SOCIETÀ ITALIANA DI FARMACOLOGIA

RELAZIONE DI FINE PERIODO

NOME E COGNOME: Lucia Lisi

UNIVERSITÀ: Università Cattolica Del Sacro Cuore

DIPARTIMENTO: Istituto di Farmacologia

TUTOR: Prof Pierluigi Navarra

TIPOLOGIA DI BORSA RICEVUTA: Contributo SIF-MSD

TIPOLOGIA DI RELAZIONE (es.: metà periodo o finale): Finale

TITOLO DELLA RELAZIONE: The effects of ARVs on ARG pathway in the brain: does the interaction play a role in microglial polarization in NeuroAids?

RELAZIONE:

State of ART

The introduction of HAART, i.e. a combination of different drugs from three or more classes of antiretroviral agents (ARVs), has significantly increased life expectancy of HIV seropositive patients, reducing both morbidity and mortality (1). Moreover HAART has significantly reduced the incidence of the most severe forms of AIDS-related dementia, whereas mild cognitive impairment is still affecting the majority of HIV infected patients: approximately 50% of HIV infected patients show signs and symptoms of neurological disorders (2).

The molecular mechanisms underlying these neurological complications are not fully elucidated. Most of the available evidence suggests that the main mechanism of neuronal injury during HIV infection is indirect, through toxins, cytokines and nitric oxide (NO) released by activated glial cells in response to residual viral replication (3). In fact, unlike other viral infections, HIV-1 does not directly infect neurons once in the brain, whereas replication is mainly observed in perivascular macrophages and microglia cells (4).

Under pathological conditions, microglia do not constitute a uniform cell population but rather comprise a family of cells with diverse phenotypes, which may be associated to beneficial or, on the contrary, to detrimental biological activities (5). Microglial activated cells can be broadly divided into classically-activated M1 cells, with cytotoxic properties, and alternatively-activated M2 cells, with phagocytic activities. Furthermore, the M2 activation can be further divided into three classes: M2a, involved in repair and regeneration; M2b, an immune-regulatory phenotype; or M2c, an acquired-deactivating phenotype (6). Each of these specific phenotypes displays a distinct pathway of activation, i.e. iNOS for M1, Arginase (ARG) for M2a and interleukin (IL)-10 for M2b activation.

A highly significant inverse correlation exists between CD4+ T cell counts and the level of ARG activity in peripheral blood mononuclear cells from naïve HIV-seropositive patients (7) and the lack of a similar correlation in HAART treated patients suggests that some ARVs might possibly modulate the activity of ARG (8). Moreover we have recently carried out a screening of different AVRs, looking at their potential pro-inflammatory effects on microglial cells. The drugs increased NO production in microglial cells activated with $Gp120_{CN54}$ and IFNγ, via a mechanism involving the inhibition of ARG I activity (9). Thus, in the framework of microglial activation, the NO-ARG pathway can be envisioned as an additional molecular target of different ARVs.
The main objectives of the present research project are the followings:

1. To characterize microglial polarization in *in vitro* model of NeuroAIDS
2. To evaluate the role of antiretroviral drugs in the regulation of microglial polarization
3. To investigate the role played by ARG pathway in the effects of ARVs on microglial cells.

To characterize microglial polarization in *in vitro* model of NeuroAIDS

Initial experiments aimed to characterize the effects of different *envolope* protein GP120 isoforms were carried out on primary cultures of rat microglial cells, prepared according to the standard protocol used in our laboratory (9). Following this procedure, microglial cultures used for the experiments are highly purified, being 95-98% positive for the specific macrophage/microglial marker CD11b [Fig. 1A-B; 9].

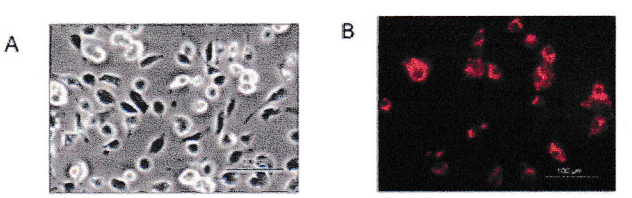

Figure 1. A) Morphology of a primary culture of rat microglia cells was observed under phase-contrast microscopy. B) Microglial cells were stained with an antibody specific for the rat CD11b surface antigen.

Microglial cells express both chemokine receptors, i.e. CCR5 and CXCR4, used by HIV-1 for entry in the host cells (10). Therefore microglial cells can be infected by all the three different phenotypes of HIV-1: (M)-tropic (R5), T-tropic (X4), and dual tropic viruses. Considering this possibility, we recently tested the effects of three different isoforms of GP120, namely two isoforms derived from R5-tropic strains ($GP120_{CN54}$ and $GP120_{BaL}$) and an isoform derived from a X4-tropic strain ($GP120_{IIIB}$). In primary cultures of rat microglia, GP120s induced the up-regulation of several M1 pro-inflammatory genes, (iNOS, IL1β, and COX1-2), although with no significant increase of their final products. As expected, the association of GP120 with IFNγ significantly enhanced GP120-mediated pro-inflammatory activity (10). In the present project we tested the effects of GP120 on different parameters of microglial polarization, in particular we studied CD68 as marker of general activation,

CD86 as marker of M1/M2 phenotypes, TGFβ and ARG-I as markers of M2 activation. Differences in the pattern of microglia activation were found in response to different isoforms of GP120.

