGD as data shows that the suppression

of the mTOR pathway allows for the *ex vivo* maintenance of human and mouse long-term HSCs^{33–35}.

This could be of great interest in view of HSCs based gene-therapy protocol for CGD.

However, the true place of rapamycin in management of CGD inflammatory complications remains to be assessed via controlled clinical trials. Such studies will help determine the real efficacy of rapamycin as compared to standard therapy (i.e. corticosteroid) and/or its potential ability to reduce the corticosteroid burden in CGD patients with inflammatory condition and corticosteroid dependence.

Contribution

A.G, I.H performed experiments and critically read the manuscript. D.M, B.N, A. LU, JS.D, F.S, C.P, S.B take care of the patients and critically read the manuscript. M.C critically read the manuscript. A.F take care of the patients, suggested experiments and critically read the manuscript. F.T designed the study, performed experiments, wrote the manuscript and take care of the patients.

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Table 1. CGD patient's characteristics

	Age at time of study (years)	Genetic	Inflammatory manifestations	Anti-inflammatory therapy at the time of the study	Antimicrobial Prophylaxis
P1	22	<i>CYBB</i>	Colitis	Prednisolone and mesalazine	Cotrimoxazole / Itraconazole
P2	2	<i>CYBB</i>	None	None	Cotrimoxazole / Itraconazole
P3	14	<i>CYBB</i>	None	None	Cotrimoxazole / Itraconazole
P4	8	<i>CYBB</i>	Colitis	Budesonide	Cotrimoxazole / posaconazole
P5	17	<i>CYBB</i>	Granulomatous Cystitis	Prednisolone	Cotrimoxazole / Itraconazole
P6	1,8	<i>CYBB</i>	None	None	Cotrimoxazole / Itraconazole
P7	4	<i>CYBB</i>	None	None	Cotrimoxazole / Voriconazole
P8	1	<i>CYBB</i>	None	None	Cotrimoxazole / Itraconazole
P9	6	<i>CYBB</i>	Colitis	Prednisolone/Azathioprine	Cotrimoxazole / Itraconazole
P10	10	<i>CYBB</i>	None	None	Cotrimoxazole / Itraconazole
P11	12	<i>NCF1</i>	Colitis	Infliximab / Methotrexate	Cotrimoxazole / Itraconazole
P12	6	<i>CYBB</i>	Colitis	None	Cotrimoxazole / posaconazole
P13	15	<i>CYBB</i>	Colitis	Prednisolone	Cotrimoxazole / Itraconazole
P14	17	<i>CYBB</i>	Colitis	Prednisone/Azathioprine	Cotrimoxazole / Itraconazole
P15	2	<i>CYBB</i>	None	None	Cotrimoxazole / posaconazole

