UNIVERSITÀ DEGLI STUDI DEL PIEMONTE ORIENTALE

School of medicine

PhD course in Sciences and Medical Biotechnology

XXX Cicle

MODULATION OF HUMAN MONOCYTE/MACROPHAGE ACTIVITY BY BDMARDS: AN IN VITRO STUDY

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Introduction

In the last fifteen years a number of biological disease-modifying anti-rheumatic drugs (bDMARDs) with proven efficacy have been developed. These drugs are structurally unrelated and have different pharmaco-dynamic and pharmacokinetic properties, although their clinical development has been largely overlapping and their differences have not been thoroughly investigated.

We have recently demonstrated that monocytes/macrophages from RA patients under sDMARD treatment with less active disease present higher PPARγ protein expression and lower MMP-9 activity than RA patients with more severe disease. In analogy, when probing monocytes from healthy volunteers *in vitro*, methotrexate and methylprednisolone increase in vitro PPARγ protein expression and inhibit LPS-induced MMP-9 activity [2].

Etanercept, abatacept and tocilizumab are bDMARDs with proven efficacy in RA [3-5] albeit with different mechanism of actions. Etanercept is a dimeric fusion protein that binds and inactivates soluble and cell-bound TNFα and lymphotoxin α [6]. Abatacept is a soluble recombinant fusion protein comprising the extracellular domain of human CTLA-4 that employs the high binding avidity of CTLA-4 for CD80/CD86 on APCs, to prevent full T cell activation [7]. Tocilizumab is a humanized anti-IL-6R antibody that prevents IL-6 from binding to its receptors, thereby blocking the pro-inflammatory effects of IL-6 [8].

We have recently observed that RA patients in treatment with tocilizumab (n=7) have higher PPARγ monocyte expression compared to healthy donors paralleling the observations with sDMARDs. Capitulating on this exploratory finding, we have now examined whether etanercept,

tocilizumab and abatacept differ in their ability to modulate human monocytes and monocytes-derived macrophages responsiveness and phenotype.

Methods

Cell culture

Human monocytes were isolated from healthy anonymous human buffy coat samples (provided by the Transfusion Service of Busto Arsizio, Varese, Italy). For cell isolation, standard techniques of dextran sedimentation and Histopaque (density = 1.077 g cm^{-3}) gradient centrifugation (400 x *g*, 30 min, room temperature) were used. Cells were then recovered by thin suction at the interface, as described previously [10=2]. Isolated cells were then re-suspended in RPMI 1640 medium supplemented with 5% fetal bovine serum (FBS), 2 mM glutamine and antibiotics; purified monocyte populations were obtained by adhesion (90 min, 37°C, 5% CO_2). Cell viability (trypan blue dye exclusion) was usually > 98%. Monocyte derived macrophages (MDM) were generated by culturing monocytes (7-10 days) in RPMI 1640 medium containing 20% FBS, glutamine and antibiotics.

Cell viability

To assess potential drug toxicity in monocytes and MDM, cell viability was evaluated using the methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay. Cells ($1x10^5$ cells) were challenged for 12h and 7 days with concentrations compatible with those found in plasma (PC) of arthritic patients: abatacept 30μg/ml [4], etanercept 2μg/ml [9], tocilizumab 1μg/ml [10]; then, the medium was replaced by the MTT assay solution (1mg/ml; 2h, 37°C 5% CO_2). Supernatant was removed

and DMSO was added in order to dissolve the purple formazan; the absorbance was read at 580 and 675 nm.

Superoxide anion (O_2^-) production

Monocytes ($1x10^6$ cells/plate) were treated for 1h with the drugs in study and then stimulated with phorbol 12-myristate 13-acetate (PMA) 10^{-6} M for 40 min. O_2^- production was evaluated by the superoxide dismutase-sensitive cytochrome C reduction assay and expressed as nmol cytochrome C reduced/10^6cells/40 min, using an extinction coefficient of 21.1 mm. To avoid interference with spectrophotometrical recordings, cells were incubated with RPMI 1640 without phenol red, antibiotics and FBS.

