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THE IMMUNOMODULATORY EFFECTS OF IRON OXIDE NANOPARTICLES (Fe_3O_4 NP) ON HUMAN NEUTROPHILS

by
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ABSTRACT

Nanoparticles (NP) are extremely small structures (1 – 100 nm) that exhibit unique physical characteristics differing from those of larger, bulk materials. As a result of these unique properties, there is a growing trend to use NP across a range of sectors. One type of NP that has been garnering attention in the field of nanomedicine is iron oxide nanoparticles (Fe_3O_4 NP). These NP possess an inherent ability to react to an external magnetic field, making them highly suitable for applications in hyperthermia and magnetic resonance imaging. However, concerns about their safety have been raised due to the lack of a comprehensive assessment of their interaction with polymorphonuclear neutrophils (PMN), a crucial cell component of the innate immune system and the inflammatory response. Many treatments based on these particles have been withdrawn from the market following concerns about severe side effects. Therefore, in this thesis, we investigated the extent to which Fe_3O_4 NP with a size of 10 nm can modulate PMN biological functions. Interestingly, our research showed that Fe_3O_4 NP increase PMN viability, meaning their ability to interfere with their spontaneous apoptosis, which is essential for homeostasis. Additionally, Fe_3O_4 NP promote the release of pro-inflammatory cytokines and increase phagocytosis, both of which are activated during the inflammatory response. However, Fe_3O_4 NP appear to have also an immunosuppressive profile, as they tend to impair oxidative burst and degranulation functions of PMN. These findings, discussed in more detail in this thesis, demonstrate that the production and use of Fe_3O_4 NP must be strictly regulated to protect populations at risk of exposure, including first-line workers involved in their mass production and patients who receive them as a therapeutic strategy for disease management.

Keywords : Inflammation; Magnetic nanoparticles; Nanosafety; Nanotoxicity; Neutrophils

RÉSUMÉ

Les nanoparticules (NP) sont des structures extrêmement petites (1 à 100 nm) qui présentent des caractéristiques physiques uniques différentes de celles des matériaux de grande taille. En raison de leurs propriétés uniques, il existe une augmentation de l'utilisation des NP dans un large éventail de secteurs d'activités. Un type de NP qui attire l'attention en nanomédecine est les nanoparticules d'oxyde de fer (Fe_3O_4 NP), qui répondent à un champ magnétique externe et peuvent être utilisées en hyperthermie et en imagerie par résonance magnétique. Cependant, des préoccupations concernant leur sécurité ont été soulevées en raison du manque d'évaluation complète de leur interaction avec les cellules de l'organisme humain, en particulier les neutrophiles polymorphonucléaires (PMN), des cellules cruciales du système immunitaire inné et de la réponse inflammatoire. De nombreux traitements basés sur ces particules ont été retirés du marché suite à des préoccupations concernant des effets secondaires graves associés avec leur utilisation. Par conséquent, dans cette thèse, nous avons étudié comment les Fe_3O_4 NP d'une taille approximative de 10 nm pourraient moduler les fonctions biologiques des PMN. De façon intéressante, notre étude a démontré que les Fe_3O_4 NP augmentent la viabilité des PMN, c'est-à-dire qu'elles ont la capacité à interférer avec leur apoptose spontanée, un élément essentiel pour l'homéostasie. De plus, les Fe_3O_4 NP induisent la libération de cytokines pro-inflammatoires et augmentent la phagocytose, deux fonctions essentielles au cours de la réponse inflammatoire. Cependant, les Fe_3O_4 NP semblent également avoir un profil immunosuppresseur sélectif, car elles tendent d'altérer les fonctions de la dégranulation et la flambée oxydative des PMN. Ces résultats, discutés plus en détail dans cette thèse, démontrent que la production et l'utilisation de Fe_3O_4 NP doivent être strictement réglementées pour protéger les populations exposées, notamment les travailleurs impliqués dans leur production en masse et les patients qui les reçoivent comme stratégie thérapeutique pour la gestion de leur maladie.

Mots-clés : Inflammation; nanoparticules de magnétite, Nanosécurité; Nanotoxicité; Neutrophiles

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LIST OF ABBREVIATIONS

AMF	Alternating magnetic field
APPs	Acute phase proteins
Bid	BH3 interacting domain death agonist
CeO_2 NP	Cerium dioxide nanoparticles
CGD	Chronic granulomatous disease
CHX	Cycloheximide
CXCR1	Chemokine receptor 1
FcγR	Fc gamma receptor
FDA	Food and Drug Administration
Fe NP	Magnetic nanoparticles
Fe_3O_4 NP	Iron oxide nanoparticles
fMLP	N-formyl-Met-Leu,Phe
GM-CSF	Granulocyte-macrophage colony-stimulating factor
G-CSF	Granulocyte-colony stimulating factor
G-CSF-R	Granulocyte-colony stimulating factor - receptor
GPCRs	G protein-coupled receptors
HCA	Human cytokine array
HKA	Human kinase array
HT	Hyperthermia
IgG	Immunoglobulin G
ITAMS	Immunoreceptor tyrosine-based activation motifs
LAL	Limulus amoebocyte lysate
LDGs	Low-density granulocytes
Lfr	Lactoferrin
LTB4	Leukotriene B4
MACS	Magnetic-activated cell sorting
MPO	Myeloperoxidase
MRI	Magnetic resonance imaging
MΦ	Macrophage
NADPH	Nicotinamide adenine dinucleotide phosphate
NE	Neutrophil elastase

NETs	Neutrophil extracellular traps
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLRs	NOD-like receptors
nm	Nanometer
NP	Nanoparticles
OELs	Occupational exposure limits
PAMPs	Pathogen-associated molecular patterns
PDI	Polydispersity index
PMA	Phorbol 12-myristate 13-acetate
PMN	Polymorphonuclear leukocytes
PRRs	Pattern-recognition receptors
PSGL-1	P-selectin glycoprotein ligand-1
ROS	Reactive oxygen species
Smac	Second mitochondria-derived activator of caspases
SRBCs	Sheep red blood cells
TEM	Transmission electronic microscopy
TGF-β	Transforming growth factor beta
TiO_2 NP	Titanium dioxide nanoparticles
TLRs	Toll-like receptors
TNF-α	Tumor necrosis factor-alpha
WB	Western Blot
WHO	World health organization
ZnO NP	Zinc oxide nanoparticles

1. SOMMAIRE RÉCAPITULATIF

1.1 Introduction

L'essor de la nanotechnologie s'est manifesté par l'exploitation des propriétés physico-chimiques que les nanoparticules (NP) expriment par rapport aux matériaux macroscopiques. Les NP sont très diverses d'un point de vue de forme, composition, méthode de synthèse et dimensions. Cependant, leur définition est basée fondamentalement sur le diamètre, qui doit être compris entre 1 et 100 nanomètres (nm). Les NP, en raison de leurs propriétés (ex. magnétisme), constituent des éléments clés pour la création d'outils innovants destinés à résoudre des problématiques dans une variété de secteurs. En effet, le domaine de la santé se présente comme une niche prometteuse pour les applications de la nanotechnologie, en particulier en ce qui concerne le traitement du cancer, où les méthodes thérapeutiques traditionnelles (c.-à-d. chimiothérapie) manquent en effet de spécificité, ce qui se traduit par des effets systémiques chez les patients.

L'utilisation prestigieuse des NP en médecine a suscité un vif intérêt, notamment grâce au rôle joué par les nanovecteurs vaccinaux à base de NP lipidiques pendant la pandémie de COVID-19. Cela a ravivé l'élan des applications des nanotechnologies après avoir connu des incertitudes quant à leur avenir. Ainsi, une attention particulière de la part des organismes subventionnaires publics et privés à la nanomédecine a été observée pendant la période post-COVID-19. Dans ce contexte, les NP de magnétite (Fe_3O_4 NP) font partie des NP les plus prometteuses en nanomédecine. Cela est principalement attribué à leurs propriétés physico-chimiques qui offrent de nombreuses applications potentielles. À titre d'exemple, les Fe_3O_4 NP possèdent une structure solide qui pourrait être utilisée pour immobiliser des anticorps ou des éléments thérapeutiques (ex. des molécules actives). De plus, leur propriété intrinsèque d'interagir à l'application d'un champ magnétique externe offre la possibilité de contrôler leur destination dans un environnement physiologique et d'être utilisées comme des nanovecteurs (i.e. Convoyeurs du médicament). Ainsi, les Fe_3O_4 NP se distinguent par leur propriété théranostique, c'est-à-dire leur capacité à être utilisées simultanément comme nanovecteur et agent de contraste. L'ensemble de ces propriétés insuffle un nouvel élan pour stimuler des applications cliniques de pointe. Par exemple, dans le domaine de l'imagerie par résonance magnétique (IRM), les Fe_3O_4 NP offrent la possibilité de remplacer les agents de contraste hypertoxiques tels que le gadolinium. De plus,

elles peuvent être utilisées dans le traitement de l'anémie ferriprive chez les patients souffrant d'insuffisance rénale chronique, ainsi elles ouvrent des perspectives pour le traitement du cancer à travers la thérapie photothermique.

Malgré ces promesses, l'utilisation des NP en nanomédecine devrait être évaluée d'un point de vue toxicologique afin d'assurer leur innocuité. En effet, au cours des dernières années, la nanotoxicologie est devenue une discipline de recherche en plein essor. Son objectif principal est d'évaluer les risques toxiques potentiels présentés par les nanomatériaux et les NP pour la santé et l'environnement. Ceci vise à établir un profil de toxicité des NP, afin de mettre en place des mesures préventives visant à protéger à la fois les producteurs et les utilisateurs de ces éléments contre leurs effets imprévisibles. En effet, des cas concrets de toxicité à l'échelle microscopique sont déjà connus, tels que les particules d'amiante et de silice qui sont reconnues pour induire une inflammation pulmonaire. À cet égard, l'exemple critique du Feraheme ®, un médicament à base de Fe_3O_4 NP commercialisé pour traiter l'anémie, est particulièrement notable. En effet, son utilisation clinique a été associée au déclenchement d'une réaction allergique grave chez certains patients. Cela souligne l'importance de prendre en considération le potentiel toxicologique de ces NP, bien qu'ils soient considérés comme des éléments compatibles en raison du rôle essentiel que le fer joue dans la physiologie de l'organisme humain. Généralement, une réaction allergique se caractérise par un profil inflammatoire résultant de l'activation de cellules immunitaires telles que les polymorphonucléaires neutrophiles (PMN), connus pour être les premières cellules à intervenir au cours de cette réponse physiologique.

Dans ce contexte, l'objectif principal de ce projet est d'évaluer la toxicité et le potentiel inflammatoire des Fe_3O_4NP envers les PMN. De plus, il vise à décrypter leur mode d'action afin de permettre leur utilisation sécuritaire dans les applications médicales.

1.1.1 Production, propriétés et caractérisation des Fe_3O_4 NP

En nanotechnologie, il existe deux approches méthodologiques pour la production des Fe_3O_4 NP: l'approche descendante et l'approche ascendante. Dans l'approche descendante, les NP sont créées à partir de structures macroscopiques en utilisant des méthodes mécaniques. Cette approche est moins sujette à la contamination en raison de l'absence de produits chimiques, mais elle est également plus coûteuse. En revanche, dans l'approche ascendante, considérée comme

une forme de chimie verte, la production des NP se réalise généralement à travers des réactions chimiques basées sur l'autoassemblage ou la co-précipitation. Bien que cette approche soit productive, son défi réside dans la production de NP de diamètres différents. En effet, le diamètre est le paramètre le plus critique dans les applications en nanomédecine.

Les NP présentent des caractéristiques différentes de celles des matériaux macroscopiques, principalement en raison de leur diamètre. En effet, au fur et à mesure que le diamètre d'un objet diminue, le nombre d'atomes à sa surface ainsi que les groupes fonctionnels augmentent, ce phénomène étant lié à une augmentation du rapport surface-volume. Cela conduit les NP à acquérir des propriétés distinctives par rapport aux matériaux macroscopiques. L'une de ces propriétés observées pour les Fe_3O_4 NP est le superparamagnétisme, qui se manifeste principalement à des diamètres inférieurs à 20 nm. En termes simples, cela signifie que la réponse des NP dépend entièrement de l'application d'un champ magnétique. Cette propriété est cruciale, car elle offre la possibilité de contrôler la magnétisation ainsi que le déplacement des NP dans des environnements physiologiques spécifiques.

Lors de la synthèse des NP, l'évaluation de l'adéquation à utiliser de telles NP repose sur leur caractérisation durant la phase de production. En effet, la microscopie électronique à transmission (TEM) est largement utilisée pour déterminer le diamètre et la forme des particules. Une autre technique couramment employée pour caractériser les NP est le Zetasizer, qui permet d'évaluer l'uniformité du diamètre des particules en calculant l'indice de polydispersité et la charge de la préparation.

1.1.2 Applications médicales de Fe_3O_4 NP

L'utilisation des Fe_3O_4NP en tant qu'agents de contraste en IRM vise à amplifier le signal lié à la présence d'une anomalie pathologique dans un tissu. En effet, plusieurs de ces agents ont été approuvés par l'Agence fédérale américaine des produits alimentaires et médicamenteux (FDA), tels que le Ferumoxide pour la détection du carcinome hépatocellulaire. Cependant, la plupart de ces agents ont été retirés en raison de leurs effets secondaires, tels que la douleur, l'hypotension et un taux élevé de faux positifs, comme c'était le cas pour le Ferumoxtran-10. Il est intéressant de noter que le seul médicament actuellement approuvé par FDA sur le marché, et qui est à base de Fe_3O_4 NP est le Feraheme®, utilisé pour traiter l'anémie. Cependant, son utilisation en tant

qu'agent de contraste n'a jamais été approuvée en raison du manque de données sur son utilisation sécuritaire dans ce contexte. D'un autre côté, la thérapie photothermique, qui repose sur la génération de chaleur pour perturber la croissance d'un tissu cancéreux, représente une utilisation prometteuse des Fe_3O_4 NP pour le traitement du cancer de la prostate et des tumeurs cérébrales. Actuellement, le seul traitement qui montre des résultats cliniques encourageants dans ce contexte est NanoTherm®, basé sur des Fe_3O_4 NP d'un diamètre de 15 nm.

1.1.3 L'évaluation de la toxicité des nanoparticules

L'exposition aux NP peut avoir différentes origines, que ce soit directement à partir de NP présentes dans des produits de consommation (ex. NP de dioxyde de titane dans les dentifrices) ou présentes sous forme d'aérosol dans les milieux de travail (ex. Les zones de production industrielle des NP). En effet, dans ce type d'environnement, l'inhalation est considérée comme la voie principale d'exposition. Cependant, l'établissement d'une limite d'exposition aux NP dans les milieux de travail est difficile en raison de leur grande variété, ainsi que le manque d'une compréhension détaillée des mécanismes associés à leur interaction avec les systèmes biologiques. D'autre part, la simple adaptation des normes de toxicité établies pour les matériaux macroscopiques ne présente pas la meilleure option, car les NP possèdent des propriétés distinctives par rapport à leurs homologues macroscopiques. Dans cette complexité, l'Organisation mondiale de la santé recommande l'utilisation d'une approche basée sur les bandes de danger. Cette approche repose sur la combinaison de plusieurs paramètres tels que le diamètre, la composition et la quantité de production, en conjonction avec un niveau de risque spécifique. Cela permet de définir un niveau de risque pour chaque préparation de NP et de mettre en place des mesures préventives adaptées.

Le risque lié à l'exposition aux NP est très préoccupant. Par exemple, l'exposition à l'amiante constitue un cas concret, bien qu'il s'agisse d'un exemple de microparticule proprement dite et non de NP. En effet, le développement du mésothéliome (cancer du poumon) est associé à l'inhalation de fibres d'amiante. Au niveau pulmonaire, les macrophages déclenchent une réponse inflammatoire pour éliminer ces microparticules, mais ils se retrouvent dans un état de "phagocytose frustrante", ce qui provoque une production massive de radicaux libres et la

persistance de l'inflammation, créant ainsi un environnement favorable au développement du cancer.

L'évaluation des risques associés aux NP est principalement basée sur l'utilisation des modèles *in vitro* et *in vivo*. Bien que l'utilisation de souris (*in vivo*) soit un modèle préférable, les divergences dans le système immunitaire entre les humains et les souris posent des interrogations. Par exemple, l'absence de l'interleukine (IL)-8 produite principalement par les PMN chez l'humain et pas chez les souris constitue une différence notable. De plus, le pourcentage de ces cellules est considérablement plus bas chez les souris par rapport aux humains. Cela pose des défis dans l'utilisation directe des connaissances obtenues à partir de modèles animaux pour les humains. Ainsi, les modèles *in vitro* basés sur l'utilisation de lignées cellulaires d'origine humaine pourraient être efficaces dans certains contextes, surtout lorsqu'il s'agit d'une évaluation préliminaire de la toxicité. Cependant, il convient de noter que leur profil de division incontrôlable pourrait entraîner une certaine persistance, ce qui pourrait biaiser l'évaluation. Par conséquent, l'utilisation d'autres modèles qui se rapprochent de la physiologie humaine est fortement recherchée. Dans ce contexte, l'utilisation de cellules primaires pourrait constituer un modèle alternatif efficace, présentant une similarité correspondante à l'organisme en question.

Au cours de l'évaluation de la toxicité des NP, leur contamination par les endotoxines constitue une préoccupation majeure. Cela est particulièrement vrai pour les NP préparées sans prendre en compte leur stérilité, ce qui risque de biaiser les résultats de la toxicité. Cependant, évaluer la contamination avant d'évaluer leur potentiel inflammatoire demeure une stratégie efficace pour éviter une mauvaise interprétation des résultats. Cela peut être réalisé en utilisant des tests qui déterminent précisément la concentration des endotoxines comme le test du lysat d'amœbocytes de limule (LAL).

1.1.4 Interaction entre les nanoparticules et le système immunitaire

L'interaction entre les NP et les cellules immunitaires peut se produire à la suite de leur passage incontrôlé à travers les barrières primaires telles que la peau et les muqueuses pour rejoindre la circulation. De manière intéressante, le système immunitaire est considéré comme le principal système à réagir à la présence de NP. Cette réaction implique une interaction entre les NP et les cellules du système immunitaire, bien que la présence de récepteurs spécifiques impliqués dans

cette reconnaissance reste à démontrer. Un élément pouvant jouer un rôle dans cette reconnaissance est l'absorption de protéines plasmatiques à la surface des NP, un phénomène connu sous le nom de biocorona. Cette biocorona est capable de masquer la véritable identité des NP, ce qui pourrait contribuer à leur reconnaissance sous forme d'haptènes capables de stimuler les cellules immunitaires. L'objectif de cette reconnaissance est de déclencher une réponse inflammatoire, une réponse physiologique propre au système immunitaire inné et est principalement assurée par les PMN et les macrophages (MΦ).

1.1.1.1 Les neutrophiles polymorphonucléaires (PMN)

Les PMN, souvent appelés neutrophiles, sont des cellules essentielles dans la réponse inflammatoire. Sur le plan cytologique, ces cellules présentent un noyau polylobé et un cytoplasme riche en granules. Ils proviennent de la moelle osseuse grâce à la différenciation des cellules souches hématopoïétiques en cellules de la lignée myéloïde, sous l'influence de divers facteurs de croissance. Le développement des PMN se caractérise par deux phases principales. La première phase est le pôle mitotique, où les cellules en développement appelées promyélocytes et myélocytes conservent leur capacité de division cellulaire. La seconde phase est le pôle post-mitotique, où les myélocytes subissent une série de différenciations pour devenir des PMN matures. Ces PMN matures sont des cellules indivisibles et ont une durée de vie relativement courte dans la circulation sanguine (moins de 24 heures), mais ils constituent de 50% à 70% de l'ensemble des leucocytes circulants. En raison de leur abondance dans la circulation sanguine, il est essentiel de maintenir leur homéostasie pour éviter des manifestations inflammatoires excessives. Cette régulation est sous le contrôle de l'axe IL-23/IL-17/G-CSF. En effet, lors de la réponse inflammatoire, les macrophages activés produisent de l'IL-23, qui à son tour stimule la sécrétion de l'IL-17 par les lymphocytes T et les cellules NK. L'IL-17 induit ensuite une augmentation de la production de facteur de croissance G-CSF par les cellules stromales de la moelle osseuse, les monocytes et les MΦ. Ce facteur joue un rôle clé dans la différenciation et la maturation des PMN. Finalement, l'élimination des PMN âgés de la circulation est caractérisée par une augmentation de l'expression de CXCR4, qui interagit spécifiquement avec CXCL12 exprimé par les cellules stromales de la moelle osseuse, dans un processus qui pourrait être décrit par le recollement neutrophilique (*PMN homing*).

1.1.1.2 L’importance de l’apoptose chez les PMN

L'apoptose joue un rôle crucial dans l'hémostasie des PMN. Étant donné que ces cellules subissent une apoptose spontanée, les facteurs qui contrôlent ce mécanisme ne sont pas encore bien définis. Cependant, des recherches suggèrent l'implication d'un mécanisme intrinsèque dans l’autoactivation de ce processus. En effet, la famille des protéines Bcl-2, qui contient à la fois des protéines pro-apoptotiques et anti-apoptotiques, semble jouer un rôle essentiel. Plus précisément, la protéine anti-apoptotique Mcl-1, fortement présente chez les PMN et ayant une demi-vie très courte, semble être impliquée dans l'activation d'une série de mécanismes induisant à l'apoptose à la suite de sa dégradation par le protéasome. En outre, les PMN expriment une série de caspases qui s'activent pendant l'inflammation, notamment la caspase-1, induisant à la pyroptose, un type de mort cellulaire associé à la libération de l'IL-1β et de l'IL-18. Bien que les PMN expriment un nombre très limité de mitochondries, leur participation à l'apoptose a été démontrée via l'implication du cytochrome c et la protéine Smac. Le rôle des ROS dans l'apoptose des PMN a été également établi. Il est intéressant de noter que les PMN dérivés des patients souffrant de granulomatose septique chronique (CGD), une maladie liée à un déficit dans le fonctionnement de la NADPH oxydase, présentent une demi-vie plus élevée que ceux dérivés de donneurs sains, suggérant ainsi l'implication de ROS dans l’apoptose des PMN. En effet, les ROS sont capables d'induire la libération de la protéine lysosomale cathepsine D, présente dans les granules primaires. Cette dernière entraîne le clivage de Bid et de la caspase-8. Ainsi, la protéine Bid interagit avec la protéine Bax, induisant la formation de pores dans la membrane externe mitochondriale, ce qui entraîne la libération du cytochrome c et la protéine Smac. Ces protéines activent la caspase-3, qui à son tour dégrade la protéine ICAD (Inhibitor caspase-activated DNase), conduisant à la fragmentation de l'ADN et donc à la mort cellulaire.

1.1.1.3 La réponse inflammatoire et les fonctions des PMN

La réponse inflammatoire se déroule en trois phases : une phase d'initiation caractérisée par le déclenchement de la réponse; une phase d'amplification où interviennent les PMN en réponse aux chimiokines libérées lors de la première phase; enfin, une phase de résolution qui implique l'élimination des cellules apoptotiques et l'activation de la réparation tissulaire pour rétablir l'homéostasie.

Le déclenchement de la réponse inflammatoire implique différents acteurs, et les mastocytes jouent un rôle critique en raison de leur position stratégique au niveau des muqueuses et des tissus. Leur rôle consiste à détecter un signal anormal et à libérer le contenu de leurs granules (dégranulation) pour initier le recrutement des PMN vers le foyer inflammatoire. Les PMN rejoignent le site inflammatoire par un processus qui comprend plusieurs étapes, regroupées sous le terme de " la cascade d'adhésion". En effet, les cellules endothéliales au niveau du site inflammatoire expriment des molécules d'adhésion telles que la sélectine P et la sélectine E, qui interagissent avec les molécules d'adhésion PSGL-1, ESL-1 et C44 exprimées par les PMN. Ces interactions favorisent la capture et le roulement des PMN le long de la paroi vasculaire pour faciliter leur adhésion. En outre, l'interaction de la sélectine E avec PSGL-1 stimule l'expression de la β2 intégrine LFA-1 (CD11a/CD18) par les PMN. Cette β2 intégrine LFA-1 va se fixer sur ICAM-1 exprimée par les cellules endothéliales, finalement cette interaction entraîne l'adhésion proprement dite des cellules, qui aboutit à la transmigration cellulaire.

Une fois arrivés au site inflammatoire, en suivant un gradient de chimiokines principalement créé par l'IL-8, les PMN assurent l'élimination de l'intrus par une série de fonctions. Celles-ci comprennent la phagocytose, la dégranulation ainsi que la production de composés réactifs de l'oxygène (ROS) dans ce que l'on appelle la "flambée oxydative". La phagocytose est le processus utilisé par les PMN et les MΦ pour éliminer les intrus (ex. NP, bactérie). Ce mécanisme est grandement facilité par le processus d'opsonisation, c'est-à-dire lorsque les intrus sont marqués par l'immunoglobuline G ou le fragment C3b du complément. La phagocytose est assurée par le biais de récepteurs Fcγ. Lorsque les récepteurs Fcγ se lient aux antigènes présents sur les particules, un complexe se forme reliant les récepteurs à des protéines cytoplasmiques appelées ITAMs. Ce complexe active ensuite la protéine Src, qui à son tour active la protéine effectrice Syk. Syk joue un rôle crucial dans la promotion du remodelage de la membrane cellulaire pour former des extensions membranaires appelées pseudopodes. Ces pseudopodes entourent et engloutissent l'élément à phagocyter, formant ainsi un phagosome. Par la suite, ce phagosome fusionne avec des lysosomes, formant un phagolysosome, où les enzymes et les substances antimicrobiennes des granules dégradent l'élément phagocyté. Le phagolysosome peut fusionner aussi avec le complexe NADPH oxydase responsable de la production de ROS. La production de ces éléments commence par le transfert d'électrons du cytosol vers l'oxygène, ce qui conduit à la formation de l'ion superoxyde (O2•–). Celui-ci est ensuite converti en peroxyde d'hydrogène (H_2O_2) qui, après sa catalyse par la myélopéroxydase, produit l'acide hypochloreux (HOCl), un puissant agent bactéricide.

En ce qui concerne la dégranulation, les PMN possèdent différents types de granules classés en primaires, secondaires, tertiaires et en vésicules sécrétoires. Les granules primaires contiennent principalement de l'élastase (NE) et de la myélopéroxydase (MPO). Les granules secondaires et tertiaires contiennent de la lactoferrine et de la gélatinase-B, tandis que les vésicules sécrétoires contiennent principalement des molécules d'adhésion. La dégranulation est liée au processus de nétose. Au cours de ce processus, les PMN libèrent un complexe sous forme de filet composé d'ADN, d'histones et de protéines issues des granules, principalement NE et MPO. Cela permet d'emprisonner l'intrus dans le filet, empêchant ainsi sa diffusion et facilitant son élimination. Ce processus est étroitement lié à la production de ROS. En effet, les patients atteints de CGD présentent une incapacité à induire ce processus, ce qui démontre l'importance de ROS dans la nétose et la dégranulation chez les PMN. Après leur intervention, les PMN entrent en apoptose, et les MΦ procèdent à leur élimination par un processus appelé éfferocytose pour prévenir l'inflammation excessive. En outre, pendant l'éfferocytose, les MΦ produisent de l'IL-10, une cytokine anti-inflammatoire qui aide à moduler la réponse immunitaire et à promouvoir la résolution de l'inflammation.

1.1.1.4 Les voies de signalisation chez les PMN

L'ensemble des fonctions des PMN au cours de la réponse inflammatoire sont régulées par des mécanismes de signalisation cellulaire qui impliquent la phosphorylation de protéines clés au sein de certaines voies de signalisation cellulaire. Ces protéines peuvent être classées en protéines kinases et en récepteurs couplés aux protéines G. Les principales protéines kinases activées chez les PMN sont PI3-K, Jak/STAT et les MAPKinases, qui sont divisées en ERK1/2, p38 et JNK. L'activation de PI3-K entraîne l'activation de la protéine effectrice Akt, qui agit en augmentant la survie des PMN. Son inhibition est associée avec l'apoptose spontanée de ces cellules. En outre, Akt joue un rôle dans l'activation des protéines impliquées dans le remodelage de l'actine du cytosquelette, favorisant ainsi la migration cellulaire. Des expériences de suppression génétique des isoformes Akt1 et Akt2 ont révélé que Akt2 est également impliqué dans la production de ROS et la dégranulation. De manière intéressante, la régulation d'Akt pourrait être assurée par p38. En effet, en absence d'activation, ces deux protéines forment un complexe stable grâce à la protéine de choc thermique 27. Ainsi, p38 joue également un rôle important dans la cascade de recrutement des PMN vers le site inflammatoire. En ce qui concerne ERK1/2, cette protéine kinase est connue pour augmenter l'expression de la protéine anti-

apoptotique Mcl-1. Alors pour la dégranulation, celle-ci est sous le contrôle des tyrosines kinases non réceptrices, notamment les protéines de la famille de Src, principalement Hck, Fgr et Lyn. Par exemple, la dégranulation des granules primaires est associée à l'activation de Hck, tandis que Fgr est associé aux granules secondaires. L'inhibition de Src par PP1 (inhibiteur pharmacologique) interfère avec le processus de dégranulation.

1.1.5 Les cytokines dans la réponse inflammatoire

La production des cytokines implique différents mécanismes, mais la voie classique impliquant le facteur de transcription NF-κB est considérée comme le mécanisme principal de production des cytokines inflammatoires. En effet, pendant la réponse inflammatoire, les cytokines produites par les cellules immunitaires mettent en œuvre différentes réponses biologiques. Ces molécules sont principalement divisées en cytokines pro-inflammatoires et anti-inflammatoires, ainsi leur production est strictement régulée pour éviter des dommages tissulaires. Par exemple, IL-1β, IL-6, IL-8, IL-17, IFN-γ et TNF-α stimulent l'inflammation, tandis que l'IL-4, IL-10 et TGF-β ont un rôle anti-inflammatoire. Il est intéressant de noter que certaines cytokines telles que l'IL-1β, TNF-α, IL-8, ainsi que les facteurs de croissance G-CSF et GM-CSF, ont principalement un effet direct sur les PMN. En retour, les PMN sont les principaux producteurs de l'IL-8, une puissante chimiokine. Il convient de noter aussi que la surproduction de cytokines pro-inflammatoires, notamment TNF-α, IL-1β, IL-6 et IL-8, est associée avec le choc septique (libération excessive de médiateurs inflammatoires en réponse à une infection). Bien que, certaines cytokines peuvent agir sur différentes cellules et induire diverses réponses biologiques (effet pléiotropique), d'autres cytokines peuvent induire des réponses biologiques similaires dans la même cellule ou différentes cellules, dans ce cas on parle de la redondance.

L'IL-1β, qui fait partie de la famille des cytokines IL-1, joue un rôle central dans la réponse inflammatoire. Son rôle est associé à la stimulation de l'expression des molécules d'adhésion nécessaires pour le recrutement des cellules au foyer inflammatoire. Sa production est activée par l'inflammasome, un complexe qui favorise la production de pro-IL-1β et pro-IL-18, qui sont ensuite activés par l'action de la caspase-1. Alors que le TNF-α a un impact significatif sur les principales fonctions des PMN, comme la phagocytose, la flambée oxydative et la dégranulation. Il stimule aussi l'expression des molécules d'adhésion nécessaires pour le recrutement des cellules. De manière intéressante, à faible concentration, le TNF-α favorise la survie des PMN,

tandis qu'à forte concentration, il induit leur mort cellulaire par apoptose. Cette dualité de fonction pourrait être considérée comme une stratégie pour induire et réguler la résolution de l'inflammation.

Alors pour l'IL-6, cette cytokine est produite comme une protéine de la phase aiguë et elle joue un rôle essentiel dans la différenciation des lymphocytes B en plasmocytes spécialisés dans la production d'anticorps. De plus, l'IL-6 joue un rôle important dans la transition de la réponse inflammatoire aiguë vers une réponse inflammatoire chronique. Cette transition est caractérisée par le recrutement principal de MΦ et de lymphocytes pendant la persistance de l'intrus.

