Chemical Science

Accepted Manuscript

This article can be cited before page numbers have been issued, to do this please use: T. HIDALGO, R. Simón-Vázquez, A. González-Fernández and P. Horcajada, *Chem. Sci.*, 2021, DOI: 10.1039/D1SC04112F.

ROYAL SOCIETY OF CHEMISTRY

rsc.li/chemical-science

View Article Online
DOI: 10.1039/D1SC04112F

ARTICLE

"Cracking the Immune Fingerprint of Metal-Organic Frameworks"

T. Hidalgo,[a,b] R. Simón-Vázquez,[c,d] A. González-Fernández[c,d] and P. Horcajada[a,] ‡

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

Human body is continuously in a never-ending chess game against pathogens. When the immune system, our natural defense tool, is weakened, these organisms are able to escape, collapsing the body contingency plan, which results in a pathological state. To overcome this checkmate status, emerging nanomedicines have been successfully employed as one of the best booming tactic for the immune response, manipulating the body defense tools for a specific recognition/elimination of pathological cells via active ingredient delivery. However, the vast majority of these drug delivery systems (DDS) has been often considered exclusively as passive vehicles. Among them, nanoscaled metal-organic frameworks (nanoMOFs) have attracted a great attention due to their versatility, allowing to carry and deliver exceptional drug payloads and to modulate their biological bypass. Nonetheless, their intrinsic immunogenicity character has been never addressed. Considering the immense possibilities that nanoMOFs offer as treatment platform, our purpose is unveiling the MOF immunological fingerprint, including an in-deep evaluation of the cellular oxidation balance, the inflammation & recruitment of immune cells and the precise Th1/Th2 cytokine profile triggered. This performance will make more feasible the design of customized immune-active MOF nanoplatforms according to targeted diseases, becoming the next ace up of the immune system sleeve.

Introduction

The use of immunotherapy to trigger the adequate cornerstones of the immune system is being a recent booming tactic to treat challenging illnesses (*e.g.* cancer, infection, autoimmune diseases) since allows to manipulate the body defense tools as a game of chess. Through the immune system's machinery, specific recognition/target or elimination of pathological tumoral cells along with refined immunological memory has been feasible.[1,2] While it is often associated with cancer, immunotherapy with monoclonal antibodies (mAbs) is also serving as a benchmark to other diseases (*e.g.* autoimmunity diseases, macular degeneration, allergies, etc.), being recently the mAbs approach against immune-check point inhibitors a real therapeutic success for many different cancer types. Moreover, cellular immunotherapy is also offering appropriated responses, such as the adoptive therapies based on engineered T cells (*e.g.* the chimeric antibody receptor T (CAR-T) cells, natural killer (NK) cells, tumor infiltrating lymphocytes, dendritic cells, etc.). In some instances, the disease progression (*e.g.* metastasis, relapse or critical therapeutic failure) manages to escape from its constant surveillance, which makes it an arduous challenge.[3] Thus, harnessing the immune response is a smart alternative to the current therapies (surgery, radiotherapy, chemotherapy).[4,5]

The clinical & preclinical trend is not just limited to a single strategy since combined multiple treatments have superior efficacy to any monotherapy. Thus, the combination of immunotherapy with other conventional treatment modalities can magnify the immune response, maximizing their therapeutic effect. In this context, emerging nanomedicines have arisen as an appealing approach, transporting the desired active pharmaceutical ingredient (API; *e.g.* adjuvants, antigens, chemodrugs) in a safe and effective manner to the target cells and/or tissues.[6] However, the vast majority of those elements has been entirely employed as passive vehicles, providing just API-protection against degradation and longer retention times in the body.[7,8] Revealing the NP inherent impact on the immune response (in absence of any APIs) will provide meaningful inputs for their *in vitro* and *in vivo* performances, approaching to more personalized nanotherapies.[2]

Among a large variety of engineered nanocarriers (*e.g.* nanoparticles-NPs, liposomes, micelles), a new class of crystalline hybrid materials known as nanoscaled metal-organic frameworks (nanoMOFs) has recently attracted a great attention in the biomedical domain.[9,10] These hybrid NPs (composed of inorganic nodes and organic polydentate linkers assembled into multidimensional periodic lattices, can be precisely designed / manipulated since their molecular level, giving rise to multifunctional smart entities, which is known as «*multifunctional efficiency*»),[11] offering several advantages as drug delivery systems (DDS): *i)* chemical and structural versatility, which permits a suitable biocompatibility upon chemical design and the potential control of their *in vivo* fate; *ii)* an ideal amphiphilic internal microenvironment, conveniently adapted to host a very broad variety of APIs (biological gases, cosmetics, enzymes, nucleic acids, drugs, etc.), releasing them in a controlled manner under physiological conditions; *iii)* easy and scalable synthesis, following green methods with high yields; i*v)* a general trend of high biocompatible profile (*eg.* lack of *in vivo* toxicity for the benchmarked mesoporous Fe trimesate MIL-100(Fe) or the microporous Zr carboxylates Uio-66(Zr)); *v)* the recent successful external surface modification in some prototypes has proven the capability to endow further multifunctional abilities such as targeting, imaging or enhanced stability (chemical/ structural or colloidal) under biorelevant conditions.[9,10,12,13,14]

The latest advances on the MOF nanocarriers have been mainly focused to their targeting *via* external functionalization and/or formulation.[9,13] However, their immunological impact has not been in the spotlight within the scientific community, still remaining totally unknown.

Most of the research reported so far on this topic is focused on cancer immunotherapy,[3,4,5] showing their significant features exclusively as passive vehicle, effectively releasing adjuvants,

[a] *Advanced Porous Materials Unit (APMU), IMDEA Energy Institute, Av. Ramón de la Sagra 3, 28935 Móstoles-Madrid, Spain.*
[b] *Institut Lavoisier, UMR CNRS 8180, Université de Versailles Saint-Quentin-en-Yvelines, 45 Av. des Etats-Unis, 78035 Versailles cedex, France.*
[c] *CINBIO, Universidade de Vigo, Immunology Group, 36310 Vigo, Spain.*
[d] *Instituto de Investigación Sanitaria Galicia Sur (IIS Galicia Sur), SERGAS-UVIGO, Spain.*
‡ Corresponding author
Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/x0xx00000x

immunomodulators or antigens.[15,16,17,18] For instance, the first MOF based vaccine using ZIF-8 loaded ovalbumin (OVA) attaching cytosine-phosphate-guanine oligodeoxynucleotide (CpG ODN) as an adjuvant prototype of able to activate a potent immune memory.[19] In terms of exploring the intrinsic immunogenicity behavior of MOFs, just some of us have preliminary evaluated the inflammatory response induced by an iron-based MOF, which was modified on their surface with polymers.[20,21] However, despite the biomedical progress made in the last 5 years, the potential MOF intrinsically active repercussion at the immune level has not been investigated in depth. This basic notion is crucial, since provide valuable information about the innate MOF and precursors features and their aroused immune reaction, which is a critical factor for boosting the multi-therapy with diverse APIs.