In particular, after 24 hours GP120s given alone are able to up-regulate CD68 and CD86 expression, albeit to a different extent, with $GP120_{CN54}$ being the most effective, at least for CD68 receptor. In contrast no effect is elicited by Gp120s on TGFβ and ARG-I gene expression [FIG 2].

Interestingly IFNγ, a well known macrophage activating cytokine, modifies microglial response to HIV GP120. Given in association with 10 UI/ml IFNγ, Gp120s altogether did not exert any effects on CD68, whereas IFNγ and $Gp120_{CN54}$ only significantly increased TGFβ gene expression [FIG 2]. The mechanisms underlying the potentiating effects of IFNγ in microglial cells are still under investigation. In conclusion, it seems that Gp120s are able to induce a classical pro-inflammatory activation (M1) without particular influence toward the M2 phenotype. $Gp120_{CN54}$ only, in association with IFNγ, leads to a full activation of microglia cells, enhancing both M1 and M2 markers.

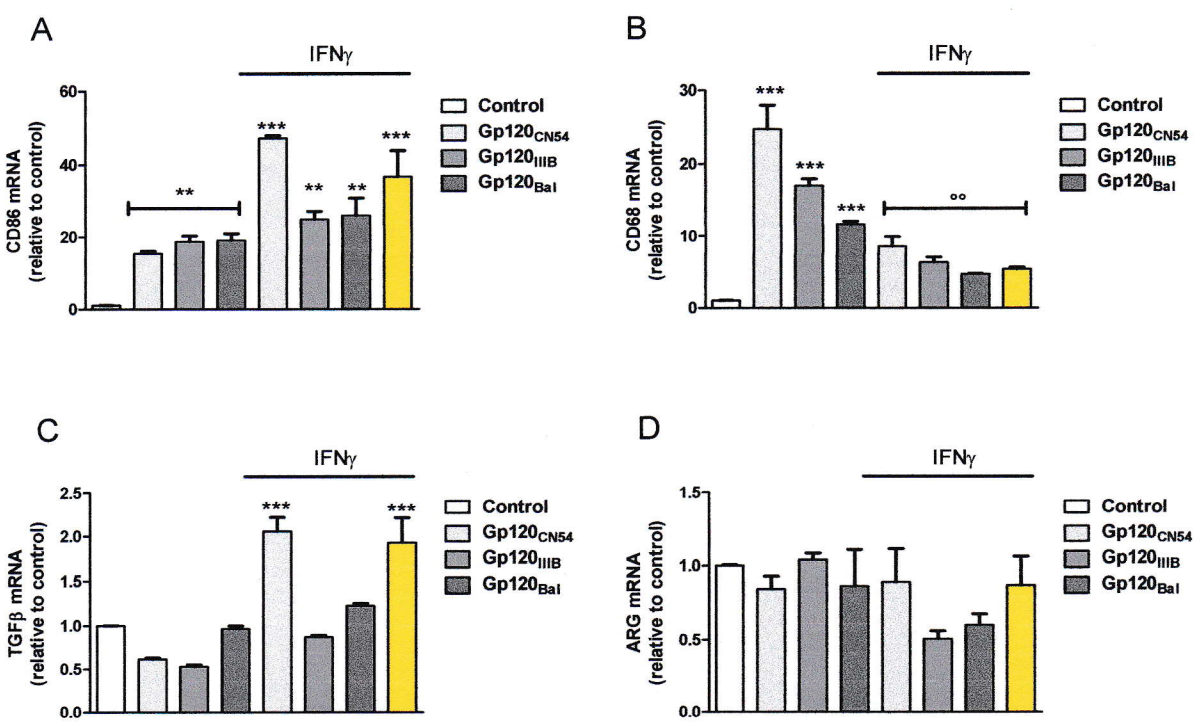

Figure 2. Effects of GP120s on M1 or M2 markers in rat microglia. (A) Total cytosolic RNA was prepared from Control, or cells treated for 24 hours with Gp120s alone or with IFNγ, and used for real time (Q)-PCR analysis of Cd86, Cd68, TGFβ and ARG-I expression. Data are expressed as fold change vs. Control, taken as calibrator for comparative quantization analysis of mRNA levels. Each sample was measured in triplicate, the experiment was repeated 2 times with similar results. Data are means ± SEM, and were analyzed by two-way ANOVA followed by Bonferroni's post-test.

To evaluate the role of antiretroviral drugs in the regulation of microglial polarization.

In order to reinforce the translational value of our findings we started to work with a human microglial cell line, CHME-5 cells [Fig 3], kindly provided by Prof. Talbot (Université du Québec, Canada).

