From a 3D-model of particle-induced granuloma-like structure

to a simple 2D-macrophage bioassay predicting granulomagenic and fibrotic activity of particles

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Abstract

Macrophages orchestrate reactive particle segregation, compact aggregates of immune cells and non-immune cells and promote fibrosis-surrounding granulomas.

We developed a simple 3D in-vitro model that mimics granuloma formation and categorizes granuloma-inducing inorganic particles. Macrophage cell line (MHS) pre-exposed for 24h to 10µg/mL of granuloma-inducing (Carbon nanotubes, CNT) or not (Carbon black,CB) particles are cocultured with fibroblasts and epithelial cells (respectively MLG and LA4 cell lines) on 0,3% agarose coated wells.

Fluorescent dyes and confocal microscopy showed that these cells in presence of CNT but not CB were organized in layered compact cellular aggregates comparable to granulomas after 7 days. The supernatant collected at 24hours (but also at 78hours and 7days) contains significantly elevated levels of the pro-fibrotic mediator TIMP1 (metallopeptidase inhibitor 1) only in granuloma-inducing conditions (CNT). The levels of other pro-granulomagenic and fibrotic mediators (such as matrix metalloproteinase 1, MMP-1; Osteopontin, OPN or the chemokine CCL2) were not increased. Our data suggest that macrophages combined to structural cells respond to granuloma-inducing particles by releasing TIMP-1 and organizing in vitro granuloma-like spheroids.

This model was further simplified, as MHS macrophages alone were sufficient for the specific release of TIMP-1 in response to granulomagenic particles. Quantification of macrophage-produced TIMP-1 is a novel and simple tool for predicting and assessing granuloma-inducing new material and airborne dust particles.

Results

We have developed a three-dimensional spheroid model whose formation is triggered by granuloma-inducing particles. Alveolar macrophages (MHS cell line $2x10^6$ macrophages) are exposed to 10 µg/mL of granuloma inducing (carbon nanotubes, CNT, (MWCNT-7, Mitsui, Tokyo, Japan)) or not (carbon black, CB (ultrafine, ENSACO 250G, Timcal)) particles [1] or with vehicle only (Dulbecco's phosphate-buffered saline (DPBS) +10% 1,2-dipalmitoylphosphatidylcholine (DPPC) +3% bovine serum albumine (BSA)). Macrophages are exposed to particles overnight in flask Cellstar cell culture flask 50mL, Greiner bio one) containing 2,5 mL of medium (Dulbecco's modified eagle medium (DMEM) + 10% fetal bovin serum (FBS) + 1% antibiotic-antimycotic (AA)). Washed macrophages are then co-cultured (50.000 cells) with lung epithelial cells (LA4 cell line, 50.000 cells) and lung fibroblasts (MLG cell line, 50.000 cells) in a 0,3% agarose gel (UltraPure Agarose, Invitrogen, prepared with distilled water, 40µL) -coated 96-plates (Cellstar cell culture 96 well flat bottom, Greiner bio one). The supernatant was collected 24 hours, 78 hours and 7 days after co-culture to evaluate granulomagenic and fibrotic responses.

First, we assessed cellular organization in granuloma-inducing conditions (CNT) or not (CB) by evaluating the repartition of macrophages, epithelial cells and fibroblasts under bright-field (Labovert, Leitz) and confocal (LSM800, Zeiss) microscopes. During co-culture, cells are seeded at low density to form a monolayer on the agarose gel. One week after co-culture, cells in vehicle or CB not-granuloma-inducing conditions form similar uniform cell patches (Figure 1.A). However, when macrophages are pre-exposed with CNT, cells form compact and organized granuloma-like spheroids (Figure 1.A). Furthermore, the high level of organization of the spheroids in granuloma-inducing conditions is highlighted when the three cell lines are stained with different cell dyes (Figure 1.B, carboxyfluorescein succinimidyl ester (CFSE, BD Horizon) for macrophages in green, CellTracker deep red (Thermofisher) for epithelial cells in red, CellTracker orange CMRA (Thermofisher) for fibroblasts in blue). In vehicle and CB conditions, cell patches are two-dimensional (Supplementary video vehicle and video carbon black) and contain a homogenous collection of the three cell types. However, CNT-activated macrophages organize cells in 3D spheroids (Supplementary video carbon nanotubes), with an epithelial and fibroblasts core (Figure 1.B, in red and blue respectively), surrounded by a corona of macrophages (Figure 1.B, in green).