Bearing this in mind, we decided to evaluate the immunogenicity of a selection of three different nanoMOF platforms: i) two cubic-zeotype mesoporous metal (Fe^{3+} or Al^{3+}) trimesates MIL-100(Fe, Al) (MIL stands for Material of Institut Lavoisier) with a very important mesoporosity (surface area S_{BET} ~ 2400 $m^2 \cdot g^{-1}$, pore volume V_p ~ 1.2 $cm^3 \cdot g^{-1}$),[22] being highlighted as an efficient DDS with lack of *in vitro* and *in vivo* toxicity;[23] and ii) the cubic microporous zinc 2-methyl-imidazolate ZIF-8(Zn) (ZIF for Zeolitic Imidazolate Framework) that can be described by a space-filling packing of regular truncated octahedral (S_{BET} ~ 1800 $m^2 \cdot g^{-1}$, V_p ~ 1.2 $cm^3 \cdot g^1$),[24] highly selected as suitable MOF-based device for immunotherapy.[18,19] In all instances, we characterized their ability to induce human cytokine production and complement activation, together with their potential cytotoxicity and production of reactive oxygen species (ROS).

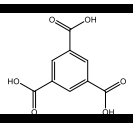
Considering the immense possibilities that MOFs offer as therapeutic platform (*e.g.* high porosity, versatile structure and biosafe character), shedding light on the specific MOF role and their constituents on the cellular homeostasis, could tip the balance towards the generation of a therapeutic effect according to a targeted pathological dysfunction (*e.g.* cancer, infections, allergies, autoimmune diseases). In other words, MOFs could be used as potential immunoactivator or immunomodulatory carrier, able to induce immune activation or tolerance under a pathological or undesirable activation («*tunable immune response*»). Therefore, unveiling the native MOF immunological fingerprint will make more feasible the design of a targeted immune active MOF nanoplatform for an efficient combined therapy.

Results and Discussion

NanoMOF characterization and biosafety profile

Despite the recent tendency to explore novel MOF applications on the biomedical field, where MOFs are mainly oriented as potential drug vehicles, the repercussion of their intrinsic impact on the immune system is still unknown. Since the physicochemical properties of nanocarriers will highly impact their affinity to different biological structures (*e.g.* proteins, cells, tissues, nucleic acids), to their efficacy and/or biodistribution (in other words, their biomedical performance),[25,26] we have firstly fully characterized the nanoMOFs (XRPD, DLS, TEM, surface chemistry and colloidal stability, etc.; see experimental section and Supporting Information-SI for synthetic and characterization details) prior any immunological encounter.

Table 1. Particle size and ξ-potential of MIL-100(Fe, Al) and ZIF-8 NPs in different physiological media together with their composition and specific surface area.

	Media	MIL-100 (Fe)	MIL-100 (Al)	ZIF-8 (Zn)
Composition	Metal	Fe	Al	Zn
	Ligand			
Size (nm) (PdI)	H_2O	153 ± 53 (0.3)	218 ± 28 (0.2)	110 ± 48 (0.2)
	PBS	177 ± 17 (>0.3)	209 ± 41 (>0.3)	227 ± 26 (>0.3)
	RPMI	145 ± 38 (0.3)	248 ± 50 (>0.3)	284 ± 22 (>0.3)
ξ-potential (mV)	H_2O	-25 ± 4	-7 ± 3	+96 ± 0
	PBS	-32 ± 0	-16 ± 1	-27 ± 1
	RPMI	-31 ± 2	-10 ± 2	-9 ± 2
BET surface ($m^2 \bullet g^{-1}$)*		1530	1510	1400

* Brunauer-Emmett-Teller (BET) surface area

The resulting materials displayed a nanometric particle size in aqueous solution (hydrodynamic diameter from DLS ~ 150, 220 and 110 nm for MIL-100(Al), MIL-100(Fe) and ZIF-8(Zn), respectively; see **Table 1**; which is in agreement with the microscopic observation acquired by TEM – **Figure S1**), preserving in all cases their crystalline structure (**Figure S2**) and textural properties (Table 1) as previous reported data.[27,28] However, it should be pointed out that the slight increase of the hydrodynamic diameter in the case of MIL-100(Al) compared with MIL-100(Fe) could be related to an aggregation effect due to the proximity to more neutral ζ-potential values (absence of enough electrostatic repulsions). This growth effect was also reflected in ZIF-8(Zn) NPs in comparison with the previous reported one (28 *vs.* 110 nm),[24] which is mainly associated to the nature of the used media for its dispersion (EtOH *vs.* H_2O, respectively), maintaining also their polydispersity index (PdI ~0.2).

Regarding the above-mentioned ζ-potential outcomes, the fluctuation of the nanoMOF surface charge here observed should be related to the diverse proportions of the partially coordinated cations *vs.* linkers exposed to the physiological media. For instance, the more negative ζ-potential values displayed by MIL-100(Fe) NPs compared to its aluminium analogue (-25 ± 4 *vs.* -7 ± 3 mV, respectively; Table 1) could be due to the higher amount of carboxylate/carboxylic acid *vs.* cation and/or the presence on surface of Fe-F (fluorine coming from a washing step in the MIL-100(Fe) preparation). Similarly, despite the contrary ζ-potential value obtained on the external ZIF-8(Zn) surface, the high positive charge (+96 ± 0 mV) could be explained by the same trend: a large proportion of cations or a higher presence of protonated ligand since the pH of the aqueous solution is lower than the pka of the imidazolate (6.0 *vs.* 7.0 and 14.9).[29,30]

Bearing in mind the high-impact of the surrounded media on the NP stability, and hence, on their biological affinities, biodistribution and

efficacy,[25,26] the nanoMOF particle size and ζ-potential were investigated under diverse simulated physiological conditions: from a simple phosphate buffer solution (PBS) to a more complex medium consisting on supplemented cell culture media (RPMI, Table 1). In all cases, the nanometric range was maintained, exhibiting an average size close to 160 & 225 & 207 nm for MIL-100(Fe & Al) and ZIF-8 NPs, respectively. However, ZIF-8(Zn) NPs underwent a notable size increase in presence of more complexed media (from 110 ± 48 nm in H_2O to 227 ± 26 or 284 ± 22 nm in PBS or RPMI, respectively; Table 1). This destabilizing effect is related with the tremendous ζ-potential fluctuation, shifting from a highly positive charge in H_2O to a lower negative character in PBS and RPMI (+96 ± 0 *vs.* -27 ± 1 & -9 ± 2 mV, respectively). This dramatic conversion has been already observed in other nanoMOF prototypes due to the formation of a superficial corona composed by phosphate groups and/or other salts/proteins from the media.[20,27] Overall, the colloidal stability of the tested nanoMOFs in these biorelevant media makes them suitable candidates for the assessment of their immunological recognition.

Prior to explore the associated immune-fingerprint of these nanoMOFs and their future implications, their toxicological character needs also to be evaluated. On this basis, a macrophage cell line (J774.A1) was selected as an appropriate model of the first defense line in the immune system against pathogens (involved in the *innate* immune response).[31,32] Remarkably, an absence of toxicity was observed by the MTT method[33] for both MIL-100 (Fe & Al) and ZIF-8(Zn) NPs after 24 h of incubation even at very high concentrations (1.2 mg·mL^{-1}; **Figure S3**). Despite that previous data suggested a higher cytoxicity tendency induced by ZIF-8(Zn) than MIL-100(Fe) NPs (may be as a consequence of a potential competition between Zn^{2+} *vs.* Fe^{2+} *vs.* Ca^{2+} through ion channels and/or deoxyribonucleic acid (DNA) damage), [34,28] as well as the often associated cytotoxicity effect of diverse cationic carriers,[35] these outcomes are in good agreement with the lack of severe toxicity observed in other cell lines[9,21,34] and with previous *in vivo* data.[10,12] Therefore, the biofriendly profile obtained from these nanoMOFs enabled further investigations about their self- immunoactive activity. In other words, shedding light on the interaction between MOFs &/or its precursors with the immune constituents could orientate towards the generation of a specific therapeutic activity, providing valuable data starting from their particular affinities with the biological surroundings, type of internalization pathways according to the cellular source until their influence on specific chemical reactions such as catalytic or oxidative processes. Thus, in the next section, the MOF recognition by essential actors of the innate immune system will be addressed by means of i) the cellular oxidation balance *via* the reactive oxidative stress production (ROS), ii) the complement activation and iii) the cytokine secretion pattern.