1.1.6 Les effets des nanoparticules sur les fonctions des neutrophiles

Un ensemble d'études ont démontré la capacité des NP à altérer les fonctions biologiques des PMN. Par exemple, les NP de fer ont montré leur capacité à perturber l'adhésion des PMN dans un modèle de recrutement des PMN *in vitro*. De plus, les NP de palladium ont été observées pour induire une légère augmentation de l'adhésion des PMN sur un substrat cellulaire de cellules endothéliales. Une observation relative à la chimiotaxie a révélé que les NP de fer augmentent l'expression des récepteurs de chimiokines CXCR1 et CXCR2, qui sont fortement exprimés par les PMN à la suite de leur activation par l'IL-8 produite pendant la réponse inflammatoire. En ce qui concerne la phagocytose, les NP de dioxyde de titane, d'oxyde de zinc et de dioxyde de cérium ont montré leur capacité à activer et à améliorer l'internalisation des globules rouges du mouton (SRBCs) opsonisés avec des IgG par les PMN. En ce qui concerne la production des ROS, les NP ont montré leur induction dans les cellules par deux mécanismes. Le premier implique la réaction de Fenton, particulièrement en présence des NP métalliques, et le second implique l'activation de la machinerie classique de production de ROS par le complexe NADPH oxydase. La capacité des NP à influencer la dégranulation a également été démontrée dans de nombreuses études. En effet, les NP d'or ont été observées pour augmenter l'activité de la métalloprotéinase 9 (MMP-9). D'autres types de NP, tels que l'oxyde de cérium, dioxyde de titane et oxyde de zinc (ZnO NP) ont été observés pour augmenter l'expression de la myéloperoxydase (granules primaires), la MMP-9 (granules tertiaires) et l'albumine (vésicules sécrétoires). En ce qui concerne l'activation de la synthèse *de novo* des protéines, cela a également été observé chez les PMN en réponse aux NP. Par exemple, les ZnO NP ont démontré la capacité à interférer avec l'apoptose spontanée des PMN par ce mécanise. En effet, l'effet anti-apoptotique de ZnO

NP a été renversé par le prétraitement des cellules avec le cycloheximide, un puissant inhibiteur de la synthèse *de novo*. L'ensemble de ces exemples montre que l'interaction entre les NP et les PMN entraîne des effets immunomodulateurs sur l'ensemble des fonctions biologiques de ces cellules, qui sont cruciales pendant la réponse inflammatoire.

1.2 Hypothèse et objectifs de la thèse

L'innocuité des Fe_3O_4NP destinées à être utilisées dans diverses applications médicales n'a jamais été l'objet d'une évaluation de toxicité approfondie, spécifiquement avec les PMN, des cellules clés de la réponse inflammatoire. En effet, les effets immunomodulateurs de ces NP, soient qu'ils activent ou inhibent le bon fonctionnement de ces cellules, constituent un défi pour leur utilisation clinique. L'exemple de Feraheme®, basé sur ces NP, démontre une association avec le choc anaphylactique et son administration chez certains patients, ce qui souligne clairement la nécessité d'étudier leurs effets immunomodulateurs sur les PMN.

Hypothèse : La Fe_3O_4 NP pourrait altérer la biologie des PMN humains en fonction du sexe.

Objectifs : Évaluation des effets modulateurs des Fe_3O_4NP sur les fonctions des PMN et le décryptage de certains mécanismes moléculaires impliqués dans la réponse des PMN aux Fe_3O_4 NP.

1.3 Méthodologie de recherche

1.3.1 Source des Fe_3O_4NP

Les NP sont disponibles commercialement (Sigma, ID : 725358). Selon le fabricant, les Fe_3O_4NP ont une forme sphérique avec un diamètre d'environ 10 nm, déterminés par TEM. Le niveau de contamination des NP par les endotoxines sera évalué à l'aide d'une trousse commerciale (le test LAL) afin de garantir l'absence de contamination. La caractérisation des NP sera effectuée à l'aide de TEM pour confirmer leur diamètre et leur forme.

1.3.2 L'origine et isolement des PMN et leur traitement avec les Fe_3O_4 NP *in vitro*

Les PMN seront obtenus à partir de sang de donneurs sains et non-fumeurs âgés de 20 à 65 ans. Une infirmière effectuera la prise de sang après avoir expliqué de manière éclairée le consentement à chaque donneur. Il convient de noter que ce projet de recherche a reçu l'approbation du comité d'éthique de l'INRS (CER-15-397). Un maximum de 100 mL de sang sera prélevé. À partir du sang total, les PMN seront isolés par une méthode standard d'isolement. En résumé, les tubes de sang seront centrifugés et le plasma sera éliminé. Les érythrocytes seront sédimentés en ajoutant du dextran à 6 % pendant ≃ 20 minutes. Ensuite, le surnageant contenant les leucocytes (lymphocytes, monocytes et granulocytes) sera recueilli, et la séparation des lymphocytes et des monocytes (PBMCs) des granulocytes sera réalisée à l'aide d'un gradient de Ficoll. Les érythrocytes résiduels seront lysés par un choc osmotique avec de l'eau pendant une très courte période de temps (< 15 secondes). Le culot final obtenu sera normalement composé de granulocytes, principalement des PMN (~98 %) et des éosinophiles (~2 %). La concentration cellulaire sera ajustée à 10 x 10^6 cellules/mL.

1.3.3 Interférence

Un test d'interférence des Fe_3O_4 NP sera réalisé pour chaque fonction testée. Pour ce faire, nous ajouterons systématiquement une condition expérimentale dans laquelle seules les NP seront introduites. En effet, l'évaluation de l'interférence des NP est essentielle, car elles peuvent perturber certains types de signaux de détection (lumière, fluorescence, etc.) utilisés dans diverses techniques en raison de leurs propriétés optiques et magnétiques. Ceci pourrait potentiellement altérer et fausser indirectement les résultats.

1.3.4 Test de viabilité cellulaire et d'apoptose

Les PMN seront incubés avec les Fe_3O_4 NP à différentes concentrations (0-500 µg/mL) pour des périodes pouvant aller jusqu'à 24h. Tout d'abord, un test d'exclusion du bleu de trypan sera effectué pour déterminer si les NP induisent la nécrose chez les PMN. En parallèle, des préparations cytologiques sur des lames colorées avec un kit de coloration (Hema 3) nous

permettront d'évaluer le taux d'apoptose des PMN, qui est facilement observable en microscopie optique. Le noyau polylobé caractéristique des cellules normales (vivantes) devient pycnotique (ronde) chez les cellules apoptotiques. De plus, ce test pourra fournir des informations détaillées telles qu'un changement de taille et de forme des cellules. Au cours de ces expériences, nous ajouterons systématiquement deux conditions expérimentales témoins, la cytokine anti-apoptotique GM-CSF et la lectine de plante Viscum album agglutinin-1, (VAA-I) un agent pro-apoptotique puissant.

1.3.5 Internalisation

L'évaluation de l'internalisation des Fe_3O_4 NP sera réalisée par deux méthodes. La première repose sur l'utilisation de TEM, au cours de laquelle les PMN à une concentration de 10×10^6 cellules/mL seront incubés avec 100 µg/mL de Fe_3O_4 NP pendant 24h. Ensuite, les cellules seront fixées avec 2,5 % de glutaraldéhyde pendant 15h à 4 °C. Après une série de lavages, les cellules seront soumises au service de microscopie pour leur préparation et leur visualisation au moyen de la microscopie électronique. L'autre méthode sera basée sur la coloration du fer internalisé par les cellules en utilisant la technique de Perls. Après une incubation de 24h, les cellules seront étalées sur des lames et fixées avec du méthanol pendant 7 minutes. Ensuite, elles seront placées dans une solution contenant du ferrocyanure de potassium et de l'acide chlorhydrique pendant 10 minutes. Les lames seront ensuite montées à l'aide d'une solution de pararosaniline. Dans cette méthode, le ferrocyanure de potassium réagira avec les ions ferreux pour former un complexe insoluble bleu appelé bleu de Prusse, détectable dans les cellules ayant internalisé les Fe_3O_4 NP par microscopie optique.

1.3.6 Flambée oxydative

La production des ROS sera évaluée par une mesure spectrométrique, après le marquage des cellules avec la sonde fluorogénique 2',7'-dichlorofluorescéine (H_2DCFDA). Les cellules seront incubées en présence de la sonde pendant 20 minutes, tout en étant protégées de la lumière. Ensuite après incubation avec différents agonistes, la cinétique de la fluorescence sera mesurée à des intervalles de temps de 5, 15, 30 et 60 minutes à l'aide d'un spectrophotomètre. Le fMLP

(un facteur de chimiotactisme) sera utilisé comme témoin positif, tandis que le peroxyde d'hydrogène sera employé comme un témoin technique positif.

1.3.7 Dégranulation

La dégranulation sera étudiée en utilisant la technique de zymographie, qui permet de déterminer l'activité enzymatique des protéines libérées lors de la dégranulation. Pour ce faire, les cellules seront incubées en présence ou en l'absence de Fe_3O_4NP ou de fMLP (témoin positif) pendant des périodes de 5, 15, 30 et 60 minutes. Les surnageants des différents conditions seront collectés après centrifugation à 13 000 rpm pendant 10 minutes à 4 °C. Pour chaque condition, un volume de 10 µL de surnageant sera mélangé avec un tampon contenant 40 % de glycérol, du Tris-HCL 1 M, du SDS 8 % (pH 6,8) et déposé sur un gel d'acrylamide à 10 % contenant 0,2 % de gélatine (substrat de digestion). Après l'électrophorèse, le gel sera lavé deux fois pendant 30 minutes avec 2,5 % de Triton X-100, puis incubé dans un tampon contenant du Tris-HCL 50 mM, du NaCl 150 mM et du CaCl2 5 mM pendant environ 15 heures. Ensuite, le gel sera coloré avec une solution à 0,1 % de bleu de Coomassie et décoloré avec une solution décolorante composée de 25 % de méthanol et de 7,5 % d'acide acétique. Enfin, la densité des bandes claires indiquant la digestion enzymatique sera analysée à l'aide du logiciel ImageJ.

1.3.8 Phagocytose

Dans cette démarche, les globules rouges de mouton (SRBCs), disponibles commercialement, seront opsonisés en les incubant avec des anticorps anti-SRBCs pendant 45 minutes. Les cellules seront incubées en présence ou en absence de Fe_3O_4NP ou de GM-CSF (témoin positif) pendant 30 minutes. Par la suite, les SRBCs opsonisés seront incubés avec les PMN pendant 30 minutes. Les SRBCs non phagocytés seront lysés par un choc osmotique. Après une cytocentrifugation, des lames seront préparées et colorées à l'aide de la coloration Hema 3. Finalement, la phagocytose sera évaluée par la microscopie optique en comptant le nombre de PMN ayant phagocyté au moins un SRBC. Dans une autre série d'expériences, les cellules seront prétraitées avec les inhibiteurs Piceatannol (30 µM) et PP1 (50 µM) pendant 30 minutes, avant d'être incubées avec les différents agonistes et les SRBCs opsonisés.

1.3.9 Adhésion cellulaire

L'évaluation de la capacité des PMN à adhérer a été éffectuée en utilisant la lignée de cellules endothéliales humaines EA.hy926 comme substrat cellulaire. Après incubation, les PMN traités avec Fe_3O_4 NP ou TNF-α (témoin positif) pendant 30 minutes seront marqués avec 5 µM de calcéine-AM, une sonde fluorescente. Ensuite, ces PMN marqués seront déposés sur les cellules endothéliales, qui auront une confluence > 75 %. Après une série de lavages, le nombre de PMN ayant adhéré aux cellules endothéliales sera déterminé en utilisant la microscopie à fluorescence.

1.3.10 Nétose

Pour évaluer la formation de NETs (*neutrophil extracellular traps*), les cellules (10×10^6 cellules/mL) seront traitées en présence ou en absence de Fe_3O_4 NP ou du témoin positif pendant 90 minutes. Ces traitements seront effectués sur des lames préalablement prétraitées avec de la poly-L-lysine. Ensuite, les cellules seront colorées avec la solution Hema 3 et observées par microscopie optique.

1.3.11 Évaluation de cytokines par la technique du microréseaux d'anticorps

En utilisant un kit spécifique (R&Dsystems, ID: ARY005B), nous évaluerons la production d'une variété de cytokines impliquées dans la réponse inflammatoire (36 analytes). Pour ce faire, les surnageants de cellules incubées avec les Fe_3O_4 NP ou HBSS pendant 24h et provenant de 10 donneurs, seront préparés et mélangés pour créer un pool d'analyse. La présence d'une sécrétion de cytokine sera révélée par la formation d'une tache noire dans une position précise sur la membrane d'analyse correspondante à une cytokine spécifique, selon une grille de référence fournie avec le kit. La densitométrie des taches sera évaluée à l'aide du logiciel ImageJ.

1.3.12 Évaluation de cytokines par la technique ELISA

L'évaluation de la sécrétion de cytokines (TNF-α, IL-1β, IL-6 et IL-8) sera réalisée à l'aide de la technique ELISA en utilisant des kits commerciaux. En résumé, après une incubation de 24h en présence ou en absence de Fe_3O_4 NP, les surnageants des cultures cellulaires seront récupérés et centrifugés à 4 °C, puis utilisés pour la détection des cytokines pro-inflammatoires à l'aide des kits ELISA correspondants. Dans ces expériences, le LPS (1 µg/mL) sera utilisé comme témoin positif.

1.3.13 Détection des protéines phosphorylées par la technique de microréseaux d'anticorps

Cette analyse sera effectuée en utilisant un kit spécifique (R&D Systems, ID : ARY003C) capable de détecter 37 analytes principalement associés à la phosphorylation des protéines kinases. Pour ce faire, des lysats cellulaires traités avec des Fe_3O_4 NP ou HBSS, provenant de plusieurs donneurs, seront préparés pour former un pool d'analyse. Il convient de noter que la lyse cellulaire sera réalisée à l'aide d'une solution de lyse enrichie en inhibiteurs des phosphatases et des protéases.

1.3.14 Immunobuverdage

L'analyse de la phosphorylation des protéines des MAPkinases telles que (p38, ERK1/2), la protéine Syk, Akt et Src, ainsi que l'analyse de la phosphorylation des protéines totales et l'expression de Mcl-1, sera effectuée par la technique d'immunobuvardage de type Western. Pour p38, ERK1/2, Syk, Akt, et Src, les cellules (4 x 10^6 / 100 µL) seront incubées avec les Fe_3O_4 NP pendant des courtes périodes de temps (1, 5, 15 ou 30 minutes), sauf pour Mcl-1 où les cellules seront incubées pendant 24h. Les cellules seront récupérées après centrifugation à 4 °C et lysées dans une solution de lyse contenant des inhibiteurs de phosphatases (PhosSTOP™) et de protéases (Protease Inhibitor Cocktail Set I ; Protease Inhibitor Cocktail Set III). Pour la protéine Syk en particulier, les cellules seront prétraitées avec 0.2 µl/mL de di-isopropyl fluorophosphate (DFP) pour empêcher sa dégradation, avant d'être incubées avec les Fe_3O_4 NP. En général, un volume de 20 µL de lysat cellulaire (≃ 8 × 10^5 cellules) sera utilisé pour l'électrophorèse sur gel

SDS-PAGE à 10 %. Après la migration, les protéines seront transférées sur une membrane de polyvinylidene difluoride (PVDF) prétraitée avec du méthanol. Ensuite, la membrane sera bloquée avec une solution de TBS-Tween contenant 5 % de BSA. Après des lavages, l'anticorps primaire, dilué selon les recommandations du fournisseur, sera ajouté à la membrane et incubé pendant 15h à 4 °C. Ensuite, la membrane sera lavée à nouveau et incubée avec un anticorps secondaire (anti-souris ou anti-lapin IgG HRP, selon l'anticorps primaire), dilué à 1:20000 dans une solution de TBS-Tween + 5 % de BSA, pendant une heure. La membrane sera lavée à nouveau et l'expression des protéines sera détectée à l'aide d'un système de détection (ChemiDoc), et la densité des bandes protéiques sera analysée à l'aide du logiciel ImageJ. Dans ces expériences, le LPS (1 µg/mL) et le GM-CSF (65 ng/mL) seront utilisés comme témoins positifs.

1.3.15 Synthèse *de novo*

Dans ce projet, l'évaluation de la synthèse *de novo* des protéines repose sur une approche pharmacologique utilisant le cycloheximide (CHX), un agent qui inhibe la synthèse *de novo* des protéines en se liant aux ribosomes et en empêchant la traduction des ARNm. Pour ce faire, les cellules seront prétraitées avec 1 µg/mL ou 10 µg/mL de CHX avant d'être incubées avec Fe_3O_4 NP ou le GM-CSF (témoin positif). L'évaluation de ce processus sera effectuée en déterminant le pourcentage d'apoptose par cytologie après 24h d'incubation.

1.4 Résumé court des articles

1.4.1 Article 1

Titre: Évaluation de la réponse des neutrophiles aux nanoparticules de fer (Fe_3O_4 NP): Des évidences que les Fe_3O_4 NP possèdent un profil pro-inflammatoire**.**

Les nanoparticules d'oxyde de fer (Fe_3O_4NP) attirent une attention importante pour différentes applications médicales. Cependant, une toxicité potentielle dans leur utilisation a été rapportée et plusieurs paramètres doivent être encore étudiés pour atteindre une efficacité thérapeutique

maximale avec un minimum d'effets indésirables. L'inflammation est l'un des effets indésirables les plus fréquemment rapportés à la suite d'une exposition aux NP, et des données contradictoires existent concernant le fait que les Fe_3O_4 NP possèdent des activités pro-inflammatoires et anti-inflammatoires. L'objectif de cette étude est de déterminer l'effet direct des Fe_3O_4NP sur la biologie des neutrophiles, des cellules clés dans l'inflammation. Des neutrophiles humains fraîchement isolés ont été incubés *in vitro* avec des Fe_3O_4 NP et plusieurs fonctions ont été étudiées. En utilisant la microscopie électronique à transmission, il a été constaté que les Fe_3O_4 NP étaient internalisées par les neutrophiles. Ces NP ne déclenchent pas la flambée oxydative, mais ils augmentent la capacité des neutrophiles à adhérer aux cellules endothéliales humaines ainsi qu'à améliorer leur fonction phagocytaire. L'utilisation d'une approche par microréseaux d'anticorps a révélé que les Fe_3O_4NP induisent la production de certaines cytokines pro-inflammatoires, dont la chimiokine IL-8 (CXCL8), ce qui a été confirmé par ELISA. Les Fe_3O_4NP ont été trouvées de retarder l'apoptose spontanée des neutrophiles, indépendamment du sexe. En utilisant une approche pharmacologique, nous avons démontré que les Fe_3O_4NP retardent l'apoptose par un mécanisme dépendant de la synthèse *de novo* des protéines et via différentes voies de signalisation cellulaire. Les données obtenues dans cette étude indiquent que les Fe_3O_4 NP peuvent altérer la biologie des neutrophiles humains et qu'ils possèdent certains effets pro-inflammatoires, en particulier en ce qui concerne leur capacité à retarder l'apoptose et à induire la production de cytokines pro-inflammatoires. Par conséquent, les Fe_3O_4 NP peuvent réguler de l'inflammation en modulant les fonctions des neutrophiles humains.

1.4.2 Article 2 (Revue de littérature)

Titre: La modulation des cellules immunitaires primaires par les nanoparticules d'oxyde de fer (IONs).

La nanotechnologie suscite de plus en plus d'intérêt ces dernières années et l'utilisation potentielle de nanomatériaux tels que les nanoparticules (NP) continue de croître dans divers secteurs d'activité. Parmi les NP les plus utilisées, on trouve les nanoparticules d'oxyde de fer (IONs), ces NP ont suscité un intérêt croissant de la part de la communauté scientifique et des industriels en raison de leurs propriétés superparamagnétiques qui permettent leur utilisation dans de nombreux domaines, y compris la médecine. Cependant, certains effets indésirables des IONs et des risques pour la santé humaine font l'objet de rapports de plus en plus dans plusieurs

études. Bien que de nombreuses études *in vivo* aient signalé que les IONs induisent une immunotoxicité dans des modèles animaux, il n'est pas clair comment les IONs peuvent altérer la biologie des cellules immunitaires humaines. Dans cette revue, une analyse des travaux de recherche concernant l'interaction entre les IONs et les cellules immunitaires primaires a été abordée. Cette revue souligne également l'importance de l'utilisation de cellules immunitaires primaires dans l'évaluation des risques liés aux NP en tant que stratégie fiable pour encourager des approches alternatives pour limiter l'utilisation des animaux en recherche. En mettant tous ces éléments ensemble, les observations rapportées dans cette revue contribuent à obtenir une idée globale de la manière dont les IONs altèrent le système immunitaire humain, surtout que l'inflammation est fréquemment rapportée comme un effet indésirable des IONs.

1.5 Discussion

La compréhension des mécanismes associés à la toxicité des NP sur le fonctionnement des cellules du système immunitaire est essentielle pour établir des mesures de protection appropriées lors de leur production, utilisation et élimination dans des contextes professionnels et cliniques. Dans cette thèse, nous avons spécifiquement étudié la réponse des PMN aux Fe_3O_4 NP afin d'évaluer leur potentiel inflammatoire. Les Fe_3O_4 NP utilisées étaient de forme sphérique, non enrobées et avaient un diamètre d'environ 10 nm, déterminés par TEM. Nos résultats ont montré que ces NP étaient capables de moduler les fonctions biologiques des PMN. En particulier, elles ont été trouvées d'interférer avec l'apoptose spontanée des PMN, par une relation dose-réponse. L'effet le plus marqué a été observé à la concentration de 100 µg/mL. De plus, nous n'avons pas observé de différence entre la réponse des cellules dérivées d'hommes et de femmes, suggérant que le dimorphisme sexuel ne joue pas de rôle dans cette interférence avec l'apoptose spontanée.

Cette observation confirme la capacité de ces NP à moduler l'apoptose des PMN, ce type d'interférence a déjà été documentée dans de nombreuses autres études. Par exemple, les NP d'oxyde de zinc (ZnO NP) ont été montrées d'interférer avec l'apoptose spontanée des PMN, conduisant à une augmentation de la viabilité cellulaire. Cependant, il est important de noter que d'autres NP, comme celles d'argent (Ag) et d'or (Au), ont été observées pour accélérer ce processus chez les PMN. Ainsi, la concentration la plus élevée utilisée dans notre étude, soit 100 µg/mL, est approximativement comparable à la concentration qu'un patient peut recevoir en

clinique à partir d'une fiole à dose unique de Feraheme®, un médicament à base de Fe_3O_4NP enrobées avec le dextran. En effet, il a été estimé que la concentration de Feraheme® qu'un patient reçoit *in vivo* est d'environ 102 µg/mL. De plus, une concentration de 100 µg/mL est fréquemment utilisée dans les études d'évaluation de la toxicité des NP *in vitro*. D'une manière intéressante, l'observation de l'interférence des Fe_3O_4 NP avec l'apoptose spontanée des PMN montre une tendance similaire à celle du facteur de croissance GM-CSF, un agent anti-apoptotique bien connu et nécessaire à la différenciation des PMN pendant la granulopoïèse. Il est important de noter que cette interférence a été renversée en inhibant le mécanisme de la synthèse *de novo* des protéines par le cycloheximide, ce qui indique que ce processus est impliqué dans la réponse des PMN aux Fe_3O_4 NP. En effet, une observation similaire a été déjà démontrée avec les ZnO NP. Cette modulation d'apoptose des PMN par Fe_3O_4NP est directement attribuée aux Fe_3O_4NP plutôt qu'à d'autres artefacts tels que les endotoxines, car ces particules ont été évaluées à l'aide du test LAL et il a été constaté qu'elles étaient relativement exemptes de contamination par les endotoxines. De plus, lors de l'incubation d'un échantillon de la solution initiale de ces NP dans un milieu de culture bactérienne sur gélose pendant 72h, aucune croissance bactérienne n'a été observée, ce qui confirme leur stérilité. En outre, l'analyse par TEM a révélé que les PMN sont capables d'internaliser efficacement les Fe_3O_4 NP dans des structures similaires à des phagosomes, ce qui indique une interaction directe entre ces particules et les cellules. L'ensemble de ces observations suggère que les effets modulateurs observés sont directement liés aux Fe_3O_4NP eux-mêmes. Bien que le mécanisme exact d'internalisation de Fe_3O_4NP ne soit pas encore complètement élucidé, leur charge négative déterminée par le Nanosizer suggère qu'un mécanisme actif tel que la phagocytose pourrait être impliqué. En effet, les NP chargées négativement sont moins susceptibles d'être incorporées à la surface cellulaire par rapport à celles chargées positivement, qui ont des taux plus élevés d'internalisation cellulaire non spécifique.

La voie Syk associée aux récepteurs Fcγ est connue comme une voie clé utilisée par les cellules phagocytaires pour internaliser les particules opsonisées. Curieusement, l'analyse par immunobuvardage de la phosphorylation de la protéine Syk en présence de Fe_3O_4 NP a démontré son activation. De manière intéressante, il a été observé que les Fe_3O_4 NP améliorent également la phagocytose des globules rouges de mouton (SRBCs) de manière similaire au GM-CSF, un puissant activateur de la phagocytose chez les PMN. Cela suggère que les Fe_3O_4 NP pourraient influencer positivement la capacité des PMN à phagocyter, en utilisant potentiellement

la voie Syk pour améliorer cette fonction. Le traitement avec le Piceatannol, un inhibiteur spécifique de la protéine Syk, a entraîné une diminution de la capacité phagocytaire des PMN vis-à-vis des SRBCs, même en présence des Fe_3O_4 NP, ce qui confirme dans une certaine mesure l'implication de la voie Syk dans la phagocytose améliorée par les Fe_3O_4 NP. D'autre part, la protéine kinase Src, qui se trouve en amont de la voie Syk, a montré aussi son activation en présence des Fe_3O_4 NP. Cette activation de Src peut contribuer à la régulation de la voie Syk et à l'amélioration de la fonction phagocytaire observée chez les PMN. En effet, l'inhibition de Src par un inhibiteur pharmacologique spécifique connu sous le nom de PP1 a démontré une diminution significative de l'internalisation des SRBCs par les PMN prétraités avec les Fe_3O_4 NP Ces résultats suggèrent que la voie de signalisation impliquant Src joue un rôle crucial dans la phagocytose améliorée des SRBCs en présence des Fe_3O_4 NP. Il est intéressant de noter que la phagocytose est un mécanisme inhabituel pour des particules du diamètre inférieur à 100 nm. Cependant, les résultats suggèrent que la phagocytose pourrait être le principal mécanisme d'internalisation des Fe_3O_4 NP, surtout que des observations par TEM ont démontrées que les Fe_3O_4 NP ont tendance à s'agréger et à former des particules d'un diamètre supérieur à 1 µm. En effet, Il est connu que les particules à l'échelle du micromètre (> 5 µm) sont généralement internalisées par les cellules du système phagocytaire mononucléé par la phagocytose. En outre, notre observation est en accord avec des résultats des études antérieures qui ont montré que d'autres types de NP, tels que les TiO_2 NP, CeO_2 NP et ZnO NP, peuvent également améliorer la phagocytose des SRBCs chez les PMN par un mécanisme dépendant de la voie Syk. Cependant, nous n'avons pas étudié les aspects mécanistiques de ce processus avec les Fe_3O_4 NP. Par conséquent, on recommande l'utilisation de terme "endocytose", qui englobe la pinocytose, l'endocytose médiée par les récepteurs et la phagocytose. En effet, il reste encore des questions à résoudre, notamment si cette activation de la phagocytose est due à des protéines absorbées à la surface des NP c'est-à-dire la biocorona ou uniquement aux propriétés intrinsèques des particules elles-mêmes. Malgré cela, les PMN qui internalisent les Fe_3O_4NP conservent toujours leur fonction phagocytaire dépendante du récepteur Fcγ envers les SRBCs. En effet, les Fe_3O_4NP et les SRBCs ont été observées simultanément dans le cytoplasme des PMN prétraités avec les Fe_3O_4NP, ce qui suggère que les mécanismes de phagocytose classique des PMN ne sont pas altérés par la présence de ces NP. Cela pourrait indiquer que les Fe_3O_4 NP n'entravent pas les voies de signalisation et les processus requis pour l'ingestion des particules opsonisées.

Les effets modulateurs des Fe_3O_4 NP sur les PMN vont au-delà de l'apoptose et de la phagocytose, car il a également été constaté qu'elles ont un impact sur l'adhésion, la flambée oxydative (ROS) et la production de cytokines. Ces mécanismes sont tous cruciaux lors de la réponse inflammatoire. Notamment, l'activation des PMN par les Fe_3O_4NP module la production de cytokines pro-inflammatoires. En effet, les Fe_3O_4 NP favorisent la libération de l'IL-8, MIP-1α/β et CXCL-1, comme a été confirmé par l'analyse des cytokines grâce à la technique de microréseaux d'anticorps (HCA). Il est important de noter que la libération de l'IL-8 pourrait ne pas être uniquement attribuable à la présence de NP, mais pourrait également être le résultat de la présence d'une contamination des NP par les endotoxines, ou tout simplement au déclenchement d'une réponse immunitaire à une infection asymptomatique chez le donneur de sang. Par conséquent, il est essentiel d'évaluer d'autres cytokines pro-inflammatoires pour confirmer le profil inflammatoire des NP en question. Dans notre étude, l'application de la technique HCA s'est avérée être une approche pratique pour la détection d'un large éventail de cytokines pro-inflammatoires et anti-inflammatoires. Cependant, il est important de noter que la sensibilité de la méthode HCA est relativement limitée en comparaison avec la technique ELISA, principalement en raison de la nécessité d'utiliser un volume plus important de surnageant de culture cellulaire. Par conséquent, certaines cytokines telles que l'IL-6, IL-1β et TNF-α n'ont pu être détectées via la méthode HCA, contrairement à la technique ELISA. En effet, l'utilisation d'ELISA a permis de confirmer que les Fe_3O_4 NP induisent la production de TNF-α, IL-1β, IL-6 et IL-8. La production de cytokines induite par les Fe_3O_4 NP affecte les fonctions biologiques des PMN, comme en témoigne l'observation d'une augmentation de la capacité d'adhérence des PMN sur le substrat de cellules endothéliales de la veine ombilicale humaine (EA.hy926). Cette augmentation de l'adhérence semble être corrélée à la production de TNF-α, qui peut agir de manière autocrine pour réguler l'expression de molécules d'adhérence cellulaire telles que Mac-1 (CD11b/CD18) et LFA-1 (CD11a/CD18) à la surface des PMN, favorisant ainsi leur adhérence au substrat cellulaire.

En ce qui concerne la flambée oxydative, les Fe_3O_4 NP n'induisent pas le mécanisme conventionnel d'activation de la machinerie de production des ROS via le NADPH oxydase. Ceci suggère que ces particules activent les PMN par un mécanisme différent de celui employé lors de la réponse aux micro-organismes. Il est important de noter que la production de ROS est corrélée avec l'apoptose des PMN. Cette corrélation implique que l'effet anti-apoptotique observé avec les Fe_3O_4 NP pourrait être lié à leur incapacité à stimuler la production de ROS et donc à induire le stress oxydatif. Cette observation est cohérente avec une autre étude portant sur des

Fe_3O_4 NP d'environ 10 nm, non enrobées, qui a également rapporté des résultats similaires. En outre, une autre étude a démontré que lorsque les PMN sont incubés avec les Fe_3O_4 NP en présence de *Staphylococcus aureus*, la prolifération et la viabilité de cette bactérie augmentent de manière significative. Cela suggère que la présence de ces particules interfère avec le mécanisme de production des ROS au sein des cellules, entraînant ainsi une survie accrue des bactéries. En d'autres termes, les cellules deviennent incapables d'éliminer efficacement les bactéries. Il est plausible que certaines protéines intrinsèques jouent un rôle dans cet effet modulateur. En effet, les PMN expriment des protéines liées au métabolisme du fer, telles que la transferrine (captation du fer), la ferritine (stockage du fer) et la ferroportine (libération du fer). De plus, des protéines de capture du fer telles que la lipocaline, la gélatinase de neutrophile associée à la lipocaline, la calprotectine et la lactoferrine sont également présentes dans leurs granules. Ces protéines jouent un rôle essentiel dans la lutte contre les infections bactériennes en régulant le métabolisme du fer. En particulier, la lactoferrine (Lfr), qui est principalement exprimée par les PMN (3 µg / 10^6 cellules) confère à ces cellules la capacité de gérer efficacement une surcharge en fer. En effet, la présence abondante de cette protéine chélatrice de fer pourrait offrir une protection contre d'éventuels effets cytotoxiques des Fe_3O_4NP. Cette hypothèse est renforcée par le fait que la surcharge en fer est liée à la réaction de Fenton, où les ions ferreux (Fe^{2+}) réagissent avec le peroxyde d'hydrogène (H_2O_2) pour générer des radicaux hydroxyles (•OH) qui induisent un stress oxydatif par la peroxydation lipidique. De manière intrigante, une étude a démontré que la Lfr, en plus de son activité chélatrice, elle possède également la capacité de capturer les radicaux •OH. Cela suggère que cette protéine pourrait agir comme un élément chélateur et cytoprotecteur contre les Fe_3O_4 NP en neutralisant la réaction qui conduit à la production des radicaux •OH.