Tissue inhibitor of metalloproteinase 1 (TIMP-1) is an important mediator in the early response to granuloma-inducing particle [2]. TIMP-1 is a key mediator of extracellular matrix remodeling by blocking metalloproteinase involved in degradation of extracellular matrix. This abnormal matrix turnover is found during the development of fibrotic granulomas [3-5]. In our co-culture model, after 24h the amount of TIMP-1 released (ELISA, DuoSet ELISA mouse TIMP-1, R&D Systems) is significantly elevated, specifically in granuloma-inducing conditions (Figure 2.A). However, the target of TIMP-1, metalloproteinase 1 (MMP-1), remains at unchanged levels (Figure 2.A, Mouse MMP-1 ELISA Kit, elabscience), resulting in unbalanced mediators of extracellular matrix remodeling.

In addition to TIMP-1 [6-8], other mediators of fibrotic granulomas have been quantified after 24hours. CCL2 is a pro-inflammatory cytokine also stimulating collagen-deposition by fibroblasts [9,10]. Similarly, osteopontin (OPN) is implicated in inorganic particles-induced lung lesions and induces inflammation, granuloma and fibrosis [11-14]. In contrast to TIMP-1, the levels of CCL2 and OPN (ELISA, DuoSet ELISA mouse CCL2 and OPN, R&D Systems) were not significantly modified by granuloma-inducing CNT in our model (Figure 2.A).

Thus, among mediators quantified, only TIMP-1 is specifically released in granuloma-inducing conditions. Moreover, TIMP-1 is released in a time-dependent manner and further accumulates in the supernatant up to seven days after co-culture (Figure 2.B). Our data showing the specific release of TIMP-1 in granuloma-inducing conditions suggest that TIMP-1 is released as an extracellular matrix remodeling mediator.

Because TIMP-1 release and spheroid formation are triggered by particle-exposed macrophages, we next investigated the importance of adding the two other cell lines when assessing the granuloma-inducing potential of particles. Interestingly, this model can be simplified using a macrophage only approach. Macrophages already release important levels of TIMP-1 exclusively when pre-exposed to granuloma-inducing particles (Figure 3). Oppositely, inflammatory and fibrotic mediators such as CCL2 and OPN are not specifically released in response to CNT. Thus, our data indicate that a simple MHS cell line exposed to particles could be used as a predictive tool for discerning granuloma-inducing (like CNT) and not-granuloma-inducing (like CB) particles via the quantification of TIMP-1.

In conclusion, we have developed a 3D- granuloma-like spheroid model using three different lung cell lines and granulomagenic inorganic particles. This model was then successfully simplified to a simple 2D-macrophage bioassay predicting granulomagenic and fibrotic activity of particles via the quantification of TIMP-1.