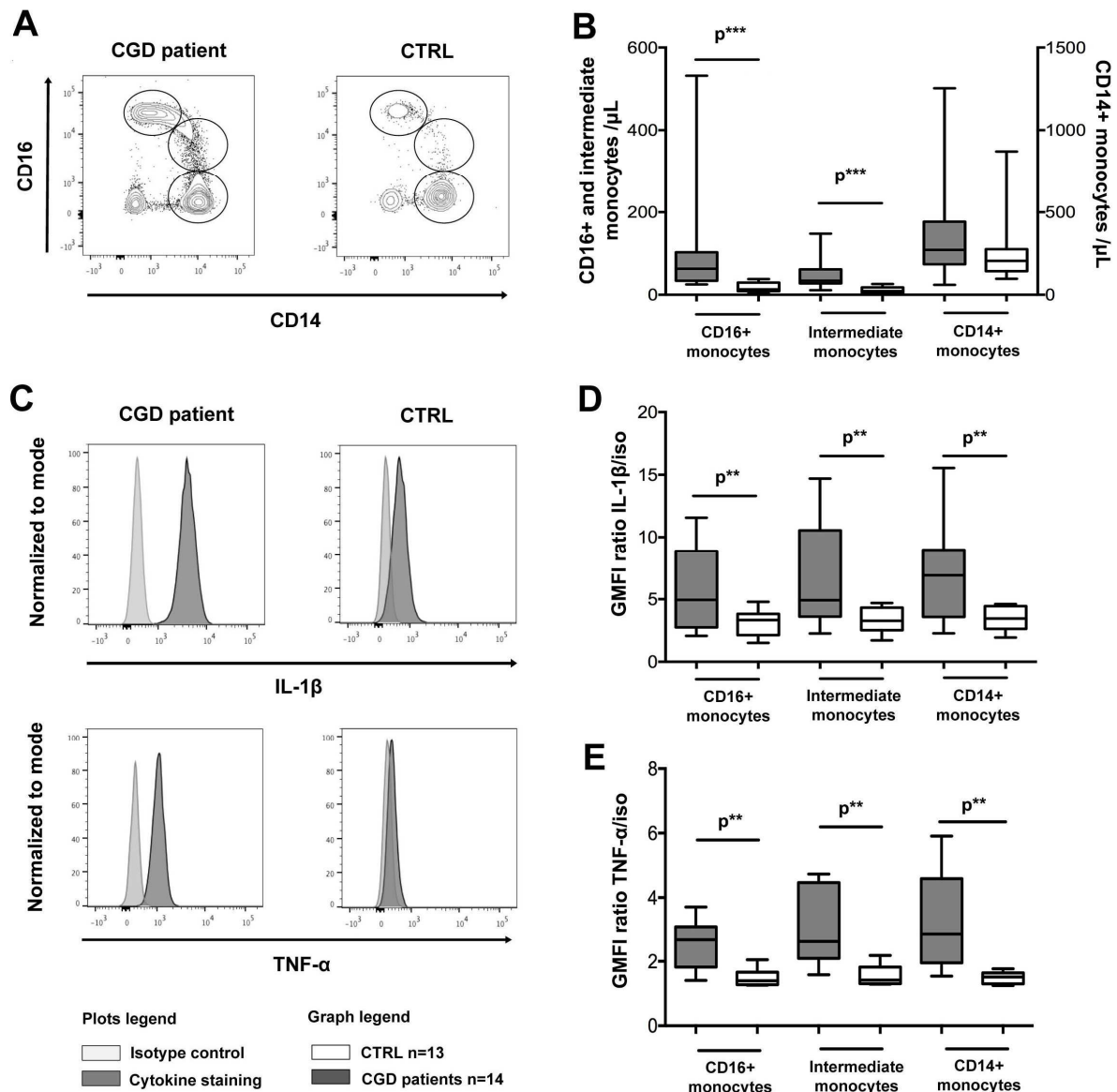


Fig 1. Analysis of circulating monocytes subsets in CGD patients.

(A) Representative dot plot of CD16/CD14 gating on circulating monocytes in CGD patients versus healthy controls. (B) Absolute numbers of circulating CD16+, intermediate and CD14+ monocytes in CGD patients (n=14) versus healthy controls (n=15). (C) Representative images of intracytoplasmic staining for IL-1 β (upper panel) and TNF- α (lower panel) in CGD patients versus healthy controls. (D) Geometric mean of fluorescence intensity (GMFI) ratio between IL-1 β and the isotype control in CGD patients (n=14) versus healthy controls (n=15). (E) GMFI ratio between TNF- α and the isotype control in CGD patients (n=14) versus healthy controls (n=15). p* < 0.05; p** < 0.01, p*** < 0.001; (Mann Whitney T-test).

Extremities of the whiskers boxes represent minimum and maximum values. Bottom and top of the box represent the first and third quartiles, respectively. The horizontal bar in the box represents the median value.