D'un point de vue mécanistique, il a été constaté que les Fe_3O_4 NP induisaient des événements de phosphorylation dans les PMN, comme avait déjà été établi pour les ZnO NP. Principalement, la voie Akt est bien connue pour son rôle dans l'inhibition de l'apoptose spontanée des PMN. Cependant, les Fe_3O_4 NP n'induisent pas son activation. Malgré cela, elles sont toujours capables d'interférer avec l'apoptose constitutive des cellules, ce qui suggère l'implication d'autres voies de signalisation cellulaires. En outre, il est important de noter qu'Akt est également nécessaire pour l'activation du complexe NADPH oxydase, lequel conduit à la production de ROS et à la nétose. Cette observation est cohérente avec le fait que les Fe_3O_4NP n'induisent pas la phosphorylation de la protéine Akt, et par conséquent, elles ne déclenchent pas la production de ROS ni la nétose. En effet, la nétose est principalement associée à la libération de ROS. De plus,

la nétose est associée à la dégranulation. Il est intéressant de noter que les Fe_3O_4NP après leur incubation avec les PMN, n'induisent pas la libération de MMP-9, qui se trouve dans les granules tertiaires. Cela pourrait indiquer une éventuelle altération de ce mécanisme, mais il est difficile de déterminer si cela est dû à l'internalisation des particules elles-mêmes ou à l'activation de voies de signalisation spécifiques favorisant l'inhibition. Cependant, il est important de noter que l'absence de nétose infère également avec l'absence de la libération de myéloperoxydase et d'élastase, qui sont des protéases emmagasinées dans les granules primaires. L'ensemble de ces observations suggère que la libération à la fois des granules primaires et tertiaires pourrait être compromise en présence des Fe_3O_4 NP. De plus, la dégranulation peut impliquer la libération du contenu des granules directement dans les phagosomes. Par conséquent, dans le cas des Fe_3O_4 NP internalisées, il est possible que les granules fusionnent avec les phagosomes où les Fe_3O_4 NP ont été internalisées, plutôt que d'être libérées à l'extérieur de la cellule. Cependant, dans le cadre de notre étude, il n'est pas évident de savoir si les Fe_3O_4 NP ciblent l'ensemble des granules ou non. Une étude antérieure a révélé que l'activation des protéines Hck et Fgr, appartenant à la famille des kinases tyrosines de type Src, est associée à la libération des granules primaires et secondaires, respectivement. De manière intéressante, dans notre étude, une activation de Src a été observée, mais les raisons pour lesquelles une telle activation ne favorise pas l'exocytose des granules ne sont pas claires. Il est important de noter que notre étude a été réalisée avec des cellules en suspension, tandis que l'étude de référence a été spécifiquement menée sur des PMN adhérants au fibrinogène. De plus, d'autres voies de signalisation cellulaire jouent également un rôle crucial dans la promotion de la dégranulation. Par exemple, l'activation de la voie p38 a été observée chez les PMN stimulés par le fMLP, un puissant activateur de la libération des granules secondaires et tertiaires chez les PMN. En revanche, l'activation de la protéine ERK 1/2 ne semble pas être impliquée dans l'exocytose des granules. Dans le cadre de notre étude, il a été observé que les Fe_3O_4 NP n'activent pas de manière significative les voies de signalisation p38 et ERK 1/2 contrairement au GM-CSF utilisé comme témoin positif (voir ANNEXE II, Figure 1.18.6-1). Cette observation suggère une corrélation potentielle entre l'absence de la phosphorylation de p38, ERK 1/2 et l'absence de dégranulation en présence des Fe_3O_4 NP. Cependant, bien que la dégranulation puisse ne pas être clairement observée pour la MMP-9, il n'est pas possible de l'exclure complètement pour les autres types de granules. En effet, l'augmentation de l'adhésion des PMN observée en présence des Fe_3O_4 NP pourrait être potentiellement liée à la libération de TNF-α. Comme discuté précédemment, la libération de TNF-α pourrait agir de manière autocrine pour favoriser la libération de vésicules sécrétoires contenant des molécules d'adhésion. Pour déterminer si une

telle modulation spécifique existe, l'étude de la libération de sérum-albumine humaine, un marqueur spécifique des vésicules sécrétoires, serait utile, ce qui malheureusement n'a pas été fait dans notre étude.

L'analyse des protéines phosphorylées à l'aide de la technique de microréseaux d'anticorps (HKA) est capable de détecter des sites de phosphorylation sur 37 protéines différentes, ce qui représente une approche utile pour comprendre les mécanismes de signalisation cellulaire par lesquels les Fe_3O_4 NP modulent les fonctions des PMN. De manière intéressante, la présence des Fe_3O_4 NP induit une augmentation significative des facteurs de transcription β-Caténine et p53. Il est important de noter que la β-Caténine est connue pour jouer un rôle dans la régulation de la voie de signalisation canonique Wingless/Integrated (Wnt) au sein des cellules souches hématopoïétiques, qui sont à l'origine de toutes les cellules sanguines. Son rôle est impliqué dans la promotion de l'expression des récepteurs du facteur de stimulation G-CSF, qui sont essentiels pour la différenciation adéquate des cellules myéloïdes en granulocytes, notamment en PMN. Bien que la recherche sur cette voie chez les PMN soit très limitée, il est important de noter que les Fe_3O_4 NP semblent être capables d'induire la libération de G-CSF par les PMN, comme a été observé dans l'analyse des cytokines effectuée via la technique de microréseaux d'anticorps. Cette observation pourrait suggérer un effet potentiel des Fe_3O_4 NP dans la régulation de la production de G-CSF ainsi que l'expression de son récepteur via la voie de signalisation de la β-Caténine. En ce qui concerne p53, il est bien établi que son activation se produit en réponse au stress cellulaire, ce qui conduit à sa phosphorylation par des MAPkinases, notamment p38 et JNK. Il est intéressant de noter que l'activation de p53 favorise l'expression de Mcl-1, une protéine anti-apoptotique critique chez les PMN. Dans le cadre de notre étude, nous avons observé une tendance dans l'augmentation de l'expression de Mcl-1 en présence des Fe_3O_4NP, ce qui pourrait être en corrélation avec l'expression de p53 mise en évidence par la technique HKA. D'autre part, une étude distincte a démontré que les Fe_3O_4 NP enrobées d'acide polyacrylique étaient capables aussi d'induire l'activation de p53 chez les PMN. Cependant, contrairement à notre étude, ces particules se sont révélées d'induire l'apoptose. Finalement, dans notre étude, il n'est pas évident de savoir la voie de signalisation précise impliquée dans l'activation de NF-κB pour favoriser la libération des cytokines pro-inflammatoires. Une étude intéressante portant sur les monocytes exposés aux Fe_3O_4NP enrobées de dextran a révélé que ces particules induisaient la libération de TNF-α et IL-1β en activant les voies de signalisation JNK et ERK1/2, en effet ces deux voies sont connues pour être impliquées dans la promotion de l'activation de NF-κB . Il est important

de noter que l'activation de la voie JNK est également associée à la production de ROS et à la nétose, particulièrement chez les PMN traités par le LPS.

À la lumière de l'ensemble des résultats obtenus dans ce projet de thèse, nous formulons l'hypothèse que les Fe_3O_4 NP favorisent l'expression de p53 au sein des PMN. Cette activation à son tour, active plusieurs mécanismes de signalisation qui contribuent à renforcer la disponibilité de la Lfr. Par conséquent, la dégradation de Mcl-1 est empêchée, ce qui entraîne une augmentation de la viabilité des PMN ainsi que leur capacité à persister à la surcharge en fer induite par la présence des Fe_3O_4 NP.

En résumé, nos résultats démontrent que les Fe_3O_4 NP modulent la réponse des PMN d'une manière qui est à la fois immunostimulante (ex. libération de cytokines pro-inflammatoires) et immunosuppressive (ex. altération de la flambée oxydative). Leur impact sur l'apoptose spontanée des PMN suggère qu'elles pourraient contribuer à la persistance des PMN dans un environnement inflammatoire. Cependant, cette observation ne devrait pas être perçue uniquement comme un inconvénient. En réalité, la capacité des Fe_3O_4 NP à activer les PMN pourrait être exploité dans des applications prometteuses, telles que le développement d'adjuvants vaccinaux. En effet, des études antérieures ont montré que certaines NP augmentent significativement la production d'anticorps par rapport aux adjuvants classiques à base d'aluminium. De plus, la capacité des Fe_3O_4 NP à améliorer la phagocytose pourrait être utilisée comme une stratégie innovante pour renforcer la fonction phagocytaire des PMN chez les patients souffrant d'infections récurrentes, notamment ceux atteints de déficiences immunitaires. Cependant, il convient de souligner que des études supplémentaires sont nécessaires avant de poursuivre de telles applications prometteuses. En effet, il n'est pas encore évident quel rôle d'autres facteurs, tels que la biocorona, jouent dans la médiation des effets modulateurs observés des Fe_3O_4 NP chez les PMN. Bien qu'il soit clair que ces NP interagissent avec le sérum et les différents éléments présents dans un milieu physiologique, il reste encore à déterminer dans quelle mesure ce "changement d'identité " est impliqué dans les effets observés, ce qui nécessite des investigations approfondies et indépendantes.

1.6 Les limites de l’étude

L'interprétation des résultats de ce projet de thèse doit être faite en tenant compte de certaines contraintes et considérations importantes. Premièrement, il est essentiel de noter que les Fe_3O_4 NP utilisées ont été achetées en suspension dans l'eau. Cependant, il est évident que leur stabilité dans les milieux de culture cellulaire est compromise, car elles ne sont pas enrobées. En conséquence, elles ont tendance à former des agrégats à l'échelle du micromètre plutôt que de maintenir leur forme nanométrique. Par conséquent, les cellules pourraient être exposées à des microparticules (>1000 nm) plutôt qu'à des NP (<100 nm), ce qui pourrait potentiellement conduire à des résultats différents. En effet, les micrographies obtenues par TEM montrent clairement que les Fe_3O_4 NP internalisées sont présentes à la fois sous forme individuelle et sous forme d'agrégats dans le cytoplasme des cellules. Deuxièmement, il est important de noter que l'évaluation des ions ferreux n'a pas été effectuée dans notre étude. Ces ions pourraient potentiellement être générés après l'internalisation des Fe_3O_4 NP par les cellules et pourraient contribuer aux effets observés, plutôt que les Fe_3O_4 NP intactes elles-mêmes. En effet, les NP métalliques sont connues pour pouvoir subir une dissolution dans les lysosomes après leur internalisation par les cellules. Troisièmement, il faut noter que les expériences menées dans cette étude n'ont pas suivi des méthodes normalisées pour l'évaluation de la toxicité des nanomatériaux, telles que les procédures opérationnelles standard basées sur les directives de l'Organisation de coopération et de développement économiques (OCDE) ou de l'Organisation internationale de normalisation (ISO). Cette situation s'explique principalement par le manque de protocoles normalisés spécifiques à l'évaluation de la toxicité des NP, en particulier en ce qui concerne leur interaction avec le système immunitaire. Par conséquent, le partage de données précieuses peut être entravé, sans nécessiter de consacrer les ressources pour la mise en place d'études similaires dans l’avenir. Enfin, il est crucial de noter que la variation des réponses cellulaires provenant de différents donneurs a été observée lors des analyses. Cela peut être attribué à des facteurs incontrôlables tels que le ratio de PMN aux éosinophiles après l'isolement, le régime alimentaire des participants, leur niveau d'activité physique, etc. Cette variation nécessite une interprétation prudente des données et souligne l'importance d'augmenter le nombre d'échantillons analysés pour renforcer la robustesse des conclusions.

1.7 Conclusion

Le développement et l'utilisation des Fe_3O_4 NP dans divers domaines est motivé par leurs propriétés physico-chimiques, ce qui les rend très attrayantes pour des applications en nanomédecine. Cependant, notre recherche a soulevé des préoccupations quant à leur innocuité. Nous avons démontré que les Fe_3O_4 NP d'un diamètre d'environ 10 nm sont internalisées par les PMN qui sont considérés comme la première ligne cellulaire de défense de notre organisme. Nos résultats ont également montré que ces NP peuvent moduler les fonctions biologiques des PMN, notamment l'apoptose spontanée, la production de cytokines pro-inflammatoires et l'activation de voies de signalisation cellulaire telle que la voie Syk impliquée dans la phagocytose. Il est bon de rappeler ici que la réponse inflammatoire, dans laquelle les PMN jouent un rôle crucial, est une réaction de défense physiologique normale qui peut ne pas être initialement considérée comme étant un état pathologique en présence des Fe_3O_4 NP. Cependant, une exposition prolongée à ces particules, dans des scénarios d'exposition chronique tels que les milieux de travail où elles sont produites en grandes quantités, pourrait potentiellement conduire à une activation immunitaire pathologique persistante. Cette situation pourrait avoir des répercussions négatives sur la santé des travailleurs, leur qualité de vie et leur productivité, entraînant des charges économiques pour la société (ex. taux élevé d'absentéisme) et mettant à l'épreuve le système de santé. Par conséquent, il est impératif de mettre en œuvre des stratégies de précaution telles que des approches de conception sécuritaire (*safe-by-design*) durant la production, l'utilisation et l'élimination de ces NP pour limiter leurs effets potentiellement nuisibles sur l'organisme humain et l'environnement.

1.8 Perspectives

En perspective, une exploration approfondie d'autres mécanismes permettra d'améliorer notre compréhension de l'effet immunomodulateur observé. Quelques orientations potentielles pour des recherches futures pourraient être les suivantes :

- Examiner le rôle des protéines de la biocorona dans la réponse des PMN aux Fe_3O_4 NP.
- Évaluer le rôle de Lfr dans la contrebalance des effets de Fe_3O_4 NP sur les PMN.

- Identifier les protéines induites par le mécanisme de synthèse *de novo* en présence de Fe_3O_4 NP.
- Analyser des Fe_3O_4 NP de diamètres plus petits ou de formes différentes (ex. 5 nm ; cubiques) afin de déterminer si la forme, le diamètre ou la composition qui joue le rôle le plus crucial dans l'immunomodulation des PMN.
- Examiner les fonctions biologiques des PMN dérivés des travailleurs exposés aux Fe_3O_4 NP dans les milieux de travail.
- Explorer le potentiel des Fe_3O_4NP en tant qu'adjuvants dans le développement de vaccins, en évaluant leurs effets modulateurs sur d'autres types de cellules immunitaires, comme les éosinophiles, les macrophages, les cellules dendritiques et les lymphocytes.
- Évaluer l'impact de l'internalisation des Fe_3O_4 NP par les PMN sur l'efférocytose par les macrophages, afin de comprendre dans quelle mesure ces particules peuvent interférer avec la résolution de la réponse inflammatoire.

2 INTRODUCTION

2.1 Background

In 1960, scientists began discussing the potential for intervening at the nanometer scale (approximately 10 atoms) to arrange atoms and create new materials with specific properties that could have a broad range of applications (Whatmore 2006). This ambitious idea was a breakthrough for many fields, following Feynman's statement "There is plenty of room at the bottom". As a result, nanotechnology emerged as a new field of science dedicated for studying miniaturization (Feynman 1992). Investing in nanotechnology has become a national priority for many countries, as it offers potential solutions to various issues, including water pollution (Talebian et al. 2021), cancer treatment (Jin et al. 2020), and microbial resistance to antibiotics (Lee, Ko, and Hsueh 2019). Indeed, nanotechnology has demonstrated its effectiveness in addressing outbreak challenges, particularly in vaccine development, as seen most recently during the COVID-19 pandemic. Lipid nanoparticles (NP), for example, have played a significant role in the development of vaccines against SARS-CoV-2. These particles effectively mimic the viral structure and facilitate the delivery of specific molecules (e.g., mRNA) to intracellular compartment. Once inside the cell, they reach to be translated into viral proteins, preparing the host immune system to mount a robust immune response against the virus during an infection. The success of this application has revitalized the field of nanomedicine, which had experienced a period of self-doubt, especially regarding its applications in oncology (i.e., cancer treatment) as many promising applications have not yet reach relevant clinical translation (Park 2019). Nonetheless, many people still believe that nanotechnology will have a positive impact on their lives, particularly in the field of medicine (Joubert et al. 2020).

NP are a central focus of nanomedicine, particularly magnetic NP. Scientists are indeed drawn to these particles due to their unique physical properties. For instance, the core hardness of magnetic NP can provide support for active molecules like antibodies, and their magnetism allows for targeted delivery to specific sites. In fact, the clinical translation of magnetic NP is already in progress, with applications in hyperthermia, magnetic resonance imaging (MRI), the diagnosis of cancer cells, as well as the treatment of anemia (Geppert and Himly 2021; Frenea-Robin and Marchalot 2022; Grauer et al. 2019). Despite the promising progress in using NP for translational purposes in nanomedicine, many scientists have raised concerns about their potential risks. Even though much research has been focusing mainly on addressing the short-term side effects of NP,

there is still a lack of research in understanding the mechanism of action associated with their potential toxic effects. Furthermore, before considering benefits, prioritizing risk assessment is crucial. Paradoxically, for many years, the risk assessment of NP was overlooked until scientists introduced nanotoxicology as a novel branch of science that deals specifically with various aspects related to NP risk assessment (Oberdörster, Oberdörster, and Oberdörster 2005).

Understanding the fate of NP in biological systems is crucial, as it is linked to the well-known paradigm of asbestos and silica particles of tiny size that induce an abnormal inflammatory response in the lung (Riediker et al. 2019). Therefore, it is essential to comprehend the mechanisms by which NP interact with immune cells. This understanding will help to establish preventive measures and more stringent regulations if they entail more risks than benefits. For example, magnetic NP have been approved for the treatment of anemia in patients suffering from chronic kidney disease under the brand name of Feraheme® (Fishbane et al. 2012). However, its clinical use has been linked to severe and potentially life-threatening allergic reactions in some patients, which raises concerns about its safety (Rubin 2015). This example highlights the importance of considering the toxicological aspects of using NP in nanomedicine, especially their potential to induce an inflammatory response in which polymorphonuclear leukocytes (PMN) play a crucial role. Therefore, the main objective of this thesis, which is based on a research study project, is to address this issue and understand how magnetic NP, specifically iron oxide nanoparticles (Fe_3O_4 NP) modulate the biological functions of PMN.

This thesis is divided into several sections. The first section is a literature review that explores the characteristics of magnetic NP and why they are of interest for use in nanomedicine. The second section focuses on the potential risks of exposure to NP and their interactions with PMN in the context of the inflammatory response. The third section presents a published article by our research group that examines the effects of Fe_3O_4 NP on PMN. This will be followed by a general discussion and provide perspectives for future research. Finally, the thesis will be concluded with an appendix section in which we present our published review article that offers an up-to-date overview of the effects of magnetic nanoparticles on other primary immune cells.

Note: *In this thesis, iron oxide nanoparticles refer specifically to Fe_3O_4 NP. However, magnetic/iron nanoparticles (Fe NP) refer to any particles that are iron-based, whether coated or not with other components.*

3 LITERATURE REVIEW

3.1 Iron nanoparticles (Fe NP)

3.1.1 Definition of nanoparticles

Nanoparticles (NP) are the tiniest materials that can be produced at the scale of a nanometer (nm). It can be challenging to establish a definitive definition of NP as their properties, such as size, chemical composition, shape, dimensionality, and origin (i.e., method of synthesis), can vary significantly (Figure 1) Despite this, size, origin, and composition are extensively used for their classification (Strambeanu et al. 2015). However, size is considered the most commonly used criterion in defining NP, and it should be limited to 100 nm in two or three dimensions (Auffan et al. 2009; Haase, Tentschert, and Luch 2012). Notably, nanoscale entities can occur naturally, such as ferritin in the biological system, which has a size of 12 nm and is used by cells to store iron (Stanley 2014). On the other hand, anthropogenic (human-made) NP are produced using a controlled process to achieve specific properties, as is the case with many inorganic NP including magnetic NP (Mansoori 2005).

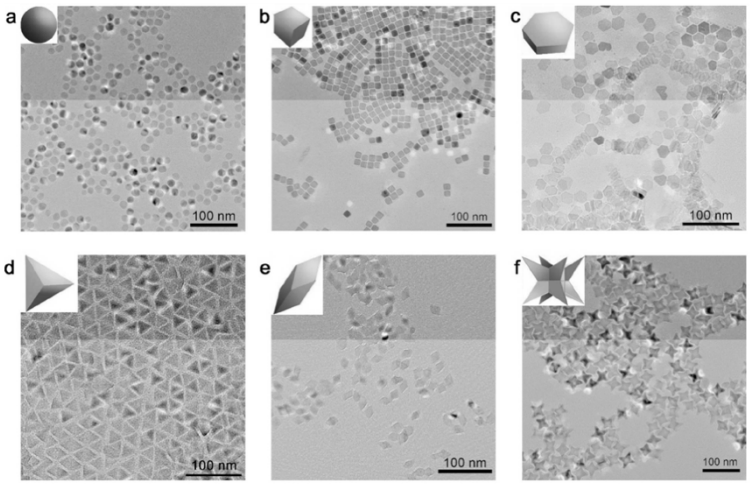

Figure 1: TEM images of monodispersed manganese-doped iron nanoparticles with different shapes.

This figure shows the diversity of shapes that NP cloud have, in this case of manganese (Mn)-doped iron (Fe) NP with Mn/Fe ratio of about 1/6 (a) spheres (diameter of 15 nm), (b) cubes (side length of 12 nm), (c) plates hexagonal (side length of 12 nm and thickness of 5 nm), (d) tetrahedrons (regular, side length of 25 nm), (e) rhombohedra (oblique parallelepiped, side length of 13.5 nm with a tilt angle of 60 degree) and (f) octapods. Adapted from (Yang et al. 2018).

3.1.2 Iron nanoparticles synthesis

NP can be produced by two main approaches: The bottom-up approach and the top-down approach. In the bottom-up approach, NP are synthesized using chemical reactions in which defined chemicals react to produce NP in a self-assembly manner. On the other hand, the top-down approach uses mechanical methods (i.e., mechanical pressure) to reduce bulk materials into smaller particles. This approach has the advantage of being eco-friendly due to its chemical solvent-free process, but it tends to be more costly than the bottom-up approach (Dhand et al. 2015). Nevertheless, both approaches are used to synthesize magnetic NP (Fe NP). The bottom-up approach is based on the co-precipitation method, which is the most commonly employed and cost-effective technique for the high-yield synthesis. However, this technique produces a broad particle size distribution, which is less desirable since size is considered to be the most critical parameter for nanomedicine applications (Dadfar et al. 2020). The top-down approach for Fe NP production relies on the ball milling technique, which involves scattering solid particulate into smaller particles in a rotating cylindrical chamber using a specific grinding media. The size of the produced particles by this method can range from 12 to 20 nm (de Carvalho et al. 2013). In fact, the size of the final product significantly depends on the speed by which the cylinder rotates, the composition of the grinding media (i.e., hardness of the balls), the ratio of the milled material to the grinding media, as well as temperature and pressure. A change in any of these factors can significantly affect the size of the final product (Calderón Bedoya et al. 2023). Furthermore, controlling the shape of NP can be achieved by adjusting the amount of the stabilizer added during the synthesis process. For example, in the case of oleic acid added to the main iron oleate complex (oleic acid mixed with iron chloride), it is used for producing spherical but not cubic Fe NP. Interestingly, increasing the concentration of oleic acid diminishes the particle growth rate, resulting in spherical-shaped Fe NP (Zhen et al. 2011).

In general, scientists are interested in three types of Fe NP: maghemite (γ-Fe_2O_3), magnetite (Fe_3O_4), and hematite (α-Fe_2O_3). Among these, magnetite is the most promising for use in nanomedicine due to its proven biocompatibility. However, there is still limited evidence regarding its potential toxicity (Lodhia et al. 2010). The diversity in NP shape, size, and composition is closely related to the different methods used for their production, as well as the concentration of precursor materials. This vast range of possibilities means that there is a virtually unlimited variety of NP that can be synthesized. As a result, scientists are always working to develop more reliable methods that can produce Fe NP with well-defined sizes and shapes. This would help to ensure

consistency in production from batch to batch and enable more reliable use of these NP in a variety of settings.

3.1.3 Properties of iron nanoparticles

NP exhibit unique properties compared to their bulk materials counterparts due to their reduced size. This phenomenon is particularly interesting since the fundamental building blocks of bulk materials are atoms that interact to create the physical and chemical properties of the material. As the size of a material decreases, the number of exposed atoms and functional groups on the surface increases due to the increase in the surface-to-volume ratio. Therefore, when the particle size drops below 10 nm, the surface area of the material increases exponentially. For example, a spherical iron crystal with a size of 1000 nm has approximately 1% of its atoms on the surface, while a similar particle with a size of 1 nm has more than 85% of its atoms exposed on the surface (Kenneth J. Klabunde 2009).

Superparamagnetism is one of the critical properties of Fe NP that has attracted scientists for biomedical applications. Interestingly, the size of Fe NP is crucial, as they display superparamagnetic properties only when their size is below 20 nm (Dadfar et al. 2020; Kaur et al. 2016). Specifically, superparamagnetic NP are characterized by remanence, which is the residual magnetic effect after magnetization, and coercivity, which is the magnetic effect necessary to neutralize the magnetization that quickly become negligible in absence of an alternating magnetic field (AMF). In other words, superparamagnetism is the complete dependence of Fe NP on AMF. Without an AMF, superparamagnetic particles cannot exhibit any magnetic behavior, which limits their movement to a specific location. This property is crucial in nanomedicine applications because it offers a reliable way to control and direct these particles towards a specific organ or tissue (Maldonado-Camargo, Unni, and Rinaldi 2017). Moreover, the ability of superparamagnetic NP to generate heat when exposed to an AMF is attributed to Néel and Brownian relaxation phenomena. The AMF induces a ‘’rotation’’ process known as the Néel relaxation mechanism, resulting in the flipping of the particle magnetic moment vector without altering the orientation of the particle itself. In addition, under the AMF the particles undergo a ‘’friction’’ process known as the Brownian relaxation mechanism, which is associated with the rotation of the particle against viscous forces (Maldonado-Camargo, Unni, and Rinaldi 2017). These mechanisms facilitate the release of heat upon removal of the AMF, making it a promising approach for cancer treatment through hyperthermia (Kolhatkar et al. 2013).

3.1.4 Characterization of iron nanoparticles

To ensure that Fe NP are suitable for their intended applications, their characterization is crucial during the early production phase. Transmission electronic microscopy (TEM) is the most reliable method to determine the size and shape of NP. This technique provides a measurement of the total particle size, which corresponds to the core of the Fe NP. On the other hand, dynamic light scattering (DLS) is a cost-effective technique that determines the hydrodynamic size of NP in a dispersion medium such as Milli-Q water. This method not only determines the core size and its monolayer but also analyzes the surface-bound layer that contains ions with the opposite charge of the measured particles (Dadfar et al. 2020; Lodhia et al. 2010). Furthermore, the Zetasizer, which determines the hydrodynamic size of particles, can also provide information on the polydispersity index (PDI), a value that reflects the degree of size uniformity among particles, ranging from 0 to 1, with a lower PDI value indicating a more homogeneous particle size distribution. Therefore, a PDI value closer to 0 suggests a more uniform particle preparation (Danaei et al. 2018). Moreover, the zeta-potential, which can also be determined using the Zetasizer, is used to determine the charge of the particles. This property provides information about the tendency of particles to flocculate, which is a weak non-covalent aggregation, in a dispersion medium. Generally, absolute values greater than - or + 30 mV in a suspension indicate good colloidal stability and a low tendency for flocculation (Vassallo et al. 2023). In other words, high zeta potential values, either positive or negative, imply highly charged particles that do not flocculate due to the similar electric charges that generate a repulsion force. However, with a decrease in the zeta potential value (approaching to 0 mV) , the attraction of particles overcomes the repulsion force, increasing their flocculation and impairing their stability (Samimi et al. 2019).

3.2 Applications of iron nanoparticles

3.2.1 General applications

Fe NP offer a broad spectrum of applications; from an environmental perspective, they can be used in wastewater treatment to separate heavy metals, as this approach is of great potential due to its effectiveness and low cost. Indeed, the introduction of Fe NP in metal-polluted water triggers a swift interaction due to their magnetism with metals, which helps to adsorb a tremendous amount of metal elements quickly on their surface (Matei et al. 2016). Additionally, these particles can be used as a reliable, cost-effective detection system to reveal toxic compounds, which offers

a valuable tool for monitoring specific contaminants with high selectivity and sensitivity (Falak and Huh 2023). Interestingly, the use of Fe NP in the food industry is increasing, such as in food packaging, as they can be used as a tiny packaging layer that reacts as an oxygen scavenger, which limits oxygen effects on food, as oxygen is well known to reduce the shelf life of food products (Góral et al. 2023).

3.2.2 Medical applications

While Fe NP have various applications, their most promising uses are observed in biomedical fields, particularly in magnetic resonance imaging (MRI) and hyperthermia.

1.1.1.5 Magnetic resonance imaging

MRI is a non-invasive imaging technique commonly used in clinical settings to detect pathological conditions in soft tissues such as the liver and brain. Despite its benefits, MRI has limited sensitivity in revealing certain diseases due to weak image contrast (Estelrich, Sánchez-Martín, and Busquets 2015). To improve disease detection, contrast agents are often used. One of the most commonly used contrast agents is gadolinium-based chelates, which is a T1 contrast agent. However, the safety of this complex has been called into question, particularly in individuals with renal impairment, as it tends to bioaccumulate in tissues, including the brain and bone structure. The toxicity of this complex is attributed to its ions (Gd^{3+}), which can compete with calcium in biological mechanisms (Rogosnitzky and Branch 2016).

The mechanism by which contrast agents act in MRI is based on shortening the relaxation time of water protons in tissues. Essentially, when a tissue is exposed to a radio frequency pulse under a magnetic field, the water protons of the tissue are excited. As time elapses, the excited protons return to their ground state (relaxation time), which is associated with a release of energy that differs between tissues (normal versus pathological), and this energy is recorded to create an MRI image. Contrast agents promote energy release by enhancing the interaction between the contrast agent metal ions and the water molecules protons. In other words, the rate by which excited protons return to equilibrium is known as T1 and T2. T1 signal results in a brighter contrast (positive contrast) improvement, while the T2 signal results in a dark contrast (negative contrast) enhancement. In both cases, the use of contrast agents improves the sensitivity of MRI (Jeon et al. 2021). T1 contrast agents are highly preferred in clinical settings as they are easy to identify

and do not disturb the background of the MRI images, which helps to avoid false-positive detection in some pathological conditions like bleeding. Some clinically approved T2 contrast agents based on Fe NP have been withdrawn due to this reason (Shen et al. 2017). Despite that, Fe NP are considered a ‘’better’’ alternative to highly toxic contrast agents, like gadolinium. In fact, several Fe NP-based contrast agents have been approved by the Food and Drug Administration (FDA). Their use in MRI is attributed to their long circulating half-life, which can considerably reduce patients exposure to repetitive doses in a short time. Additionally, they can be easily functionalized with other molecules, such as probes, which can greatly enhance their imaging potential (Weissleder et al. 1989; Jin et al. 2014).

Ferumoxide was one of the earliest Fe NP used as a contrast agent, approved in 1996 for imaging of hepatocellular carcinoma. Interestingly, normal liver tissue tends to accumulate Fe NP, leading to a negative signal, while tumor tissue does not internalize these particles, resulting in a positive signal that can be easily detected (Thakor et al. 2016). While the number of Fe NP approved for MRI applications was growing, such as Ferumoxil for gastrointestinal tract imaging, Ferumoxides and Ferucarbotran for liver imaging, and Ferumoxtran-10 for lymph node imaging, many safety concerns and lack of efficacy were raised. As a result, Ferumoxil, Ferumoxide, Ferucarbotran, and Ferumoxtran-10 are all withdrawn from the market. For instance, Ferumoxides use was associated with hypotension and pain, primarily in the lumbar region, with an overall incidence rate of 10%. Ferucarbotran main adverse effects were vasodilation and paresthesia (i.e., abnormal skin sensation), while Ferumoxtran-10 was withdrawn due to a high false positive rate that could be harmful to disease management (Wang 2011). It is surprising to know that the only Fe NP that are still approved is Ferumoxytol (Feraheme®). The FDA has only approved its use for treating anemia in chronic kidney disease patients. Moreover, due to its long blood-circulating half-life of approximately 15h, the use of these particles as a contrast agent in imaging is attracting significant attention (Daldrup-Link 2017). Nonetheless, there has not been an official approved statement yet regarding their use in this context (Thakor et al. 2016; Geppert and Himly 2021). This is likely because of the lack of comprehensive clinical data about their safety. Indeed, some patients have reported hypersensitivity reactions to Ferumoxytol, but the reason behind such allergic reaction is still unknown (Pai and Garba 2012).