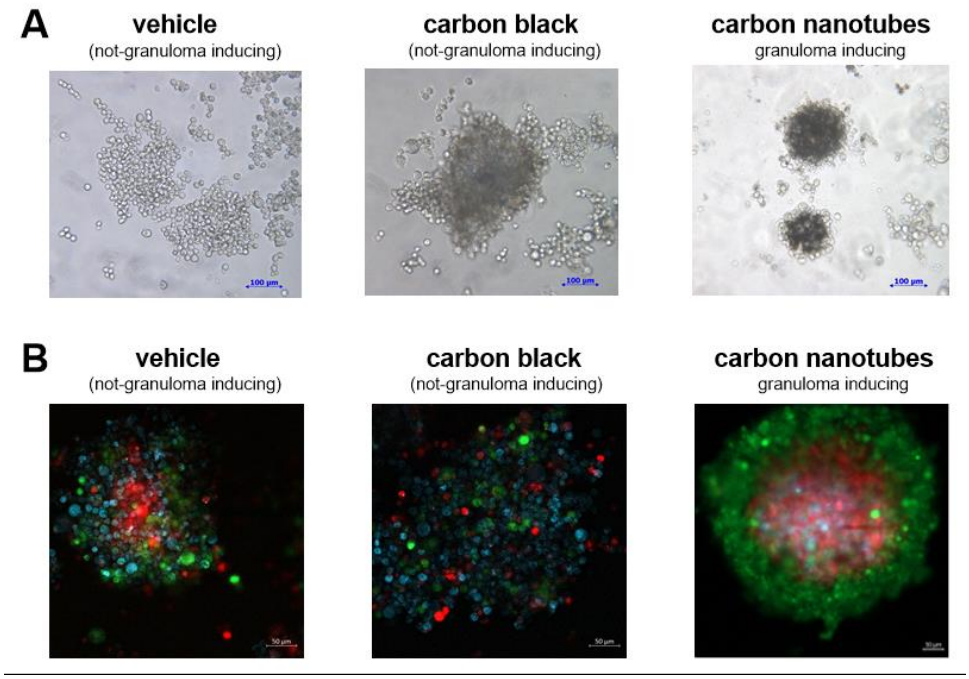

Figure 1: Macrophages (MHS cell line) organize fibroblasts and epithelial cells (MLG and LA4 cell lines) to form compact granuloma-like spheroids only in response to granuloma-inducing particles (carbon nanotubes), and not other particles or vehicle.

A: Representative optical microscopy images illustrating uniform cell patches in vehicle and not-granuloma-inducing conditions (carbon black, CB), and compact organized granuloma-like spheroids in response to granuloma-inducing conditions (carbon nanotubes, CNT) seven days after the co-culture of LA4, MLG and pre-exposed MHS cell lines. **B:** Representative confocal microscopy images of the 7 day-coculture of LA4, MLG and pre-exposed MHS cell lines stained using fluorescent cell dyes (in red, blue and green respectively), in cell patches (vehicle, CB) or layered-organized spheroids (CNT).