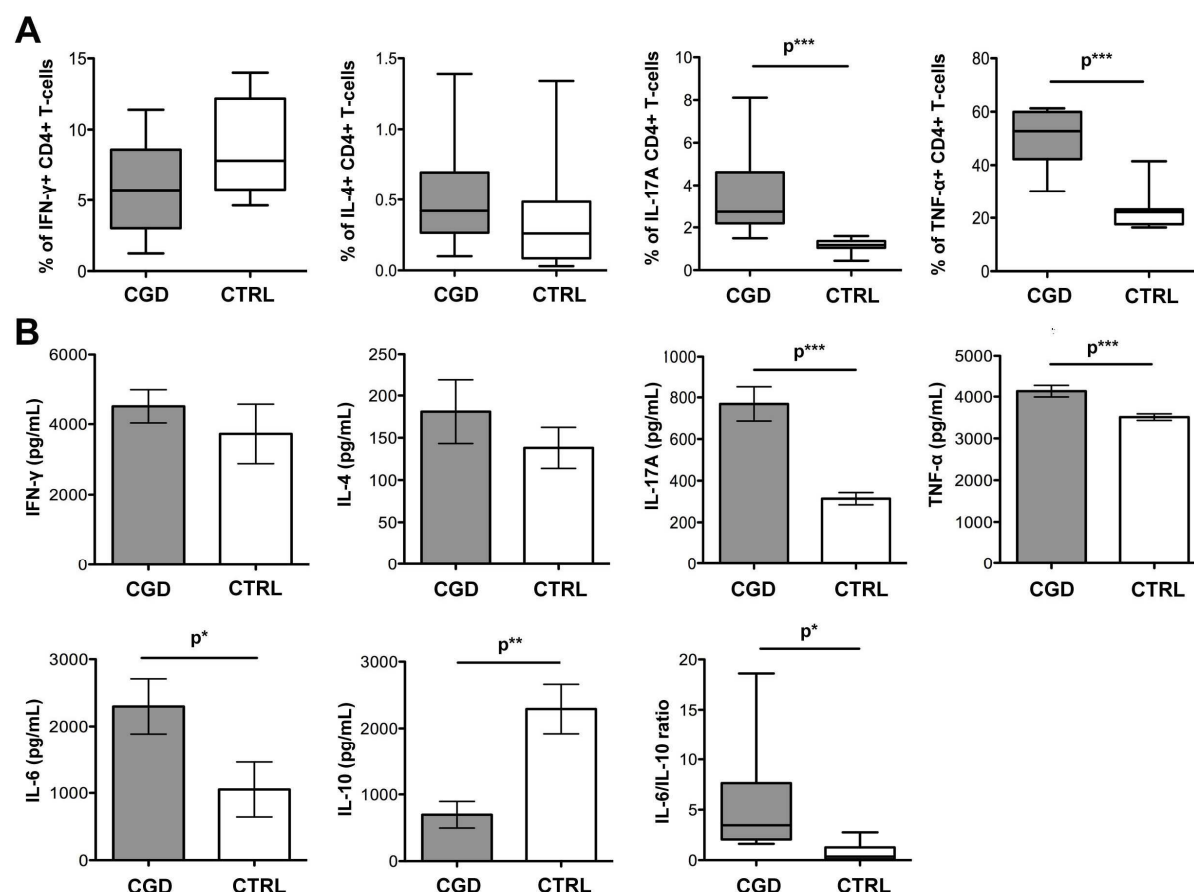


Fig 2. Cytokine profile of CD4⁺ T-cells from CGD patient is skewed towards Th17.

(A) Percentage of IFN- γ , IL-4, IL-10 IL-17A and TNF- α secreting CD4⁺ T-cells evaluated by intracellular staining after PMA/ionomycin stimulation in CGD patients (n= 10) and healthy control (n= 8) were stimulated with antiCD3/CD28 for 48 H. Supernatants were then collected for cytokine analysis by cytometric bead assay (MacsPLEX). **(B)** Purified total CD4⁺ T-cells obtained from CGD patients (n= 10), and control (n= 6) were stimulated with antiCD3/CD28 for 48 H. Supernatants were then collected for cytokine analysis by cytometric bead assay (MacsPLEX).

p* < 0.05; p** < 0.01, p***<0.001; (Man-Whitney T-test).

Extremities of the whiskers boxes represent minimum and maximum values. Bottom and top of the box represent the first and third quartiles, respectively. The horizontal bar in the box represents the median value.

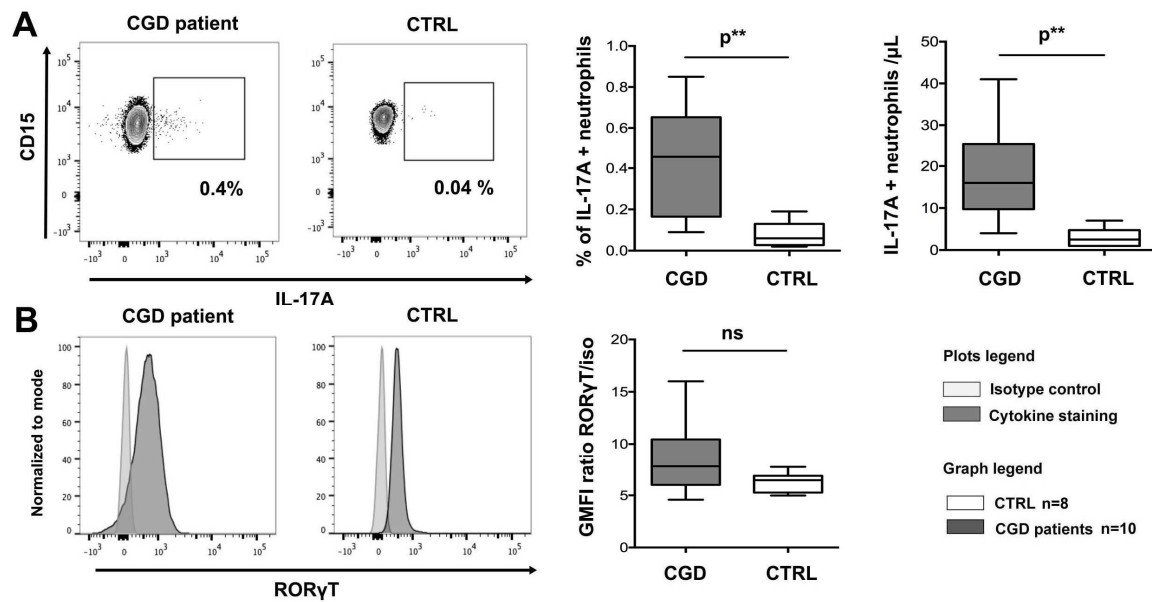


Fig 3. Expression of IL-17A and ROR γ t in human peripheral blood neutrophils from CGD patients or healthy controls.

(A) Intracytoplasmic IL-17A staining of total neutrophils in blood of CGD patients (n=10) or healthy controls (n=8). Representative scatter-plot (left panels) and proportion and absolute number of IL-17A secreting neutrophils in blood (right panels). **(B)** Intracytoplasmic ROR γ t staining of total neutrophils in blood of CGD patients or healthy controls. Representative scatter-plot (left panels) and proportion and absolute number of ROR γ t secreting (right panels).

$p^* < 0.05$; $p^{**} < 0.01$, $p^{***} < 0.001$; (Man-whitney T -test).

Extremities of the whiskers boxes represent minimum and maximum values. Bottom and top of the box represent the first and third quartiles, respectively. The horizontal bar in the box represents the median value.