1.1.1.6 Hyperthermia therapy

In recent years, hyperthermia (HT) has emerged as a promising intervention for cancer treatment. This approach involves using Fe NP exposed to an alternating magnetic field (AMF) to generate heat. The heat produced can reach temperatures ranging from 41 to 48 °C, which can disrupt cancer cell proliferation by inhibiting DNA synthesis, leading to cell death. Moreover, increasing temperatures could make cancer cells more vulnerable to the effects of radiation and various chemotherapeutic drugs. Thus, HT can serve as an effective supplementary intervention to standard cancer therapies (Kaur et al. 2016). Recently, researchers have produced Fe NP that can generate a heat performance of 50 °C when exposed to an AMF, significantly increasing their effectiveness in HT clinical applications (Demessie et al. 2022). HT has demonstrated promising clinical outcomes in the treatment of brain cancer, prostate cancer, and esophageal cancer. The only Fe NP approved for HT are marketed under the brand name NanoTherm®. These 15 nm particles are coated with aminosilane and approved for treating glioblastoma (an aggressive and fast-growing brain tumor) and prostate cancer (Rodríguez et al. 2022).

3.3 Exposure and risk assessment of nanoparticles

3.3.1 Exposure to nanoparticles

The exposure of humans to NP can originate from various sources, including products that contain nanomaterials, nano-based drugs used in clinical settings, and unbound NP that become aerosolized in the workplace environment for example. Many studies have confirmed the existence of occupational exposure, and exposure to Fe NP is particularly common during reactor cleaning processes. Even when a local ventilation system is present, workers can still be exposed to Fe NP in their breathing zone, with exposure levels reaching up to 335 $\mu g/m^3$ in the absence of such a system (Debia et al. 2016). Such exposure can occur through inhalation, absorption through the skin or eyes, or ingestion. Indeed, inhalation is the primary route of exposure in occupational settings, owing to the large surface area of the lungs (Mihalache et al. 2017; Sarah Z. Wang 2016). Interestingly, a predictive model has suggested that particles with a diameter of 20 nm tend to accumulate deeply in the lungs alveolar region, whereas larger particles (>10 µm) are primarily trapped in the upper respiratory tract, such as the pharynx (Buzea, Pacheco, and Robbie 2007).

In the clinical setting, the primary route of exposure to Fe NP is intravenous administration. In fact, this approach has already been used with Ferumoxytol, an Fe NP-based drug with a size of 17–31 nm, prescribed for treating iron deficiency anemia in patients who cannot tolerate oral iron agents. Furthermore, this method is commonly employed in MRI and cancer treatments. Generally, the Fe NP used in clinical setting are coated with carbohydrate components like dextran to enhance their stability and slow the release of free iron into the bloodstream, which is highly beneficial for patients with renal impairment (Huang et al. 2022).

3.3.2 Risk assessment of nanoparticles

The complexity of establishing standardized occupational exposure limits (OELs) for NP is a complex issue. The European Chemicals Agency defines OELs as a set of regulatory values that indicate safe levels of exposure (health-based) for a chemical substance in the air of a workplace to prevent workers from inhaling chemicals and reduce their chemical exposure (ECHA 2023). It is evident that this definition is addressed to chemical exposure and not to NP. In fact, for NP, there is no defined and standardized tool due to their diversity, the lack of scientific understanding of their behavior in biological organisms, and the absence of reliable techniques for nanoscale measurements (Mihalache et al. 2017).

Moreover, directly translating established toxicological regulatory assessments for chemical compounds to NP may not guarantee their safety, because NP exhibit physical properties that are significantly different from their bulk counterparts (Buzea, Pacheco, and Robbie 2007). As a result, the complexities associated with the diversity of size, shape, and other characteristics of NP make it challenging to predict their potential toxic effects. In the absence of firm toxicological evidence, the World Health Organization (WHO) recommends a control banding approach (WHO 2017). This low-cost intervention method combines NP composition parameters, such as size, shape, and surface activity, with their dustiness (dry versus wet) and the produced quantity. Each combination is linked to a risk level ranging from 1 to 4, which helps define a risk level score for each NP preparation (Paik, Zalk, and Swuste 2008).

3.3.3 Potential risks associated with exposure to nanoparticles

It is undeniable that humans are exposed to anthropogenic NP, making it essential to understand their fate in the body. The case of asbestos tiny particles is a well-known example model of the potential risks that can be presented by NP (Whatmore 2006). Asbestos exposure has been linked to lung cancer (mesothelioma), with the pathological mechanism underlying this type of cancer related to macrophages (MΦ). Normally, MΦ would eliminate tiny asbestos fibers in the alveoli via phagocytosis, but their long diameter prevents complete engulfment. As a result, MΦ undergo a process called 'frustrated phagocytosis,' leading to sustained generation of reactive oxygen species (ROS) and persistence of the inflammatory response, ultimately resulting in cancer development (Riediker et al. 2019). Furthermore, exposure to tiny silica particles is associated with silicosis (pulmonary fibrosis), which results from a persistent inflammatory response induced by alveolar MΦ (Mischler et al. 2016).

The potential risks associated with NP exposure are correlated with their size, shape, and composition. One of the earliest studies assessing the toxicity of NP focused on titanium NP (TiO_2 NP) with sizes of 20 nm and 250 nm. After a subchronic inhalation toxicity study (approximately 3 months of exposure), bronchoalveolar lavage of exposed rats showed cell infiltration primarily composed of PMN and MΦ, indicating the establishment of inflammation. Notably, particles measuring 20 nm were found to be more hazardous than those measuring 250 nm (Oberdörster, Ferin, and Lehnert 1994). In another study, rats instilled with carbon black NP with a size of 14 nm showed a significant increase in the number of PMN in their lungs. Interestingly, similar particles with a size of 320 nm did not exhibit any inflammatory activity (Brown et al. 2000). Moreover, a study demonstrated that TiO_2 NP with a fiber-like shape are more hazardous for MΦ than spherical ones, as they have shown to induce a specific type of cell death (Hamilton et al. 2009). Although Fe NP are considered to be biocompatible, their safety cannot be guaranteed due to the fact that biocompatibility does not imply a lack of toxicity. Biocompatibility, is defined as the ability of a biomaterial (e.g., an artificial cardiac pacemaker) to function as intended for medical therapy without causing any unwanted local or systemic effects in the recipient. Conversely, toxicity refers to the capacity of a biomaterial/particles to adversely impact the normal physiological functions of cells, tissues, and organs in a particular organism (Li et al. 2012). In other words, certain NP may exhibit biocompatibility while still being able to influence the biological activities of cells, such as the ability of some NP to trigger immune cell suppression or

activation. To clarify that, Feraheme® is an iron-based NP that has been approved for the treatment of anemia in patients with chronic kidney disease. However, it has been found to be unsafe for some patients despite it is based on a biocompatible element (iron), as some patients have developed serious and potentially life-threatening allergic reactions following its intravenous injection (Rubin 2015). Another example, is an iron-based NP known under the brand name NanoTherm®, which has been developed for cancer treatment by hyperthermia. The use of these NP is associated with a high accumulation of pro-inflammatory cells, specifically PMN in the treated tumor area. This presents a double-edged sword situation because while the presence of PMN can trigger a robust immune response against tumor cells and help control cancer progression (Grauer et al. 2019), their activation suggests that these NP may have the potential to modulate the profile of immune cells towards a pro-inflammatory phenotype.

3.3.4 Toxicity assessment of nanoparticles

Although animal models are commonly used to evaluate the toxicity of NP, the inflammatory response differs between mice and humans because of the intricate pathways involved. This variation can be attributed to the absence of certain cytokines, such as IL-37 and the chemokine IL-8, in mice that are normally produced by primary human PMN. Furthermore, the percentage of PMN in mice is considerably lower than in humans (Table 1). As a result, it is challenging to directly apply the toxicity outcomes obtained from animal models to humans (Hidalgo et al. 2019). On the one hand, *in vitro* models are commonly utilized to investigate the ability of NP to modulate immune cells due to their cost-effectiveness and easy maintenance when compared to *in vivo* models. Immortalized cell lines, which have unlimited cell division, are extensively used *in vitro*. However, they may not be perfectly suitable for risk assessment studies since some cell characteristics might be altered (e.g., expressions of immune markers) with each cell division. Moreover, they may react differently than normal cells and are prone to mycoplasma contamination, resulting in potential biases in toxicological outcomes. On the other hand, primary cell lines are highly sought after as they are capable of producing a response that can be replicated *in vivo*, which makes them a more reliable alternative to immortalized cell lines (Boraschi et al. 2021).

One of the primary challenges in conducting toxicological studies on NP is the presence of endotoxin contamination, specifically lipopolysaccharide (LPS), which can lead to inaccurate interpretation of toxicological outcomes. This means that the observed inflammatory response

effect could be wrongly attributed to LPS instead of NP (Boraschi et al. 2017). For instance, a study revealed that endotoxin-contaminated particles induced the production of pro-inflammatory cytokine IL-1β, while endotoxin-free particles of similar characteristics did not stimulate any response in human primary monocytes (Mangini et al. 2021). Therefore, it is essential to evaluate endotoxin contamination in NP. Indeed, the Limulus amoebocyte lysate (LAL) assay is commonly used for this purpose (Mangini et al. 2021).

Table 1: Main differences between murine and human neutrophils

Property	Murine neutrophils	Human neutrophils
Percentage of neutrophils in peripheral blood	10–25%	50–70%
Neutrophil size	8.64 ± 0.14 μm	10.39 ± 0.19 μm
Nuclear morphology	Ring-like	Segmented
Neutrophil granule contents	Defensins absent; low expression of BPI, MPO, β-glucuronidase, lysozyme, alkaline phosphatase, and arginase-1	Defensins present; high expression of BPI, MPO, β-glucuronidase, lysozyme, alkaline phosphatase, and arginase-1
Chemokine and chemokine receptor expression	CCL6, CCL9, CXCL15, CCL12 in mice, but not humans	CXCR1, CXCL8, CXCL7, CXCL11, CCL13. CCL14, CCL15, CCL18, CCL23, CCL24/CCL26 in humans, but not mice
Neutrophil antigen expression	Express Gr-1 and Ly-6G	Absent
Different Fc receptor expression	Do not express FcαRI or FcγRI	Express FcαRI; inducible expression of FcγRI
Affinity of fMLF receptor	Low	High

This table illustrates that there are several distinct characteristics between human and murine neutrophils. For instance, human neutrophils express numerous cytokines, including CXCL-8, which is not expressed in murine neutrophils. Additionally, human neutrophils exhibit a higher expression of granule proteins compared to murine neutrophils. Adapted from (Hidalgo et al. 2019).

3.4 The Inflammatory response

Inflammation is a spectrum process that involves different components of the innate immune system. Typically, inflammation is considered to be a physiological response to external stimuli such as microorganisms and physical injury (i.e., sterile inflammation). However, if it persists as a result of permanent exposure to stimuli, it can lead to pathological conditions such as tissue damage. Therefore, tight regulation of the inflammatory response is necessary for defending and promoting tissue repair after eliminating foreign material and damaged cells (Figure 2).

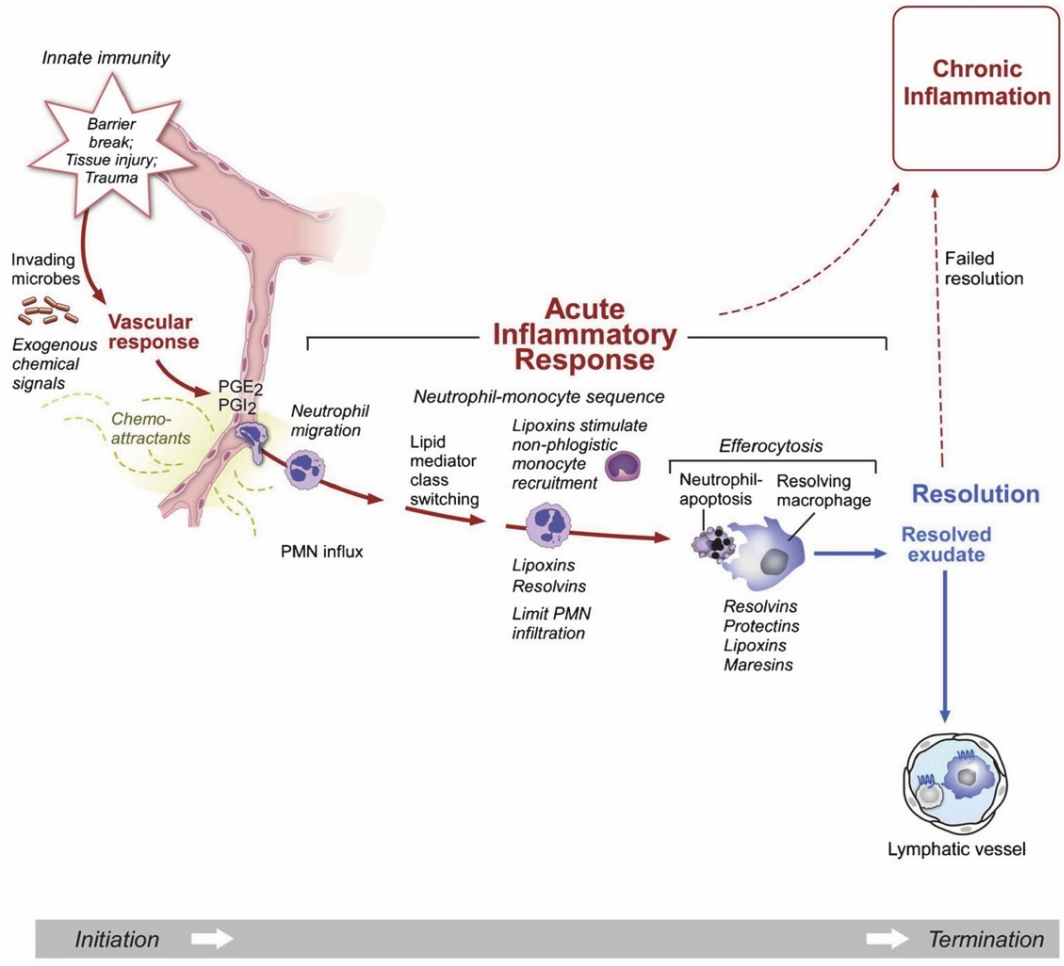

Figure 2: The sequence of events of the inflammatory response.

The inflammatory response takes place following tissue injury caused by physical, chemical, or microbial agents. This response begins with a vascular reaction that involves vasodilator prostaglandins (PGE2, prostaglandin E2; PGI2, prostacyclin). Subsequently, innate immune cells, mainly neutrophils (PMN) present in the blood circulation, intervene by following a chemotactic gradient of chemokines to reach the

inflammatory site in a cascade process known as neutrophil migration, which defines the acute phase of the inflammatory response. Then PMN, through their various functions (e.g., phagocytosis, degranulation), eliminate intruders (e.g., bacteria) and then undergo apoptosis in order to be cleared by macrophages in a process known as efferocytosis. Failure of this process can lead to chronic inflammation, that can induce a sustained tissue injury. Adapted from (Suthahar et al. 2017).

Inflammation has two arms to deal with foreign particles: resistance and adaptation. The resistance arm is synonymous with acute inflammation. However, if the foreign materials persist, the adaptation arm becomes dominant, leading to a chronic inflammatory response. The transition between both arms is linked to the magnitude and duration of the response (Medzhitov 2021). Inflammation is typically triggered by the detection of pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) through pattern-recognition receptors (PRRs) such as Toll-like receptors (TLRs) and NOD-like receptors (NLRs). PAMPs are markedly expressed within microorganisms, while DAMPs are produced by endogenous molecules (Jean-Marc Cavaillon 2018). Moreover, the inflammatory response is characterized by five cardinal signs, including redness, swelling, heat, pain, and loss of function. Each sign is associated with a specific step in the inflammatory process. Heat, redness, and swelling are linked to vasodilation induced primarily by histamine, which is necessary to increase blood flow and related plasma leakage into the extracellular matrix, thereby facilitating immune cells transmigration to the inflammatory site. On the other hand, pain is more related to the release of bradykinin, which also acts as a vasoactive substance produced through a complex mechanism involving plasma proteins like factor XII, prekallikrein, and kininogen proteins (Oschatz et al. 2011). Furthermore, this protein increases the sensitivity of nerve endings, which results in loss of function due to neurological reflex impairing in the inflamed tissue.

The inflammatory response consists of overlapping phases, and mast cells play a critical role in initiating it due to their abundance in most tissues and mucosal surfaces. These cells function as an early detection system that responds quickly to harmful foreign particles, producing various cytokines that mobilize PMN to the affected tissue (Johnston and Kubes 2001). During the acute phase, PMN are the first cells to intervene and eliminate the stimulus agent in response to mast cell inflammatory mediator release. If the stimulus agent persists, inflammation involving MΦ and lymphocytes may occur.

To reach the inflammatory site, PMN must cross the endothelium using adhesion molecules such as selectins (L-selectin and PSGL-1) and integrins. Endothelial cells express P-selectin and E-selectin, with P-selectin molecules presented to PMN by endothelial cells and activated platelets following inflammatory mediator release by mast cells (Dole et al. 2005; Germolec et al. 2018). P-selectin and E-selectin interact with P-selectin glycoprotein ligand-1 (PSGL-1) and L-selectin expressed by PMN, respectively, to facilitate PMN rolling. Integrins, on the other hand, promote cell-cell and cell-extracellular matrix interactions, facilitating PMN adhesion and transmigration between endothelial cells to reach the inflammatory site (Jean-Marc Cavaillon 2018).

The resolution of inflammation is a complex process that is controlled by immune cells, cytokines, and active molecules, including lipoxins and resolvins. MΦ are the primary cells involved in the resolution of inflammation, as they phagocytose apoptotic PMN and produce anti-inflammatory mediators such as IL-10, which promotes inflammatory suppression and thus promoting homeostasis. In addition to IL-10, some acute-phase proteins (APPs) such as α-1 antitrypsin also have significant anti-inflammatory properties (Netea et al. 2017). PMN clearance is essential during inflammatory resolution. Interestingly, after reaching an amount of internalized microbes/particles in the inflammatory site, PMN are prone to undergo phagocytosis-induced cell death (PICD) following activation of the nicotinamide adenine dinucleotide phosphate (NADPH) multimeric complex to destroy ingested microbes. PICD prompts MΦ to eliminate apoptotic PMN, which involves their assistance in promoting PMN cell death by producing cell-death-inducing ligands, primarily Fas ligand (FasL) and tumor necrosis factor (TNF)-α which they interact with Fas and TNFR1 PMN receptors, respectively (Murphy and Caraher 2015). The FAS-induced cell death mechanism involves the interaction of PMN Fas receptor with FasL expressed by MΦ, resulting in caspase-8 processing and cleavage of caspase-3. Consequently, this death mechanism is associated with mitochondrial cytochrome c and Second mitochondria-derived activator of caspases (Smac) release and involves the pro-apoptotic BH3-interacting domain death agonist (Bid) protein. The other mechanism of cell death involves TNF-α; however, the pro-apoptotic effect of this pro-inflammatory cytokine is correlated with its concentration and duration of cell stimulation. Interestingly, PMN response to TNF-α does not likely engage the canonical mitochondrial death pathway (i.e., the release of cytochrome c); however, it is demonstrated that TNF-α stimulation induces a rapid rise of reactive oxygen species (ROS) production, which is considered to be the underlying mechanism by which this cytokine induces apoptosis (Figure 3). Intriguingly, PMN derived from chronic granulomatous disease (CGD) patients, which have

impaired NADPH complex, do not undergo apoptosis under TNF-α stimulation, inferring the importance of ROS in this cell death mechanism (Geering and Simon 2011a).

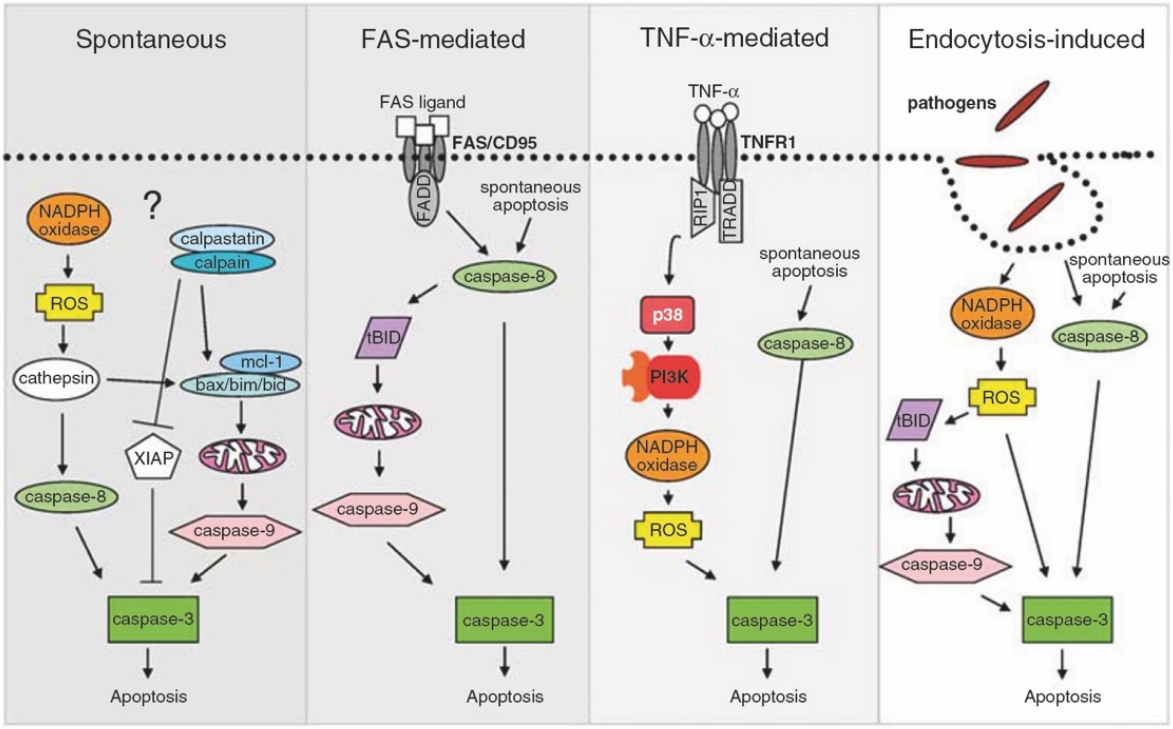

Figure 3: Modalities and molecular mechanisms of PMN apoptosis.

PMNs are known to have a high turnover due to spontaneous apoptosis, a mechanism that is still not fully understood. Furthermore, these cells can undergo an 'active' apoptosis process through a Fas-mediated and TNF-α-mediated mechanism. Interestingly, after phagocytosis of pathogens, PMNs can also enter apoptosis through a ROS-mediated mechanism. Adapted from (Geering and Simon 2011b).

3.4.1 Cytokines in the inflammatory response

Cytokines are proteins that act as messengers, facilitating communication between immune cells and promoting specific biological responses in other cells. They can function in either autocrine or paracrine mode and are characterized by their pleiotropic effect and redundancy. The former refers to the ability of a particular cytokine to elicit varying responses in different cells, while the latter describes the ability of multiple cytokines to generate similar responses in either the same

or different cells (Nicola 1994). Cytokines are essential for establishing a successful immune response, but their balance (Figure 4) and network are strictly regulated to prevent tissue damage.

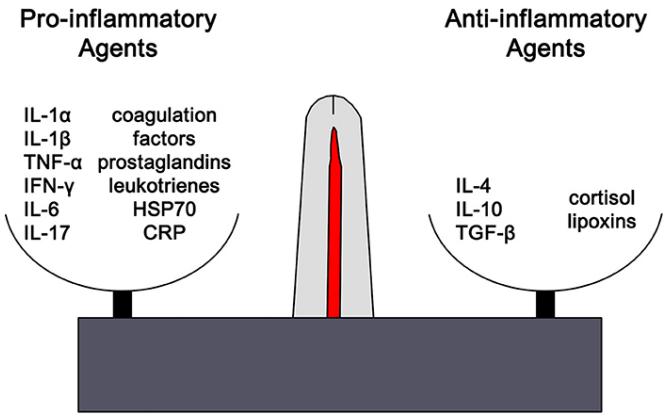

Figure 4: The key mediators involved in regulating the inflammatory response.

The outcome of any inflammatory response depends on the balance between pro-inflammatory and anti-inflammatory factors, each of which is regulated by different cytokines and hormones. For instance, IL-1β, IL-6, and TNF-α promote inflammation, while IL-10 and TGF-β inhibit it. Adapted from (Bennett et al. 2018).

Cytokines play a key role in regulating inflammation. While some cytokines such as IL-1β, IL-6, IL-8, IL-17, IFN-γ, and TNF-α promote inflammation, others including IL-4, IL-10, and TGF-β act as anti-inflammatory mediators. The balance of cytokines is tightly regulated, and dysregulation can contribute to the development of various diseases (Medzhitov 2021). Proinflammatory cytokines such as IL-1β, TNFα, IL-8, and growth factors G-CSF and GM-CSF mainly target PMN, which by themselves are capable of producing cytokines through both preformed granules and *de novo* synthesis mechanism (Jean-Marc Cavaillon 2018).

TNF-α and IL-1β are considered to be the early pro-inflammatory cytokines produced during inflammation as they play a central role in immune regulation; moreover, their release is associated with promoting the production of late pro-inflammatory cytokines, principally IL-6 and IL-8. The overproduction of TNF-α, IL-1β, IL-6 and IL-8 is potentially associated with sepsis (Gierlikowska et al. 2021). Specifically, IL-6 is recognized as a critical marker of this response

(Küster et al. 1998). Moreover, an increase in the IL-8 plasma concentration is presumably correlated with mortality during sepsis response (Marty et al. 1994).

IL-1 family has 11 different members of cytokines; the most prominent one is IL-1β, as this cytokine is known to stimulate the expression of adhesion molecules necessary for the recruitment of immune cells to the inflammatory site (Germolec et al. 2018). Interestingly, the production of IL-1β is contributed generally to the inflammasome activation. This intracellular complex is required to activate the pro-caspase-1, which is a crucial mediator in innate immunity; Indeed, activation of the inflammasome complex following NLRP1 and NLRP3 oligomerization promotes the production of pro-inflammatory cytokines pro-IL-1β and pro-IL-18 and their activation by caspase-1 [79].

On the other hand, TNF-α promotes a broad range of PMN functions, primarily by stimulating the expression of cell adhesion molecules, phagocytosis, respiratory burst, and degranulation (Yan and Novak 1999). This cytokine is also involved in upregulating the expression of the major histocompatibility complexes I and II, which are necessary for the adaptive immune response (Germolec et al. 2018). At low concentrations (≦1 ng/mL), TNF-α promotes PMN survival. However, at high concentrations (≧ 10 ng/mL), it induces apoptosis in these cell, which is thought to be another mechanism by which the inflammatory response is resolved (van den Berg et al. 2001). Indeed, as the response becomes prominent, the increase in TNF-α may act as a positive feedback loop for its resolution (Murphy and Caraher 2015). Nevertheless, it has been found that TNF-α can be produced by PMN and promote the production of IL-8, which acts to enhance PMN survival in an autocrine fashion (Cowburn et al. 2004).

Both IL-1β and TNF-α have autocrine effects that mainly activate MΦ (Lombardo et al. 2007; Jayaraman et al. 2013). Additionally, TNF-α and IL-1β can promote the expression of Fc gamma receptors (FcγRs) in PMN, which is essential for the phagocytosis of opsonized particles (Gierlikowska et al. 2021). Furthermore, when their production is sufficiently high, they act on hepatocytes to promote the release of APPs (Gruys et al. 2005). It is worth mentioning that IL-1β can be produced by PMN but does not directly act on PMN themselves; rather, it promotes their recruitment to the inflammatory site by upregulating the expression of adhesion molecules and stimulating the production of chemokines in cells adjacent to the inflammatory site (Pyrillou, Burzynski, and Clarke 2020).

Another well-known pro-inflammatory cytokine is IL-6, which can also be produced by hepatocytes as APPs (Gruys et al. 2005). IL-6 is essential for the differentiation of B cells into plasma cells, which are specialized in antibody production (Germolec et al. 2018). Additionally, IL-6 reduces the availability of iron by promoting hepcidin production in the liver (Germolec et al. 2018). This restricts iron absorption in the intestine and inhibits ferroportin, preventing iron release from cells. As a result, bacterial proliferation is hindered due to the limited access to iron (Germolec et al. 2018). Furthermore, IL-6 is involved in the transition from an acute to a chronic inflammatory response by enhancing the survival of MΦ and lymphocytes, which are key players in chronic inflammation (Germolec et al. 2018).

3.4.2 Chemokines in the inflammatory response

Chemokines play a crucial role in promoting cell migration during the inflammatory response. They are produced by various cells, including stromal cells in the bone marrow and endothelial cells. They are characterized by the presence of four conserved cysteines and are divided into four subfamilies based on the position of two of these cysteines. Indeed, these cysteine residues are considered the molecular signature of chemokines. The subfamily of chemokines with two conserved cysteines separated by only one amino acid (C-X-C) is the primary chemoattractant for PMN, with CXCL8 (IL-8) being the most prominent member. In contrast, the CC subfamily, such as CCL3 (MIP1-α) and CCL4 (MIP1-β), is the main chemoattractant for monocytes and is referred to as inflammatory chemokines since they attract PMN and monocytes during the inflammatory response (Jean-Marc Cavaillon 2018). On the other hand, dual-function chemokines are expressed in normal tissues during homeostasis, but their release is more prominent during inflammation. Chemokines can interact with similar receptors (ligand redundancy), and a specific receptor can interact with many chemokines (receptor promiscuity). Chemokines bind to receptors that belong to the rhodopsin-like family of G protein-coupled receptors (GPCRs), which are coupled to G protein heterodimers. Once activated, downstream signaling pathways activate phosphoinositide 3-kinase, which activates molecules that support cell migration, such as the actin cytoskeleton, resulting in cell polarity and pseudopodia extension necessary for cell motility (Jean-Marc Cavaillon 2018).

Table 2: The main cytokines and chemokines involved in the inflammatory response and their respective function.

Mediator	Source	Function
Cytokines		
Interleukin 1 (IL-1)	Macrophages, dendritic cells, T and B cells	Activates T and B lymphocytes Increases production of other cytokines and acute-phase proteins Induces adhesion molecules
Interleukin 2 (IL-2)	Activated T cells, B cells	Growth and activation of T and B cells Growth and activation of NK cells and macrophages Induces production of pro-inflammatory cytokines
Interleukin 6 (IL-6)	Macrophages, dendritic cells, B cells, activated T cells	Multiple effects on T cells Myeloid cell development Regulation of acute-phase proteins
Interleukin 17 (IL-17)	T_H17 cells, NK cells, NK T cells, macrophages	Recruits monocytes and neutrophils Induces production of many other cytokines, chemokines, and prostaglandins Enhances allergic inflammatory responses
Interleukin 33 (IL-33)	Macrophages, mast cells, dendritic cells, fibroblasts, epithelial cells	Stimulates production of Th2-associated cytokines from T helper cells, mast cells, eosinophils, and basophils
Interferon gamma (IFNγ)	T cells, NK cells, epithelial cells, fibroblasts	Increases MHC expression Enhances CTL, NK, and macrophage activity Stimulates production of IL-1 and TNFα
Transforming growth factor beta (TGF-β)	Macrophages, megakaryocytes, chondrocytes	Inhibits cytokine production and activity Inhibits B-cell proliferation Stimulates wound healing
Tumor necrosis factor alpha (TNFα)	Macrophages, dendritic cells, lymphocytes, mast cells	Increases MHC expression Activates macrophages Enhances tumor cell killing
Chemokines		
Macrophage chemoattractant protein-1 (MCP-1) (CC family)	Endothelial cells, epithelial cells, fibroblasts, monocytes	Attracts monocytes Activates macrophages and T cells Stimulates histamine release
RANTES (CC family)	T cells, endothelial cells, platelets	Attracts macrophages
Gro (α, β, γ MSGA) (CXC family)	Macrophages, fibroblasts	Attracts PMS, angiogenesis
Interleukin 8 (IL-8) (CXC family)	Macrophages, lymphocytes	Mobilizes PMN from bone marrow
Fractalkine (CX_3C family)	Endothelial cells, microglia macrophages	Attracts T cells, monocytes, and PMN in the brain

This table represents various cytokines and chemokines involved in the inflammatory response. Each of these cytokines plays a distinct role or acts on different cells to induce specific functions. IL-8, TNF-α, IL-6, and IL-1 are known to be pro-inflammatory cytokines. Adapted from (Germolec et al. 2018).

3.5 Polymorphonuclear leukocytes (PMN)

PMN are key cell players in inflammation and have the shortest lifespan in the bloodstream. They are characterized by their multilobulated nucleus and cytoplasm containing granules that play a crucial role in eliminating pathogens. Interestingly, PMN are fully differentiated cells and do not undergo cell division, and their cell cycle is arrested in the G0 phase (Geering and Simon 2011a). PMN are phagocytic cells and can carry out phagocytosis even without opsonins. In addition, their ability to produce ROS during the oxidative burst is critical for destroying foreign materials (Boraschi et al. 2017). The impairment of NADPH oxidase and proteolytic granules is associated with chronic granulomatous disease (CGD) and Chédiak-Higashi syndrome, respectively (Hidalgo et al. 2019).