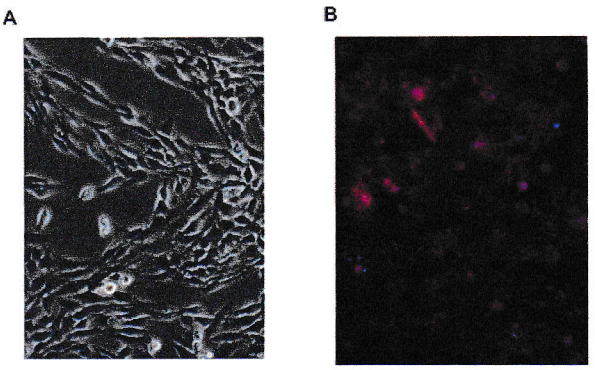

Figure 3. Morphology and F-actin immunostaining of CHME-5 cells. (A) Morphology of CHME-5 cell line, observed by phase-contrast microscopy. (B) Cells were grown on coverslips for 24 h, and morphology was evaluated by staining of F-actin with phalloidin-TRIC and fluorescence microscopy.

CHME-5 cells are able to polarize toward both M1 and M2 phenotype *in vitro.* We tested different stimuli, different times of incubation in order to assess iNOS (M1) activity or ARG (M2) activity. First, cells were incubated with known M1 polarizing stimuli and iNOS activity was evaluated. As shown in Fig. 4, iNOS activity was significantly increased by a mixture of proinflammatory cytokines (TNF α , IFN γ , IL1 β - named TII, which is a classical tool to induce M1 status). Likewise, TII significantly increased iNOS expression after 4h (approximately 400 fold higher than control) and expression levels remained elevated at 24 hours (40 fold) (Fig. 5). Other classical inducers of M1 status, namely 1 ng/ml LPS, 10 ng/ml glycoprotein GP_{120} with or without IFNγ, and each of the above cytokines given alone, failed to stimulate NO release by CHME-5 (Fig. 4), and consistently GP_{120} alone did not increase the expression of iNOS (Fig. 5). Thus, TII was chosen as pro-inflammatory (M1-polarizing) stimulus.

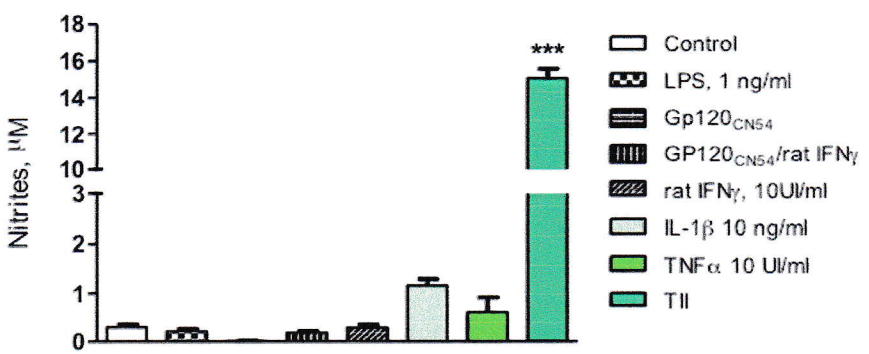

Figure 4. Effects of M1 polarizing stimuli on iNOS activity in human microglial CHME-5 cells. CHME-5 cells were incubated for 48h in presence of several pro-inflammatory and M1-polarizing stimuli, including bacterial endotoxin lipopolysaccharide (LPS), the HIV-envelope protein gp120, rat interferon γ (IFNγ), the human cytokines interleukin-1β (IL-1β) and tumor necrosis factor α (TNFα). A cytokine mixture (TII) containing the main M1 pro-inflammatory cytokines, TNFα, IL-1β, IFNγ, each used at the indicated concentrations, was also tested in the system. NO production was assessed indirectly by measurement of nitrite accumulation in the incubation medium (Griess method). Data are means ± SEM (n = 6), and were analyzed by one-way ANOVA followed by Bonferroni's post hoc test. ***P < 0.001 vs Control.

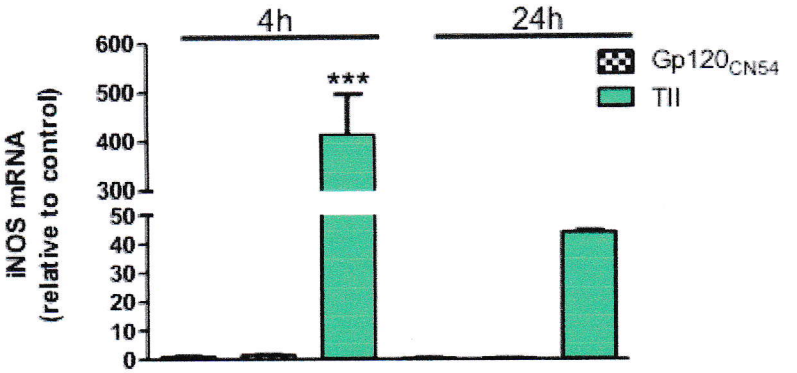

Figure 5. Effects of M1 polarizing stimuli on iNOS expression in human microglial CHM E-5 cells. Total cytosolic RNA was prepared from Control, or CHME-5 cells treated with TII or Gp120 as indicated in figure, and used for real time (Q)-PCR analysis of iNOS expression. Data are expressed as fold change vs. Control, taken as calibrator for comparative quantitation analysis of mRNA levels. Each sample was measured in triplicate, the experiment was repeated 2 times with similar results. Data are means ± SEM, and were analyzed by two-way ANOVA followed by Bonferroni's post hoc test. ***P < 0.001 vs control.