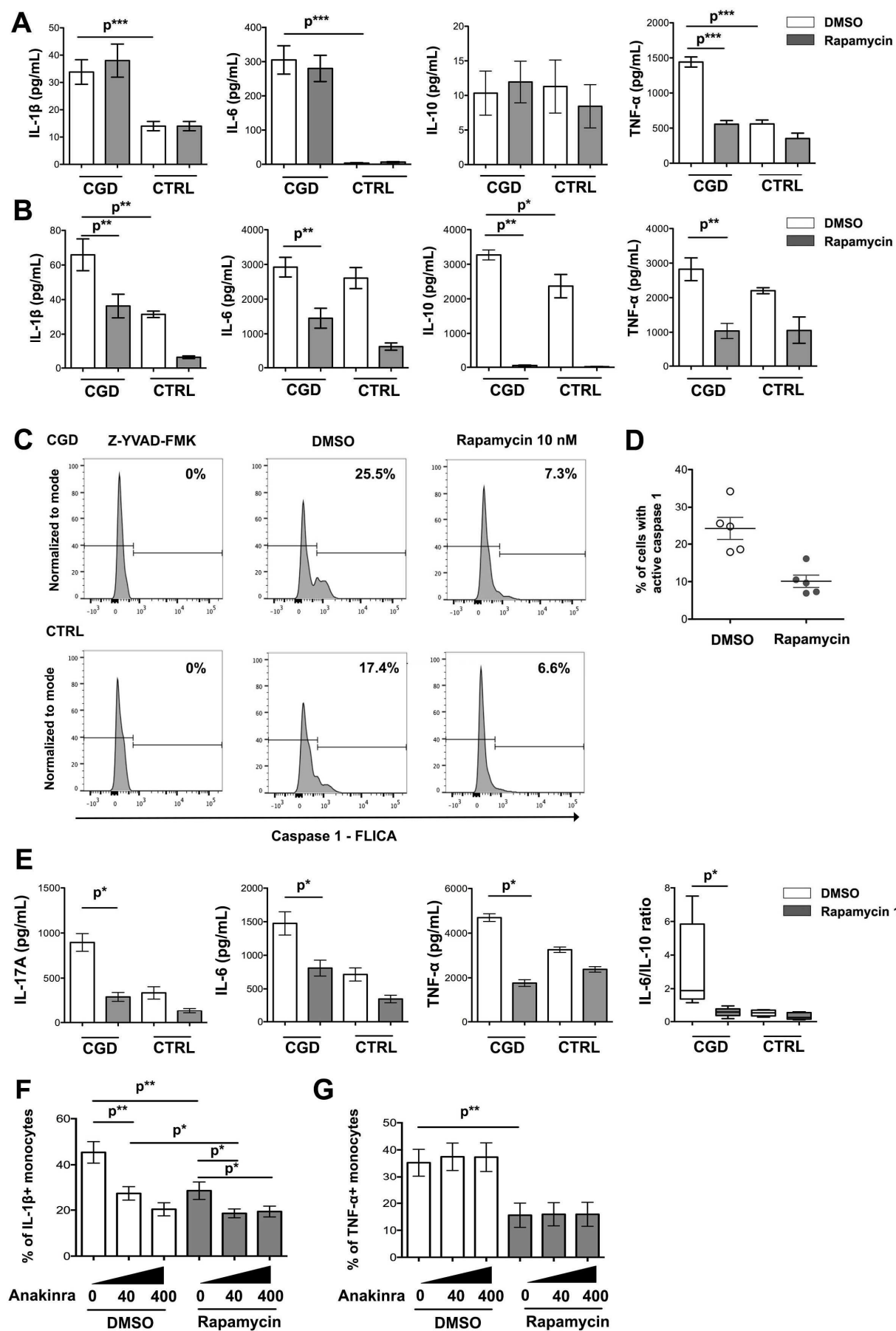


Fig 4. Reversion of CGD inflammatory status by rapamycin.

(A) CD14⁺ monocytes from CGD patients (n=15, 18 samples) and healthy controls (n=7, 7 samples) were differentiated into macrophages with rapamycin (grey bars) or without (DMSO, open bars). Supernatant were collected at day+6 after differentiation and cytokines were analyzed by cytometric bead assay (MacsPLEX) or ELISA. **(B)** Monocyte-derived macrophages (MDM) from CGD patients (n=12) and healthy controls (n=5) were stimulated 24h with 100 ng/mL LPS in the presence of 10 nM rapamycin (grey bars) or not (DMSO, open bars). Supernatant were collected for cytokines analysis through cytometric bead assay (MacsPLEX) or ELISA. **(C)** MDM from CGD patients and healthy controls were primed with LPS and stimulated 30 min with adenosine triphosphate (ATP) in the presence or not of 10 mM rapamycin. Caspase 1 activity was determined by a fluorescent inhibitor of active caspase-1 (FLICA). Numbers above bracketed lines indicate percentage of cells with active caspase-1. **(D)** Active caspase-1 in MDM from 5 CGD patients treated or not with 10 nM rapamycin quantified by caspase-1 FLICA. **(E)** Purified total CD4⁺ T-cells obtained from CGD patients (n= 6), and control (n= 5) were stimulated with anti-CD3/CD28 for 48 H in the presence of 10 nM rapamycin (grey bars) or not (DMSO, open bars). Supernatants were then collected for cytokine analysis by cytometric bead assay (MacsPLEX). **(F and G)** CD14⁺ monocytes from CGD patients (n=8) were treated 24h with 10nM rapamycin and increasing dose of anakinra (i.e. 0, 40 and 400 ng/mL) before 4h stimulation with LPS. Monocytes were then analyzed for IL-1 β **(F)** and TNF- α secretion **(G)**. p* < 0.05; p** < 0.01, p***<0.001; (Man-Whitney T-test or Wilcoxon paired T-test)