3.5.1 Origin and heterogeneity

Granulopoiesis is the mechanism of granulocytes production (including PMN, eosinophils, and basophils) in hematopoietic organs, primarily in the bone marrow and to a lesser extent in the spleen, by hematopoietic stem cells. After differentiation, the multipotent progenitor cell gives rise to the common myeloid progenitor (CMP), which differentiates into the granulocyte-macrophage progenitor (GMP) and the megakaryocyte-erythrocyte progenitor (MEP). The GMP is further divided and differentiated into monocytes and granulocytes.

The first progenitor that gives rise to PMN is the neutrophil promyelocyte, which is characterized by a round nucleus and dark cytoplasm. This cell proliferates and differentiates into myelocytes, which is the last stage in which the cell can divide. Promyelocytes and myelocytes are considered to be in the mitotic pool. After that, myelocytes enter the postmitotic pool, during which they mature into functional cells (Figure 5*)*. This postmitotic stage begins with the differentiation of myelocytes into metamyelocytes, which are characterized by a kidney-shaped nucleus and clear cytoplasm. These neutrophil precursors mature into banded cells with a horseshoe-shaped nucleus, which can only be seen in blood circulation during the acute inflammatory response. Under normal homeostatic conditions, banded cells mature into functional PMN with a fully-segmented nucleus. Interestingly, mature PMN account for 50% to 70% of whole blood leukocytes, and they are known for their high expression of FcγR (CD16) and L-selectin adhesion molecules (Hidalgo et al. 2019).

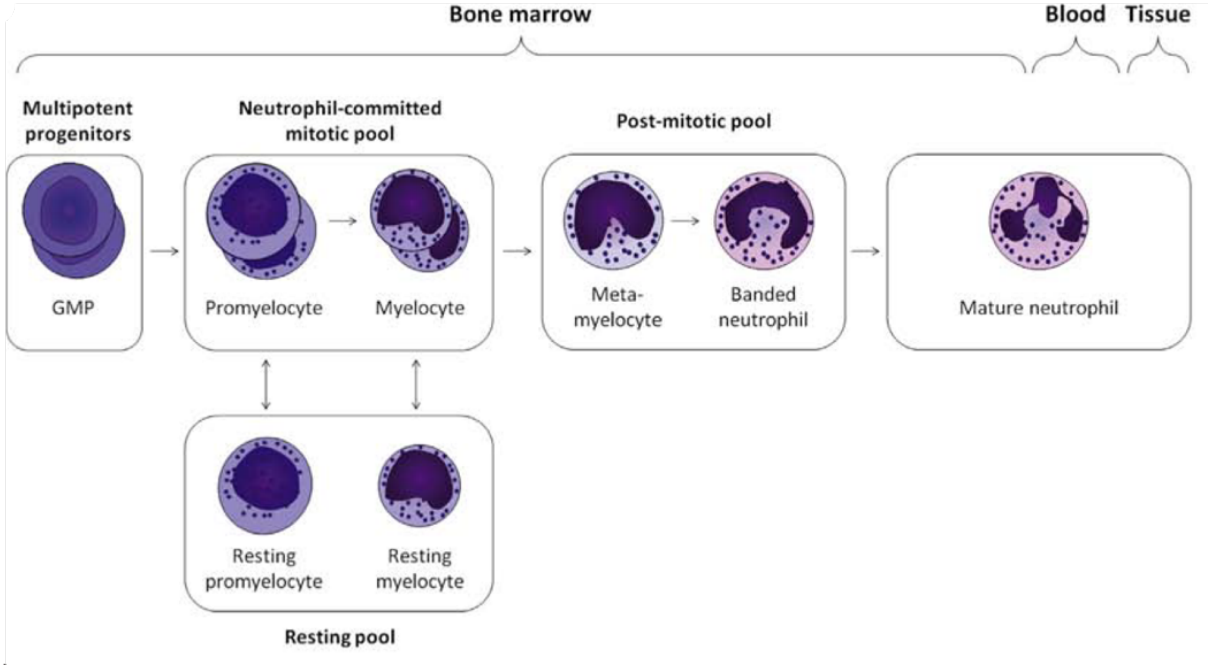

Figure 5: Depicted model for neutrophil differentiation stages in the bone marrow.

The granulocyte-macrophage progenitor (GMP) cells under specific growth factors in the bone marrow are differentiated into mitotic pool cells, promyelocytes, then myelocytes, which will originate in the formation of the undivided cells known as metamyelocyte followed by banded cells; these last cells undergo a maturation process that will form a fully segmented mature neutrophil mainly found in blood circulation and tissues. Adapted from (Hidalgo et al. 2019).

The formation of granules in PMN during granulopoiesis occurs at the level of promyelocytes and continues until the segmented stage. Interestingly, this production follows a model known as "targeting by the timing of biosynthesis", which refers to the sequential production of different granule proteins. For example, myeloperoxidase (MPO) and neutrophil elastase (NE) are localized in the azurophilic/primary granules produced in the promyelocyte stage. In contrast, adhesion molecules, principally CD11a/CD18 (LFA-1) and CD11b/CD18 (Mac-1), are made in fully mature PMN and stored in secretory vesicles, which are the first to be released during cell activation (Martin 2023). The heterogeneity of PMN has been identified in specific contexts, but the pure existence of PMN subsets is still controversial. Their short lifespan and limited transcriptional activity are classically believed to limit their capacity to adopt new functional properties. However, in cancer, PMN have shown significant phenotypic and functional

divergence. Two subsets, N1 (CD49d+, CD11b-) and N2 (CD49d-, CD11b+) have been identified (Jean-Marc Cavaillon 2018). N1 is a pro-inflammatory subset with anti-tumor activity, activated by TGF-β blockade, while N2 (CD49d-, CD11b+) is an anti-inflammatory subset that is activated by TGF-β (Gierlikowska et al. 2021). Moreover, a distinct pro-inflammatory PMN subset, known as low-density granulocytes (LDGs), can be purified from the mononuclear cell fraction during cell isolation. These cells are detected in some autoimmune diseases, such as antineutrophil cytoplasmic antibody-associated vasculitis. In fact, LDGs have a higher capacity to produce pro-inflammatory cytokines and neutrophil extracellular traps (NETs) than normal density PMN (Martin 2023).

3.5.2 Neutrophils homeostasis

PMN are continuously produced in the bone marrow, and their production and maturation are regulated by the IL-23/IL-17/G-CSF axis. During the inflammatory response, activated MΦ release the pro-inflammatory cytokine IL-23, which induces IL-17 production by T cells and natural killer cells. IL-17, in turn, increases the release of granulocyte-colony stimulating factor (G-CSF) by bone marrow stromal cells, monocytes, and MΦ (Stark et al. 2005). G-CSF is a crucial regulator of PMN production, responsible for the differentiation and proliferation of PMN precursors in the bone marrow. After their release, PMN may stay alive from ten hours to five days, depending on the context. When PMN are activated, their number and lifespan increase to respond effectively to external stimuli. It is thought that PMN form various pools in the body. The well-known pool is in the bloodstream, while a marginal pool is thought to be in most other organs, such as the lung, liver, and spleen. It is likely that PMN are involved in regulatory functions in each compartment (i.e., pool), but the mechanism is not yet understood. However, it is confirmed that the lung contains a large number of marginalized PMN due to its large surface area and high expression of adhesion molecules in its capillary structures, which could allow rapid PMN transmigration during inflammation (Gierlikowska et al. 2021). Finally, aged PMN are eliminated from circulation following an increased expression of the chemokine receptor 4 (CXCR4), which interacts with CXCL12 expressed in the bone marrow. This process is known as PMN homing, and it helps them return to the bone marrow, where they will be eliminated (Overbeeke, Tak, and Koenderman 2022).

3.5.3 Apoptosis in neutrophils

PMN undergo spontaneous or constitutive apoptosis, but the exact mechanism that triggers this process is still unclear. Specific inhibitors for the main signaling pathways in PMN, such as MAPKs (Mitogen-activated protein kinases), did not increase their viability compared to untreated cells, suggesting that the constitutive apoptosis is independent of signaling pathway activation (Geering and Simon 2011a). Therefore, spontaneous apoptosis is likely regulated by an intrinsic mechanism involving the anti-apoptotic B cell lymphoma-2 (Bcl-2) family of proteins, which contains pro- and anti-apoptotic proteins. Among them, myeloid cell leukemia-1 (Mcl-1), an anti-apoptotic protein, which is thought to play a critical role since it has a short lifespan and is prone to ubiquitination and proteasomal degradation in these cells (Murphy and Caraher 2015).

Apoptosis plays a crucial role in maintaining the balance between neutrophilia and neutropenia. In both cases, tissues might suffer damage or infection persistence, respectively. PMN can undergo extrinsic and intrinsic apoptosis pathways, which depend on the activation of effector caspases. In fact, PMN express many of these proteins, mainly caspases-1, -3, -4, -6, -7, -8, -9, and -14 (Geering and Simon 2011a). Interestingly, caspase-1, -4, -5, and -12 are activated during the inflammatory response via inflammasome activation. Furthermore, NLRP3 inflammasome activates caspase-1, leading to a particular inflammatory cell death, known as pyroptosis (Boraschi et al. 2017). Despite having a minimal number of mitochondria, these organelles are still efficient in participating in apoptosis of PMN by releasing cytochrome c and Smac. Moreover, an increased level of expression of the apoptotic protein protease-activating factor 1 compensates for the limited presence of mitochondria (Geering and Simon 2011a).

Oxidative stress is known to activate apoptosis in PMN. Indeed, most molecular pathways that lead to PMN apoptosis are associated with an overproduction of ROS, which is commonly observed when the antioxidant defense mechanisms are impaired. Additionally, PMN derived from CGD patients lacking a functional NADPH oxidase exhibit a higher cell viability rate than PMN derived from healthy donors, suggesting the role of ROS in PMN apoptosis (Geering and Simon 2011a). This is supported by the fact that ROS can trigger the release of lysosomal proteases such as cathepsin D, which is present in primary granules. These proteases can cleave BH3 interacting domain death agonist (Bid) and caspase-8. Subsequently, Bid interacts with Bcl-2-associated X protein (Bax) to form pores in the mitochondrial outer membranes, releasing cytochrome c and Smac. These proteins cleave caspase-3, which in turn activates caspase-

activated DNase and results in chromosomal DNA fragmentation and cell death. It is worth noting that, the activation of caspase-3 also accelerates the degradation of Mcl-1, shifting the cell balance toward a pro-apoptotic profile that accelerates apoptosis (Murphy and Caraher 2015).

3.6 Neutrophils biological functions

3.6.1 Adhesion

The recruitment of PMN to an inflammatory site is a multistep process, known as the neutrophil recruitment cascade, which includes tethering, rolling, adhesion, crawling, and paracellular or transcellular migration (Diapedesis). This process begins at the surface of endothelial cells in contact with the inflammatory site. Upon stimulation, endothelial cells expose their adhesion molecules to allow PMN recruitment (Gierlikowska et al. 2021).

During the first step, endothelial cells engage P-selectin and E-selectin, which interact with selectin ligands expressed by PMN. The interaction of endothelial selectins with P-selectin glycoprotein ligand-1 (PSGL-1), E-selectin ligand 1 (ESL-1), and CD44 expressed by PMN promotes tethering and rolling. Specifically, E-selectin interaction with PSGL-1 promotes the activation and expression of the β2 integrin LFA-1 (CD11a/CD18), which binds to ICAM-1 expressed by endothelial cells and helps PMN to arrest onto the vascular structure. The cell arrest is further strengthened by the interaction of the β2 integrin Mac-1 (CD11b/CD18) with ICAM1/2 expressed on the endothelial surface promoting adhesion (Figure 6). Finally, PMN extravasate the endothelial lining by diapedesis ‘’crawling/transmigration’’ (Martin 2023). In fact, PMN transmigration involves adhesion molecules PECAM-1 and junctional adhesion molecule (JAM) and mainly occurs at endothelial cell borders. PECAM-1 is expressed in endothelial cell junctions and PMN surface. After their extravasation, PMN migrate into the inflammatory site via a gradient of chemoattractants, in which IL-8 produced by activated immune and/or epithelial cells plays a prominent role (Jean-Marc Cavaillon 2018).

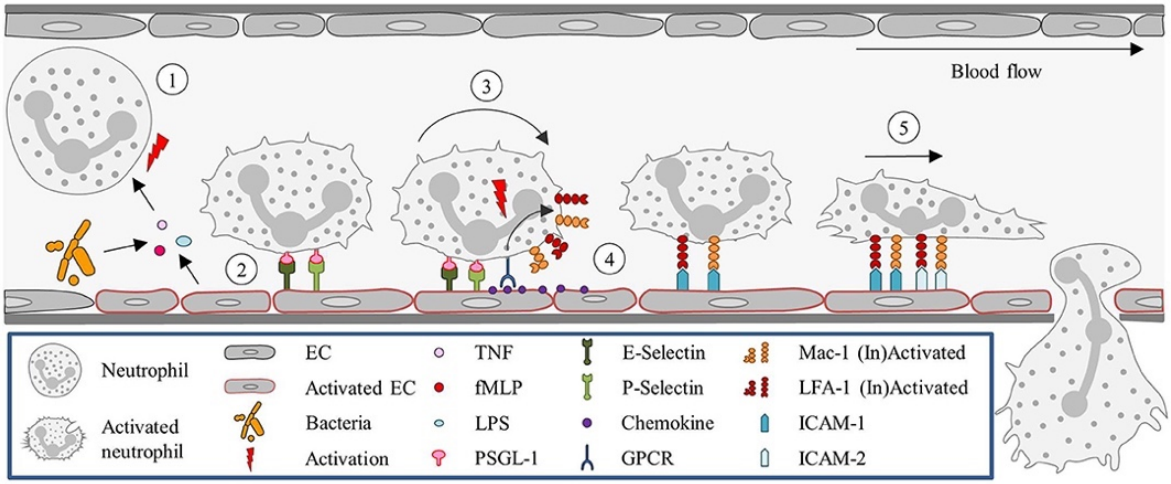

Figure 6: The neutrophil recruitment cascade during the inflammatory response.

(1) PMN are primed when exposed to inflammatory agents, such as cytokines (e.g., TNF), DAMPs, PAMPs, or through interaction with activated endothelial cells (ECs). This priming step induces the expression of P-selectin glycoprotein ligand-1 (PSGL-1) on PMN surface, which is necessary to initiate the recruitment cascade. (2) This initiation involves the interaction of PSGL-1 with P- and E-selectin expressed on the surface of ECs, a step known as tethering. (3) Subsequently, the rolling of PMN is also largely regulated through selectin signaling. (4) Following the rolling phase, PMN firmly adhere to ECs by their integrins (LFA-1 and Mac-1) which are activated by a G protein-coupled receptors (GPCRs) under the influence of chemokines presented on the endothelium surface. (5) Following firm adhesion, PMN then crawl along the endothelium, by the interaction of Mac-1 and LFA-1 with ICAM-1 and ICAM-2 molecules expressed by ECs. They continue this process until they reach a transendothelial migration site, where they transmigrate to migrate into the inflammatory site. Adapted from (Maas, Soehnlein, and Viola 2018).

3.6.2 Migration

The process of cell migration starts with adhesion and involves the guided migration of PMN towards the inflammatory site by a chemical gradient of chemoattractants. These chemoattractants, known as chemokines, are bound to glycosaminoglycans or heparan sulfate on the luminal surface of the endothelium (Cicchetti, Allen, and Glogauer 2002).

Chemoattractants are designed to promote PMN migration and are involved in their priming; they assist in transitioning PMN into a pre-activated state, which is essential for their readiness to combat external stimuli. Chemoattractants are classified into chemokines (e.g., IL-8), chemotactic lipids (e.g., LTB4), formyl peptides (e.g., N-formyl-Met-Leu-Phe 'fMLP'), and complement

anaphylatoxins (C3a and C5a). Interestingly, each of these chemoattractants orchestrates PMN migration hierarchically. In other words, they intervene at a well-defined time during cell migration. The end chemoattractants, such as IL-8 and fMLP, are dominant over intermediary ones as they are expressed in the core of the inflammatory site. LTB4 is considered an intermediate chemoattractant that helps in PMN recruitment at the beginning of the inflammatory response.

The surface expression of specific receptors regulates cell migration. Chemokine receptor 1 (CXCR1) is predominantly expressed by PMN and is a key receptor for their trafficking. It is activated by CXCL6 and CXCL8 (IL-8). Moreover, PMN also express CXCR2, and both receptors play a role in PMN homeostasis. Remarkably, activated PMN and aged PMN are characterized by a reduced expression level of CXCR1/2, which is opposite to the case of naïve (i.e., inactivated) PMN. Interestingly, this decrease in the expression of CXCR1/2 is apparently associated with PMN homing, as stromal cells in the bone marrow express high levels of CXCR1/2 ligand known as CXCL12 (SDF-1), which might facilitate their return into the bone marrow. On the other hand, formyl peptides possess specifically a formyl methionine in their NH2 terminal structure. They are generally released by bacteria during their degradation and act on the formyl peptide receptor 1 (FPR1), predominantly expressed by PMN (Metzemaekers, Gouwy, and Proost 2020; Petri and Sanz 2018).

3.6.3 Phagocytosis and oxidative burst

Phagocytosis is a fundamental mechanism by which PMN can eliminate bacteria, apoptotic cells, and cell debris. This process is enhanced when microbial agents are opsonized with immunoglobulin G (IgG) or the C3b fragment of the complement system. These are recognized by FcγR and complement receptor 3, respectively. Typically, phagocytosis is followed by NADPH oxidase activation, a complex composed mainly of p40phox, p47phox, p67phox, and Ras-related C3 botulinum toxin substrate 2 (Rac2), which are present in the cytosol and assemble following cellular activation by various stimuli. This complex mainly migrates to fuse with proteolytic granules; however, a small fraction can also attach to cell membranes, producing ROS during the oxidative burst to harm foreign intruders. Interestingly, PMN have a well-equipped antioxidant system that intervenes to scavenge the produced ROS in exaggeration to protect themselves from oxidative stress (Geering and Simon 2011a).

NADPH oxidase synthesizes the superoxide anion (O_2•–) by promoting the transfer of electrons from the cytosol to oxygen. Then, O_2•– is prone to be converted by superoxide dismutase to hydrogen peroxide (H_2O_2), which is not technically a free radical. However, H_2O_2 is catalyzed by MPO to produce hypochlorous acid, a powerful bactericidal component (Martin 2023). Moreover, H_2O_2 can be converted into highly reactive hydroxyl radicals (•OH) in the presence of iron via the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} +$ •OH + OH-). Interestingly, •OH produced by this reaction can react with lipid molecules to form lipid peroxyl radicals that act on other lipids by inducing their oxidation in a process known as lipid peroxidation (Stockwell 2022).

3.6.4 Degranulation and netosis

PMN are highly packed with granules that are released during the inflammatory process at the site of inflammation (Table 3). This process can be attributed to presorted granules or *de novo* synthesis. In fact, degranulation is a common feature in many inflammatory disorders, and it is an effective mechanism in killing invasive microorganisms. PMN granules can be classified into primary granules (azurophilic granules), secondary granules (specific granules), tertiary granules (gelatinase granules) , and secretory vesicles. Primary granules contain the most toxic mediators, such as neutrophil elastase (NE) and myeloperoxidase (MPO). The secondary and tertiary granules have overlapping contents where lactoferrin (Lfr) and gelatinase B (MMP-9) are stored, respectively (Lacy 2006).

Table 3: The main sets of granules in human PMN.

Protein	Key function	Granule	Lineage stage of onset
MPO	MPO uses hydrogen peroxide to generate secondary oxidants necessary to destroy pathogens. MPO also plays a role in NETosis	Azurophilic granules	Promyelocyte
Neutrophil elastase	Roles in NETosis, adhesion, ECM degradation. Plays roles in non-oxidative pathways of destroying pathogens, both intracellular and extracellular	Azurophilic granules	Promyelocyte
Cathepsin G	NETosis, adhesion, ECM degradation. Plays roles in non-oxidative pathways of destroying pathogens, both intracellular and extracellular	Azurophilic granules	Promyelocyte
Proteinase 3	NETosis. Plays roles in non-oxidative pathways of destroying pathogens, both intracellular and extracellular	Azurophilic granules	Promyelocyte
Lysozyme	Killing of bacteria by hydrolysis of cell wall peptidoglycan, ECM degradation	Azurophilic granules	Promyelocyte
NSP4	ECM degradation	Azurophilic granules	Promyelocyte
Azurocidin 1	Antibacterial functions	Azurophilic granules	Promyelocyte
Alpha-defensins	Anti-microbial functions and roles in NETosis	Azurophilic granules	Promyelocyte
Flavocytochrome b (gp^{91phox})	Phagocytosis	Specific granules	Promyelocyte
Lactoferrin	Role in NETosis and anti-bacterial properties.	Specific granules	Myelocyte
OLFM-4	Bacterial infections against *S. aureus*	Specific granules	Myelocyte
Resistin	Chemoattractant	Specific granules	Myelocyte
CD177	Adhesion, extravasation, antimicrobial functions	Specific granules	Myelocyte
NGAL	Antimicrobial functions	Specific granules	Myelocyte
MMP-9	Migration through extracellular matrix	Gelatinase granules	Metamyelocyte
Gelatinase	Migration through basement membrane	Gelatinase granules	Metamyelocyte
Mac-1	Phagocytosis, adhesion, crawling, transmigration and diapedesis of vessel wall	Gelatinase granules	Metamyelocyte
LFA1	Rolling, adhesion, transmigration and diapedesis of vessel wall	Secretory vesicles and ficolin-1 granules	Band
VLA-4	Rolling, tethering, adhesion, crawling, transmigration and diapedesis of vessel wall	Granule unknown	Unknown

This table provides an overview of PMN granules, their main functions, and localization. PMN possess four main sets of granules known as: Primary granules (azurophilic granules), secondary granules (specific granules), tertiary granules (gelatinase granules) and secretory vesicles which are released upon cell activation during the inflammatory process. For example, MMP-9 is released to facilitate extracellular matrix degradation, which is essential for cells to migrate to the inflammatory site. On the other hand, MPO plays a crucial role in the production of hypochlorous acid, a highly effective bactericidal molecule against pathogens. Adapted from (McKenna et al. 2021).

On the other hand, netosis produces NETs, which are structures composed mainly of chromatin fibers and granule proteins, mainly NE and MPO. Indeed, NETs are released by PMN as an external degranulation process to trap invading foreign materials in a net-like structure, preventing their dissemination and facilitating their destruction and elimination (Brinkmann et al. 2004). Interestingly, netosis is impaired in PMN derived from CGD patients, which suggests the essential role of ROS in this process (Geering and Simon 2011a).

3.6.5 *De novo* synthesis of proteins

Activation of PMN is associated with cellular events that are necessary during their recruitment to the inflammatory site. One of the interesting cytoplasmic events is the induction of *de novo* synthesis of proteins. However, this event occurs in a restricted fashion; in other words, PMN are known to have little transcriptional activity, and their capacity to activate this process is highly correlated with the stimuli to which they are exposed. Nevertheless, cytokines and other antimicrobial components have been demonstrated to be produced to some extent by *de novo* synthesis. For example, GM-CSF and TNF-α activate this process; Indeed, GM-CSF has shown to induce *de novo* synthesis of the anti-inflammatory cytokine IL-1 receptor antagonist (Beaulieu et al. 1992). Moreover, the chemokine MIP-1α is produced by *de novo* following stimulation of PMN by LPS (Sollberger, Amulic, and Zychlinsky 2016); Furthermore, LTB4, a chemoattractant molecule, is produced by PMN in response to pro-inflammatory cytokines also via *de novo* synthesis (Metzemaekers, Gouwy, and Proost 2020). This process is also involved in cytoskeleton modulation; In fact, IL-4 has been shown to be an activator of *de novo* protein synthesis to induce actin production (Girard, Paquin, and Beaulieu 1997). Conversely, the release of NETs has shown not to be associated with this process upon stimulation of PMN with Phorbol 12-myristate 13-acetate (PMA) (Sollberger, Amulic, and Zychlinsky 2016).

3.6.6 Signaling mechanisms in neutrophils

The biological functions of PMN are mainly regulated by a plethora of signaling pathways that are activated by various stimuli, following the phosphorylation of key downstream proteins. The downstream pathways of pro-survival receptors in PMN are principally divided into tyrosine kinases, which can be activated by growth factors (e.g., GM-CSF), and those that can be activated by G protein-coupled receptors (GPCRs), for instance, by chemoattractants such as LTB4. The main tyrosine kinases activated in PMN are phosphoinositide 3-kinase (PI3-K), the Janus kinase/signal transducers and activators of transcription (Jak/STAT) pathway, and the mitogen-activated protein kinase (MAPK) pathways, which are divided into the extracellular signal-regulated kinase (ERK) pathway and the MAPK p38 pathway (Geering and Simon 2011a).

PI3-K activity is generally assessed by its downstream effector Akt also known as protein kinase B, which has three isoforms: Akt1, Akt2, and Akt3. Akt activation increases the lifespan of PMN,

and it enhances the expression of the anti-apoptotic protein Mcl-1. Conversely, its inhibition is associated with PMN spontaneous apoptosis (Geering and Simon 2011a). Additionally, Akt plays a role in cell migration by inducing the activation of actin-binding proteins involved in cytoskeletal remodeling, which is necessary for cell migration (Zhang et al. 2013). Furthermore, Akt implication in the oxidative burst is associated with the phosphorylation of $p47^{phox}$ which acts as an adaptor for NADPH oxidase assembly. Interestingly, a knockout study of Akt isoforms 1 and 2 confirmed the involvement of Akt2 in cell migration, superoxide production, and degranulation (Chen et al. 2010).

On the other hand, ERK is known to enhance Mcl-1 activity. However, p38 seems to be activated in both scenarios in the presence of pro-survival or pro-apoptotic stimuli. In other words, its role in the control of PMN survival is unclear (Geering and Simon 2011a). Nevertheless, p38 can regulate the activity of Akt. Indeed, this activation involves the heat shock protein 27, which forms a stable complex with Akt in unstimulated PMN (Zhang et al. 2013). Moreover, p38 is known to play an essential role in the recruitment cascade of PMN (Petri and Sanz 2018). Additionally, ERK and p38 activation regulate netosis. The activation of NADPH and the production of superoxide anion have been shown to promote phosphorylation of p38 and ERK, which then activate NETs release (Keshari et al. 2013).

In regard to degranulation, it has shown to be controlled by cytosolic enzymes (nonreceptor tyrosine kinases) associated with Src kinase proteins Hck, Fgr, and Lyn. Indeed, Hck is associated with primary granule release, whereas Fgr is correlated with secondary granule releases. Interestingly, PMN from knockout mice of Hck, Fgr, and Lyn have shown a deficiency in secondary granules release, which was associated with weak p38 activity, suggesting that Src acts upstream of the p38 pathway. Furthermore, PP1, a specific Src kinase inhibitor, impairs the release of primary granules, secondary granules, and secretory vesicles, confirming the importance of Src in promoting degranulation in PMN (Lacy 2006).

The regulation of cytokine production in PMN is controlled by transcription factors, primarily nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). This factor is associated with a regulatory inhibitory protein called IkBα, which is highly expressed in unstimulated PMN compared to other immune cells (Castro-Alcaraz et al. 2002). When PMN are activated by external stimuli, IkBα is degraded, which activates a canonical pathway responsible for pro-

inflammatory cytokine transcription. Moreover, this enhances the expression of adhesion molecules and cell viability of PMN (Liu et al. 2017).

In regard to phagocytosis, the interaction of IgG-coated particles with FcγR promotes the activation of cytoplasmic immunoreceptor tyrosine-based activation motifs (ITAMs) via the activation of Src tyrosine kinase. This leads to the activation of Syk, which regulates the mobilization of adapter molecules that promote lipid modifications and actin remodeling. This results in a localized reorganization of the membrane and actin rearrangement, leading to the formation of membrane protrusions in which the particles are engulfed (Gierlikowska et al. 2021; Jean-Marc Cavaillon 2018). On the other hand, phagocytosis of opsonized particles with C3b by complement receptor necessitates a double activation of PMN by formyl-peptides, which are common signatures of pathogens, and by extracellular matrix components (fibronectin or laminin). This results in the activation of phosphorylation events that lead to particle engulfment (Jean-Marc Cavaillon 2018).

3.7 Interaction of nanoparticles with the innate immune system

The innate immune system acts as the first line of defense of the human body in response to the invasion of foreign materials. Since biological barriers such as the skin may not be sufficient to prevent the entry of such materials, effector cells such as monocytes/MΦ and PMN intervene to combat them (Boraschi et al. 2017). Many studies have examined the influence of NP on the immune response, as the immune system is considered the primary system associated with toxicological outcomes related to NP exposure (Alsaleh and Brown 2020). Although the phagocytic ability of immune cells to internalize NP is well established, the sensing mechanism is not yet fully understood. An interesting study showed that carbon nanotube NP can induce primary MΦ chemokine secretion by pattern-recognition receptors (PRRs) called toll-like receptors (TLRs) 2/4-dependent pathway. This suggests that some NP may express a nanoparticle-associated molecular pattern that immune cells can easily detect (Mukherjee et al. 2018). When NP come into contact with the biological environment, they can undergo chemical and physical transformations, such as aggregation, and can interact with plasma proteins (i.e, Albumin) to form a biocorona (Casals et al. 2010). This phenomenon is typically more pronounced in hydrophobic NP than in hydrophilic ones. Such alterations in the identity of NP may impact their eventual destination, leading to changes in their recognition by immune cells (Portilla et al. 2023).

In some cases, adsorbed proteins can misfold on the NP surface, resulting in the presentation of danger signals that could trigger an inflammatory response (Boraschi et al. 2017). Consequently, the potential of NP to induce an immune response may be related to their binding to protein structures that enable them to function like hapten molecules (Fadeel 2019).

3.7.1 Inflammation and nanoparticles at a glance

In the following paragraphs, we provide a general overview of the role that Fe NP may play in inflammation by referring to studies conducted *in vitro*, *in vivo,* and *ex vivo*.

Endothelial cells play a crucial role in the recruitment phase of the inflammatory response, as they are in direct contact with the bloodstream. These cells can be affected by intravenously administered Fe NP, potentially leading to alterations in vascular barrier integrity. In fact, a research study using human aortic endothelial cells (HAECs) found that Fe NP could cross the cell membrane and induce a decrease in cell viability. Additionally, an increase in the release of the pro-inflammatory chemokine IL-8 and the expression of ICAM-1 was observed. Furthermore, when these cells were co-cultured with monocyte-like cells U937, an increase in cell adhesion was confirmed. Interestingly, Fe NP were found to significantly increase the release of ROS by U937 and nitric oxide by HAECs. Moreover, the internalization of Fe NP by U937 was observed, and their dissolution under the pH conditions of U937 lysosomes was detected using inductively coupled plasma-mass spectrometry. This dissolution was found to play a role in increasing ROS levels and, consequently, oxidative stress, similar to what was observed for ferric chloride used as a control (Zhu et al. 2011). Another study demonstrated that human umbilical vein endothelial cells (HUVECs) are capable of internalizing large amounts of both Fe_3O_4 NP coated with dextran or citrate. They found that citrate-coated NP were more cytotoxic. Furthermore, it was discovered that the treatment of HUVECs with Fe_3O_4 NP altered their cytoskeleton network and their capacity to mature and differentiate to form tube-like structures, which is a critical step in angiogenesis (Wu et al. 2010)

A study that aimed to assess the potential of Fe NP to be used as an intrinsic strategy to control cancer growth found that adenocarcinoma cells co-incubated with MΦ and Ferumoxytol, a drug-based Fe NP, showed a decrease in tumor cell growth by inducing apoptosis mediated by ROS production. Interestingly, this effect was attributed to the activation and polarization of MΦ to a

pro-inflammatory phenotype (M1), which was confirmed by a significant increase in TNF-α and CD86, classical markers of the M1 phenotype (Zanganeh et al. 2016).

The morphology of Fe NP can also play a key role in inflammasome activation, a critical component of the inflammatory response. In a study, researchers have assessed four Fe NP with similar aspect ratios (size) and surface charges but different morphologies (octopod, plate, cubic, and spherical). Interestingly, they found that all of these NPs induce inflammasome activation in bone marrow-derived macrophages and human THP-1 cells. However, the octopod and plate-shaped Fe NP exhibited a greater effect on inflammasome activation compared to the cubical and spherical Fe NP. This was demonstrated by an increase in caspase-1 activity, which was associated with an increase in IL-1β release and pyroptosis, a typical cell death process associated with inflammasome activation (Liu et al. 2018; Miao, Rajan, and Aderem 2011). Interestingly, the cytotoxicity of these Fe NP was also correlated with their morphology. In other words, octopod Fe NP had the most cytotoxic effect, followed by plate-shaped, cubic, and then spherical Fe NP. It is worth mentioning that all of these NP induce ROS production, which is associated with lysosomal damage (Liu et al. 2018).