Matrix Metalloproteinase (MMP)-9 gene expression and activity

Total mRNA was extracted from monocytes and MDM cells using GenElute Mammalian Total RNA Miniprep Kit (Sigma Aldrich, MO, USA) according to manufacturer's instructions. First strand of cDNA was synthesised from 1μg of total RNA using ImProm-II RT system (Promega). Real-time PCR was performed using GoTaq qPCR Master Mix (Promega) on an SFX96 Real-Time System (Biorad, Segrate, Italy). S18 ribosomal protein was used to normalize PCR product levels.

MMP-9 activity was evaluated by Novex Gelatin Zymography (Invitrogen), according to the manufacturer's instructions. Monocytes were pre-treated with drugs for 6h and then stimulated with LPS 500 ng/ml for 1h. PG was used at 10μM for 6h and 7 days; PPARγ antagonist GW9662 was used at 10μM for 1h. Supernatants were recovered and 10 μl were mixed with 10 ml Novex Tris-glycine SDS Sample Buffer (2X). Samples were run on a 10% Novex Zymogram Gelatin

Gels (Invitrogen); protein bands were analysed with an image densitometer (Versadoc, Bio-Rad, USA).

PPARγ protein expression

PPARγ protein expression was evaluated in human monocytes and MDM by Western blot, as described previously. Cells were evaluated for their constitutive expression and after 6h and 7 days challenge with the drugs at study. Cells were scraped off and lysed in radio-immunoprecipitation assay (RIPA) buffer. Protein samples (30 μg) were analysed by SDS-PAGE (10% acrylamide) and electro-blotted on nitrocellulose membranes. Immunoblots were performed using a cocktail comprising polyclonal rabbit anti-human PPARγ (Abcam, UK) and monoclonal mouse anti-human β-actin (Abcam, UK) antibodies. Chemiluminescence signals were analysed under non-saturating conditions with an image densitometer (Versadoc, Bio-Rad, USA). Semi-quantitative evaluation of PPARγ protein was performed by calculating the ratio between its expression and the expression of the reference housekeeping protein, β-actin. Results were expressed as fold increase relative to control, un-stimulated cells.

Flow Cytometry

The phenotype of monocytes and macrophages were analysed by direct staining with FITC-conjugated anti-CD14, -CD16, -CD36; RPE-conjugated anti-CD86 and -CD163; PerCp-coniugated anti-CD206 monoclonal antibodies (eBioscience, UK). Cells were then processed by a FACS Calibur flow cytometer (BD and analysed by CellQuest Software (BD). Monocytes and MDM were gated in a side scatter (SSC)/forward scatter (FSC) plot with the scatter gate for monocytes/macrophages. Adding to such region the co-expression of CD14/CD16, CD14/CD36,

CD14/86, CD14/163 and CD14/206, a comparison between cells treated with the drugs at study and those not treated were analysed. Final data were expressed as percentage of positive events for each surface marker on the total $CD14^+$ events.

Statistical analysis

All experiments were repeated using cells isolated from at least 5 separate donors. All statistical analyses were performed using the software GraphPad Prism 5.0. Data are expressed as mean ± standard error of mean (s.e.m.). Comparison among groups was analyzed by one-way analysis of variance between groups (ANOVA) followed by Student's *t* test. $P < 0.05$ was considered statistically significant.

Results

Effects of drugs on cell viability

In order to avoid confounding effects attributable to cell senescence or drug toxicity, we evaluated the effects of abatacept, etanercept and tocilizumab on cell viability. Under conditions detailed in the Methods section, there was no reduction in monocyte and MDM viability after 12h (monocytes) or 7 days (MDM; data not shown) of the incubation periods.

Drugs reduce PMA-induced burst

Exposure of monocytes to phorbol 12-myristate 13-acetate (PMA) 10^{-7} M for 40 min led to a significant increase in reduced cytochrome C (about 29 nmol), as expected. Abatacept, etanercept and tocilizumab all significantly inhibited PMA-induced bursts. Yet, abatacept was more efficacious at inhibiting PMA-induced bursts compared to the other two drugs (Fig. 1a).