NanoMOF immune fingerprint: an innate & adaptive immunity tour.

Innate immunity tour: Chess opening

The exogenous intervention of engineered nanomaterials into the human body entails their participation in the modulation of **cellular redox homeostasis**: a moderate concentration of **ROS** can act as a second messenger for physiological regulation (activating the immune system), while excessive ROS may overwhelm the antioxidant cell capacity, generating cellular toxicity, and consequently, triggering cell death. Thus, understanding the nanoMOF impact on the cellular redox status could guide the therapeutic effect to a specific pathological dysfunction (*e.g.* cancer, infections, allergies, autoimmune diseases).[36] It has been reported that the immune recognition of metal/metal oxide NPs could be associated not only with a potential nanotoxicity (due to the metal leaching, increasing the ROS production), but also to a positive immunogenicity role of the released ions.[37,38]

Given that we are proposing three nanoMOFs based on different cations (Fe^{+3}, Al^{+3} and Zn^{+2}), their repercussion on the cellular oxidation balance should be investigated. To address this point, a human promyelocytic leukemia cell line (HL-60) was selected since it has been shown to modulate ROS production through a dose-dependent response.[21, 39] Two different doses (25 and 250 μg·mL^{-1}) of MIL-100(Fe, Al) and ZIF-8(Zn) NPs were put in contact with the HL-60 cells at different time points (1, 4, 8 and 24 h), comparing with three different controls: i) a positive control (C+), cells incubated with PMA (ROS inducer); ii) the basal control (C_{basal}), the intrinsic oxidation state of HL-60 cells (in the absence of ROS inducer but with the ROS reactant) and iii) a negative control (C-), cells in media without any treatment (neither the ROS inducer or the ROS reactant; see the Experimental section). Remarkably, no ROS induction was detected at short times (≤ 8h) regardless the NPs concentration with the exception of the highest concentration of MIL-100(Al) NPs (250 μg·mL^{-1}), which exhibited a slight increase of the oxidative stress (**Figure 1**). On the contrary, at longer incubation times (24 h), ROS production rose in all cases at high dose (250 μg·mL^{-1}), being more prominent in MIL-100(Al) NPs (even at the lowest concentration). This oxidative stress, promoted by the Al-trimesate, was higher than its Fe-analogue or Zn based NPs, displaying an oxidative strength tendency of Al > Fe ~ Zn. Therefore, all the tested nanoMOFs induced moderate ROS at 24 h, being stronger for the MIL-100(Al) NPs; this performance could be beneficial to enhance their potential immunotherapeutic effect since the immune system can be smoothly activated («*friendly warned*»), as previously proposed for other nanoparticulated systems.[29,38] In fact, it is not the first occasion that ROS production by innate immune cells has been related with a good *in vivo* adjuvancy since an immune activation mechanism is triggered.[40]

Consequently, the nature of the metal seems to be a crucial parameter since the redox homeostasis could be tampered.[36,41] Although the mechanism is not well-described, these metals favor the formation of superoxide radical formation [mainly superoxide anion (O_2•−), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and hydroxyl radical (•OH)].[36] In our particular case, despite the non-redox character of Al compounds, MIL-100(Al) NPs have proven to be a powerful *in vitro* and *in vivo* pro-oxidant,[42,43] promoting both iron auto-oxidation and ROS formation by their binding with the superoxide radical anions.[44,45] Most of the Al^{+3} present in the human organism is not free in solution, but forms stable complexes with low/high molecular mass biomolecules, being around 90% of the aluminium in the blood serum bounded to the transferrin protein.[46] Concerning the Fe^{+3}, it is widely reported that iron oxides (*e.g.* Fe_3O_4, Fe_2O_3) can induce the ROS production through the Fenton reaction (catalyzing the H_2O_2 reaction), showing high reactivity with biological molecules such as lipids, proteins and DNA.[47,48] In fact, iron is generally bound to specific proteins, leaving few free iron cations available for Fenton reaction (*e.g.* inducing ferroptosis).[49] In our particular case, the moderate ROS levels of MIL-100(Fe) NPs are also in agreement with an increase of the *in vitro*[34] and *in vivo*[12] oxidative stress, previously described by some of us, demonstrating a totally reversible effect after 2 weeks of its intravenous administration with a high dose (up to 220 mg·kg^{-1}). Finally, the redox-inert Zn^{+2} is the most abundant metal in the brain, being also an essential component to various enzymes and transcription factors involved in the regulation of key cellular functions (DNA replication, repair of DNA damage, cell cycle progression and apoptosis).[41] The

depletion of zinc may enhance DNA damage by impairing DNA repair mechanisms, generating free radicals: its high solubility and easy nitrogen- or oxygen- coordination lead to the formation of chelates with many biomolecules involved in the oxidative balance homeostasis, resulting in their inactivation and then, induction of ROS.[50] For instance, Zn^{+2} is associated with the inhibition of the important antioxidant enzyme glutathione reductase.[51,52] Besides, the zinc competition with other redox active metals (such as copper or iron) may also play a role in oxidative stress-mediated damage since Zn^{+2} may bind and protect sulfhydryl groups belonging to proteins. In contrast, other authors have also proposed a possible antioxidant and anti-inflammatory effect of this cation associated to i) the potential activation of the antioxidant enzyme superoxide dismutase (SOD1 and 3), which possesses Zn and Cu in its active metal site and ii) to the inhibition of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, involved in the free radicals production.[53,54]

Therefore, the moderate oxidative stress generated by ZIF-8(Zn) NPs would favor to design a dual functionality (oxidant & antioxidant behavior) according to the immune system demand. In other words, the presence of additional metals and potential action of the selected nanoMOFs will be determined by the particular cellular status and/or pathological environment to be treated. Thus, previous knowledge on each clinical condition will lead to more precise nanoMOF therapies.