Supplemental data**Methods****Purification of total CD4⁺ T lymphocytes**

Peripheral blood mononuclear cells were separated by Ficoll-Paque (GE Healthcare) from fresh blood from patients or from healthy donors. CD4⁺ T lymphocytes were then purified by immunomagnetic depletion with the human memory CD4⁺ T cell Isolation Kit II (Miltenyi Biotec). Median purity of CD4⁺ T cells was 95% (92-97).

Staining of CD4⁺ memory T cells

Purified CD4⁺ T lymphocytes were stained with BD Horizon BV421 anti-CCR6, anti-CD45RO FITC, anti-CCR4 PE (all from BD Bioscience), CXCR3 PEVio770, CD4 APC and CD3 APCVio770 (from Miltenyi Biotec)

CD4⁺ T cell stimulation

Purified CD4⁺ T cells were incubated in TEXMACS GMP medium (Miltenyi Biotec), for 6 h with PMA (100 ng/ml; Sigma), and ionomycin (500 ng/ml; Sigma). Brefeldin (10 µg/ml eBioscience) was added 1h30min after the start of stimulation. After 6 h of stimulation, cells were harvested, washed and stained as described below.

Intracytoplasmic staining

Intracytoplasmic staining of CD4⁺ T cells was performed on purified CD4⁺ T cells using the live/dead kit (Invitrogen), the Cytofix/Cytoperm plus kit (BD Biosciences) and the following antibodies: anti-CD3 APC, CD4 FITC (from BD Biosciences) anti-IFN-γ PE or FITC, anti IL-4 PE anti IL-6 PE, anti-IL10 PE, anti-TNFα PE (from Miltenyi Biotec) and anti-IL17A PE (clone BL168 from Biolegend)

Intracytoplasmic staining of monocytes was performed on isolated peripheral blood mononuclear cells using the live/dead kit (Invitrogen), the Cytofix/Cytoperm plus kit (BD Biosciences) and the following antibodies: anti-CD16 FITC (BD biosciences), anti-CD14 VioBlue, anti-HLA-DR PerCP, anti IL-6 PE, anti-IL10 PE, anti-TNFα PE (all from Miltenyi Biotec), anti-IL1β PE (from eBioscience). Intracytoplasmic staining of neutrophils was performed on fresh blood using the PerFix-nc kit (Beckman Coulter) and the following antibodies: anti-CD16 FITC, anti CD15 PacificBlue (Beckman Coulter), anti-IL17A PE (eBioscience). Intracytoplasmic staining of monocytes derived macrophages was performed using the live/dead kit (Invitrogen), the Cytofix/Cytoperm plus kit (BD Biosciences) and the following antibodies: anti-CD86 PE (BD biosciences), anti-HLA-DR PercP, anti CD11b APC, anti IL-6 PE, anti-IL10 PE, anti-TNFα PE (all from Miltenyi Biotec), anti-IL1β PE (from eBioscience), and anti-phosphoS6 S235/236 (eBioscience)

Analysis of CD4⁺-T cell cytokines production

Purified CD4⁺ T cells were cultured in TEXMACS GMP medium (Miltenyi Biotec) were cultured in 48-well plates (Falcon) at a density of 1×10^6 cells/ml for 48 h in X-VIVO 20 serum free medium (Lonza) in the presence of Dynabeads CD3/CD28 T cell expander (Invitrogen). After 48 h, supernatants were collected and frozen at -80°C. GM-CSF, IFN-α, IFN-γ, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-17A, TNF-α were measured by the MACSPlex cytokine 12 KIT (Miltenyi Biotec) according to the manufacturer's instructions.

Macrophage differentiation

Monocytes were isolated from PBMCs by negative selection using the Monocyte Isolation Kit II (Miltenyi Biotec; 130-091-153) according to the manufacturer's instructions. Mean purity of monocytes and macrophages was more than 96% as determined by fluorescent-activated of monocytes was 94% (93-97). Monocytes were cultivated in X-VIVO-20 medium containing 100 ng/mL of recombinant human Granulocyte-Macrophage Colony-Stimulating Factor (rh GM-CSF; Peprotech) for 6 days with 10 nM rapamycin (Sigma) or DMSO (Cryosure)

Analysis of monocytes derives macrophage cytokines production

After 6 days of differentiation, monocyte-derived macrophages were primed with 100 ng/mL LPS (Sigma E.Coli O26:B6) for 24H before supernatant harvest. GM-CSF, IFN-α, IFN-γ, IL-6, IL-10, IL-12p70, TNF-α were measured by the MACSPlex cytokine 12 KIT (Miltenyi Biotec) according to the manufacturer's instructions. IL-1β, IL-23 and IL-18 were measured by Human IL-1 beta ELISA Ready SET-Go! (2nd Generation), Human IL-23 ELISA Ready SET-Go! (both from eBioscience), and IL-18

ELISA kit (Thermofischer) respectively.