Effects of drugs on PPARγ protein expression

Cells exposed to tocilizumab showed a dramatically increased expression of PPARγ compared to vehicle-treated cells both in monocytes and in MDM. A modest, yet significant increase was observed also when cells were treated with abatacept, while etanercept was unable to modulate the expression of the receptor (Fig. 1b).

MMP-9 expression and activity

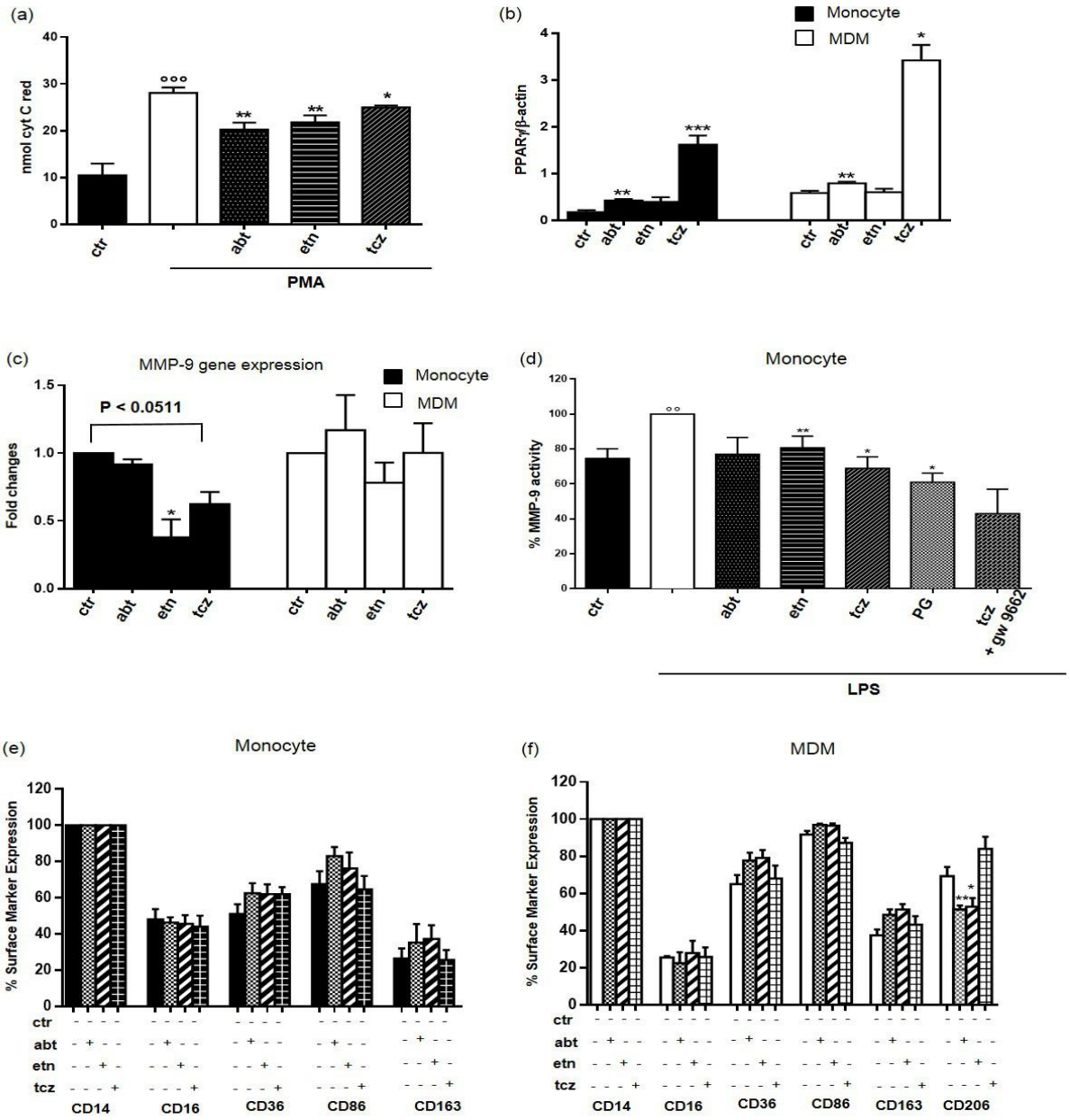
Gene expression of MMP-9 was reduced only by etanercept and tocilizumab in monocytes. In MDM, instead, the three treatments were unable to induce significant changes (Fig. 1c). We also evaluated the ability of drugs to modulate LPS-induced activity of MMP-9 in monocytes. As