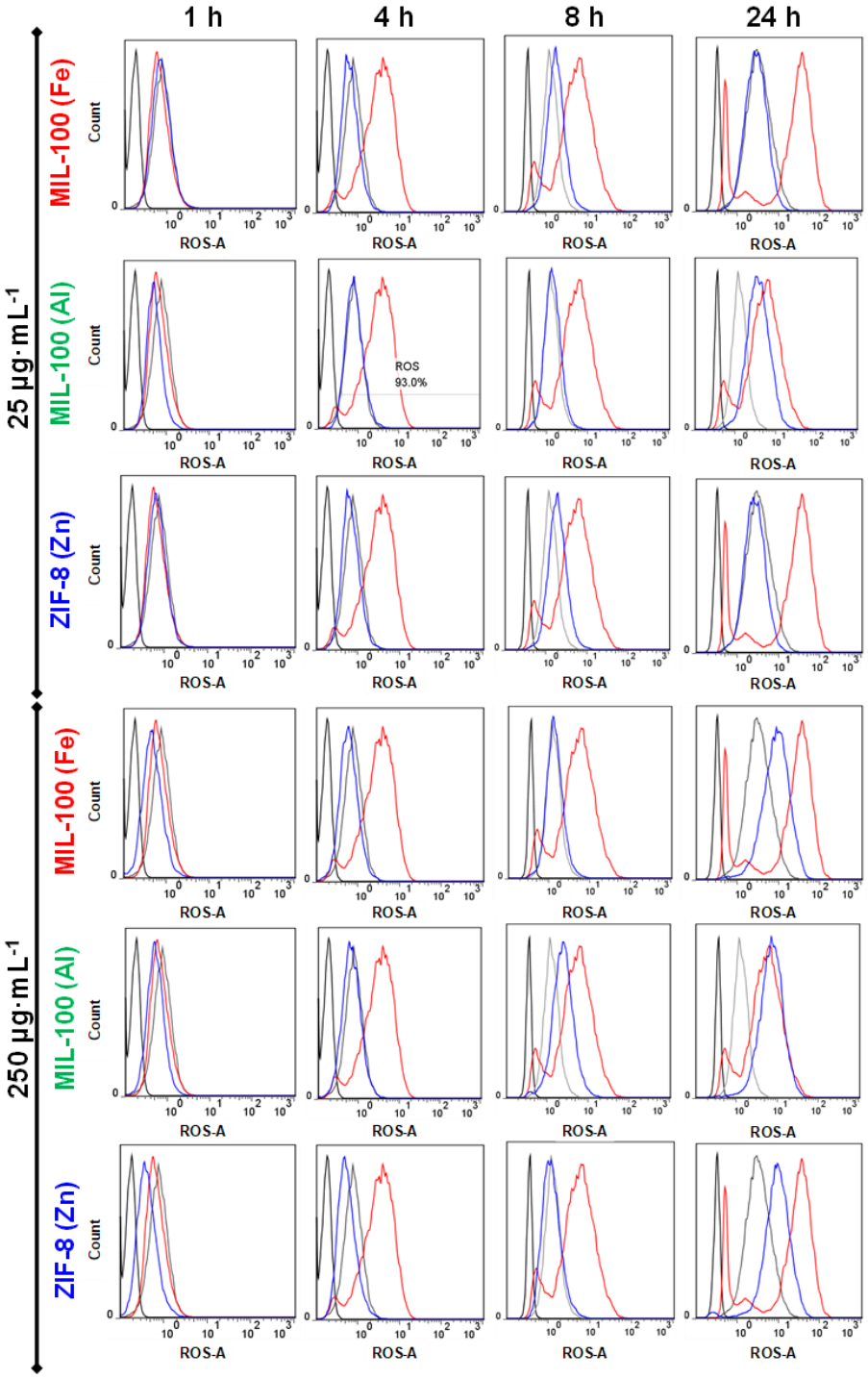


Figure 1. ROS production in HL60 cells incubated with MIL-100(Fe) (top), MIL-100(Al) (middle) and ZIF-8(Zn) NPs (bottom) at two different concentrations (25 and 250 µg·mL^{-1}; marked with a blue line). Basal (cells), negative (cells + ROS reagent) and positive control (cells + ROS reagent + ROS inducer) are disclosed in black, grey and red lines, respectively. Note that these data, corresponding to one of the triplicates obtained in four independent experiments (n = 12) are totally representative from the whole results.

Innate immunity tour: the pivotal point in the Middlegame

On the other hand, apart from the mentioned activation of innate immune cells during a pathogen invasion (*e.g.* macrophages, natural killers-NK cells, innate lymphoid cells), humoral factors are also triggered. This is the case of the **complement system**, a complex network of plasma proteins, which can elicit highly efficient and tightly regulated inflammatory and cytolytic immune responses to infectious organisms, tissue damage by physical, chemical, or neoplastic insults, and other surfaces identified as '*non-self*'.[37,55] It has been proven that the contact with nanomaterials can activate this system through three pathways (classical, lectin or alternative), leading to particle opsonization and clearance.[56] Typically, the degradation of the central factor C3 promotes the membrane attack complex (MAC) to create pores in the lipid bilayers (accelerating the tissue danger and inflammation) as well as the production of anaphylotoxins, which behave as inflammatory alert signals attracting immune cells to the zone.[57] In this context, the ability of nanoMOFs to mediate the complement pathway and, in turn, the inflammation process and recruitment of immune cells were investigated. A pool of human sera from three different donors were put in contact with two different concentrations (25 and 250 μg·mL^{-1}) of MIL-100(Fe, Al) and ZIF-8(Zn) NPs, evaluating by Western blot the degradation of the common factor C3, a protein that fulfills a pivotal role in the three complement cascades (see Experimental section). Overall, there was no induction of the complement cascades at high concentration (250 μg·mL^{-1}), regardless the MOF nature. Nonetheless, it should be noted that both MIL-100(Al) and ZIF-8(Zn) NPs slightly stimulated this system at low concentration (25 μg·mL^{-1}; **Figure 2**), being even higher in the case of MIL-100(Al) NPs. The ROS and complement activation (both relevant adjuvant mechanisms) observed with these nanoMOFs are in agreement with the induction effect of alum, the first and most widely used adjuvant in vaccines.[58] Hence, these findings make them potentially attractive as good adjuvants, heading to the next level of immune surveillance: the activation of the adaptive immunity, which can be combined with specific antigens.