Potentialiation of rapamycin by anakinra

Whole blood from CGD patient was incubated 24 hours with 10 nM rapamycin or DMSO and increasing dose of anakinra (i.e. 0, 40 and 400 ng/mL) (Swedish Orphan Biovitrum) before 4 hours stimulation with LPS in presence of brefeldin A. Intracytoplasmic staining of monocytes was performed on fresh blood using the PerFix-nc kit and the following antibodies: anti-CD14 VioBlue, anti-TNF α PE (all from Miltenyi Biotec), anti-IL1 β PE (from eBioscience).

Caspase-1 activation assay

Monocyte-derived macrophages were primed with 1 μ g/mL LPS for 3 hours before addition of adenosine triphosphate (ATP; 2mM, Sigma) from Roche. Caspase-1 activity was assessed by fluorescent-flow cytometry using caspase-1 FLICA (Abd Serotec) according to the manufacturer's instructions. MDM cells treated with 10 μ M of Caspase 1 inhibitor Z-YVAD-FMK (ab141388, Abcam) were used as a negative control.

Immunofluorescence Detection of LC3

CD14+ monocytes were differentiated on cover slips in X-VIVO-20 medium containing 100 ng/mL of recombinant human Granulocyte-Macrophage Colony-Stimulating Factor (rh GM-CSF; Peprotech) for 6 days with 10 nM rapamycin (Sigma) or DMSO (Cryosure). After 6 days, cells were washed with PBS and fixed with 4% paraformaldehyde in PBS for 15 min. After each step, the cover slips were rinsed three times with PBS. Cells were incubated for 20 min with PBS containing 0.1M glycine and permeabilized in 0.5% Triton X-100 in PBS for 15 min. Cells were incubated for 30 min with the PBS-BSA solution. Cells were then labeled with anti-LC3A/B (ab128025, Abcam), followed by washes with PBS-BSA solution and incubation with Alexa488 Goat anti-Rabbit (Invitrogen). Slides were counterstained with 0.1 μ g/ml of DAPI (4', 6'-diamidino-2-phenylindole) and mounted in Fluorsave (Calbiochem). Slides were viewed by epifluorescence microscopy (Axioplan ZEISS), and images were taken by a cooled charge-coupled device (CCD) camera. Grayscale images were processed using Adobe Photoshop CS5. (San Jose, CA).

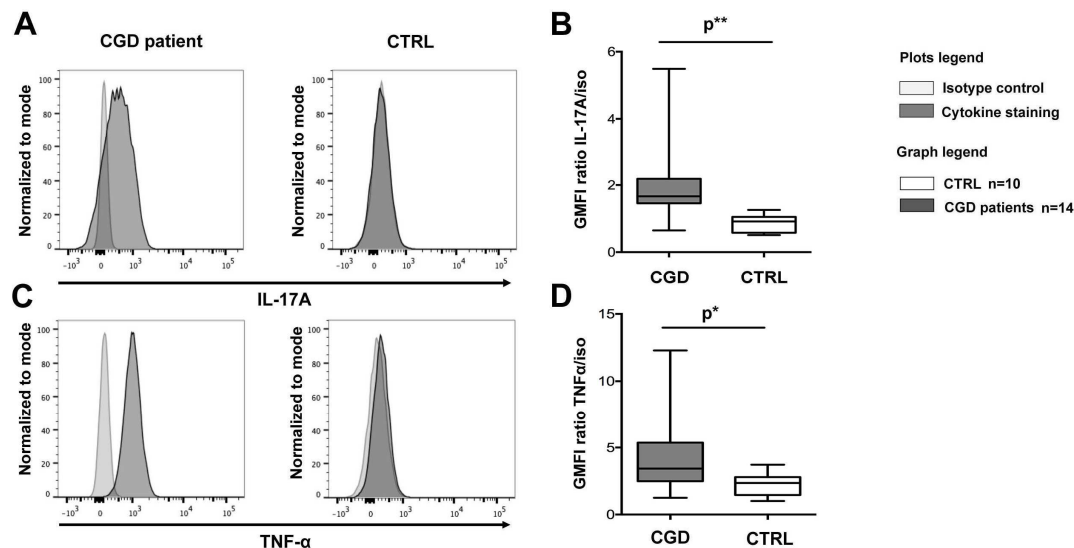


Fig E1. TH17 Cytokine profile of CD4⁺ T-cells from CGD patient.

Representative intracellular staining of IL-17A (**A**) and TNF-α (**C**) in unstimulated CD4⁺ T-cell of CGD patient and healthy control. Intracellular content of IL-17A (**B**) and TNF-α (**D**) in unstimulated CD4⁺ T-cell of CGD patient (n=14) and healthy controls (n=10) evaluated by the ratio between the geometric mean of fluorescence intensity (GMFI) of the specific staining versus isotype control.

p* < 0.05; p** < 0.01, p*** < 0.001; (Man-Whitney T-test)

Extremities of the whiskers boxes represent minimum and maximum values. Bottom and top of the box represent the first and third quartiles, respectively. The horizontal bar in the box represents the median value.