The Th2 cytokine, interleukin (IL)-4, has been characterized as a potent inducer of the alternative M2 polarization status in macrophages, thus it was tested in our experimental model. Increased ARG activity was detected in human CHME-5 microglial cells exposed to 20 ng/ml IL-4 for 48 hours (Fig. 6).

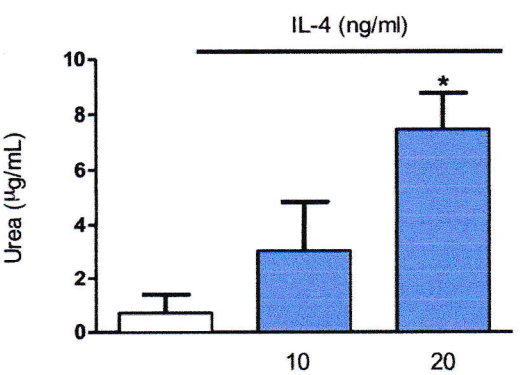

Figure 6. Effects of recombinant human IL-4 on CHME-5 activation. CHME-5 cells were treated for 48 hours with IL-4 and urea levels were measured in the incubation medium. Data were analyzed by one-way ANOVA followed by Bonferroni 's post hoc test. * P < 0.05 vs. Control.

In a subsequent series of experiment we tested the effect of both stimuli (TII or IL-4) on iNOS and ARG expression and activity. As shown in Fig. 7, IL-4 significantly increased ARG activity (Fig. 7B), without significant induction of its expression at 4 hours (Fig. 7A). Conversely, TII tended to reduce ARG expression and activity in this experimental setting (Fig. 7), thus suggesting that the inhibition of ARG activity in part contributes to enhance iNOS activity in response to M1 polarizing stimuli. On the other hand, we found that TII significantly increased the expression and activity of iNOS, while IL-4 had no modulatory effect (Fig. 8).

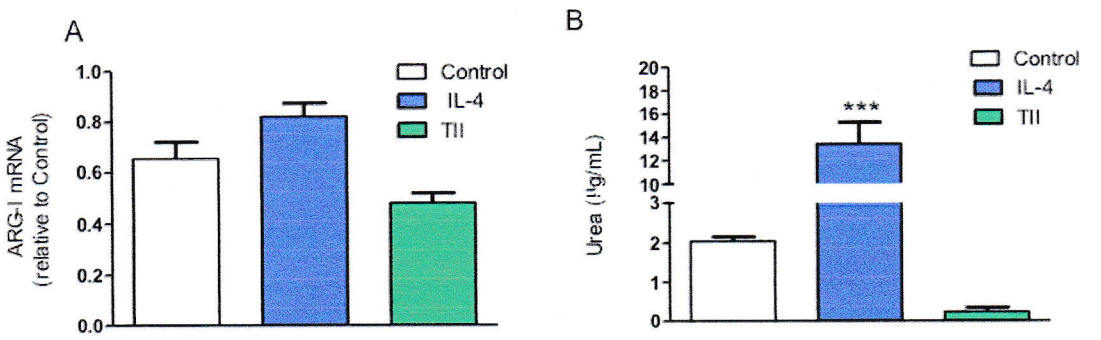

Figure 7. Effects of the two different polarizing stimuli on ARG expression and activity in human microglial CHME-5 cells. (A) Total cytosolic RNA was prepared from Control, or CHME-5 cells treated for 4 hours with IL-4 and TII, and used for real time (Q)-PCR analysis of human ARG-I expression. Data are expressed as fold change vs. Control, taken as calibrator for comparative quantitation analysis of mRNA levels. Each sample was measured in triplicate, the experiment was repeated 2 times with similar results. Data are means ± SEM, and were analyzed by two-way ANOVA followed by Bonferroni's post-test. (B) CHME-5 cells were treated for 72 hours with IL-4 or TII and urea levels were measured in the incubation medium. Data were analyzed by one-way ANOVA followed by Bonferroni 's post hoc test. *** $P < 0.001$ vs. Control.