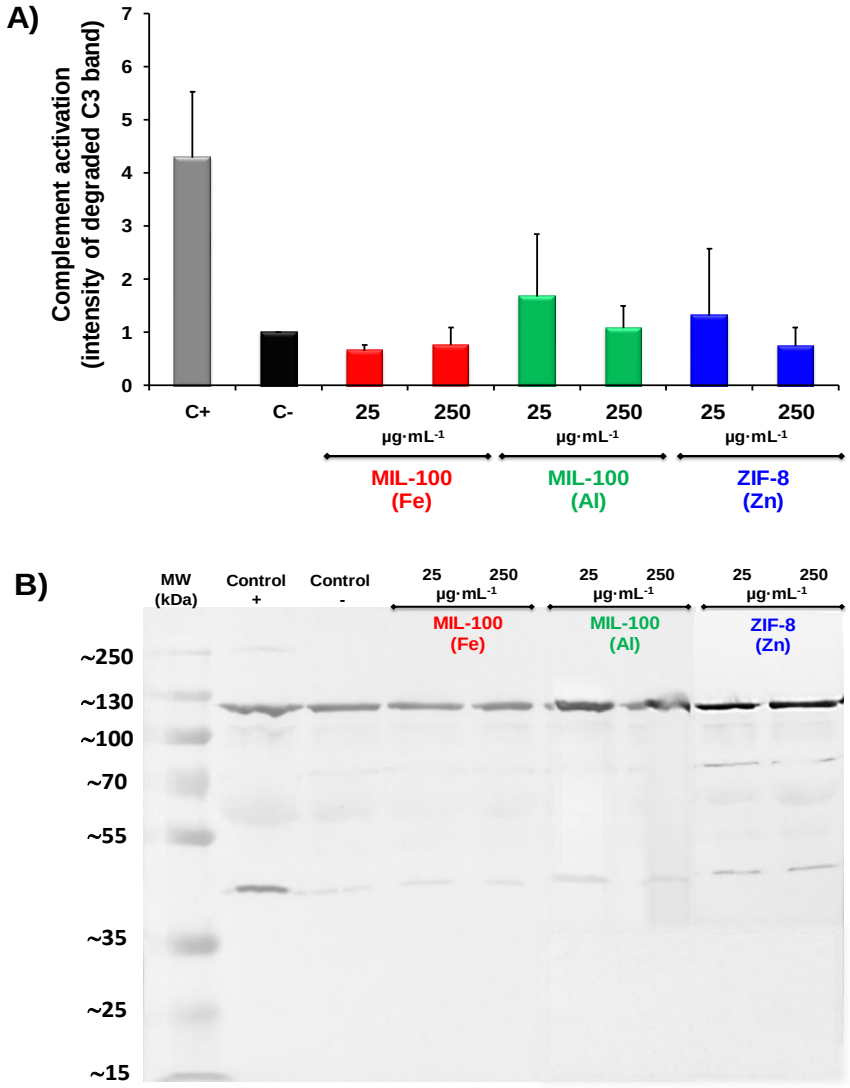


Figure 2. Top: Complement activation, represented by determining the intensity of the band at 43 kD, corresponding to the degraded C3 factor, and compared with the band at 115 kD, corresponding to the intact protein. The samples were normalized with respect to the negative control. **Bottom:** Complement activation data for MIL-100(Fe), MIL-100 (Al) and ZIF-8 (Zn) NPs determined by Western blot (WB) using a specific C3 antibody to measure the degradation of the protein. Note that these data correspond to one example of the duplicates obtained in three independent experiments (n=6, 3 different WB), totally representative from the whole results, and the error bars correspond to the standard deviation. All data were tested by one-way ANOVA test ($P<0.05$, considered statistically significant).

DOI: 10.1039/D1SC04112F

Adaptive immunity tour: best tactics for an Endgame

As stated, this recognition by the innate immune system (*e.g.* macrophage recognition or activation of the complement cascade) is a critical point; diverse parameters can stimulate this response in different sensing pathways, which are designed to determine the class of infecting pathogens (based on their localization, viability, replication or virulence) and to be translated into signals (extracellular factors: cytokines -*CK*-) that, together with the antigen presentation to T cells, will contribute to initiate an appropriate specific *adaptive immune response*. Note here that for a suitable adjuvant/vaccine task is generally expected to elicit a specific and long-term immune response,[59,60] keeping active the specific immune memory (T & B cells, long live plasma cells) with the main aim of maintaining the «*immune warning status=checkmate*» until the pathological battle will be over.

Moreover, a lack of inflammatory signals or the presence of regulatory factors during antigen presentation can promote *tolerogenic* responses, suppressing immune reactions (*e.g.* modulation of inflammation, restricting migration of self-reactive immune cells),[61] which could be a great scenario for combined immuno- and chemo-therapeutic nanocarriers for autoimmune and allergic diseases, among others.

The transition from innate to adaptive immunity requires the antigen processing and T cell presentation by antigen-presenting cells (APCs): dendritic cells (DC) are able to trigger naïve T cells that, with the already activated macrophages and B cells (effectors of the antibody production), can promote the activation of helper T cells (*CD4+*), crucial for a specific immune response and immunological memory.[5,6]

However, it should be mentioned that the immunological scenario and its consequent action rely on the type of disease. For instance, i) in a tumoral environment, a high presence of immunocompromised cells is observed, where the therapeutic approach aims to reverse this immunosuppression by stimulating the immune system; or ii) facing viral pathogens (*e.g.* SARS-Cov-2), the current vaccine treatments aim to induce both B & T cells responses, either by the generation of neutralizing antibodies and anti-viral specific helper &/or cytotoxic T cells (*CD8+*) as well as long live memory cells;[62] iii) on the contrary, the autorreactivity and inflammatory processes concerned to autoimmune and autoinflammatory diseases, the induction of a tolerance response is required. Consequently, revealing the intrinsic immunogenicity of nanomaterials can be exploited to modulate the immune response: understanding the molecular action mechanisms of different cytokines in the context of a specific disease could contribute to develop more targeted anti-cytokine/cytokine therapy («*innovative nanotherapeutic immune-modulating strategies*»).[6]

To shed light on the type of the **adaptive immune response** elicited by the nanoMOF, and thereby the potential role as therapeutic carriers on diverse diseases, two different concentrations (25 and 250 $\mu g \cdot mL^{-1}$) were incubated with human peripheral blood mononuclear cells (PBMCs) from three voluntary donors (see Experimental section; **Table 2**) for the determination of their cytokine profile. It should be noted that the PBMC fraction is mainly composed by lymphocytes (70-90% including T, B & NK cells) and monocytes (10-30%), generally activated in response of external stimulus such as the nanoMOFs or positive controls (selected here as C+: lipopolysaccharide –*LPS*–and lectin phytohemagglutinin –*PHA*–), known as human cytokine activators. In particular, the cytokine profile was here represented as the average of the three donors' values for each nanoMOF in comparison with the negative control (C-) together with the number of donors included within this variation (Table 2), for greater clarity.

View Article Online
DOI: 10.1039/D1SC04112F

Table 2. Summary of the cytokine production.

		NPs dose (μg·mL^{-1})	Positive control (LPS & PHA)	MIL-100 (Fe)	MIL-100 (Al)	ZIF-8 (Zn)
Th1 cytokines	IL-12p70	25	**3/3** (10^2-10^3)	**3/3** (10^2-10^3)	**2/3** (10-10^2)	**3/3** (10-10^2)
		250				
	INF-γ	25	**3/3** (10^3-10^4)	**2/3** (10^2-10^3)	**3/3** (10-10^2)	**2/3** (10-10^2)
		250		**3/3** (10^2-10^3)	**3/3** (10^2-10^3)	**2/3** (10^2-10^3)
	IL-2	25	**2/3** (10^2-10^3)	**2/3** (10-10^2)	**2/3** (10-10^2)	**3/3** (10-10^2)
		250		**3/3** (10-10^2)		**2/3** (10-10^2)
Anti-inflammatory cytokine	IL-10	25	**2/3** (10^3-10^4)	**2/3** (10^3-10^4)	**2/3** (10^3-10^4)	**2/3** (10^2-10^3)
		250			**2/3** (10^2-10^3)	**2/3** (10^3-10^4)
Pro-inflammatory cytokines	IL-6	25	**3/3** (>10^5)	**3/3** (10^2-10^3)	**3/3** (>10^5)	**2/3** (>10^5)
		250				
	IL-8	25	**2/3** (10^3-10^4)	**2/3** (10^2-10^3)	**2/3** (10^3-10^4)	**3/3** (10^3-10^4)
		250		**3/3** (10^2-10^3)		
	IL-1β	25	**3/3** (10^2-10^3)	**3/3** (10^3-10^4)	**2/3** (10^3-10^4)	**2/3** (10^3-10^4)
		250			**3/3** (10^3-10^4)	
	TNF-α	25	**3/3** (10^3-10^4)	**2/3** (>10^5)	**3/3** (10^4-10^5)	**2/3** (10^4-10^5)
		250		**3/3** (>10^5)	**2/3** (>10^5)	**3/3** (10^4-10^5)
	TNF-β	25	**3/3** (10^3-10^4)	**2/3** (10^2-10^3)	**3/3** (10-10^2)	**2/3** (10-10^2)
		250		**3/3** (10^2-10^3)	**2/3** (10-10^2)	

*Secretion of Th2 cytokines (IL-4 and IL-5) was not observed with in MIL-100(Fe), MIL-100(Al) and ZIF-8 (Zn) NPs. The values correspond to the variation of the cytokines concentration (in pg·mL^{-1}) in comparison with the negative control (10, 10^2, 10^3, 10^4or >10^5 times) obtained from 3 different donors (1/3, 2/3 or 3/3), with the representation of the activation showed in the positive control (LPS and PHA, acting as inducers of the cytokines production).