Another study assessed the effect of morphology (rod-shaped and spherical Fe NP) on mouse macrophage cells (RAW264.7). They found that both types of NP were internalized by the cells. However, the rod-shaped Fe NP were more cytotoxic than the spherical ones, as assessed by the soluble tetrazolium assay and LDH leakage assay. The analysis of pro-inflammatory cytokines showed a significant increase in TNF-α but not in IL-1β or IL-6. Moreover, both Fe NP demonstrated the capacity to induce ROS, and pretreatment of cells with the antioxidant N-acetylcysteine revealed a significant decrease in the ROS level (Lee et al. 2014).

In a pilot study that aimed to assess the effects of metal NP in the workplace environment, specifically in the welding area where iron is the major detected metal (Thanachoksawang et al. 2022), it was found that blood sample analysis from welders who did not wear protective equipment was positively associated with an increase in the pro-inflammatory cytokine IL-6 but not IL-8. Interestingly, in this study, they also found a significant increase in oxidative stress, detected by a decrease in the urinary secretion of 8-Hydroxy-2'-deoxyguanosine (8-OHdG), which correlates with DNA damage due to oxidative stress and an impaired mechanism of DNA repair necessary to remove 8-OHdG from DNA (Omari Shekaftik and Nasirzadeh 2021). The same researchers observed that exposure of human bronchial epithelial cells (BEAS-2B) to Fe NP

increased ROS production, 8-OHdG (i.e., DNA damage), and apoptosis, as measured by the activity of caspase-3 and 7. Interestingly, wearing a protective mask has been shown to decrease IL-6 and increase the urinary level of 8-OHdG, indicating a sign of DNA repair (Thanachoksawang et al. 2022).

In the same context, another study assessed lung tissue sections from welders who were also smokers and found a significant presence of Fe NP deposited in the alveolar lumen and in the fibrous tissue, as revealed by Perls Prussian blue staining. Moreover, immunohistochemistry staining of MΦ, as indicated by CD68, also showed an increased presence when compared to control patients (Andujar et al. 2014). Interestingly, exposure of MΦ (THP-1) *in vitro* to Fe NP present in welding fumes has shown an increase in the production of IL-8, IL-1β, IL-6, TNF-α, CCL-2, CCL-3, and CCL-4. Significantly, the exposure of these cells to Fe NP in addition to cigarette smoke did not demonstrate any synergistic effect. Moreover, Fe_3O_4 NP were found to be less reactive in terms of the amount of cytokines produced, but they were still able to significantly release IL-8, IL-1β, and IL-6, but not TNF-α (Andujar et al. 2014).

As Fe NP can be found as an essential component associated with air pollution and work environment (Thanachoksawang et al. 2022; Andujar et al. 2014), an *in vivo* study has shown that a single intratracheal instillation of these particles (spherical with a size of 50 nm) in BALB/c mice induces an increase in PMN and lymphocytes in the airway submucosa, along with a significant increase in eosinophils in bronchoalveolar lavage fluid (BAL). This was accompanied by different related cytokines and chemokines, such as IL-5, eotaxin, TNF-α, and murine KC, which corresponds to IL-8 in human PMN (Yue et al. 2022). Using the same administration method, Fe_3O_4 NP were found to induce inflammation, as analyzed by assessing BAL, confirming a significant increase in IL-6 and TNF-α at day one post-administration. Additionally, a decrease in the intracellular level of reduced glutathione (GSH) has been observed, suggesting potential oxidative stress. Histologically, microgranulomatous changes in the alveolar space have been observed at day 14 and 28 post-administration. These structures are formed by a collection of immune cells that struggle to eliminate the intruders, which can be considered a marker for an ongoing chronic inflammatory process (Park et al. 2010).

3.7.2 Neutrophils and interaction with nanoparticles

PMN are capable of interacting with and responding to the presence of NP. Indeed, the ability of NP to induce phosphorylation events in PMN has been established. ZnO (Zinc oxide) NP have been shown to activate PMN by inducing a plethora of tyrosine phosphorylation events (Goncalves and Girard 2014). Additionally, TiO_2 NP, ZnO NP, and CeO_2 NP were found to enhance the phagocytosis of sheep red blood cells (SRBCs) and latex beads in PMN by inducing phosphorylation of the Syk pathway (Babin, Goncalves, and Girard 2015).

NP have the potential to alter the recruitment cascade of PMN. Studies have shown that PMN treated with Fe NP can affect the rolling step of their recruitment cascade. They increase the rolling velocity on the E-selectin substrate by 37% compared to control buffer-treated cells. However, the cells are still capable of arresting in the presence of E-selectin and ICAM-1 substrate in response to IL-8 stimulation by inducing CD18 (β2-integrin) expression. Nevertheless, the duration between the rolling and onset of cell arrest is increased (Garcia et al. 2020). In addition, another study has demonstrated that palladium (Pd) NP slightly increase the adhesion of PMN onto human endothelial cells (Kwemo et al. 2020).

Various studies have demonstrated that NP have the ability to alter cell migration. For instance, MΦ pretreated with Fe NP were shown to have a decreased ability to transmigrate in a chemotaxis chamber model compared to cells treated with the chemoattractant agonist fMLP (Müller et al. 2007). Another study found that Fe NP could alter the expression of CXCR1 and CXCR2 in PMN. Specifically, incubation of cells with these particles increased the expression of CXCR1 and CXCR2 by 26% and 15%, respectively (Garcia et al. 2020). In addition, Pd NP were found to increase cell migration in the Boyden chamber model, but did not have the capacity to induce PMN infiltration in the mouse air pouch model (Kwemo et al. 2020).

Phagocytosis is considered the primary mechanism of NP internalization by PMN and MΦ. Nevertheless, other mechanisms such as macropinocytosis, caveolin- or clathrin-dependent mechanisms, which are encompassed in endocytosis, are also involved. During phagocytosis, internalized NP are driven into phagosome-like structures where they are exposed to destructive cell machinery (e.g., lysosomes) that may cause nonclassical toxicity known as trojan-horse-type, during which NP dissolve and release ions due to the acidic environment of phagolysosomes. The size, shape, and surface coating are critical factors in NP uptake. Additionally, the protein

corona can also play a role in the mechanism of NP engulfment. Indeed, protein adsorption, mostly by opsonins, could facilitate NP uptake. It is noteworthy that phagocytic cells exhibit faster uptake of spherical particles than worm-like particles. This dissimilarity in uptake is believed to be connected to the lower energy requirements for actin remodeling required by cells to internalize simple-shaped NP compared to complex ones (Boraschi et al. 2017; Fadeel 2019).

Assessment of NP internalization is essential to confirm NP toxicity; however, cellular uptake does not necessarily result in cell death. One study has demonstrated that Ag NP can induce apoptosis following their internalization by PMN (Poirier et al. 2014). In contrast, silica-coated Fe NP have been shown to be relatively inert despite being considerably internalized by MΦ (Kunzmann et al. 2011). Furthermore, the ability of NP to enhance this process in PMN has already been demonstrated for TiO_2 (Titanium) NP, ZnO NP, and CeO2 (Cerium dioxide) NP (Babin, Goncalves, and Girard 2015).

As phagocytosis is related to NADPH oxidase activation, NP may be able to induce ROS production. Indeed, oxidative stress has been considered a key marker of NP toxicity (Fadeel and Garcia-Bennett 2010). Interestingly, NP have been shown to have the capacity to induce the production of ROS by two mechanisms. The first is the oxidative potential of metallic NP to produce ROS by the Fenton reaction. The second is through the activation of inflammatory cells, mainly PMN and MΦ which promote ROS production via NADPH oxidase. In this context, Ag NP have been shown to induce ROS production in PMN (Liz et al. 2015). However, ZnO NP and Pd NP have been demonstrated to interact with PMN without promoting ROS release (Goncalves and Girard 2014; Kwemo et al. 2020).

Degranulation and netosis are correlated with inflammation, and therefore, it is fundamental to assess the capacity of NP to induce both processes. Indeed, the ability of NP to alter degranulation has already been documented in many studies. For example, a study showed that Au NP with both positive and negative charges could increase the activity of MMP-9 released by PMN, as determined by zymography assay (Durocher et al. 2017). Another study found that TiO_2 NP, CeO_2 NP, and ZnO NP can increase degranulation in PMN; they showed an increased expression of MPO (azurophil/primary granules), MMP-9 (gelatinase granules), and albumin (secretory granules). Interestingly, TiO_2 NP and CeO_2 NP were found to induce a more significant increase in the enzymatic activity of MMP-9 compared to ZnO NP, indicating that different NP may have varying effects on this process in PMN (Babin et al. 2013b). In regard to netosis, a

study has shown that Ag NP can induce the release of NETs through a ROS-dependent mechanism (Liz et al. 2015). However, it has been demonstrated that netosis can also be activated in a ROS-independent manner when PMN are stimulated through the CXCR2 receptor (Marcos et al. 2010)

The number of studies analyzing the capacity of NP to induce *de novo* synthesis in PMN is limited. Nonetheless, it has been demonstrated that ZnO NP can interfere with PMN apoptosis by increasing *de novo* protein synthesis. Remarkably, the anti-apoptotic effect of ZnO NP was reversed when cells were treated with cycloheximide (CHX), a potent inhibitor of the translation process (i.e, *de novo* synthesis). This suggests that the effect of ZnO NP on PMN apoptosis is dependent on *de novo* protein synthesis (Goncalves and Girard 2014). On the other hand, Ag NP with a size of 20 nm has been shown to be pro-apoptotic and to inhibit this process in metabolically labelled PMN with Redivue Pro-Mix L-[^{35}S] (Poirier et al. 2014). These studies confirm the potential presence of a specific protein translation process that can interfere with or promote apoptosis, depending on the stimulus to which PMN have been exposed.

3.8 Conclusion

The majority of studies focusing on the assessment of Fe NP toxicity have primarily centered on MΦ. This preference is mainly due to the fact that these cells are known to be present in all tissues, play a role in both inducing and resolving inflammation, and can be easily isolated from blood samples (Dalzon et al. 2020). Furthermore, in contrast to PMN, the ability of MΦ to maintain their viability for a longer period of time makes them an attractive cell population for NP drug delivery systems within cells for the treatment of diseases, thus attracting more researchers to study their interactions with NP (Vishnevskiy et al. 2021).

Despite polymorphonuclear neutrophils (PMN) constituting over 65% of human leukocytes and playing a key role in inflammation, they have been somewhat neglected in nanotoxicology studies (Keshavan et al. 2019). Indeed, primary PMN are a challenging model to work with, primarily due to their short lifespan and their high susceptibility to priming and activation during isolation, as well as their significant inter-donor variability (Verdon et al. 2021).

Regardless of these challenges, in this research project we aim to understand the interaction of primary PMN with spherical, uncoated Fe_3O_4 NP with a size of approximately 10 nm. The reason for choosing these characteristics is that Fe NP under a size of 20 nm exhibit superparamagnetism, a property which is highly sought after in nanomedical-based applications, and can be significantly affected by the shape and size of the NP (Zhen et al. 2011; Dadfar et al. 2020; Lodhia et al. 2010). Moreover, spherical NP are easily produced and have good penetration and retention rates, compared to other NP shapes (Montiel Schneider et al. 2022). However, uncoated Fe_3O_4 NP tend to aggregate. Despite this, we have chosen to utilize them in their uncoated state to gain a deeper understanding of their actual behavior with cells, as the presence of a coating could potentially influence the final outcomes. This approach will contribute to our comprehension of their interactions, ultimately aiding in the enhancement of biocompatibility and ensuring their safe use across various applications.

4 HYPOTHESIS AND OBJECTIVES

The use of iron oxide nanoparticles in their chemical form Fe_3O_4 has attracted the attention of scientists in recent years, mainly due to their intrinsic physicochemical properties that offer a wide range of applications, particularly in medicine. However, the safety of these NP has not been thoroughly assessed, especially with PMN, which are the primary cells recruited during the inflammatory response and play a crucial role in the body defense system. The immunomodulatory potential effects of Fe_3O_4 NP on PMN could be a setback in their biomedical applications, as it has been documented in cases of anaphylactic shock associated with the use of Feraheme®, an iron oxide NP-based drug, in some patients. Therefore, the main objective of this thesis is to assess the capacity of Fe_3O_4 NP to modulate the biological functions of PMN.

Hypothesis:

I. Fe_3O_4 NP could potentially interfere with human PMN biological functions in a sex-dependent manner.

Objectives:

I. Assessment the modulatory effects of Fe_3O_4 NP on PMN biological functions.

II. Deciphering the mechanisms by which Fe_3O_4 NP modulate some of PMN biological functions.

5 RESEARCH ARTICLE

Interaction between iron oxide nanoparticles (Fe_3O_4 NP) and human neutrophils: Evidence that Fe_3O_4 NP possess some pro-inflammatory activities.

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Contribution of the author:

I, Prof. Denis Girard, confirm that Abdelaziz Saafane contributed as a primary author of this publication. I collaborated with Abdelaziz to design the study, and he conducted the entire set of experiments and their analysis. Furthermore, Abdelaziz assisted me in the writing process by suggesting and contributing to various parts of the article, including the introduction and materials

and methods sections. Additionally, Abdelaziz oversaw the review process of the article before submission and has been available to answer questions raised by the reviewers after submission.

5.1 Resumé

Les nanoparticules de magnétite (Fe_3O_4 NP) présentent un grand potentiel d'utilisation dans diverses applications médicales. Cependant, des préoccupations liées à une éventuelle toxicité associée à leur utilisation émergent et plusieurs paramètres doivent encore être étudiés afin d'atteindre une efficacité thérapeutique maximale avec le minimum d'effets indésirables. L'inflammation est l'un des effets indésirables les plus fréquemment signalés lors de l'exposition aux nanoparticules. L'utilisation de divers modèles inflammatoires révèle l'existence de données contradictoires concernant le profil pro-inflammatoire ou anti-inflammatoire des Fe_3O_4 NP.

L'objectif de cette étude est de déterminer l'effet direct des Fe_3O_4 NP sur la biologie des neutrophiles, des cellules clé de l'inflammation. Des neutrophiles humains fraîchement isolés ont été incubés *in vitro* avec des Fe_3O_4 NP, et plusieurs fonctions cellulaires ont été étudiées. En utilisant la microscopie électronique à transmission, il a été constaté que les Fe_3O_4 NP étaient internalisées par les neutrophiles. Ces NP ne déclenchent pas à eux seuls la flambée oxydative, mais ils augmentent la capacité des neutrophiles à adhérer aux cellules endothéliales humaines ainsi qu'à améliorer la phagocytose. Une approche basée sur la technique de microréseaux d'anticorps a révélé que les Fe_3O_4 NP induisent la production de certaines cytokines, notamment la chimiokine IL-8 (CXCL8) qui a été confirmée par la technique ELISA. Les Fe_3O_4 NP ont été trouvées pour retarder l'apoptose spontanée des neutrophiles humains, indépendamment du sexe. En utilisant une approche pharmacologique, nous avons démontré que les Fe_3O_4 NP retardent l'apoptose par un mécanisme dépendant de la synthèse *de novo* des protéines et via différentes voies de signalisation cellulaire. Les données obtenues indiquent que les Fe_3O_4 NP peuvent altérer la biologie des neutrophiles humains et qu'ils possèdent certains effets pro-inflammatoires, en particulier en raison de leur capacité à retarder l'apoptose et à induire la production de cytokines pro-inflammatoires. Par conséquent, les Fe_3O_4 NP peuvent moduler l'inflammation en ciblant des fonctions clés des neutrophiles humains.

5.2 Abstract

Iron oxide nanoparticles (Fe_3O_4 NP) are important for different medical applications. However, potential toxicity has been reported and several parameters must still be studied to reach highest therapeutic efficacy with minimal undesired effects. Inflammation is one of the most reported undesired effects of NP exposure in a variety of inflammatory models and conflicting data exist regarding whether Fe_3O_4 NP possess pro- or anti-inflammatory activities. The aim of this study was to determine the direct effect of Fe_3O_4 NP on the biology of neutrophil, a key player cell in inflammation. Freshly isolated human neutrophils were incubated *in vitro* with Fe_3O_4 NP, and several functions have been studied. Using transmission electronic microscopy, Fe_3O_4 NP were found to be ingested by neutrophils. These NP do not induce a respiratory burst by themselves, but they increase the ability of neutrophils to adhere onto human endothelial cells as well as enhance phagocytosis. An antibody array approach revealed that Fe_3O_4 NP induce the production of some cytokines, including the chemokine IL-8 (CXCL8), which was confirmed by ELISA. Fe_3O_4 NP were found to delay spontaneous neutrophil apoptosis regardless of sex of the donor. Using a pharmacological approach, we demonstrate that Fe_3O_4 NP delay apoptosis by a *de novo* protein synthesis-dependent mechanism and via different cell signaling pathways. The data indicate that Fe_3O_4 NP can alter the biology of human neutrophils and that they possess some pro-inflammatory effects, particularly based on their capacity to delay apoptosis and to induce the production of pro-inflammatory cytokines. Therefore, Fe_3O_4 NP can regulate inflammation by targeting human neutrophil functions.

5.3 Introduction

The emergence of nanomedicine has brought a lot of interest for using nanoparticles (NP) as prominent elements for many biomedical applications, mostly as nanovectors for drugs (Patra et al. 2018). Among NP, the superparamagnetic iron oxide nanoparticles (SPION) have been demonstrated to possess a wide panel of utilization either in diagnostic applications such as in magnetic resonance imaging or in therapy including their use as an efficient vector in drug and gene delivery, biological sensing, and hyperthermia (Arias et al. 2018). However, the use of SPION in various biomedical applications, and others, do not exclude the possibility that these NP possess some toxicity and undesired effects including their interactions with innate immune cells, especially in the context of the inflammatory response (Malhotra et al. 2020; Liu et al. 2013). Indeed, inflammatory responses of rats following acute inhalational exposure to iron oxide nanoparticles (Fe_3O_4 NP) were previously reported based on different inflammatory markers, including the number of neutrophils (Srinivas et al. 2012). Indeed, as reported for many different agents including several NP, inflammation is more intense as the number of neutrophils is increased (Hussain et al. 2011; Liu et al. 2014; Cho et al. 2010; Larsen et al. 2010). However, regarding Fe_3O_4 NP, others reported in contrast that these NP rather down regulate the neutrophil inflammatory response based on the inhibition of chemotactic activation and by downregulating normal rolling of neutrophils to arrest under shear flow (Garcia et al. 2020). Despite such conflicting observations, the effects of Fe_3O_4 NP on neutrophil cell biology need to be studied more in depth. Neutrophils are the most abundant leukocyte in the human blood circulation and are known as key player cells in acute inflammation (Kolaczkowska and Kubes 2013). In fact, they are among the first cells to migrate at an inflammatory site and to kill microorganisms by phagocytosis and induce production of reactive oxygen species (ROS) and degranulation leading to the release of powerful antimicrobial agents. In 2004, a new mechanism by which neutrophils can kill microorganisms was identified, the release of neutrophil extracellular traps (Kim and Cheong 2020) consisting mainly of chromatin bound to cytoplasmic proteins (Brinkmann et al. 2004). Neutrophils are released from the bone marrow at an impressive rate of $\sim 5 \times 10^{10}$ cells/day in a normal adult and therefore must be rigorously under tight control to avoid adverse effects (Ward et al. 1999). Neutrophils have a limited life span (half-life of ~12h in circulation) and fortunately undergo constitutive or spontaneous apoptosis allowing to maintain a relatively stable number of cells in healthy individuals. Efferocytosis is one of the major steps for the resolution of inflammation consisting of the elimination of apoptotic neutrophils by professional phagocytes like macrophages. Although they are well-recognized to be involved in innate immunity, neutrophils

are also actively involved in adaptive immunity since they produce several cytokines and chemokines regulating various other cells (Mantovani et al. 2011). Even though iron oxide nanoparticles (IONP) have been found to induce some pro-inflammatory effects, and knowing that neutrophils are important in inflammation, the direct role of IONP on the biology of neutrophils is not well documented. Interestingly, a recent study shows that agglomerations of non-stabilized superparamagnetic IONP (SPIONs) induce NET formation and that their stabilization with human serum albumin or dextran reduced it (Bilyy et al. 2018a). This indicated that SPIONs can activate human neutrophils. In mice, neutrophils were found to be able to phagocytize magnetic mesoporous silica NP synthesized by encapsulating hydrophobic magnetic Fe_3O_4 NP in mesoporous silica spheres (Wu, Zhang, Tie, et al. 2018). The aim of this study was to better document how Fe_3O_4 NP can alter the biology of human neutrophils. We found that several, but not all, neutrophil functions are altered in response to these Fe_3O_4 NP and that these NP possess some pro-inflammatory effects.

5.4 Materials and methods

5.4.1 Chemicals

RPMI-1640, HEPES, penicillin/streptomycin (P/S), H_2O_2, *Viscum album* Agglutinin-I (VAA-I), trypan blue, Bovine Serum Albumin (BSA), Phosphate buffered saline, Dextran and rabbit IgG anti-SRBCs antibodies, the MAPK/ERK inhibitor PD098059, the p38 MAPK inhibitor SB203580, the Syk inhibitor piceatannol, the protein synthesis inhibitor cycloheximide and the tripeptide N-formyl-Met-Leu-Phe (fMLP) were purchased from Sigma Aldrich Ltd (Saint-Louis, Missouri). Recombinant human (rh) tumor necrosis factor-alpha (TNF-α) and Granulocyte- Macrophage Stimulating Factor (GM-CSF) were purchased from Peprotech Inc. (Rocky Hill, NJ, USA). Wortmannin, a PI3-K/Akt inhibitor, was purchased from EMD Biosciences (San Diego, CA). Hank's balanced salt solution (HBSS), HEPES, H_2DCFDA were purchased from Fisher Scientific (Ottawa, Canada). ELISA kits for Interleukin (IL)-8 were purchased from BD Biosciences (CA, USA) and sheep red blood cells (SRBCs) were obtained from Lampire Biological laboratories (Pennsylvania, USA).

5.4.2 Fe_3O_4 NP

The Fe_3O_4 NP were purchased from Sigma Aldrich Ltd (Saint-Louis, Missouri). According to the manufacturer, the particle size is 9–11 nm as assessed by transmission electronic transmission (TEM). The solution is at 5 mg/mL in de-ionized water and a fraction was further diluted to obtain a working stock solution of 1000 X and was used as is.

5.4.3 Characterization Fe_3O_4 NP

The NP suspension obtained from the manufacturer was examined using a Hitachi H-7100 transmission electron microscope to validate the small size and the form of these NP. The endotoxin level of the NP suspension was determined by the classical Limulus amebocyte lysate (LAL) assay using the ToxinSensor™ - Chromogenic LAL Endotoxin Assay Kit (Genscript Biotech Corp., Piscataway, NJ). Measurements were performed at a concentration of 100 µg/mL. In addition, we incubated the Fe_3O_4 NP suspension in lysogeny broth agar plates for 72h for testing sterility and observed the presence or absence of colonies as previously described (Goncalves and Girard 2014). As a positive control, we used a suspension voluntary contaminated with human hair. Pictures of the plates were taken with a Canon EOS Rebel T5 camera.

5.4.4 Neutrophil isolation

Neutrophils were isolated from venous blood of healthy volunteers by dextran sedimentation, followed by centrifugation over Ficoll-Hypaque (Pharmacia Biotech, Inc., Quebec, Canada) as described previously (Babin et al. 2013b; Gonçalves, Chiasson, and Girard 2010). Blood donations were obtained from informed and consenting individuals according to institutionally approved procedures. Cell viability was monitored by trypan blue exclusion and found to be consistently ≥ 97%. Cell purity (≥ 98%) was verified by cytology from cytocentrifuged preparations colored by Hema-3 stain set (Biochemical sciences Inc., Swedesboro, NJ). Cell viability was evaluated systematically before and after each treatment. Neutrophils were then resuspended 1 × 10^7 cells/mL in RPMI-HEPES (25 mM), penicillin (100 U/mL)/streptomycin (100 µg/mL) for all experiments, but ROS production.

5.4.5 Cellular uptake of Fe_3O_4 NP

To visualize potential cellular internalization of the NP, freshly isolated neutrophils were incubated (10×10^6 cells/mL) with 100 µg/ mL of Fe_3O_4 NP or with the equivalent volume of HBSS for 1h or 24h. Cells were then fixed overnight with 2.5% glutaraldehyde in 0.05 M sodium cacodylate buffer. After several washes in PBS with 3% sucrose, cells were fixed with 1.3% OsO_4 for 2h and embedded in Spurr resin. Thin slices were prepared with an ultramicrotome and stained with 5% uranyl acetate and filtered lead citrate. Examinations of ultrathin sections (total of 50–70 cells/sample) were examined using a Hitachi H-7100 transmission electron microscope as described (Noël, Simard, and Girard 2016; Poirier et al. 2014).

5.4.6 Detection of intracellular ROS

Cells (1×10^7 cells/mL) were suspended in HBSS containing 10 µM CM-H_2DCFDA (Invitrogen/Molecular Probes, Camarillo, CA) for 15 min at 37 ℃ as previously published [23]. Cells were then washed twice before being incubated in the presence of buffer or Fe_3O_4 NP for 5, 15 and 30 min. In these experiments, H_2O_2 (1 mM) and fMLP (1 µM) were used as a technical and positive control, respectively. ROS production was determined using a Spectra Max M5 plate reader (Molecular Devices, San Jose, CA) with an excitation/emission wavelength of 485/538 nm and was expressed as relative fluorescence units (RFU).

5.4.7 Neutrophil cell adhesion assay

The human umbilical vein cell line EA.hy926 (ATCC® CRL-2922™) was purchased from American Type Culture Collection (Manassas, VA) and was grown in RPMI-1640 supplemented with 10% fetal calf serum and antibiotics. Cell viability was systematically evaluated before and after each treatment, and mortality never exceeded 5%. These endothelial cells were used as a substratum for neutrophil cell adhesion. To do so, neutrophils were treated with buffer (Ctrl), Fe_3O_4 NP (100 µg/ mL) or TNF-α (10 ng/mL, used as a positive control) (Pelletier and Girard 2005) for 30 min and then labeled for 30 min with 5 µM calcein-AM (Molecular Probes, Inc., Eugene, OR, USA). After, the cells are washed to eliminate free calcein-AM and then incubated with endothelial cells. The number of adherent neutrophils was calculated by counting the number of fluorescent cells from five randomly selected high-power fields (Å~ 400) observed with a photomicroscope Leica

DMRE equipped with an ebq 100 dc epifluorescent condenser. Images were taken with a Cooke Sensicam High performance camera coupled to the Image Proplus® (version 4·0) program.

5.4.8 Phagocytosis of opsonized sheep red blood cells (SRBCs)

SRBCs were opsonized with a 1:200 dilution of a rabbit IgG anti- SRBC Ab for 45 min at 37 ℃ as previously published (Babin, Goncalves, and Girard 2015; Ratthé and Girard 2004). Neutrophils were treated with the following agents: buffer (negative Ctrl), 65 ng/mL GM-CSF (positive control) or the indicated concentrations of Fe_3O_4 NP and then incubated in a 1:5 ratio with opsonized SRBCs for 30 min at 37 ℃. After incubation with SRBCs, the samples were centrifuged at 200×*g* for 10 min at 4 ℃. Supernatants were discarded and an osmotic shock was performed on the pellets by resuspending the cells with 400 µL H_2O for 15 s, followed by the addition of 4.5 mL PBS. The samples were then washed twice, and the final cell pellets were suspended in 400 µL PBS 1X. Phagocytosis is expressed as the percentage of neutrophils (% phagocytosis) having ingested at least one opsonized SRBC. A total of ~200 cells/slide was evaluated in duplicate.

5.4.9 Cytokine production using the proteome profiler™ array

The human cytokine array panel A was purchased from R&D Systems Inc. (Minneapolis, MN) and all the steps for the simultaneous detection of 36 different analytes were performed within 3 months of harvesting cells, as per the manufacturer's recommendation. According to previous studies (Gonçalves, Chiasson, and Girard 2010; Durocher et al. 2017; Durocher and Girard 2016), the supernatants of 10 different experiments were harvested from HBSS-treated (control) or Fe_3O_4NP-induced human neutrophils and pooled to probe the membranes. The chemiluminescent signal from the bound cytokines/chemokines present in the supernatants was detected by Image Quant LAS 500 (GE Healthcare, Chicago, USA) and the signal intensity of each analyte (means of duplicate) was quantified by densitometry using Image J program (NIH, Maryland, USA). The 36 different analytes and the corresponding reference spots and negative control are listed in the legend of the corresponding figure.

5.4.10 IL-8 production

The concentration of IL-8 was determined using a commercially available ELISA kit (Life Technologies, Carlsbad, CA). Freshly isolated human neutrophils (n= 32) were incubated in the presence of buffer (Ctrl) or Fe_3O_4 NP as above at 37 ℃ in 5% CO_2 for 24h in a 24-well plate

containing RPMI-1640 supplemented with 10% autologous human serum. Supernatants were harvested after centrifugation and stored at - 20 ℃ before performing ELISA.

5.4.11 Assessment of neutrophil apoptosis

1×10^7 cells/mL in RPMI 1640-HEPES-P/S, supplemented with 10% heat-inactivated autologous serum were treated with buffer, Fe_3O_4 NP, the antiapoptotic cytokine GM-CSF or 1 µg/mL of the potent proapoptotic plant lectin *Viscum album* agglutinin-I (VAA-I) (Lavastre et al. 2002; Savoie et al. 2000) for 24h. In some experiments, cells were pretreated with the protein synthesis inhibitor cycloheximide (CHX) at 1 µg/mL or with the indicated concentration of inhibitors of different cell signaling pathways such as MAPkinases (PD 098059 at 5 µM; SB203580 at 10 µM), PI3 Kinase/AKT (wortmannin at 10 µM) and Syk (piceatannol at 30 µM), then apoptosis was evaluated as follows. After the incubation, cytocentrifuged samples of neutrophils were prepared, stained with the Hema-3 Stain staining kit according to the manufacturer's instructions and processed as documented previously (Lavastre et al. 2002). Apoptosis was determined by optical microscopic observations by calculating the number of cells containing one or more characteristic, round, and darkly stained pyknotic nuclei. The results were plotted from counting duplicates of 5–6 different fields for each condition representing a number of ~200 cells and were expressed as percent of apoptosis.

5.4.12 Statistical analyses

Statistical analyses were performed using repeated measures ANOVA and differences between groups were assessed using the Dunnett's Multiple Comparison Test with GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA). Statistical significance was established at $p < 0.05$.

5.5 Results

5.5.1 Characterization of Fe_3O_4 NP

Fig. 7.A illustrates the size from 20 different measures obtained from TEM observations and calculated with the Image J program. The primary size of Fe_3O_4 NP is 10.5 ± 0.9 nm (mean ± SD, n = 20) agreeing with the diameter of 9–11 nm reported by the manufacturer. Also, TEM images reveal that the NP possess a spherical shape. As assessed by the LAL assay, the Fe_3O_4 NP suspension reveals a level of endotoxin under the detection limit of 0.01 EU/mL. Since some NP could interfere with classical LAL assay for determining endotoxin level (Dobrovolskaia and McNeil 2007), we incubated the NP suspension (100 µg/mL) in lysogeny broth agar plates for 72h for further confirming sterility. As illustrated in Fig. 7.B, unlike the positive control, no colony was observed on the plate containing Fe_3O_4 NP.

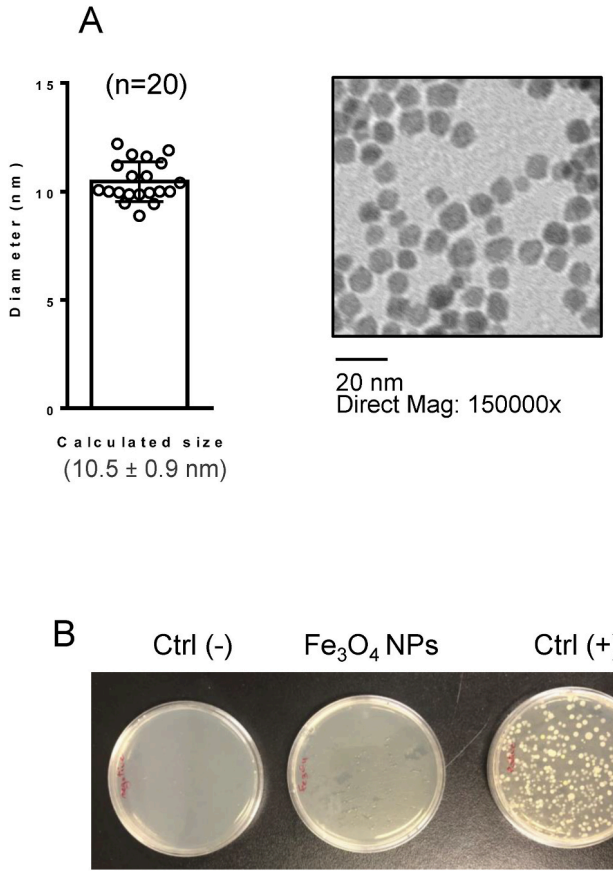

Figure 7: Characterization of Fe_3O_4 NP.