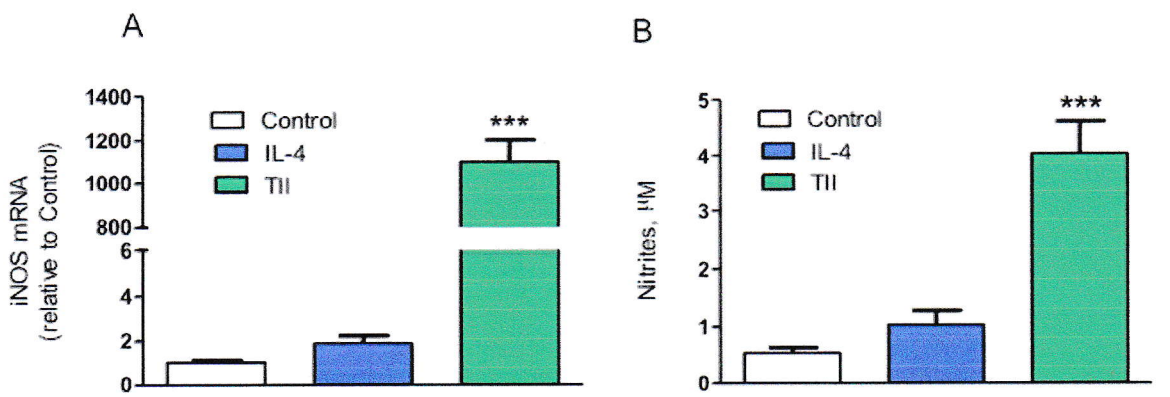

Figure 8. Effects of the two different polarizing stimuli on ARG expression and activity in human microglial CHME-5 cells. (A) Total cytosolic RNA was prepared from Control, or CHME-5 cells treated for 4 hours with IL-4 or TII, and used for real time (Q)-PCR analysis of iNOS expression. Data are expressed as fold change vs. Control, taken as calibrator for comparative quantitation analysis of mRNA levels. Each sample was measured in triplicate, the experiment was repeated 2 times with similar results. Data are means ± SEM, and were analyzed by two-way ANOVA followed by Bonferroni's post-test. ***$P < 0.001$ vs control. (B) CHME-5 were incubated for 48h in presence of IL4 or TII. NO production was assessed indirectly by measurement of nitrite accumulation in the incubation medium (Griess method). Data are means ± SEM (n = 6), and were analyzed by one-way ANOVA followed by Bonferroni's post-test.***$P < 0.001$ vs Control.

After the characterization of CHME-5 system, we studied the effects of ARVs on ARG activity in four different conditions: basal conditions (FIG 9A), after the induction of a M2 milieu by IL-4 (FIG 9B), after the induction of a M1 environment by TII (FIG 9C), and in a mixed M1/M2 paradigm, induced by the simultaneous exposure to IL-4 and TII (FIG 9D). After 72 h of exposure to stimuli, urea levels were significantly increased in all paradigms; in particular, the levels of urea, expressed as the means ± SEM of (n) replicates per group, were: a) 1.29±0.44 µg/ml (20) under basal conditions; b) 3.87±0.961 µg/ml (10) after IL-4 stimulation; c) 3.734±0.374 µg/ml (10) after TII challenge; and d) 3.31±0.76 µg/ml (10) after TII+IL-

4. In these conditions, ARVs (100 pM DRV, 100 pM ATV, 1 nM EFV and 1μM NVP) reduced the release of urea in the incubation medium by about 50% with respect to the relevant stimuli given alone (FIG 9 A-D).