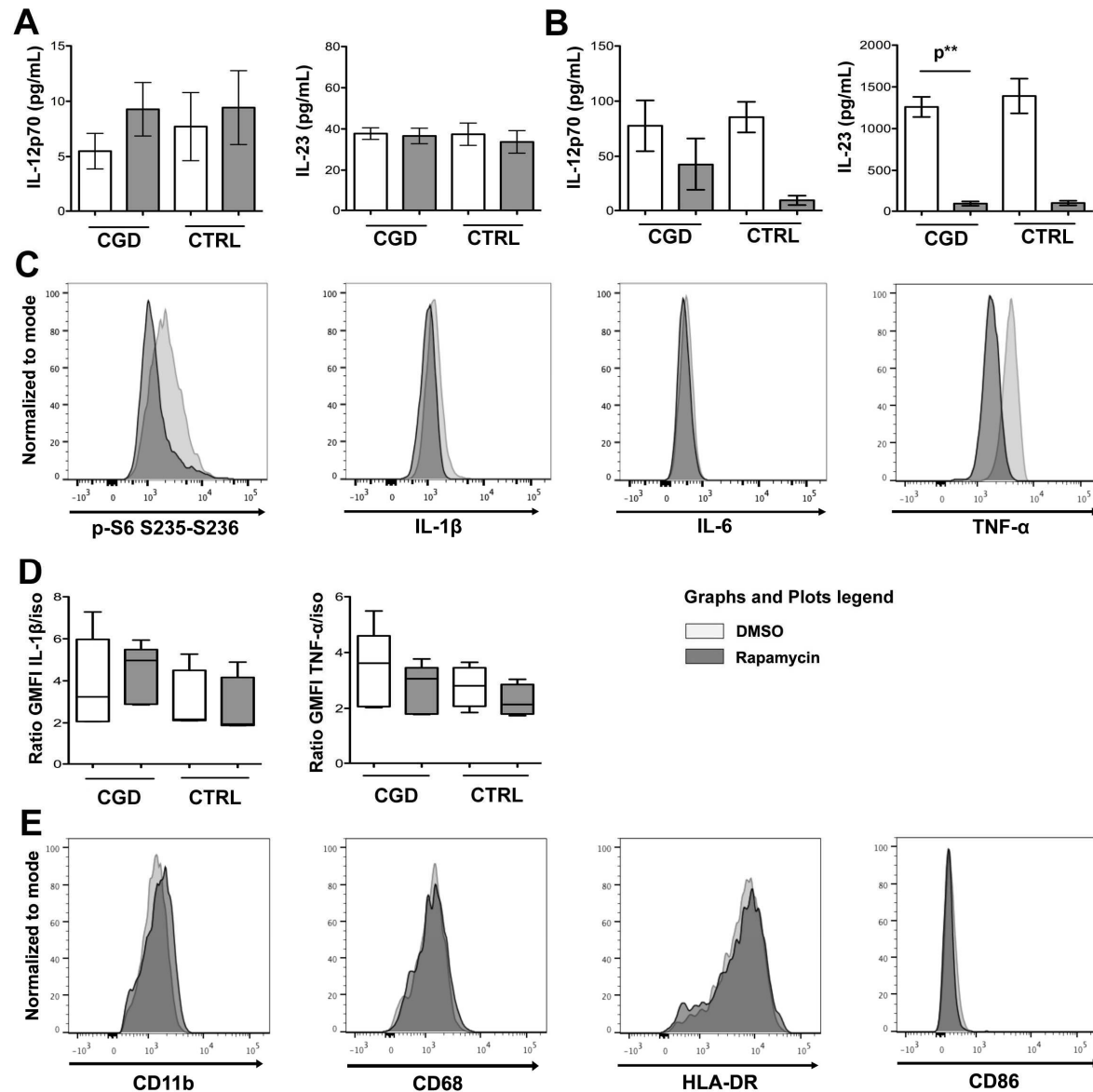


Fig E2. Effect of rapamycin on monocyte-derived macrophage from CGD patients.

(A) CD14⁺ monocytes from CGD patients (n=15, 18 samples) and healthy controls (n=7, 7 samples) were differentiated into macrophages with or without (DMSO) rapamycin. Supernatant were collected at day+6 after differentiation and cytokines were analyzed by cytometric bead assay (MacsPLEX) or ELISA. (B) Monocyte-derived macrophages (MDM) from CGD patients (n=12) and healthy controls (n=5) were stimulated 24h with 100 ng/mL LPS in the presence or not (DMSO) 10 nM rapamycin. Supernatant were collected for cytokines analysis through cytometric bead assay (MacsPLEX) or ELISA. (C) MDM from CGD patients treated or not with 10 nM rapamycin were analyzed for S235-236 phospho S6 ribosomal protein IL-1 β , IL-6 and TNF- α expression by intracytoplasmic staining. (D) Geometric mean of fluorescence intensity (GMFI) ratio between IL-1 β or TNF- α and the isotype control in MDM from CGD patients (n=5) versus healthy controls (n=4) at day+6 after differentiation. (E) Expression of CD11b, CD68, HLA-DR and CD86 by MDM macrophage from CGD patients after differentiation in the presence or absence (DMSO) of 10 nM rapamycin.

p* < 0.05; p** < 0.01, p*** < 0.001; (Man-Whitney T-test or Wilcoxon paired T-test)

Extremities of the whiskers boxes represent minimum and maximum values. Bottom and top of the box represent the first and third quartiles, respectively. The horizontal bar in the box represents the median value.