An aliquot from the original stock obtained from the manufacturer was tested by TEM to confirm the size and form (A) and sterility (B) of the NP as described in Materials and Methods. The image shows that the form of

the NP is spherical with a size close to 10 nm (see the scale). B, several Fe_3O_4 NP suspensions were incubated in lysogeny broth agar plates for 72 h for testing sterility. The illustrated example shows an absence of colonies in contrast to the positive control (Ctrl (+)).

5.5.2 Fe_3O_4 NP are internalized by human neutrophils

To confirm internalization of Fe_3O_4 NP in neutrophils, we performed TEM experiments. As shown in Fig. 8 Fe_3O_4 NP were easily observable inside cells after 1h of treatment. They penetrated inside neutrophils into vacuole-like structures (Fig. 8.B, white asterisks) and are also randomly distributed in the cytosol and in or close to the nucleus (white arrows). As expected, these small dots were not observed in untreated neutrophils (Fig. 8.A). Fig. 9 shows representative TEM images of cells after 24h of incubation with Fe_3O_4 NP. Interestingly, after 24h, Fe_3O_4 NP are still observed inside cells freely in the cytosol (white arrows), but only few NP are detectable close to the nucleus (Fig, 9.A, top panel) or in vacuole-like structures (Fig. 9.A, bottom panel, white arrowheads. The image in Fig. 9.B was selected for illustrating that a high number of Fe_3O_4 NP could be ingested by a neutrophil. The enlarged portion of the image indicated that Fe_3O_4 NP inside the neutrophil also possess a spherical form with a size close to 10 nm (black arrows) like the primary stock solution (Fig. 7.A).

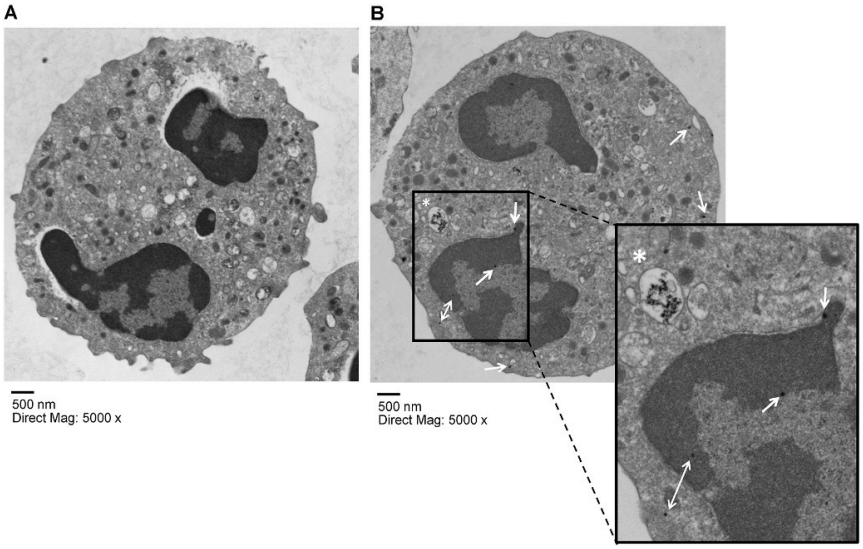

Figure 8: Fe_3O_4 NP are rapidly internalized by human neutrophils.

Freshly isolated human neutrophils were incubated in the presence of buffer (A) or 100 µg/mL Fe_3O_4 NP (B) for 1h and samples were prepared for TEM analysis as described in Materials and Methods. In contrast to controls

(A) note that NP are located everywhere in the cells (B) where they are randomly distributed in the cytosol and in (or close to) the nucleus (arrows and double arrow) and in vacuole-like structures as evidenced in the enlarged region (asterisk).

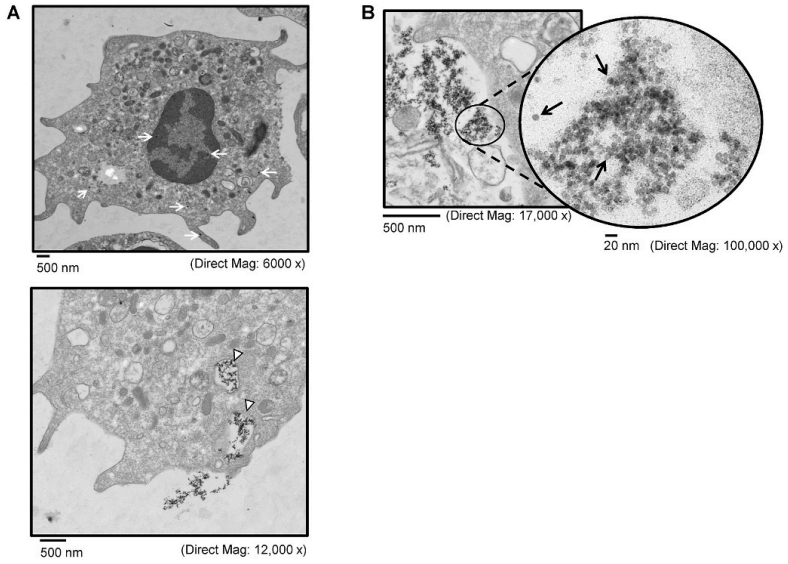

Figure 9: Fe_3O_4 NP remained inside human neutrophils for 24h.

Freshly isolated human neutrophils were incubated in the presence of 100 µg/mL Fe_3O_4 NP for 24h and samples were prepared for TEM analysis as described in Materials and Methods. The representative TEM images show that Fe_3O_4 NP are still observed freely in the cytosol (A, top panel, arrows) but fewer are detectable close to or in the nucleus. They can also be inside cells in vacuole-like structures (A, bottom panel, arrowheads). B, this TEM image illustrates that a high number of Fe_3O_4 NP could be ingested and incorporated in vacuole-like structures by a neutrophil and that the NP possess, as expected, a size close to 10 nm (black arrows, enlarged circle).

5.5.3 Impact of Fe_3O_4 NP on ROS production, cell adhesion and phagocytosis in human neutrophils

Knowing the importance of ROS production by neutrophils (Dobrovolskaia and McNeil 2007; Dahlgren, Karlsson, and Bylund 2019), we next determined the impact of Fe_3O_4 NP on this biological response. Fig. 10.A shows that Fe_3O_4 NP do not increase ROS production after 5, 15 and 30 min of treatment as compared with controls even though the positive control H_2O_2 gives the expected results. To further confirm that Fe_3O_4 NP do not induce ROS production, fMLP was used as a positive control in another cohort of blood donors (Fig. 10.A, inset). Since

superparamagnetic iron oxide NP were previously found to promote endothelial progenitor cell adhesion capacity (Yang, Tang, and Wang 2010) and since we previously documented that different NP could increase the adhesion of eosinophils (one other type of granulocytes), we next determined if Fe_3O_4 NP could also increase adhesion of human neutrophils. As illustrated in Fig. 10.B, Fe_3O_4 NP can increase the capacity of neutrophils to adhere onto human endothelial cells with a ratio of 1.5 ± 0.1 (mean ± SEM, n = 5). As expected, the positive control TNF-α gives a significant ratio of 2.2 ± 0.2. The function of neutrophil capacity to exert phagocytosis was also investigated and we found that Fe_3O_4 NP can significantly increase basal phagocytosis from 32.1 ± 2.1% (mean ± SEM, n =11) to 45.6 ± 3.3% and 54.3 ± 3.5% for cells treated with 10 and 100 µg/mL Fe_3O_4 NP, respectively (Fig. 10.C). In comparison, the cytokine GM-CSF, used as positive control, significantly increased phagocytosis to a level of 62.7 ± 3.5%.

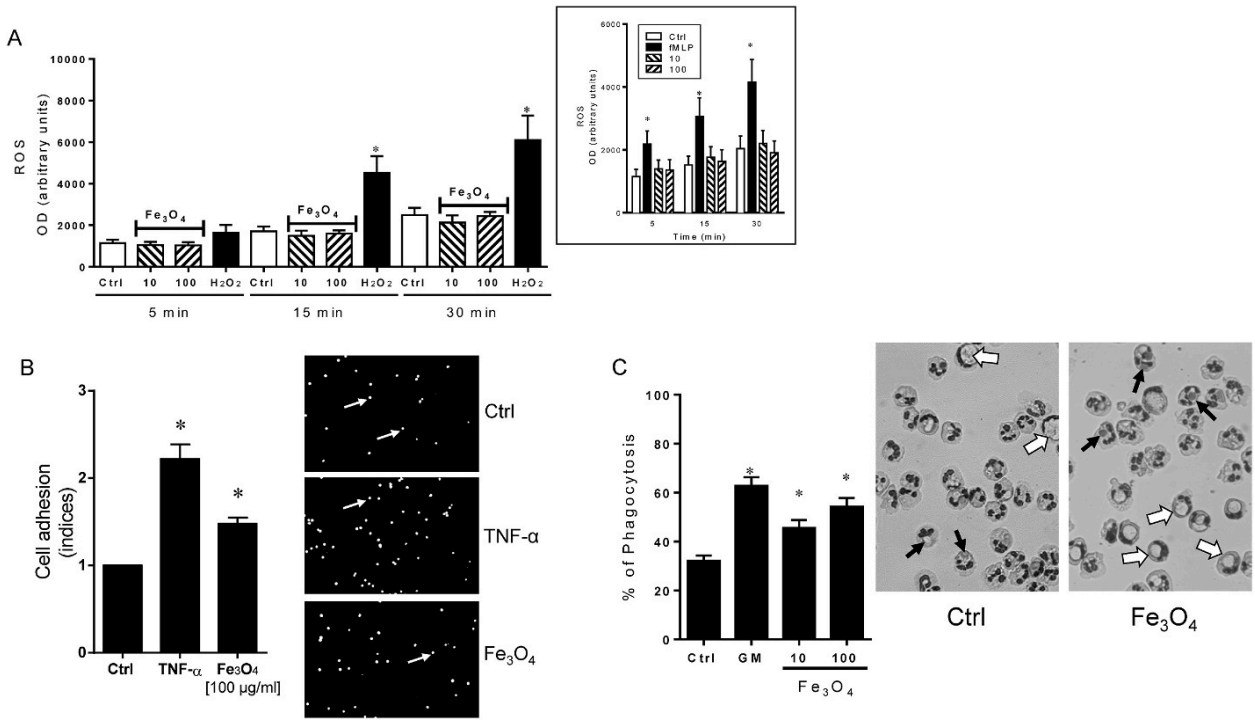

Figure 10: Effect of Fe_3O_4 NP on ROS production, adhesion, and phagocytosis.

Freshly isolated human neutrophils were incubated in the presence of buffer (Ctrl), Fe_3O_4 NP (at 10 or 100 µg/mL) or the indicated agonists, and ROS production (A), cell adhesion (B) or phagocytosis (C) was determined as described in Materials and Methods. B, arrows in the images, show a single fluorescent neutrophil adhere onto the endothelial cell substratum. C, large white arrows show a large phagolysosome and small black arrows demonstrate the presence of one SRBC. Results are mean ± SEM, n = 5 (A); n = 4 (A, inset); n = 4 (B); n = 11 (C). *, p <0.05 vs Ctrl.

5.5.4 Effect of Fe_3O_4 NP on cytokine production

Fe_3O_4 NP were found to increase the production of diverse pro-inflammatory cytokines in human peripheral blood cells and in murine neutrophils (Wu, Miao, et al. 2018; Couto, Freitas, et al. 2015). However, it is not clear if this occurs in human neutrophils. As illustrated in Fig. 11.A and Fig. 11.B, the antibody array approach reveals that Fe_3O_4 NP can increase the basal level of some analytes, including IL-8 (CXCL8), MIP1α/β (CCL3/4), and Gro-α (CXCL1). The production of IL-8 was quantified using ELISA and the results indicate that the basal production of IL-8 significantly increased from 376.3 ± 200.9 pg/mL in Ctrl cells to 1027 ± 17.3 pg/mL (mean ± SD, n = 5) in Fe_3O_4-treated neutrophils (Fig. 11.C).

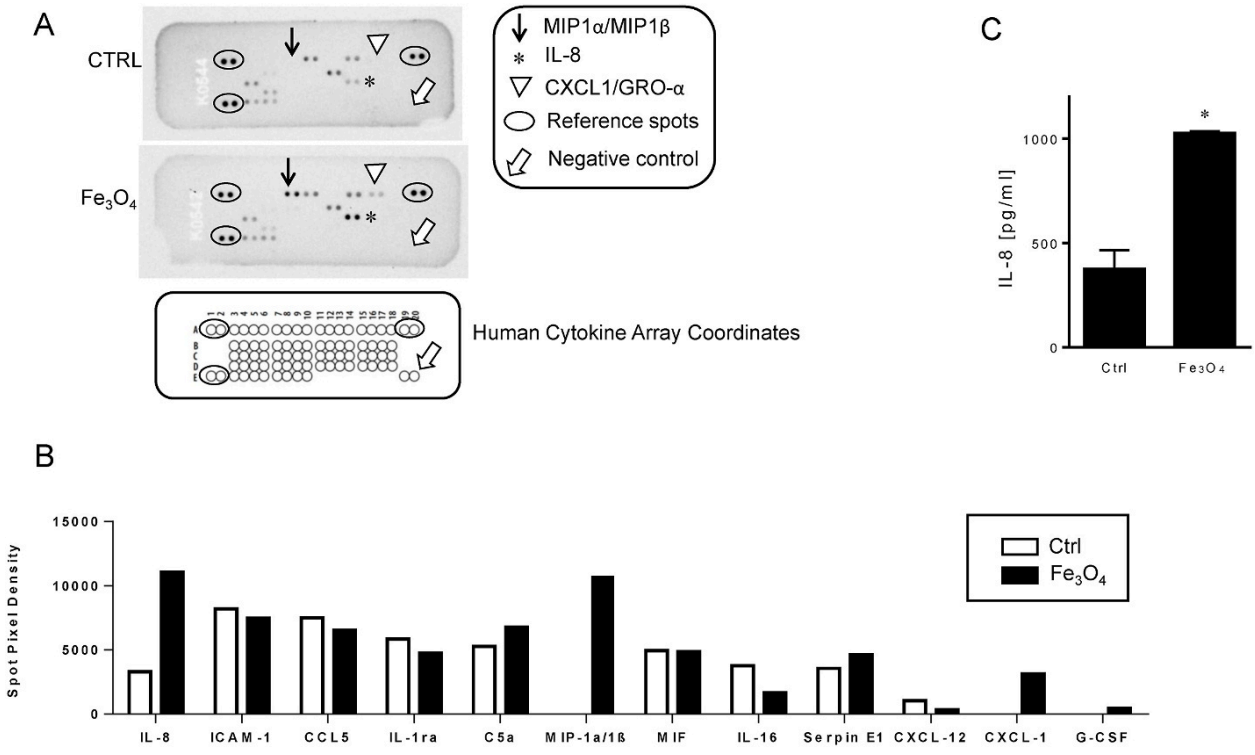

Figure 11: Effect of Fe_3O_4 NP on the cytokine production.

Freshly isolated human neutrophils were incubated in the presence of buffer (Ctrl) or Fe_3O_4 NP (100 µg/ mL) for 24h and the supernatants from ten different blood donors were pooled and used for screening purpose using an antibody array approach as described in Materials and Methods (A,B). In other experiments, freshly isolated human neutrophils were treated as above, but the supernatants were separately harvested and tested by ELISA to quantify IL-8 production (C). Results are mean ± SEM, n = 32. *, p <0.05 vs Ctrl. The Coordinates correspond to: A1-2, Reference spots; A3-4, CCL1; A5-6, CCL2; A7-8, MIP-1a/b; A9-10, CCL5; A11-12, CD40L; A13–14, C5/C5a; A-15-16, CXCL1; A17-18, CXCL10; B3-4, CXCL11; B5-6, CXCL12; B7-8, G-CSF; B9-10, GM-CSF;

B11-12, ICAM-1; B13-14, IFN-γ; B15-16, IL-1α; B17-18, IL-1β; C3-4, IL-1ra; C5-6, IL-2; C7-8, IL-4; C9- 10, IL-5; C11-12, IL-6; C13-14, IL-8; C15-16, IL-10; C17-18, IL-12p70; D3-4, IL-13; D5-6, IL-16; D7-8, IL- 17A; D9-10, IL-17E; D11-12, IL-18; D13-14, IL-21; D15-16, IL-27; D17-18, IL32α; E1-2, Reference spots; E3-4, MIF; E5-6, Serpin E1; E7-8, TNF-α; E9-10, TREM-10; and E19-20, Negative control.

5.5.5 Fe_3O_4 NP delay human neutrophil apoptosis

Fig. 12.A illustrates that Fe_3O_4 NP slightly delay neutrophil apoptosis at a concentration of 10 µg/mL but at the concentration of 100 µg/mL, a concentration routinely used for other NP (Babin et al. 2013b), Fe_3O_4 NP significantly suppressed apoptosis. Indeed, at this latter concentration, 8.7 ± 1.2% (mean ± SEM, n = 10) of cells are in apoptosis vs 44.5 ± 5.1% for Ctrl. Of note, in these conditions, Fe_3O_4 NP were more efficient as compared with the well-known neutrophil antiapoptotic cytokine GM-CSF (16.6 ± 3.6%). Because of this potent anti-apoptotic effect of Fe_3O_4 NP, and since we recently documented that some NP can differently alter the apoptotic rate according to sex (Vanharen et al. 2022), we next determined if this occurs similarly in neutrophils isolated from men and women. As illustrated in Fig. 12.B, the basal rate of spontaneous apoptosis is similar between men (39.8 ± 2.8%, mean ± SEM, n = 17) and women (43.8 ± 3.8%, n = 18). The antiapoptotic effect of Fe_3O_4 NP at the concentration of 100 µg/mL is also similar and not significantly different whether neutrophils were isolated from men (16.8 ± 2%, n = 17) or (women 12.9 ± 2,3%, n = 18). This indicates that Fe_3O_4 NP delay spontaneous apoptosis regardless of sex. Knowing that Fe_3O_4 NP act regardless of sex, we next determined if these NP could inhibit the pro-apoptotic effect of VAA-I by incubating cells with both VAA-I and Fe_3O_4 NP. As illustrated in Fig. 12.C, Fe_3O_4 NP can inhibit the proapoptotic effect of VAA-I. Indeed, although VAA-I was less potent in this cohort of donors where the spontaneous neutrophil apoptotic rate of 55.0 ± 6.5% (mean ± SEM, n = 5) significantly increased to 69.8 ± 4.4% in the presence of VAA-I, the addition of Fe_3O_4 NP to VAA-I decreased the neutrophil apoptotic rate to 37.6 ± 6.0%. In parallel, the antiapoptotic effect of Fe_3O_4 NP was confirmed where the apoptotic rate was significantly decreased to 29.8 ± 3.0%.

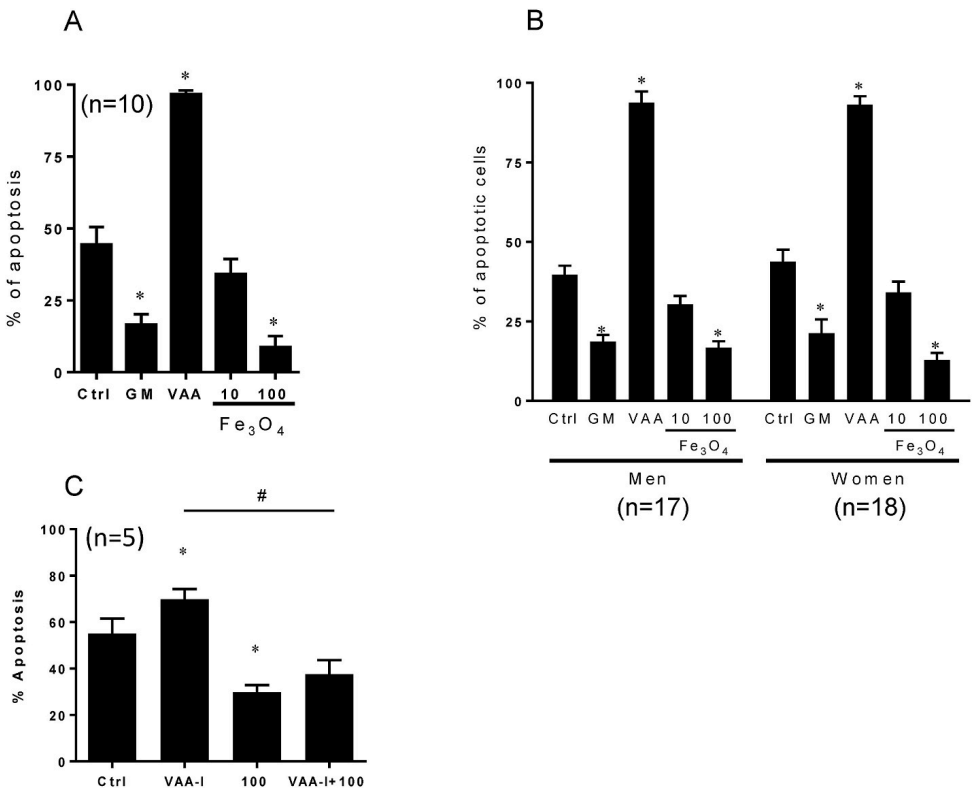

Figure 12: Fe_3O_4 NP delay human neutrophil apoptosis regardless of sex.

Freshly isolated human neutrophils were incubated in the presence of buffer (Ctrl), the antiapoptotic cytokine GM-CSF (GM), the proapoptotic plant lectin *Viscum album* agglutinin-I (VAA), or Fe_3O_4 NP (10 or 100 µg/mL) for 24h and apoptosis was determined as described in Material and Methods. A, apoptosis was determined using neutrophils isolated from different blood donors (n = 6 men and 4 women) and the data were plotted together to create the bar graph (mean ± SEM). B, apoptosis was determined using neutrophils isolated from men (n = 17) and from women (n = 18) and the graphs were created using data obtained with men (B, left part) and with women (B, right part). C, cells were incubated for 24h in the presence of buffer (Ctrl), VAA-I, 100 µg/mL Fe_3O_4 NP (100) or with both VAA-I and 100 µg/mL Fe_3O_4 NP (VAA-I + 100) and apoptosis was determined as above (results are mean ± SEM, n = 5). Note that Fe_3O_4 NP inhibit the proapoptotic effect of VAA-I. *, p <0.05 vs Ctrl.

5.5.6 Fe_3O_4 NP delay human neutrophil apoptosis by a *de novo* protein synthesis-dependent mechanism and via several cell signaling pathways

Knowing that some NP could modulate neutrophil apoptosis by a *de novo* protein synthesis-dependent mechanism (Poirier et al. 2014; Poirier, Simard, and Girard 2016), we next treated cells in the presence or absence of the potent protein synthesis inhibitor, CHX. As expected (Goncalves and Girard 2014), addition of CHX alone in neutrophil cultures does not alter the basal level of spontaneous apoptosis. Indeed, as illustrated Fig. 13.A, the apoptotic rate is 53.7 ± 2.2% and 59.2 ± 2.5% (mean ± SEM, n =6) for cells incubated in the absence or in the presence of CHX, respectively. As expected, Fe_3O_4 NP alone significantly delayed apoptosis (16.3 ± 2.8%), however, addition of CHX markedly and significantly reversed the antiapoptotic activity of Fe_3O_4 NP (51.8 ± 7.2%). This indicates that Fe_3O_4 NP suppressed neutrophil apoptosis by a protein synthesis-dependent mechanism. To better determined the mode of action of Fe_3O_4 NP, we next used a pharmacological approach with inhibitors of important cells signaling pathways known to be involved in the regulation of different functions of human neutrophils, including apoptosis. Fig. 13.B, illustrated that none of the different inhibitors used alone did not significantly alter the basal level of neutrophil apoptosis. Both wortmannin (WT) and PD098059 (PD) were found to significantly reverse the antiapoptotic effect of Fe_3O_4 NP, wortmannin (WT) being the most potent. The other inhibitors (SB and Pic) also slightly inhibit the Fe_3O_4 NP antiapoptotic effect, but this was not statistically significant. This indicates that Fe_3O_4 NP induce several cell signaling events in human neutrophils.

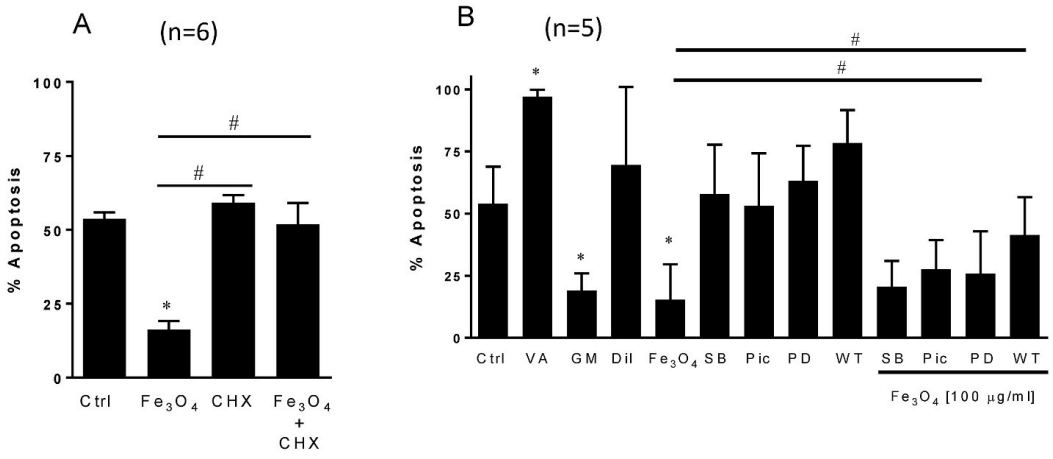

Figure 13: Fe_3O_4 NP delay human neutrophil apoptosis by a *de novo* protein synthesis dependent mechanism and via several cell signaling events.

Freshly isolated human neutrophils were pretreated 30 min with (A) the potent protein synthesis inhibitor cycloheximide (CHX) or with (B) the different indicated cell signaling event inhibitors SB203580 (SB), PD098059 (PD), piceatannol (Pic) or wortmannin (WT) prior the addition of buffer (Ctrl), the corresponding diluent (Dil, <1% DMSO) or 100 µg/mL Fe_3O_4 NP for 24h and then apoptosis was determined as described in Materials and Methods. Results are means ± SEM (A, n = 6; B, n = 4). *, p <0.05 vs Ctrl; #, p <0.05 vs Fe_3O_4 NP.

5.6 Discussion

Although the number of studies investigating the direct effect of a given NP on the biology of neutrophils is growing, there is still a lot to do in this area of research. Here, using TEM, we found that Fe_3O_4 NP with a primary size of 10 nm can be ingested by neutrophils *in vitro*. To our knowledge, this is the first study showing TEM images that clearly illustrates the presence of Fe_3O_4 NP inside human neutrophils. These results agree with a previous study in which ultrasmall superparamagnetic iron oxide NP (Ferumoxtran-10) were found to be ingested by human monocyte-macrophages *in vitro* (Müller et al. 2007). Intriguingly, it was reported that, *in vivo*, iron oxide NP with a primary size greater than 60 nm are rapidly phagocytosed by cells of the reticuloendothelial system in the liver and spleen, but not those with a smaller size. It is now clear that surface coating and size of iron oxide NP can affect their biodistribution, plasma half-life and cellular uptake (Roohi et al. 2012). Herein, in addition to their ultra-small size of 10 nm, Fe_3O_4 NP were not coated (naked). They do not induce a respiratory burst in neutrophils as evidenced by their inability to increase ROS production after treatment. These results are in agreements with others reported in human neutrophils using up to 4 mg/mL Fe_3O_4 NP demonstrating not only that these NP do not induce ROS production by themselves but that they can also inhibit fMLP-induced ROS production (Garcia et al. 2020). Fe_3O_4 NP were also reported not to induce ROS production in human monocyte-macrophages (Müller et al. 2007). In addition, ~25 years ago, magnetite particles of ~0.8 µm (Estapor® M_1-0.70-/60), although not at the nanoscale (by definition, 3D <100 nm), did not significantly promote superoxide production in human neutrophils, also in accordance with our observations. In contrast, one study reported that iron oxide NP coated and uncoated, also with a primary size of ~10 nm, were found to induce neutrophil oxidative burst (Couto et al. 2014). Of note, although several experimental conditions differ from those used in our present study (cell culture medium, cellular concentration, fluorescent probe, etc.), the most important thing is that ROS production was measured too tardily after 24h of treatment which does not necessarily correspond to a burst (normally observed after few minutes of cell activation) but rather to probably an unspecific accumulation of ROS over time. As discussed by the authors, they mentioned the possibility that other mechanisms can be involved in the ROS production other

than the typical NADPH activation, the major source of ROS production in neutrophils. In this study, we were interested in determining how Fe_3O_4 NP could alter the biology of human neutrophils by investigating several different neutrophil functions others than ROS production, such as adhesion, phagocytosis, cytokine production and apoptosis. Although Fe_3O_4 NP were found to increase, although not significantly, the capacity of neutrophils to adhere onto the human umbilical vein cell line EA.hy926, a clear trend was observed. These results agree with our previous studies demonstrating that Fe_3O_4 NP, using the same essay, significantly increased the human eosinophil cell adhesion (Murphy-Marion and Girard 2018; Chhay et al. 2018). Using endothelial progenitor cells of swine and rat origin, superparamagnetic iron oxide NP were found to promote their adhesion capacity, indicating that these NP can increase cellular adhesion of cells other than granulocytes (Yang, Tang, and Wang 2010). However, the role of Fe_3O_4 NP in cellular adhesion has not been the object of intense studies and much more needed to be performed in this area. Even though several studies investigated the ability of different types of cells to ingest NP (Baumann et al. 2013; Fröhlich 2012; Gliga et al. 2014; Singh and Ramarao 2012), there are fewer reports studying how a given NP could alter the ability of phagocytes to exert phagocytosis. In this report, Fe_3O_4 NP were found to significantly increase the capacity of human neutrophils to phagocytose opsonized SRBCs. This indicates that these NP do not prevent Fc-mediated phagocytosis even if we observed that these NP can penetrate inside human neutrophils as observed 1h and 24h after incubation. This agrees with a recent *in vivo* study revealing that neutrophils ingesting Fe_3O_4@TiO_2 NP retained the cell membrane functionality as well as their immune functions (Zhang, Zhao, et al. 2020). In the above experiments, we have studied ROS production, cell adhesion and phagocytosis that are functions requiring relatively short period of times of incubation of neutrophils with Fe_3O_4 NP. Aiming at studying functions related to inflammation and requiring longer periods of time, we also investigate here the effect of Fe_3O_4 NP on the cytokine production and apoptosis, performed after 24h after incubation of neutrophils with Fe_3O_4 NP. In this study, using an antibody array approach, we found that Fe_3O_4 NP can increase the production of different analytes, including the chemokines MIP-1α/MIP-1β, CXCL1 (GRO-α), and CXCL8 (IL-8). The increased production of IL-8 was confirmed by ELISA. This is not without any precedent and these results agree with others previously reported when neutrophils were activated with other types of NP, including TiO_2 (Gonçalves, Chiasson, and Girard 2010), Au(+) (Durocher et al. 2017), AgNP20, but not AgNP70 (Poirier, Simard, and Girard 2016) nor Pd-NP (Kwemo et al. 2020). Also, our results agree with the above *in vivo* observations reporting that Fe_3O_4 NP-treated mice have elevated concentration of several cytokines and chemokines. Of note, most of the studies in the literature reporting inflammation in iron oxide NP