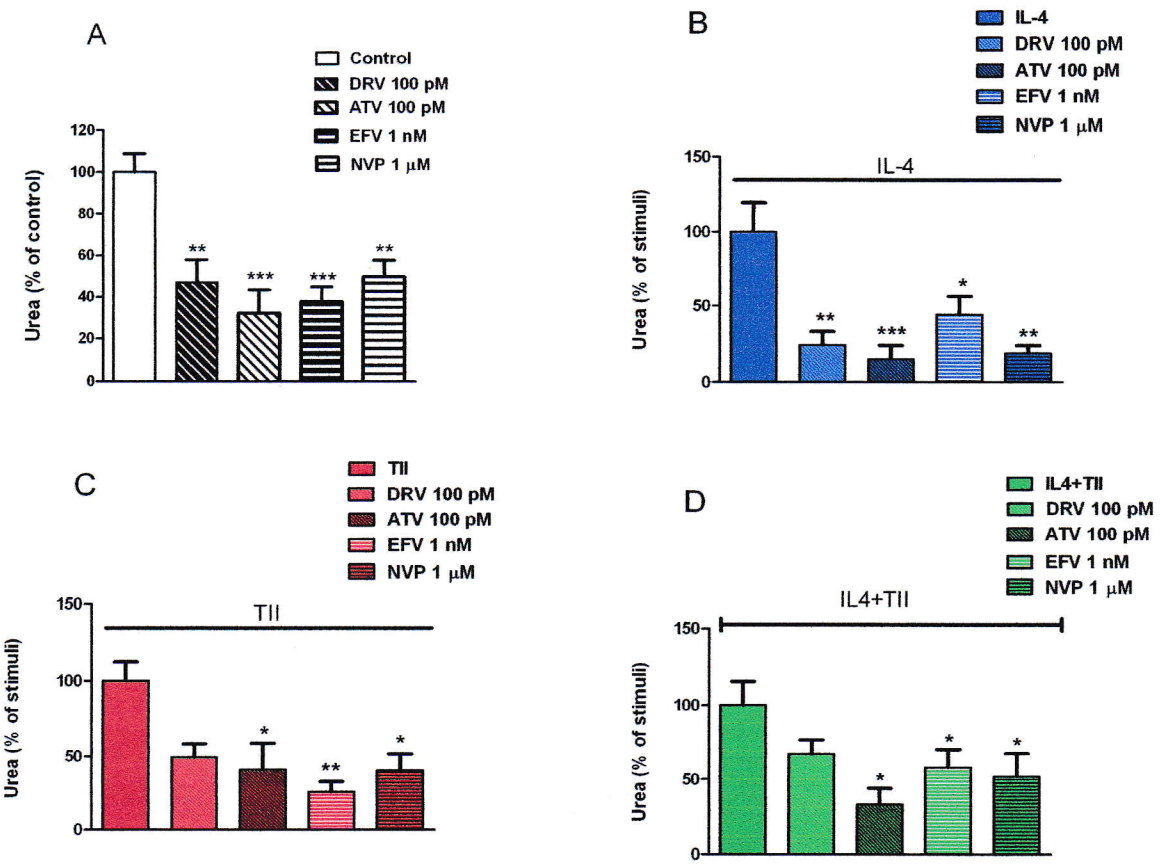

Figure 9. Effects of ARVs on ARG activity in CHME-5. In presence of ARVs 4 different conditions were investigated: basal conditions (A), after the induction of a M2 milieu by IL-4 (B), after the induction of a M1 environment by TII (C), and after simultaneous exposure to IL-4 and TII (D). After 72 hours of incubation the levels of urea, expressed as the means ± SEM of (n) replicates per group were: panel A- basal condition 100%±8.9 (15), DRV 46%±11.0 (15), ATV 31.9%±11.3 (15), EFV 37.6%±7.2 (15), NVP 49.6%±8.1 (15); panel B- IL4 100%±19 (5), IL4+DRV 24%±8.9 (5), IL4+ATV 15%±8.8 (5), IL4+EFV 44.2%±12.2 (5), IL4+NVP 18.9%±4.9 (5); panel C- TII 100%±12.4 (5), TII+DRV 49.5%±8.6 (5), TII+ATV 40.7%±17.9 (15), TII+EFV 25.%±7.2 (15), TII+NVP 40.5%±11 (5); panel D- IL4+TII 100%±15.5 (15), IL4+TII+DRV 67.3%±9.5 (10), IL4+TII+ATV 32.7%±11.1 (10), IL4+TII+EFV 58.1%±12.3 (10), IL4+TII+NVP 51.9%±15.7 (10). Data were analyzed by one-way ANOVA followed by Bonferroni's post-test. *P < 0.05 and **P < 0.01 ***P < 0.001 vs control or stimuli.

To investigate the role played by ARG pathway in the effects of ARVs on microglial cells.

Arginase (ARG) is a hydrolytic manganese-containing enzyme using L-Arginine (L-Arg) as substrate. Two arginase isoforms are expressed in mammals: ARG-I (cytosolic) and ARG-II (mitochondrial) (11). Although these isoforms are the products of distinct genes located on different chromosomes, both isoenzymes catalyze the same reaction: arginine + H_2O → ornithine + urea. Both ARG isoforms are primarily involved in the final step of urea cycle. The arginase genes display independent regulation; they are expressed in different cell types and can be induced and/or up-regulated by a wide range of agents (12). For example, ARG-I but not ARG-II is expressed under basal conditions in macrophages; in these cells ARG-I is strongly up-regulated by interleukin (IL)-4 whereas ARG-II gene expression is induced by exposure to serum and is not influenced by IL-4 (13).

To demonstrate a direct inhibitory activity of ARVs on isolated ARG preparations, we investigated the effects of the above ARVs on isolated ARG enzyme preparations obtained from CHME-5. This experimental model is a less complex system in which it is possible to study drug-enzyme interactions, and thus fast screening pharmacological compounds with direct modulatory activity on the ARG enzyme.

First of all, we carried out time-course experiments under basal conditions. Cell lysates were incubated at 37°C; Mn^{2+} and L-Arg were added to the incubation medium and ARG activity was measured after 0.5, 1, 1.5 and 2 hours of incubation. Optimal ARG activity was obtained at 2 hours [Fig 10A]. In this experimental paradigm 560 µM of the ARG inhibitor Nor-NOHA, was able to reduce ARG activity by about 30% [Fig 10B]. All subsequent experiments were carried out at 2 hours.