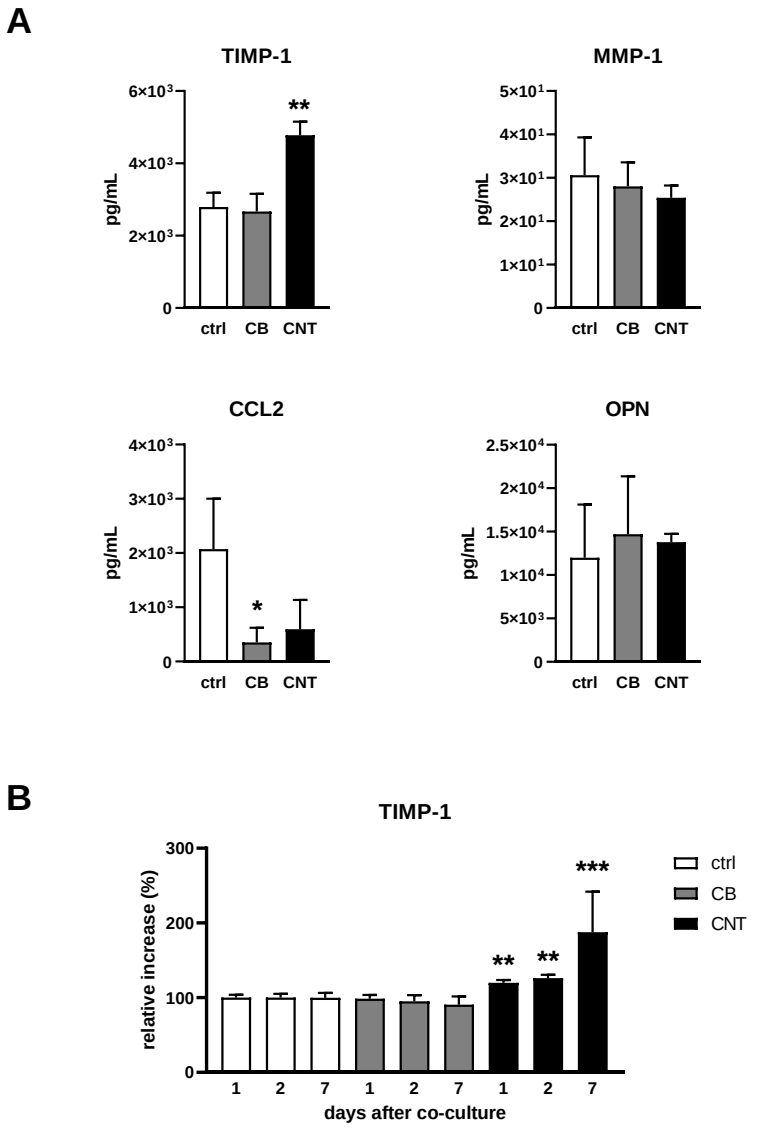

Figure 2: TIMP-1 is a mediator specifically released in response to granuloma-inducing particles by an in vitro model composed of three lung cell lines (LA4, MLG and pre-exposed MHS).

A: Mediators of granuloma formation and fibrosis (TIMP-1, CCL2, OPN and MMP-1 respectively) were measured 24 hours after co-culture of lung epithelial cells (LA4 cell line), lung fibroblasts (MLG cell line) and washed alveolar macrophages (MHS cell line) pre-activated by a 24-hour long exposition to vehicle (ctrl), granuloma-inducing (CNT) or not (CB) particles at 10µg/mL. **B:** TIMP-1 levels 1, 2 and 7 days after co-culture of LA4, MLG and pre-exposed MHS.

Bars represent means ± standard deviation (SD). Results are statistically processed by a one-way ANOVA followed by a Dunnett's test and * p<0.05, **p<0.01, *** p<0.001, **** p<0.0001 indicate significant difference from control (ctrl).

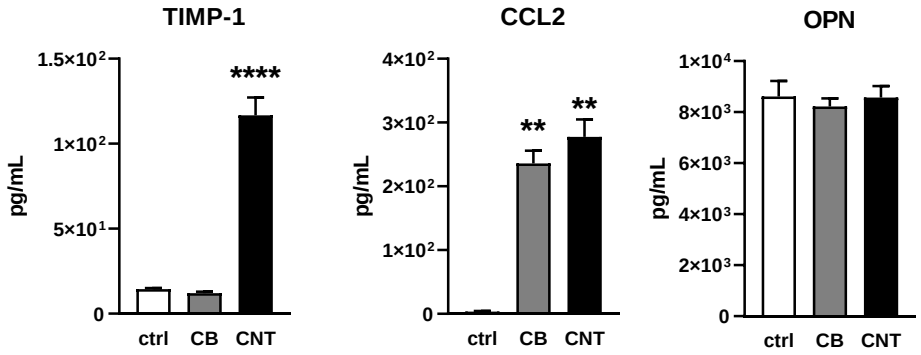

Figure 3: Macrophages (MHS cell line) alone are sufficient for the specific release of TIMP-1 in response to granuloma-inducing particles.

Mediators of granuloma, inflammation and fibrosis (TIMP-1, CCL2 and OPN respectively) measured 24 hours after the culture of washed alveolar macrophages (MHS cell line) pre-activated by a 24-hour long exposition to vehicle (ctrl), granuloma-inducing (CNT) or not (CB) particles at 10µg/mL.