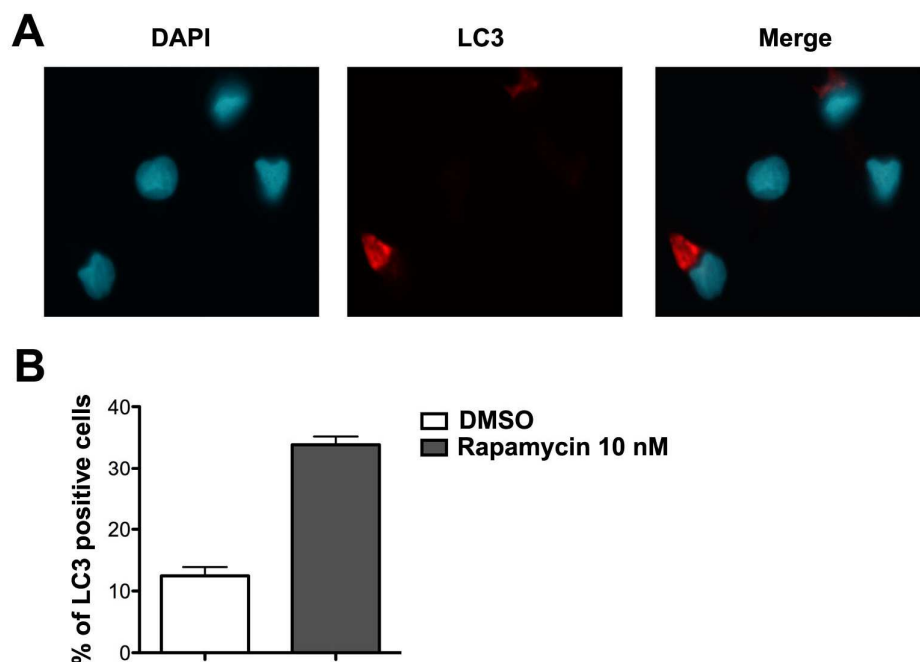


Fig E3. LC3 staining in monocytes-derived macrophage from CGD patients as a surrogate marker of autophagy induction.

(A) Representative LC3 staining in MDM from CGD patient after rapamycin (B) Percentage of cells with positive staining for LC3 with or without (DMSO) treatment with 10nM rapamycin (data representative of three experiments).

Table E1. Contribution of CGD patients to the different biological tests.

	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13	P14	P15
Age (years)	22	2	14	8	17	1.8	4	1	6	10	12	6	15	17	2
Circulating monocytes analysis															
- Phenotype		+	+	+	+	+	+	+	+	+	+	+	+	+	+
- Intracytoplasmic cytokine staining		+	+	+	+	+	+	+	+	+	+	+	+	+	+
- Potentiation of rapamycin by anakinra	+	+		+	+		+			+		+			+
CD4 + T cells analysis															
- Cytokines intracytoplasmic staining w/o stimulation	+	+	+	+	+	+	+	+	+	+	+	+		+	+
- Cytokines staining after PMA/iono	+	+	+	+		+			+	+		+	+	+	
- Cytokines secretion after CD3/CD28	+	+	+	+		+			+	+		+	+	+	
- Cytokines secretion after CD3/CD28 and rapamycin exposition				+	+	+			+	+		+			
MDM															
- Cytokines secretion at day+6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
- Cytokines secretion after LPS			+	+	+	+	+	+	+	+		+	+	+	+
- Intracytoplasmic staining at day+6			+	+					+	+	+				
- Caspase 1 activity				+	+		+		+	+					
- LC3 staining				+	+					+		+			
Neutrophil staining	+	+	+	+	+		+	+	+			+	+		

LC3 Microtubule-associated protein 1A/1B-light chain 3, LPS Lipopolysaccharide, PMA Phosphomolybdic acid, w/o without.

Table E2. Contribution of healthy control to the different biological tests.

	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13	C14	C15
Age (years)	2	37	26	4	10	14	23	48	56	21	47	30	28	7	24
Circulating monocytes analysis															
- Phenotype	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
- Intracytoplasmic cytokine staining	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
CD4 + T cells analysis															
- Cytokines intracytoplasmic staining w/o stimulation	+	+	+	+	+	+	+	+				+	+		
- Cytokines staining after PMA/iono		+		+	+	+	+	+						+	+
- Cytokines secretion after CD3/CD28	+	+		+				+						+	+
- Cytokines secretion after CD3/CD28 and rapamycin exposition		+		+	+		+	+							
MDM															
- Cytokines secretion at day+6		+			+	+	+	+						+	+
- Cytokines secretion after LPS		+			+	+	+	+							
- Intracytoplasmic staining at day+6		+			+	+	+								
Neutrophil staining	+	+		+	+		+	+						+	+

LC3 Microtubule-associated protein 1A/1B-light chain 3, LPS Lipopolysaccharide, PMA Phosphomolybdic acid, w/o without.