shown in Fig 1d, both etanercept and tocilizumab reduced significantly enzyme activity. Given that the natural agonist 15-deoxy-d-prostaglandin J2 (PG, 10^{-5} M) of PPARγ [11] also reduced MMP-9 activity (Fig. 1d), we probed whether the antagonist was able to revert the effects of tocilizumab, but this was not the case (Fig. 1d).

Expression of surface markers

Stimulation of monocytes with abatecept, etanercept or tocilizumab did not induce significant changes in the expression of the surface markers considered. A trend of increase of $CD36^+$ cells after treatment of monocytes with all drugs and an increase of both $CD86^+$ and $CD163^+$ cells in the presence of abatacept and etanercept (Fig. 1e) was observed. Differentiation of monocytes to MDM appeared to be affected by the presence of abatacept and etanercept: a significant decrease in $CD206^+$ cells was observed which was compensated by an increase in $CD36^+$ and $CD163^+$ cells (Fig. 1f).

Fig. 1 Effects of drugs on monocytes/macrophages responsiveness



Legend

Figure 1. Effects of drugs on monocyte/macrophage reponsiveness

(a) Superoxide anion production in human monocytes after pre-incubation with for 1h with the indicated drugs (ctr, control (un-stimulated cell); abt, abatacept; etn, etanercept; tcz, tocilizumab)

and then stimulated with PMA 10^{-6} M for 40 min. °°° $P < 0.001$ vs ctr; ** $P < 0.005$ and * $P < 0.05$ vs PMA. (b) Expression of PPARγ protein evaluated by the ratio PPARγ/βactin in human monocytes and MDM after treatment with the indicated drugs. *** $P< 0.001$, ** $P < 0.005$ and * $P < 0.05$ vs respective ctr. (c) real time PCR for MMP-9 in monocytes and MDM after treatment with the indicated drugs. * $P < 0.05$ vs ctr. (d) MMP-9 zymography gel activity in monocytes pre-treated with the indicated drugs for 6h and then stimulated with LPS 500 ng/ml for 1h. °° $P < 0.005$ vs ctr, ** $P < 0.005$ and * $P < 0.05$ vs LPS. (e and f) Flow cytometry analysis in monocytes (e) and MDM (f) after treatment with the indicated drugs. ** $P < 0.005$ and * $P < 0.05$ vs respective control.

Discussion

Monocytes/macrophages are involved in the pathogenesis of RA [12], and subset populations have been demonstrated to change according to disease severity and duration [13,14] and to the milieu by which are surrounded. In fact, it has been demonstrated that TNFα and IL-6 have different effects on monocyte activation and differentiation, the former inducing differentiation to dendritic cells, and the latter inducing a switch to macrophages [15]. Also, monocyte production of MMP is strongly dependent by levels of TNFα [16,17]. This paper aimed at framing the variations in functional properties of monocytes/macrophages triggered by etanercept, abatacept and tocilizumab.

As shown, all the three bDMARDs were able to blunt PMA-induced anion production in monocytes, demonstrating therefore a specific anti-inflammatory effect that may contribute to inflammation relief in RA patients.