View Article Online
DOI: 10.1039/D1SC04112A

On the whole, a substantial immune response was evidenced in presence of the three nanoMOFs: a diverse cytokine production of 10 to >10^5 times higher than the negative control was obtained (see Table 2; **Figure 4**), highlighting a general secretion of pro-inflammatory cytokines in all cases (mostly derived from activated monocytes). One example is the IL-6, secreted by activated monocytes, who participate in diverse functions such as the B cell growth or endocrine effects (*e.g.* induction of fever, production of reactive C protein on liver, etc.). In this case, similar values were obtained than the positive control, being more than 10^5 times higher than the negative control. Other significant pro-inflammatory cytokines observed here were the interleukin IL-1β (relevant cytokine for the activation of T & B lymphocytes) along with the tumor necrosis factor (TNF, α and β responsible of signaling pathways for cell survival, apoptosis, inflammatory responses or cellular differentiation), displaying levels higher than the C-, from 10^3-10^4 for IL-1β and ~10^4- >10^5 in case of TNFα (being even 10 to 10^2 times higher than the C+ in both cases), which suggest that the tested nanoMOFs significantly induce inflammation. On the contrary, high levels of the anti-inflammatory IL-10 was also observed within the same range than the positive control and ~10^3-10^4 times higher values than the negative control. Although it can be produced by different cell types such as Th2 cells or regulatory T & B cells, activated monocytes are also able to secrete in large amounts.

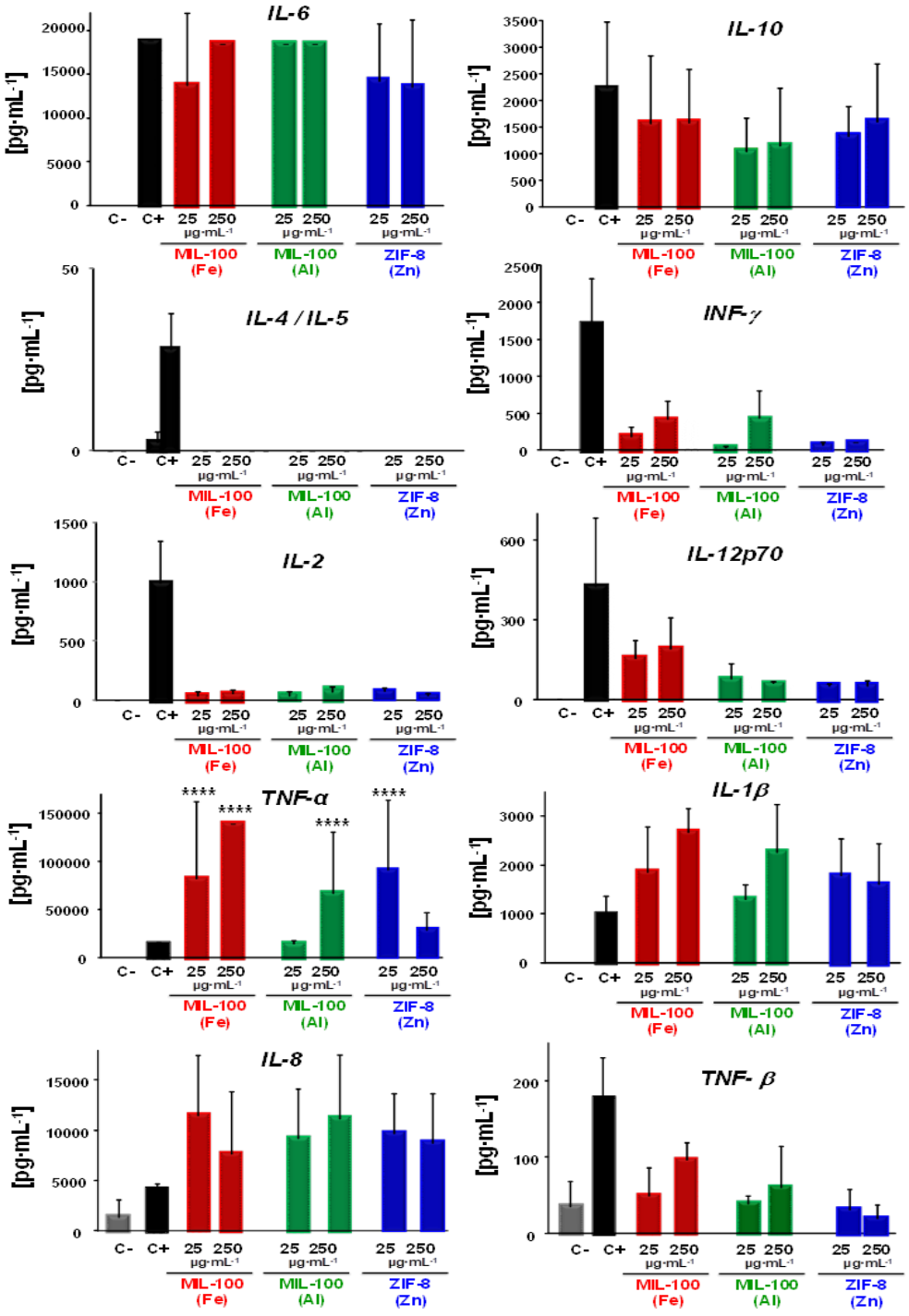


Figure 4. Individual levels of human cytokines production from peripheral blood mononuclear cells (3 different donors) after 24 h in contact with 25 or 250 µg·mL^{-1} of MIL-100(Fe), MIL-100(Al) or ZIF-8(Zn) NPs. Representing as C-: negative control (PBS) and C+: positive control (10 µg·mL^{-1} PHA + 1 mg·mL^{-1} LPS). All data were tested by two-way ANOVA and Tukey´s tests ($P<0.05$, considered statistically significant).

However, this secretion is usually delayed in presence of other pro-inflammatory factors, as shown here with the IL-6, tumor necrosis factor or INF-γ production (main Th1 cytokine implicated in the inflammation and proliferation of the macrophages). Regarding the chemokine IL-8, a potent chemoattracting agent, its levels were also raised with values ~10^3-10^4 higher than cells incubated with media for the MIL-100(Al) and ZIF-8(Zn) NPs, being 10 times lower in case of MIL-100(Fe) NPs. These findings reveal a great trend of those nanoMOFs to be recognized by the innate cells.