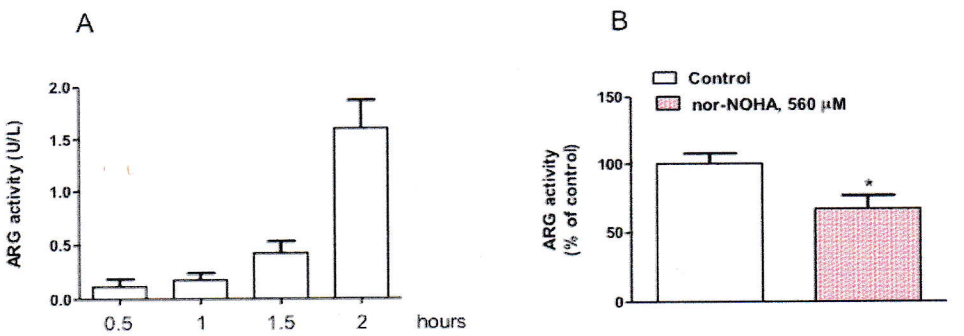

Figure 10. ARG activity in human CHME-5 microglial cell lysates. (A) One 75 cm2 flask of 80% confluent CHME-5 cells was lysed in 1000 µL of Tris-HCl with protease inhibitors. Optimal ARG activity was obtained after 2 hours of incubation, thus this time point was selected for further experiments. Data are expressed as means ± SEM (n = 4), the experiment was repeated 3 times with similar results and analyzed by one-way ANOVA followed by Bonferroni's post hoc test. ***P < 0.001 vs Control. (B) The ARG-I inhibitor, nor-NOHA, significantly reduced ARG activity. Data are means ± SEM (n=5), and were analyzed by one-way ANOVA followed by Bonferroni's post-test. *P < 0.05 vs control.

Moreover in a further series of experiments, cellular lysates were incubated at 37°C in the presence of Mn^{2+}, L-Arg, GDH and other co-factors in order to optimize both ARG and iNOS enzyme activity. Under these conditions, ARG activity was evaluated by measuring urea levels after 2 hours of incubation, whereas iNOS activity was measurable already after 20 min of incubation. In particular, the cells were pre-stimulated for 48 h with i) IL-4 or ii) TII or iii) IL-4/TII and the interplay ARG-iNOS was studied. As shown in figure 8, in all the conditions studied it was possible to measure ARG activity [Fig 11A] but obviously only after TII challenge (alone or in combination with IL-4) it was possible to measure iNOS activity as well [Fig 11B].

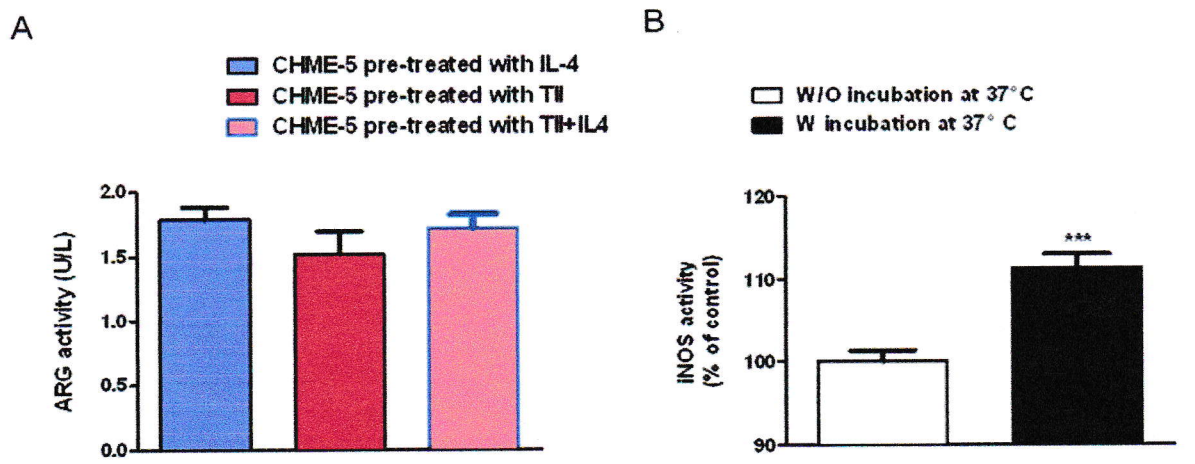

Figure 11. ARG and iNOS activity in human CHME-5 microglial cell lysates. One 75 cm^2 flask of 80% confluent CHME-5 cells as lysed in 1000 µL of Tris-HCl with protease inhibitors. (B) The iNOS activity resulted measurable only in presence of TII (alone or with IL-4). Data shown in Figure are from CHME-5 cells pre-stimulated with IL4 and TII (added in combination) for 48 h. The iNOS activity was measured after 20 min of incubation at 37°C with ENOS-100 kit according to the manufacturer's instructions. Data are means ± SEM (n=6), and were analyzed by one-way ANOVA followed by Bonferroni's post-test. ***P < 0.001 vs Control.

Since in previous experiments on intact cells we found no difference among the effects of ARVs on ARG regardless of whether or not the cells were stimulated with IL-4 and/or TII, experiments on cell lysates were conducted only in the presence of IL-4 + TII, to induce the full expression of both iNOS and ARG. Therefore, CHME-5 cells were stimulated with TII+IL4 for 48 hours before cell lysis. Thereafter the supernatants of cells lysates were incubated at 37°C in the presence of ARVs; Mn^{2+}, L-Arg, GDH and other co-factors were also added, in order to optimize both ARG and iNOS enzyme activity. Under these conditions, ARG activity was evaluated by measuring urea levels after 2 hours of incubation, whereas iNOS activity (but only for 2 samples) was measurable already after 20 min of incubation (data not shown). Similar to experiments on intact cells, ARVs (100 pM DRV, 100 pM ATV, 1 nM EFV, 1 µM NVP) were altogether able to reduce by about 50 % ARG activity (FIG 12) (14).