This common feature of the three drugs is offset by differences observed in PPARγ expression, MMP-9 activity and phenotype. First, tocilizumab depicted a strong induction of the anti-inflammatory receptor PPARγ, a feature that was not shared by the other two drugs. This effect parallels our observation on RA patients, in which PPARγ expression is significantly increased in patients treated with tocilizumab. Second, MMP-9 activity was reduced by etanercept and tocilizumab but not by abatacept in both monocytes and MDM. We hypothesized that the strong induction of PPARγ expression by tocilizumab could be linked to the significant inhibition of MMP-9 activity and expression, but the association of tocilizumab plus the antagonist of PPARγ did not abolish the effect of the anti-IL6 receptor, supporting therefore that the effect of tocilizumab on MMP-9 is PPARγ-independent. Etanercept resulted instead the most effective in reducing both MMP-9 gene expression and activity and this is in keeping with its anti-TNFα

mechanism: in fact as mentioned above [15,16], this cytokine induces MMP-9 confirming its role as pro-inflammatory mediator. Third, abatacept and etanercept, unlike tocilizumab, reduced $CD206^+$ MDM cells. Differentiation of monocytes to mature macrophages represents the first step of colonization of synovial layer by peripheral cells [19]. Therefore, functional diversity of macrophages in this area is strictly correlated to the expression of different activation markers, contributing differently to disease progression. In our in vitro experiments, the three bDMARDs considered, do not modify monocytes phenotype and do not interfere with natural differentiation of monocytes to MDM. Interestingly, in our model, we observe that MDM have a M2-like phenotype. Tocilizumab does not alter the number of $CD206^+$ cells, while etanercept and abatacept reduced their number.

In conclusion, the present study shows that the three bDMARDs tested act in a cell-autonomous manner on monocytes and macrophage. Their effects on MMP-9 activity, PPARγ expression and phenotype are significantly different and this may contribute to their peculiar anti-inflammatory actions.

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SEMINARS ATTENDED (2014-2015)

- 4th December 2014 ***"Uncovering the role of β-HPV in field cancerization: a collaboration in progress "*** School of Medicine – Palazzo Bellini – Main Lecture Hall, Prof. Girish Patel
- 5th December 2014 ***"Focus on the liver: from basics of NAFLD to hot topics in HBV & HCV infections"*** School of Medicine – Palazzo Bellini – Main Lecture Hall Prof. Rifaat Safadi M.D
- 16th December 2014 ***"From the legend of Prometheus to regenerative medicine"*** School of Medicine – Palazzo Bellini – Main Lecture Hall Prof. Antonio Musaro
- 17th December 2014 ***"Microglia microvesicles: messengers from the diseased brain"*** School of Medicine – Palazzo Bellini – Main Lecture Hall Roberto Furlan
- 19th January 2015 ***"Anticancer strategy Targeting cancer cell metabolism in ovarian cancer"*** School of Medicine – Palazzo Bellini – Main Lecture Hall Prof. Dr Yong-Sang Song, MD, PhD
- 20th January 2015 ***"Regulation of hepatocytes differentiation during the transitions between epithelial and mesenchymal states"*** School of Medicine – Palazzo Bellini – Main Lecture Hall Dr Tonino Alonzi, PhD
- 27th January 2015 ***"Myeloid cells as therapeutic target in cancer"*** Department room School of Medicine – Palazzo Bellini – Prof. Antonio Sica

- 11th March 2015 ***"Proof of principle for cell therapy: from autologous transplantation of tissue specific progenitors to gene corrected patient specific injured pluripotent stem cells "*** School of Medicine – Palazzo Bellini – Main Lecture Hall

  Darko Bosnakovski, PhD

- 7th May 2015 ***"An Integrated Approach to the Diagnosis and Treatment of Ovarian Cancer"*** School of Medicine – Palazzo Bellini – Main Lecture Hall

  Prof. John McDonald

- 14th May 2015 ***"Conflicting interests and scientific communication"*** School of Medicine – Palazzo Bellini – Main Lecture Hall

  Kathleen Ruff

- 10th September 2015 ***" Cancer through a Stem Cell Lens"*** Mary Babb Randolph Cancer Center-West Virginia University

  Prof. David T. Scadden, MD

CONGRESS ATTENDED

- 14th-16th May **- *International Congress "Contrasts in Pharmacology 2.0"***

  Centro Congressi Torino Incontra