An expected optimal scenario should be with well-balanced Th1 & Th2 response, suited to a particular immune challenge. In view of unveiling the potential type of adaptive immune response induced by these nanoMOFs, the specific influence pursued by Th1-Th2 cytokines was in depth investigated. Related with Th1 stimulation, the interplay of interleukin 2 (IL-2, involved in the T and NK cells proliferation), interferon gamma (IFNγ) and Th2 cytokine profiles (IL-4 and IL-5, main markers of Th2 cells, promoting specific cellular differentiation) showed low levels of IL-2 regardless the MOF nature (Table 2, Figure 4), being slighted higher those of IFNγ with MIL-100(Fe) and (Al) than with ZIF-8(Zn) NPs. In both cases, the levels produced by these Th1 cells were 10-10^2 times higher than those in the negative control. Similarly to IL-2, the IL12p70 production in presence of MIL-100(Al) and ZIF-8(Zn) NPs was ~10-10^2 times higher than in cells incubated with media, with the exception of MIL-100(Fe) NPs, where the values reached the positive control levels. It should be noted that, this last interleukin stimulates the Th1 profile and inhibits the Th2 response.[63] In fact, deepen into the Th2 cell impact, no secretion of IL-4 or IL-5 were detected notwithstanding the MOF topology or composition. These outputs evidence the activation of mainly the cellular response *vs.* the humoral (antibody), which can be beneficial for vaccines purposes, for instance.[57]

Overall, all nanoMOFs seem to be very well-recognized by the innate monocyte population, eliciting a potent response with the secretion of pro-inflammatory cytokines together with the chemokine IL-8. Conversely, the IL-10 release, produced by activated monocytes and other immune cells after the nanoMOFs exposure, could indicate the tendency of the cells to revert this pro-inflammatory status, showing higher IL-10 levels that might be also associated with a slight INF-γ inhibition. This CK pattern, detected on human cells reflects the type of immune response that one could expect if those nanoMOFs will be used *in vivo*.

In a nutshell, the lack of IL-4 and IL-5 (main markers of the Th2 profile), the presence of IL-2, IL12p70 and IFNγ (distinctive Th1 profile) and the induction of IL-6, IL1β and TNFα (involved in inflammatory processes) suggest that the presence of nanoMOFs can tip the balance to Th1 responses (highly recommended for anti-tumoral, anti-viral &/or intracellular bacteria responses), promoting their specific differentiation.

Conclusions

Understanding the native immunological features of nanoMOFs will make possible to customize the design of effective nanomedicines to prevent and/or treat specific pathological disorders. Each nanoMOF has a unique biological repercussion: their large versatility (type of metal/linker nature, topology, reactivity, etc.) requires specific safety profiles, considering not only the cellular but also the geno &/or immunological impact.

The nanoMOFs studied here (*i.e.* MIL-100(Al), MIL-100(Fe) and ZIF-8(Zn)) showed a high biocompatible profile with a slight activation of the complement cascade along with the ROS induction in innate cells, especially for the innate monocytes, displaying both the production of several pro-inflammatory (IL-6, TNFα and β, IL1β, IL-8) and anti-inflammatory (IL-10) cytokines. Despite all showed a very similar pattern, MIL-100(Fe) seems to induce a more Th1 immune response compared to MIL-100(Al) and ZIF-8(Zn) NPs, with a higher induction of INF-γ and IL12p70 cytokines. Moreover, it´s noteworthy the lack of Th2 response elicited by any nanoMOF, which could suggest a slight cellular response (antibody production).

Overall, the activation of innate and Th1 cells induced by these nanoMOFs make them promising adjuvant candidates for targeted immunotherapy. These findings will help to create more novel & effective immunoactive MOFs, opening new horizons not only in biomedicine (*e.g.* therapy, imaging, vaccines) but also in other economically and societally relevant fields such as environment, catalysis or sensing, in which the safety of the MOFs is a crucial parameter to be practically used.

Author Contributions

TH and PH: synthesis, characterisation cellular toxicity; TH, RSV, AGF, and PH: immunological studies (ROS, Complement, CKs). The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Conflicts of interest

"There are no conflicts to declare"

Acknowledgements

This work was partially supported by the Labex NanoSaclay financial support for the MSc studies (ANR-11-IDEX-0003-02) together with the CNRS, IMDEA Energy and Xunta de Galicia (GRC-ED431C 2020/02) funding. T.H. and P. H. acknowledge the regional Madrid founding (Talento 2018 Modality 2, (2018-T2/IND-11407), the Multifunctional Metallodrugs in Diagnosis and Therapy Network (MICIU, RED2018-102471-T) and the European Union's Horizon 2020 research and innovation programme under the Marie Sklodowska-Curie grant agreement Nº 897678. P. H. acknowledges the Spanish Ramón y Cajal Programme (grant agreement no. 2014-16823).