Bars represent means ± standard deviation (SD). Results are statistically processed by a one-way ANOVA followed by a Dunnett's test and * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ indicate significant difference from control (ctrl).

References

1 Hiéronimus, L. *et al.* Mouse innate-like B-1 lymphocytes promote inhaled particle-induced in vitro granuloma formation and inflammation in conjunction with macrophages. *Arch Toxicol*, doi:10.1007/s00204-021-03200-2 (2021).

2 Lebrun, A. *et al.* CCR2(+) monocytic myeloid-derived suppressor cells (M-MDSCs) inhibit collagen degradation and promote lung fibrosis by producing transforming growth factor-β1. *The Journal of pathology* **243**, 320-330, doi:10.1002/path.4956 (2017).

3 Gomez, D., De Lorenzo, M., Alonso, D. & Andrade, Z. Expression of metalloproteinases (MMP-1, MMP-2, and MMP-9) and their inhibitors (TIMP-1 and TIMP-2) in schistosomal portal fibrosis. *The American journal of tropical medicine and hygiene* **61**, 9-13, doi:10.4269/ajtmh.1999.61.9 (1999).

4 Ding, X. *et al.* TIMP-1 Mediates Inflammatory and Immune Response to IL-6 in Adult Orbital Xanthogranulomatous Disease. *Ocul Immunol Inflamm* **28**, 288-297, doi:10.1080/09273948.2019.1581227 (2020).

5 González-Avila, G. *et al.* Mycobacterium tuberculosis effects on fibroblast collagen metabolism. *Respiration* **77**, 195-202, doi:10.1159/000163064 (2009).

6 Sands, M. F. *et al.* Tissue inhibitor of metalloproteinase-1 modulates allergic lung inflammation in murine asthma. *Clin Immunol* **130**, 186-198, doi:10.1016/j.clim.2008.08.029 (2009).

7 Allen, J. R. *et al.* TIMP-1 Promotes the Immune Response in Influenza-Induced Acute Lung Injury. *Lung* **196**, 737-743, doi:10.1007/s00408-018-0154-2 (2018).

8 Wu, X., Guo, L. & Ye, G. Remifentanil ameliorates lung injury in neonate rats with acute respiratory distress by down-regulating TIMP1 expression. *Am J Transl Res* **12**, 6325-6334 (2020).

9 Nishida, C. *et al.* Predictive Biomarkers for the Ranking of Pulmonary Toxicity of Nanomaterials. *Nanomaterials (Basel)* **10**, doi:10.3390/nano10102032 (2020).

10 Kolahian, S., Fernandez, I. E., Eickelberg, O. & Hartl, D. Immune Mechanisms in Pulmonary Fibrosis. *American journal of respiratory cell and molecular biology* **55**, 309-322, doi:10.1165/rcmb.2016-0121TR (2016).

11 Khaliullin, T. O. *et al.* Mediation of the single-walled carbon nanotubes induced pulmonary fibrogenic response by osteopontin and TGF-β1. *Exp Lung Res* **43**, 311-326, doi:10.1080/01902148.2017.1377783 (2017).

12 Dong, J. & Ma, Q. Osteopontin enhances multi-walled carbon nanotube-triggered lung fibrosis by promoting TGF-β1 activation and myofibroblast differentiation. *Particle and fibre toxicology* **14**, 18, doi:10.1186/s12989-017-0198-0 (2017).

13 Dong, J. & Ma, Q. In Vivo Activation and Pro-Fibrotic Function of NF-κB in Fibroblastic Cells During Pulmonary Inflammation and Fibrosis Induced by Carbon Nanotubes. *Front Pharmacol* **10**, 1140, doi:10.3389/fphar.2019.01140 (2019).

14 Sabo-Attwood, T. *et al.* Osteopontin modulates inflammation, mucin production, and gene expression signatures after inhalation of asbestos in a murine model of fibrosis. *Am J Pathol* **178**, 1975-1985, doi:10.1016/j.ajpath.2011.01.048 (2011).