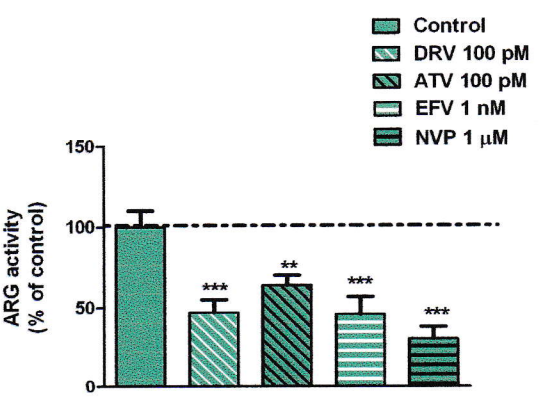

Figure 12. Effects of ARVs on ARG activity of pre-stimulated CHME-5 cell lysates. CHME-5 cells were pre-stimulated with rh-IL4 and TII for 48 h. After this framework the cells were lysed and incubated at 37°C with Mn^{2+}, L-Arg, GDH and other co-factors. After 2 hours of incubation the levels of urea, expressed as the means ± SEM of (n) replicates per group were: IL4+TII alone 100 ± 9.8 (9), IL4+TII+DRV 47.0 ± 8.1 (9), IL4+TII+ATV 64.2 ± 6.3 (9), IL4+TII+EFV 46.0 ± 10 (9), IL4+TII+NVP 30.6 ± 7.6 (9). Data were analyzed by one-way ANOVA followed by Bonferroni's post-test. ***P < 0.001 and **P < 0.01 vs IL4+TII.

References

1- May MT, Ingle SM (2011) Life expectancy of HIV-positive adults: a review. Sex Health. 8, 526-533.

2- Clifford, DB. (2008) HIV-associated neurocognitive disease continues in the antiretroviral era. Top HIV Med. 16, 94-98.

3- Valcour V, Sithinamsuwan P, Letendre S, Ances B (2011) Pathogenesis of HIV in the central nervous system. Curr HIV/AIDS Rep. 8, 54-61.

4- Yadav A, Collman RG (2009) CNS inflammation and macrophage/microglial biology associated with HIV-1 infection. J Neuroimmune Pharmacol. 4(4):430-47. Review.

5- Schwartz M, Butovsky O, Brück W, Hanisch UK (2006) Microglial phenotype: is the commitment reversible? Trends Neurosci. 29:68-74.

6- Chhor V, Le Charpentier T, Lebon S, Oré MV, Celador IL, Josserand J, Degos V, Jacotot E, Hagberg H, Sävman K, Mallard C, Gressens P, Fleiss B (2013) Characterization of phenotype markers and neuronotoxic potential of polarised primary microglia in vitro. Brain Behav Immun 32:70-85.

7- Cloke T.E., Garvey L., Choi B.S., Abebe T., Hailu A., Hancoc M., Kadolsky U., Bangham C.R., Munder M., Müller I., Taylor G.P., Kropf P. (2010a) Increased level of arginase activity correlates with disease severity in HIV-seropositive patients. J Infect Dis. 202(3):374-385.

8- Cloke TE, Abebe T, Hailu A, Munder M, Taylor GP, Müller I, Kropf P (2010) Antiretroviral therapy abrogates association between arginase activity and HIV disease severity. Trans R Soc Trop Med Hyg. 104, 746-748.

9- Lisi L, Tramutola A, Navarra P, Dello Russo C (2014) Antiretroviral agents increase NO production in gp120/IFNγ-stimulated cultures of rat microglia via an arginase-dependent mechanism. J Neuroimmunol. 266(1-2):24-32.

10- Lisi L, Tramutola A, De Luca A, Navarra P, Dello Russo C. (2012) Modulatory effects of the CCR5 antagonist maraviroc on microglial pro-inflammatory activation elicited by gp120. J Neurochem. 120(1):106-14.

11- Jenkinson C.P., Grody W.W., Cederbaum S.D. (1996) Comparative properties of arginases. Comp Biochem Physiol B Biochem Mol Biol. 114(1):107-132.

12- Caldwell R.B., Toque H.A., Narayanan S.P., Caldwell R.W. (2015) Arginase: an old enzyme with new tricks. Trends Pharmacol Sci. doi: 10.1016/j.tips.2015.03.006.

13- Louis C.A., Mody V., Henry W.L. Jr, Reichner J.S., Albina J.E. (1999) Regulation of arginase isoforms I and II by IL-4 in cultured murine peritoneal macrophages. Am J Physiol. 1999 276(1 Pt 2):R237-R242.

14- Lisi L, Laudati E, Miscioscia TF, Dello Russo C, Topai A, Navarra P. Antiretrovirals inhibit arginase in human microglia. J Neurochem. 2015 Oct 14.

Roma, 13 aprile 2016

Tutor: Prof. Pierluigi Navarra
Firma

Borsista: Dr Lucia Lisi
Firma