References

View Article Online
DOI: 10.1039/D1SC04112F

1 X. Zhong, X. Sun, *Acta Pharmacol. Sin.* 2020, **41**, 928.
2 W. Sang, Z. Zhang, Y. Dai, X. Chen, *Chem. Soc. Rev.* 2019, **48**, 3771.
3 L. Scheetz, K.S. Park, Q. Li, P.R. Lowenstein, M.G. Castro, A.S. Schewendeman, J.J. Moon, *Nat. Biomed. Eng.* 2019, **3**, 768.
4 N. Papaioannou, O.V. Beniata, P. Vitsos, O. Tsitsilonis, P. Samara, *Ann. Transl. Med.* 2016, **4**, 261.
5 M.L. Guevara, F. Persano, S. Persano, *Semin. Cancer Biol.* 10.1016/j.semcancer.2019.11.010.
6 K. Naran, T. Nundalall, S. Chetty, S. Barth, *Front Microbiol*. 2018, **9**, 3158.
7 C.T. Perciani, L.Y. Lui, L. Wood, S.A. MacParland, *ACS Nano* 2021, **15**, 7.
8 C. D'Amico, F. Fontana, R. Cheng, H.A. Santos, *Drug Deliv Transl Res.* 2021, **11**, 353.
9 J. Yang, Y.W. Yang, *Small* 2020, **16**, 1906846.
10 S. Rojas, A. Arenas-Vivo, P. Horcajada, *Coord. Chem. Rev.* 2019, **388**, 202.
11 R. Freund, U. Lächelt, T. Gruber, B. Rühle, S. Wuttke, ACS Nano, 2018, 12, 2094.
12 P. Horcajada, T. Chalati, C. Serre, B. Gillet, C. Sebrie, T. Baati, J.F. Eubank, D. Heurtaux, P. Clayette, C. Kreuz, J.S. Chang, Y.K. Hwang, V. Marsaud, P.B. Bories, L. Cynober, S. Gil, G. Férey, P. Couvreur, R. Gref, *Nature Mater*. 2010, **9**, 172.
13 S. Wuttke, M. Lismont, A. Escudero, B. Rungtaweevoranit, W.J. Parak, *Biomaterials* 2017, **123**,172.
14 S. Yuan, L. Feng, K. Wang, J. Pang, M. Bosh, C. Lollar, Y. Sun, J. Qin, X. Yang, P. Zhang, Q. Wang, L. Zou, Y. Zhang, L. Zhang, Y. Fang, J. Li, H.C. Zhou, *Adv. Mater.* 2018, **30**, 1704303.
15 Y.B. Miao, W.Y. Pan, K.H. Chen, H.J. Wei, F.L. Mi, M.Y. Lu, Y. Chang, H.W. Sung, *Adv. Funct. Mater.* 2019, 10.1002/adfm.201904828.
16 X.F. Zhong, Y.T. Zhang, L. Tan, T. Zheng, Y.Y. Hou, X.Y. Hong, G. Du, X. Chen, Y. Zhang, X. Sun, *J. Control. Release*. 2019, **300**, 81.
17 Y. Yang, Q. Chen, J.P. Wu, T.B. Kirk, J. Xu, Z. Liu, W. Xue, *ACS Appl. Mater. Interfaces.* 2018, **10**, 12463.
18 H. Zhang, J. Zhang, Q. Li, A. Song, H. Tian, J. Wang, Z. Li, Y. Luan, *Biomaterials* 2020, **245**, 119983.
19 Y. Zhang, F.M. Wang, E.G. Ju, Z Liu, Z.W. Chen, J.S. Ren, X. Qu, *Adv. Funct. Mater.* 2016, **26**, 6454.
20 E. Bellido, T. Hidalgo, M.V. Lozano, M. Guillevic, R. Simón-Vázquez, M.J. Santander-Ortega, Á. González-Fenández, C. Serre, M.J. Alonso, P. Horcajada, *Adv. Healthc. Mater* 2015, **4**, 1246.
21 T. Hidalgo, M. Giménez-Marqués, E. Bellido, J. Avila, M.C. Asensio, F. Salles, M.V. Lozano, M. Guillevic, R. Simón-Vázquez, Á. González-Fernández, C. Serre, M.J. Alonso, P. Horcajada, *Sci. Rep*. 2017, **7**, 43099.
22 A. García-Márquez, A. Demessence, A.E. Platero-Prats, D. Heurtaux, P. Horcajada, C. Serre, J.S. Chang. G. Férey, V.A. Peña-O'Shea, C. Boissière, D. Grosso, C. Sanchez, *Eur. J. Inorg. Chem*. 2012, **32**, 5165.
23 M. Giménez-Marqués, T. Hidalgo, P. Horcajada, *Coord. Chem. Rev.* 2016, **307**, 342.
24 J. Cravillon, S. Munzer, S.J. Lohmeier, A. Feldhoff, K. Huber, M. Wiebcke, *Chem. Mater.* 2009, **21**, 1410.
25 S. Mitragotri, P.A. Burkre, R. Langer, *Nat. Rev. Drug Discov.* 2014, **13**, 650.
26 E. Fattal, N. Tsapis, *Clin. Transl. Imaging* 2014, **2**, 77.
27 E. Bellido, M. Guillevic, T. Hidalgo, M.J. Santander-Ortega, C. Serre, P. Horcajada, *Langmuir* 2014, **30**, 5911.
28 R. Grall, T. Hidalgo, J. Delic, A. Garcia-Marquez, S. Chevillarda, P. Horcajada, *J. Mater. Chem. B* 2015, **3**, 8279.
29 A. Phan, C.J. Doonan, F.J. Uribe-Romo, C.B. Knobler, M. O'Keeffe, O.M. Yaghi, *Acc. Chem. Res.* 2010, **43**, 58.
30 S. Luo , P.G. Wang , J.P. Cheng, *J. Org. Chem*. 2004, **69**, 555.
31 L. Franken, M. Schiwon, C. Kurts, *Cell. Microbiol.* 2016, *18*, 475.
32 D. Hirayama, T. Iida, H. Nakase, *Int. J. Mol. Sci.* 2018, **19**, 92.
33 S. Arora, J.R. Rajwade, K.M. Paknikar, *Toxicol. Appl. Pharm*. 2012, **258**, 151.
34 C. Tamames-Tabar, D. Cunha, E. Imbuluzqueta, F. Ragon, C. Serre, M.J. Blanco-Prieto, P. Horcajada, *J. Mater. Chem. B.* 2014, **2**, 262.
35 C. Teijeiro, A. McGlone, N. Csaba, M. Garcia-Fuentes M.J. Alonso *Handbook of Nanobiomedical Research*. World Scientific Ed., 2014.
36 B. Yang, Y. Chen, J. Shi, *Chem. Rev.* 2019, **119**, 4881.
37 B. Fadeel, *Front. Immunol.* 2019, **133**, 1.
38 V. Mallikarjun, D.J. Clarke, C.J. Campbell, *Free Radical Bio. Med.* 2012, **53**, 280.
39 M.D. Ferrer, A. Sureda, A. Mestre, J.A. Tur, A. Pons, *Cell. Physiol. Biochem.* 2010, **25**, 241.
40 M. Peleteiro, E. Presas, J.V. González-Aramundiz, B. Sánchez-Correa, R. Simón-Vázquez, N. Csaba, M.J. Alonso, A. González-Fernández, *Front. Immunol.* 2018, **9**, 791.
41 M. Valko, K. Jomova, C.J. Rhodes, K. Kuča, K. Musílek, *Arch. Toxicol.* 2016, **90**, 1.
42 C. Exley, *Free Radic. Biol. Med.* 2004, **36**, 380.
43 J.I. Mujika, F. Ruipérez, I. Infante, J.M. Ugalde, C. Exley, X. Lopez, *J. Phys. Chem. A* 2011, **115**, 6717.
44 C. Exley, P. Siesjo, H. Eriksson, *Cell press* 2010, **31**,103.
45 F. Ruipérez, J. Mujika, J. Ugalde, C. Exley, X. Lopez, *J. Inorg. Biochem.* 2012, **117**, 118.
46 J. Mujika, G. Dalla Torre, X. Lopez, *Phys. Chem. Chem. Phys.* 2018, **20**, 16256.
47 H.J.H. Fenton, *J. Chem. Soc. Trans.* 1894, **65**, 899.
48 M. Wlaschek, K. Singh, A. Sindrilaru, D. Crisan, K. Scharffetter-Kochanek, *Free Radic. Biol. Med.* 2019, **133**, 262.
49 X. Qiana, J. Zhangb, Z. Gud, Y. Chenc, *Biomaterials* 2019, **211**, 1.

View Article Online
DOI: 10.1039/D1SC04112F

50 Y. Chang, M. Zhang, L. Xia, J. Zhang, G. Xing, *Materials* 2012, **5**, 2850.
51 M.G. Bishop, R. Dringen, S.R. Robinson, *Free Radic. Biol. Med.* 2007, **42**, 1222.
52 R. Ryu, Y. Shin, J.W. Choi, W. Min, H. Ryu, C.R. Choi, H. Ko, *Exp. Brain Res.* 2002, **143**, 257.
53 A.S. Prasad, *Curr. Opin. Clin. Nutr.* 2009, **12**, 646.
54 Y. Song, S.W. Leonard, M.G. Traber, E. Ho, *J. Nutr.* 2009, **139**, 1626.
55 M.G. Netea, A. Schlitzer, K. Placek, L.A.B. Joosten, J.L. Schultze, *Cell Host & Microbe* 2019, **25**, 13.
56 M.A. Dobrovolskaia, S.E. McNeil, *Handbook of Inmunological properties of Engineered Nanomaterials*. Eds. World Scientific Publishing Co. Inc., 2012.
57 J. Szebeni, D. Simberg, Á. González-Fernández, Y.Barenholz & M.A. Dobrovolskaia, *Nat. Nanotechnol.*, 2018, 13, 1100.
58 T.R. Ghimire, *SpringerPlus* 2015, **4**, 181.
59 R.S. Oakes, E. Froimchuk, C.M. Jewell, *Adv. Therap.* 2019, **2**, 1800150.
60 A. Iwasaki, R. Medzhitov, *Nature Immunology* 2015, **16**, 343.
61 J.M. Gammon, C.M. Jewell, *Adv. Healthcare Mater.* 2019, **8**, 1801401.
62 G. Canedo-Marroquín, F. Saavedra, C.A. Andrade, R.V. Berrios, L. Rodríguez-Guilarte, M.C. Opazo, C. Riedel, A.M. Kalergis, *Front. Immunol.* 2020, **11**, 569760.
63 M. Elsabahy, K.L. Wooley, *Chem. Soc. Rev.* 2013, **42**, 5552.