treated animals, reported not only increased concentrations of a variety of cytokines and chemokines, but also an increased number of leukocytes, merely neutrophils. Therefore, it is tempting to speculate, at least for MIP-1α/MIP-1β, CXCL1 (GRO-α), that neutrophils may be, at least partly, responsible for such an increased concentration of these chemokines. Of note, IL-8 is not expressed in rodents like mice, but some equivalent chemokines have been proposed. It will be important in future to better determine the participation of neutrophils in cytokine production under similar conditions. Several NP have been demonstrated to induce cytotoxicity and apoptosis in a variety of cell types, including human neutrophils (Noël, Simard, and Girard 2016; Poirier et al. 2014; Alarifi et al. 2017; Govender et al. 2013; Zhao et al. 2013; Zhang et al. 2012; Pujari-Palmer et al. 2016). However, the anti-apoptotic activity of NP is less documented, especially in primary human cells. In this study, Fe_3O_4 NP clearly delay human neutrophil apoptosis. This is not without any precedent for NP since we previously documented that TiO_2 NP also delay neutrophil apoptosis (Gonçalves, Chiasson, and Girard 2010) as well as ZnO NP (Goncalves and Girard 2014) and Ag NP with a diameter of 70 nm (Poirier, Simard, and Girard 2016). Further, our results agree with others reporting that non-coated iron oxide NP delay neutrophil apoptosis (Couto et al. 2014). Although sexual dimorphisms exist in innate immunity (Jaillon, Berthenet, and Garlanda 2019) and that sex-specific alteration in neutrophil apoptosis has been reported at least in one study (Molloy et al. 2003), there are still a lot to do in this area. Here, by performing several numbers of different experiments with neutrophils isolated from men ($n \geq 16$) and women ($n = 18$), we demonstrated that Fe_3O_4 NP markedly delay neutrophil apoptosis regardless of sex. This correlates well with one *in vivo* study reporting that no sexual dimorphism was observed in Fe_3O_4-treated male and female rats in terms of cytotoxic, inflammatory, and oxidative stress (Srinivas et al. 2012). In contrast to other types of NP (Chen et al. 2013; Han et al. 2020; Shvedova et al. 2016), and to the best of our knowledge, there is no *in vivo* or *in vitro* study reporting sexual dimorphism in response to Fe_3O_4 NP. In fact, it was estimated that only ~20% of basic research in the general sciences performed experiments in parallel with both male and female animals (Ray et al. 2020b), or with primary cells isolated from both sexes, as we did in this study. In addition, too many studies do not simply address differences between sexes within their analyses. We believe that it is mandatory to incorporate sex-based analysis, whenever possible, into future experiments aiming at understanding how sex could be an important variable in NP-induced toxicity and inflammation. The anti-apoptotic effect of Fe_3O_4 NP was markedly reversed by CHX showing the importance of protein synthesis as a mechanism involved in this biological function. To date, such a mechanism has only been reported by us in two other studies. First, ZnO NP were also found to be anti-apoptotic by a protein synthesis-

dependent mechanism (Goncalves and Girard 2014). Moreover, as for Fe_3O_4 NP, ZnO NP do not induce ROS production in neutrophils. Second, silver NP with as primary size of 70 nm (AgNP70) were also found to delay neutrophil apoptosis but, intriguingly, they do not act by a protein synthesis-dependent mechanism and do not induce ROS production (Poirier, Simard, and Girard 2016). Of note, in parallel, we demonstrated that the same NP, but with a size of 20 nm (AgNP20) were in contrast proapoptotic. Taken together, these observations highlight the fact that not all NP act the same way and how complex is their mode of action. The exact nature of the proteins that are involved in NP-induced suppression of neutrophil apoptosis needs to be determined. Using a panel of pharmacological inhibitors, we demonstrated that Fe_3O_4 NP delay human neutrophil apoptosis by at least by two different cell signaling pathways, MAPK and PI3-K/Akt. Our results correlate with those reported by others showing that iron oxide NP (coated and/or non-coated) can activate cytokine production via transforming growth factor beta (TGF-β)-activated kinase (TAK1), p38 MAPK) and c-Jun N-terminal kinases (JNK) in human peripheral blood cells (Couto, Freitas, et al. 2015), activation of macrophages by activation of p38 MAPK, p44/p42 MAPK (Mulens-Arias et al. 2015) and activation of the MAPK pathway in primary human monocyte cells (Wu, Miao, et al. 2018). In our experimental conditions, PD and Pic inhibitors were found to slightly, but not significantly, inhibit the antiapoptotic effect of Fe_3O_4 NP. Regarding the delaying effect of neutrophil apoptosis induce by other NP, we previously documented that ZnO NP induce tyrosine phosphorylation events, although we did not identify exactly the cell pathways involved (Goncalves and Girard 2014). However, the antiapoptotic effects of TiO_2 NP were found to be associated with activation of extracellular signal-regulated kinases 1/2 (Erk-1/2) and p38 MAPK (Gonçalves, Chiasson, and Girard 2010). In summary (Fig. 14), Fe_3O_4 NP were found to enter inside human neutrophils but do not promote ROS production. However, they can enhance the ability of neutrophils to adhere onto endothelial cells, increase phagocytosis of opsonized SRBCs and induce the production of the potent chemokines MIP-1α/MIP-1β, CXCL1 (GRO-α), and CXCL8 (IL- 8). The antiapoptotic activity of Fe_3O_4 NP is the most remarkable effect observed in this study and this occurs regardless of sex, by a mechanism involving *de novo* protein synthesis and by activation of several cell signaling pathways. Therefore, Fe_3O_4 can be added to a growing list of NP that can alter the biology of human neutrophils and this as to be considered if the aim is to develop or ameliorate therapeutic strategies based on their use. In addition, because of their potential pro-inflammatory activities, peoples manipulating large quantities of Fe_3O_4 NP and others that are repeatedly exposed must take appropriated precautions.

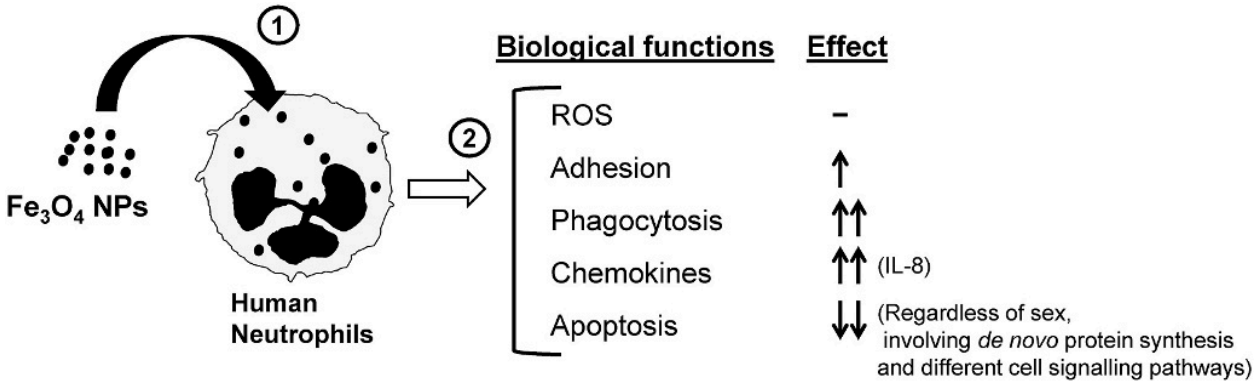

Figure 14: A schematic diagram summarizing how Fe_3O_4 NP can alter the biology of human neutrophils.

(1) Fe_3O_4 NP are uptaked by neutrophils and can be in vacuole-like structures or can be randomly distributed in the cytosol and near or in the nucleus. (2) Production of ROS is not induced by the Fe_3O_4 NP whereas they can increase neutrophil cell adhesion onto endothelial cells, phagocytosis of opsonized SRBCs and chemokine production including IL-8. In contrast, Fe_3O_4 NP delay human neutrophil apoptosis regardless of sex and the mechanism is complex and involves *de novo* protein synthesis and several cell signaling pathways.

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Declaration of competing interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

6 SUPPORTING DATA (UNPUBLISHED)

6.1 Methodology for detecting phosphorylated p38, Erk1/2, Akt, and Syk :

Isolated cells (PMN) were incubated (4 x 10^6 / 100 µL) with buffer (Ctrl), GM-CSF (65 ng/mL) or LPS (1µg/mL) for 5 min or 100 µg/mL of Fe_3O_4 NP for 1, 5 and 15 min at room temperature. After incubation, cells were lysed in 1X Laemmli's sample buffer (0.25 M of Tris–HCL (pH 6.8), 8% SDS, 40% glycerol and 20% 2-ME), and aliquots of extracts corresponding to 8 × 10^5 cells were loaded onto 10% SDS-PAGE gels, followed by electrophoresis and then transferred onto polyvinylidene fluoride membrane for the detection of p-p38, p-ERK, p-Akt or p-Syk. Membranes were blocked for 1h at room temperature in TBS-Tween containing 5% BSA. After washing, the anti-phospho specific antibodies were added at a final dilution of 1:1000 (1:500 for p-syk). The membranes were kept overnight at 4 °C, then washed with TBS-Tween and incubated for 1h at room temperature with HRP secondary antibody, diluted to 1:20000 in TBS-Tween 5% BSA, followed by several washes. Protein expression was revealed using HRP Substrate. Membranes were stripped with ReBlot Plus Strong and reprobed to confirm equal loading of proteins at the non-phosphorylated state and then by using anti-GAPDH (1:1000). Chemiluminescence was revealed with a chemiDoc (Gonçalves, Chiasson, and Girard 2010). Note that, for p-Syk detection cells were pretreated with 0.2 µl/mL di-isopropyl fluorophosphate to prevent its degradation before incubation with different agonists (Mócsai et al. 2000)

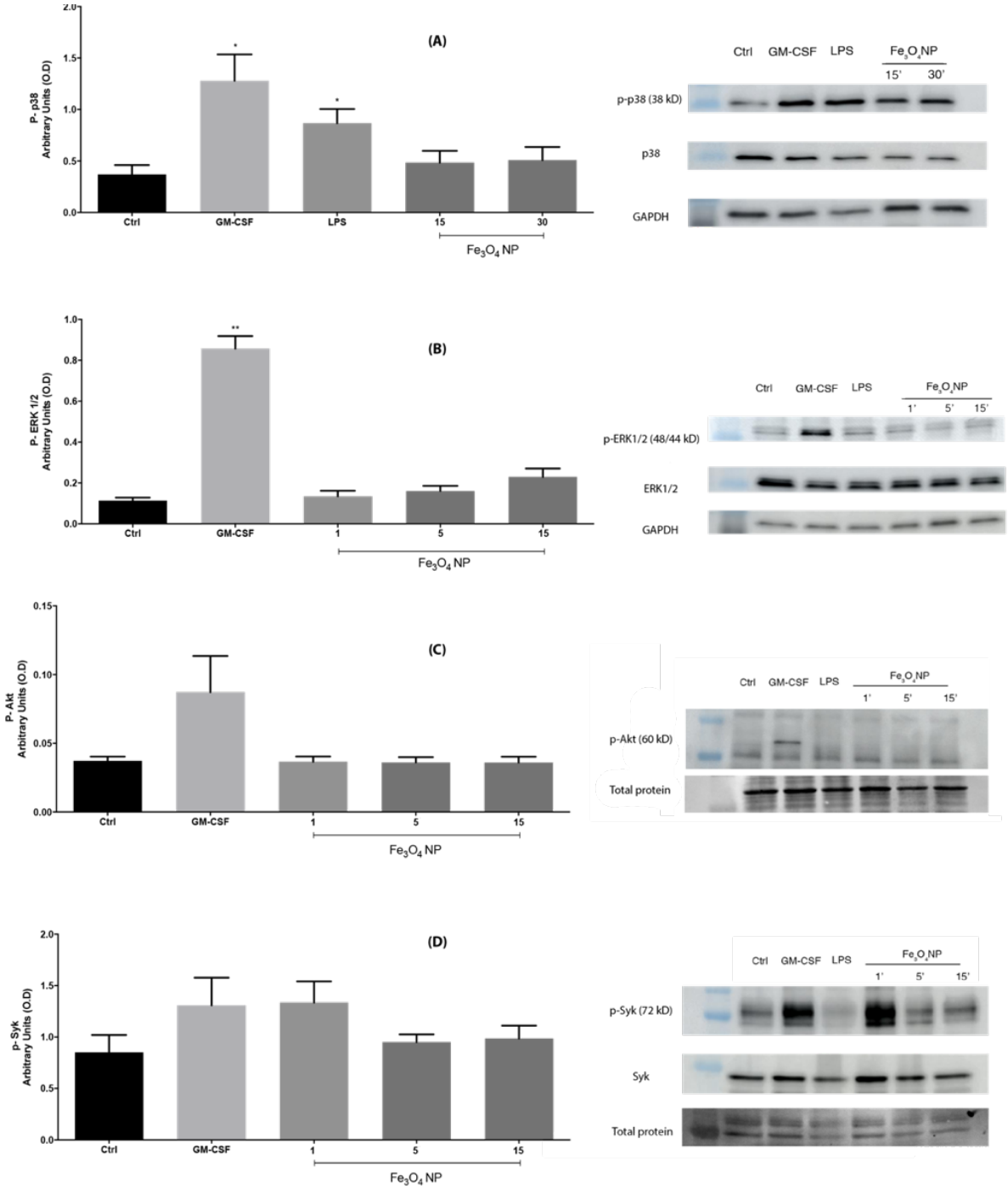


Figure 15: p38, Erk1/2, Akt, and Syk phosphorylation in human neutrophils.

Freshly isolated human neutrophils were incubated for 1, 5, and 15 min in the presence of a buffer (Ctrl), GM-CSF, LPS, or Fe_3O_4 NP (100 µg/mL). The iron particles induced a slight increase in the phosphorylation of p38 (A; n=9) and Erk1/2 (B; n=5), but not Akt (C; n=4). Additionally, an increase in Syk was observed (D; n=3). Statistical analysis was performed using Tukey's test (± SEM).

6.2 Methodology for assessing the effect of Src and Syk inhibition on SRBCs phagocytosis

Sheep Red Blood Cells (SRBCs) were opsonized with a 1:200 dilution of a rabbit IgG anti-SRBC Antibody for 45 min at 37 °C. Neutrophils were treated with the following agonists: buffer (negative Ctrl), 65 ng/mL GM-CSF (positive control) or Fe_3O_4NP (100 µg/mL) for 30 min, and then incubated in a 1:5 ratio with opsonized SRBCs for 30 min at 37 °C. In some experiments, cells were treated with the Src inhibitor PP1 (50 µM), or Syk inhibitor piceatannol (30 µM) for 30 min prior to incubation with the agonists. After incubation with SRBCs, the samples were centrifuged at 200 × g for 10 min at 4°C. Supernatants were discarded and an osmotic shock was performed on the pellets by resuspending the cells with 400 µl H_2O for 15 secondes, followed by the addition of 4.5 mL PBS 1X. The samples were then washed twice and the final cell pellets were suspended in 100 µL PBS 1X. Phagocytosis is expressed as the percentage of neutrophils (% phagocytosis) having ingested at least one opsonized SRBC. A total of ~200 cells/slide were evaluated in duplicate.

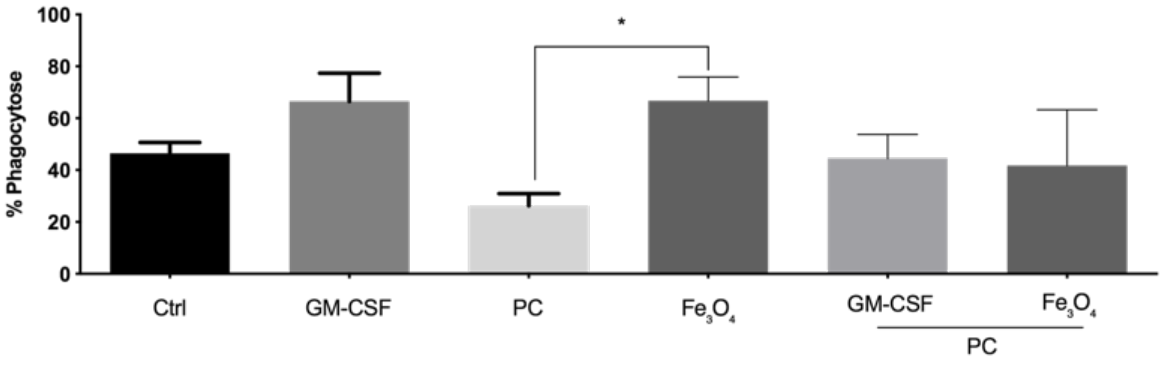


Figure 16: Fe_3O_4 NP increase the phagocytic capacity of neutrophils through a mechanism that involves the Syk pathway.

Human neutrophils were pretreated with piceatannol for 30 minutes and then with different agonists before being incubated with opsonized sheep red blood cells. The percentage of phagocytosis was determined by cytology. Statistical analysis was performed using Tukey's test (± SEM, n=4). *, p<0.05 vs PC.

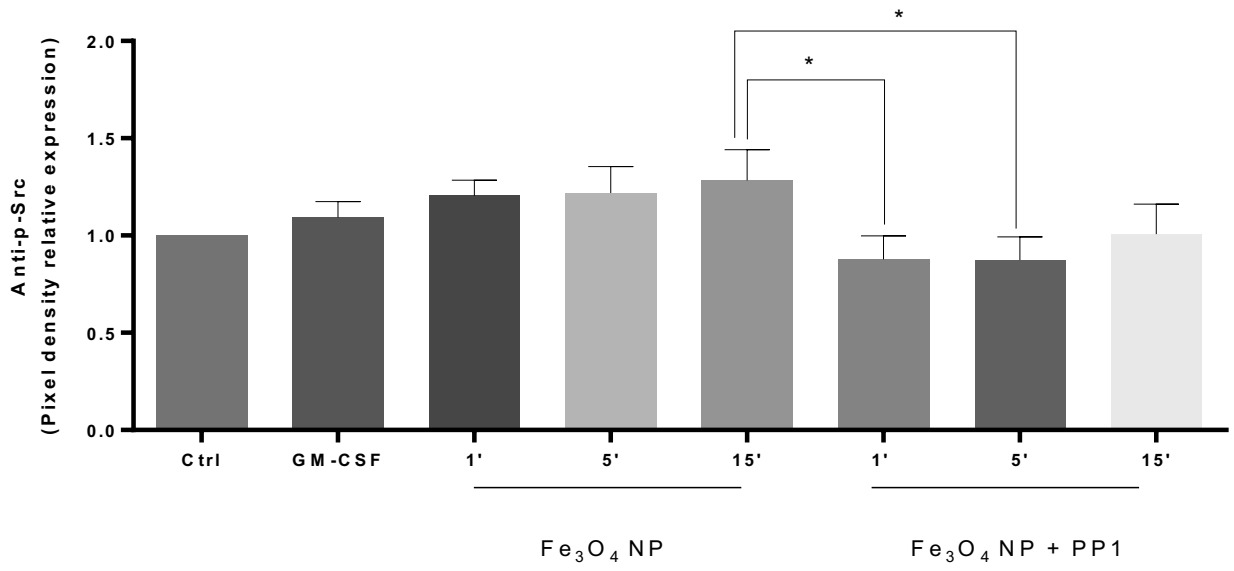
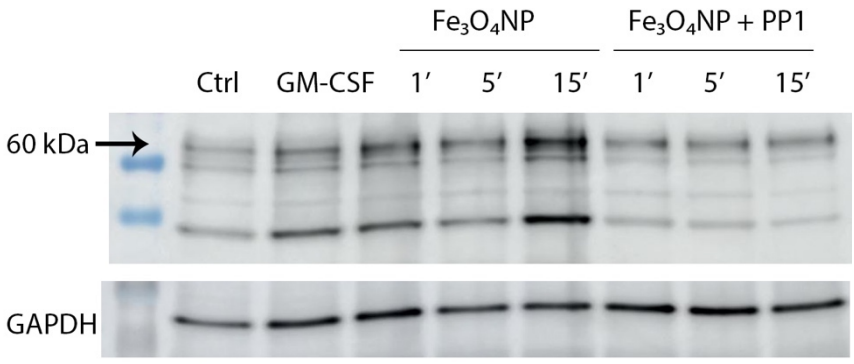


Figure 17: Fe_3O_4 NP increase phosphorylation of Src in PMN.

Cells were incubated with Buffer and GM-CSF for 15 minutes, and with Fe_3O_4 NP at a concentration of 100 µg/mL for 1, 5, and 15 min. In another set of experiments, cells were pre-treated for 10 min with PP1 , a selective inhibitor of Src, before being treated with Fe_3O_4 NP. (n=7). Statistical analysis was performed using Tukey's test (± SEM, n=7). *, p<0.05 vs Fe_3O_4 NP (15').



Representative image of one of the analyzed blot of p-src (60 kDa)

6.3 Methodology for phospho-kinase array analysis

The phospho-kinase array was conducted using a mixed pool of supernatants from seven donors after incubating their cells (1 x 10^6 cells per sample) with either a control buffer (Ctrl) or 100 µg/mL of Fe_3O_4 NP for 8 minutes. Following the manufacturer's protocol, neutrophils were then incubated in Lysis Buffer 6 from the ARY003C kit by R&D Systems, which was supplemented with 10% PhosStop (ID: 4906845001), as well as 4% each of protease inhibitor cocktails I (ID: 535142) and III (ID 535140) from SigmaAldrich. Cells were centrifuged at 4 °C and the resulting supernatants were stored at -70°C and used within six months. The expression of a protein (indicated by a gray or black spot) was observed on the membrane following revelation using a ChemiDoc, and the densitometry of the spots was analyzed using ImageJ to assess the level of protein phosphorylation (Dumitru et al. 2012).

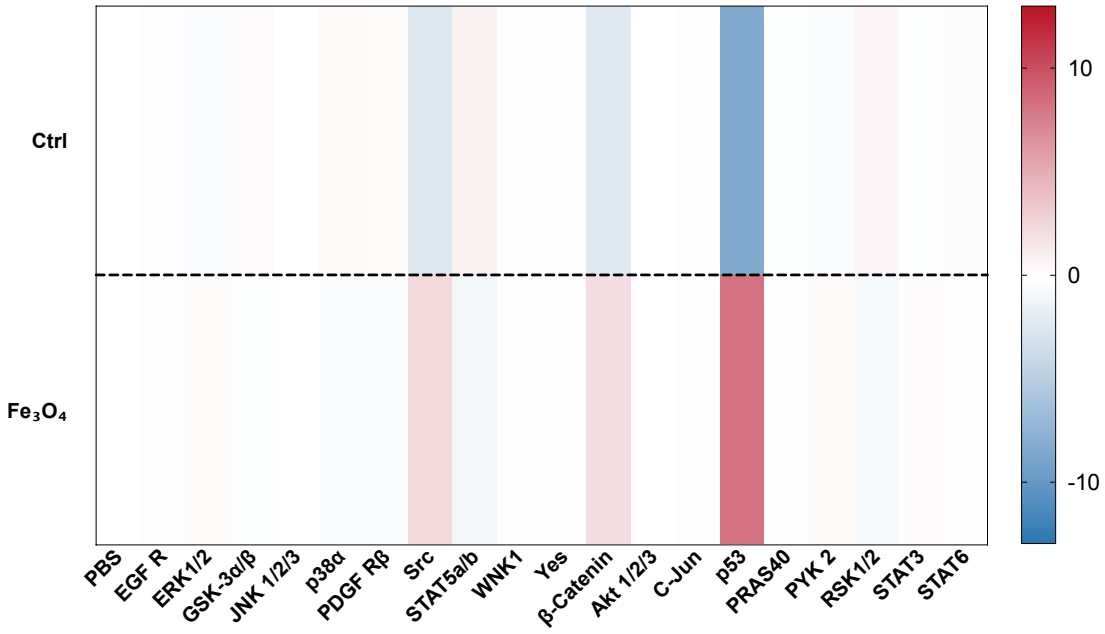


Figure 18: Fe_3O_4 NP can induce the phosphorylation of Src, β-Catenin and p53 factors in human neutrophils.

Freshly isolated human neutrophils were incubated for 8 min in the presence of buffer (Ctrl) or 100 µg/mL of Fe_3O_4 NP. Then, cells were lysed in a lysis buffer supplemented with anti-phosphatases/anti-proteases, and collected lysates were conserved at -70 ℃ for less than six months until analysis. For each condition, the cells lysates of 7 donors were pooled to make one test sample and assessed by kinase array assay. The heatmap plot was performed by using GraphPad Prism 8; A value of 0 indicate the baseline of different phosphorylated proteins, the maximum value is 13 whereas the smallest value is -13.

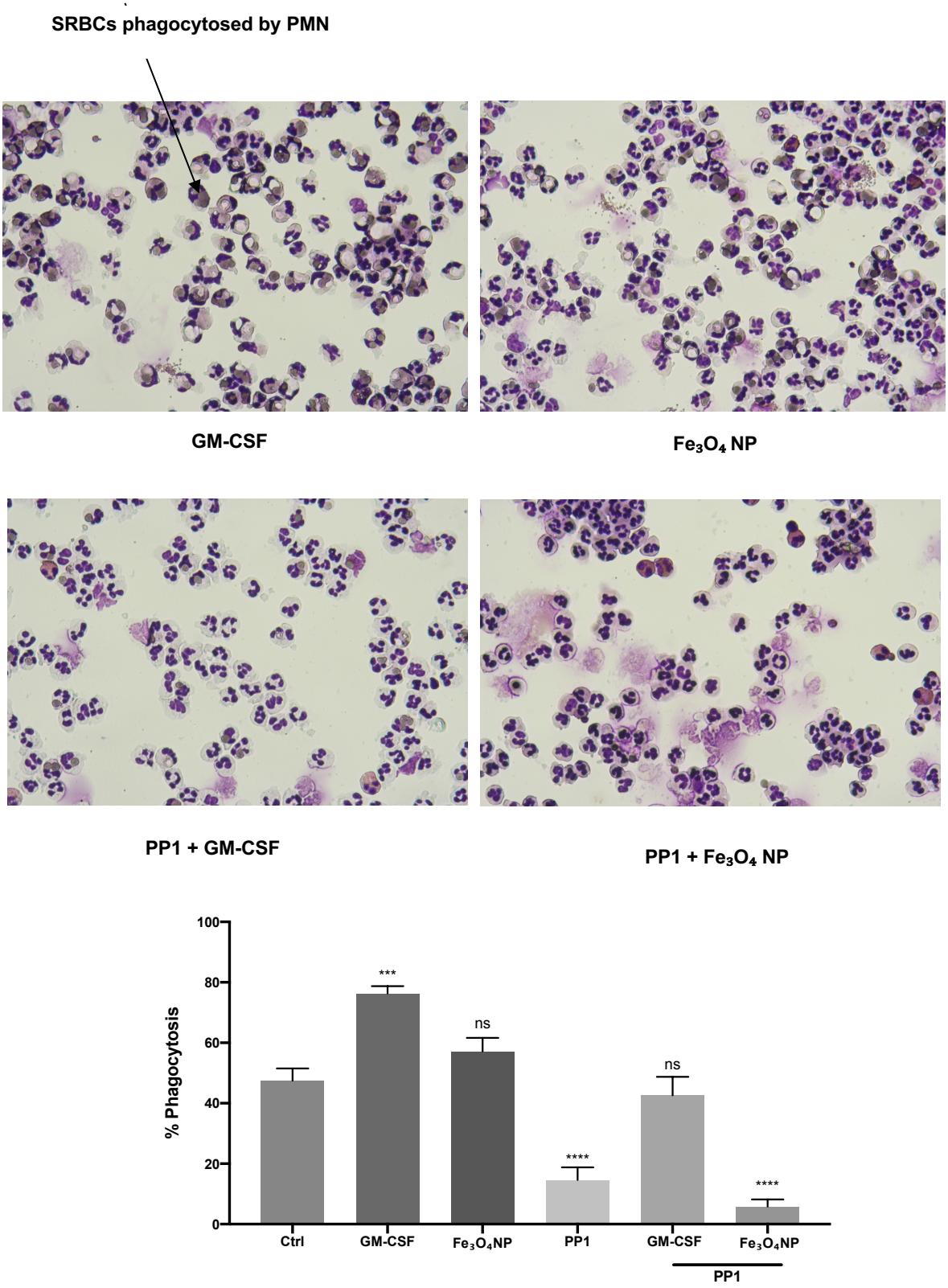


Figure 19: Inhibition of Src by PP1 limits the capacity of PMN to internalize SRBCs following their exposure to Fe_3O_4NP.

Human neutrophils were directly incubated in GM-CSF, or Fe_3O_4 NP. Alternatively, cells were pre-incubated with PP1 for 30 minutes, followed by incubation with GM-CSF or Fe_3O_4 NP for 30 minutes. Subsequently, the cells were incubated with opsonized SRBCs. The number of phagocytosed SRBCs was assessed using two slides for each condition, randomly counting at least 200 cells for each slide. Statistical analysis was performed using Tukey's test (± SEM). *, p<0.05 vs Ctrl (n=4).

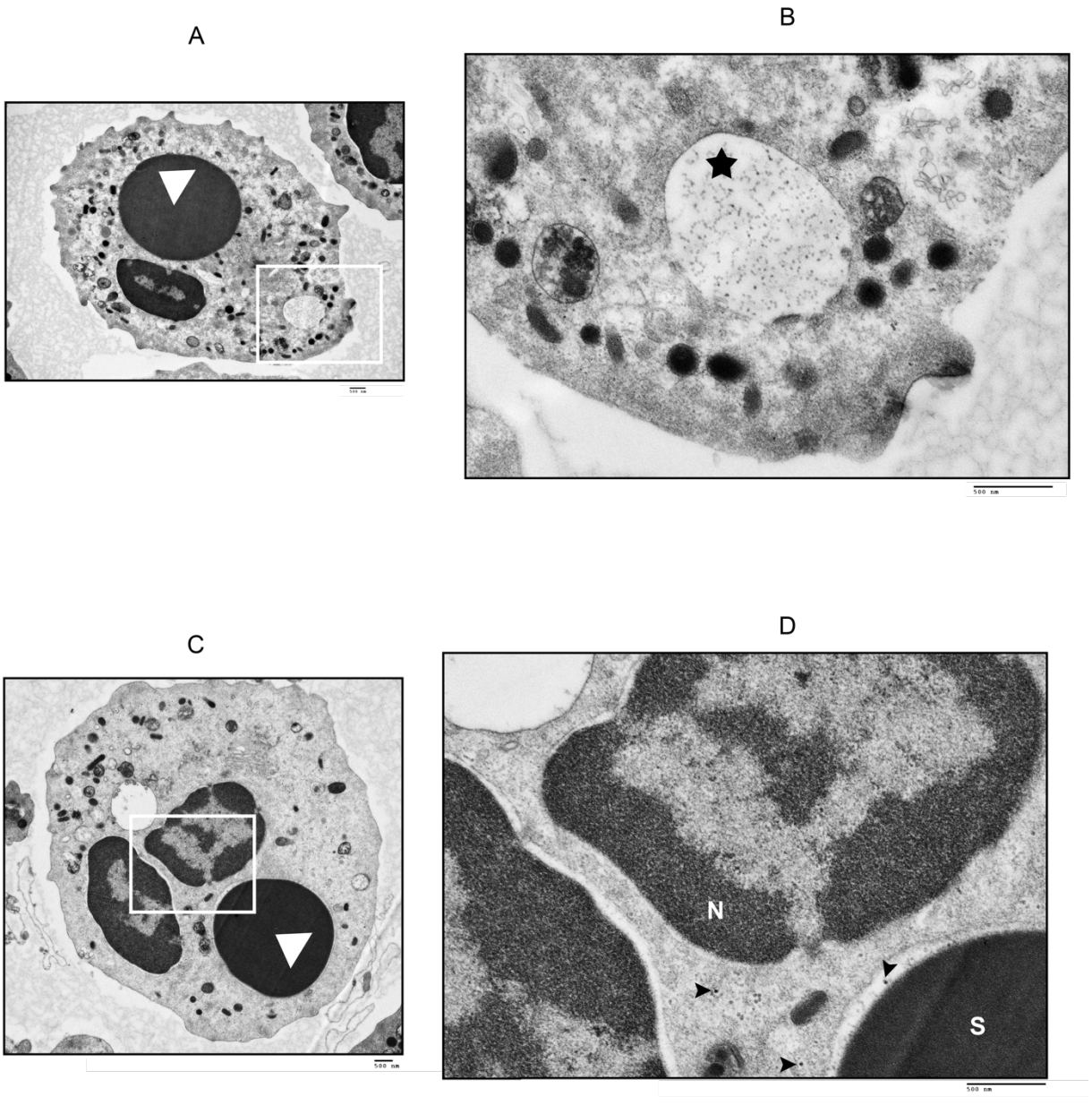


Figure 20: Fe_3O_4 NP do not alter the capacity of neutrophils to phagocytize sheep red blood cells (SRBCs).

In the whole-cell micrograph on the left (A and C; X5000), a sheep red blood cell is indicated by a white triangle and is seen near the cell nucleus. The magnification of the white frame in micrograph A on the right (B; X17000)

shows a vacuole-like structure indicated by the black star, in which Fe_3O_4 NP are enclosed. On the other side, micrograph D shows the magnification of the white frame in micrograph C, indicating free Fe_3O_4 NP (black arrowhead) in the cytoplasm between a red blood cell (S) and the nucleus (N).

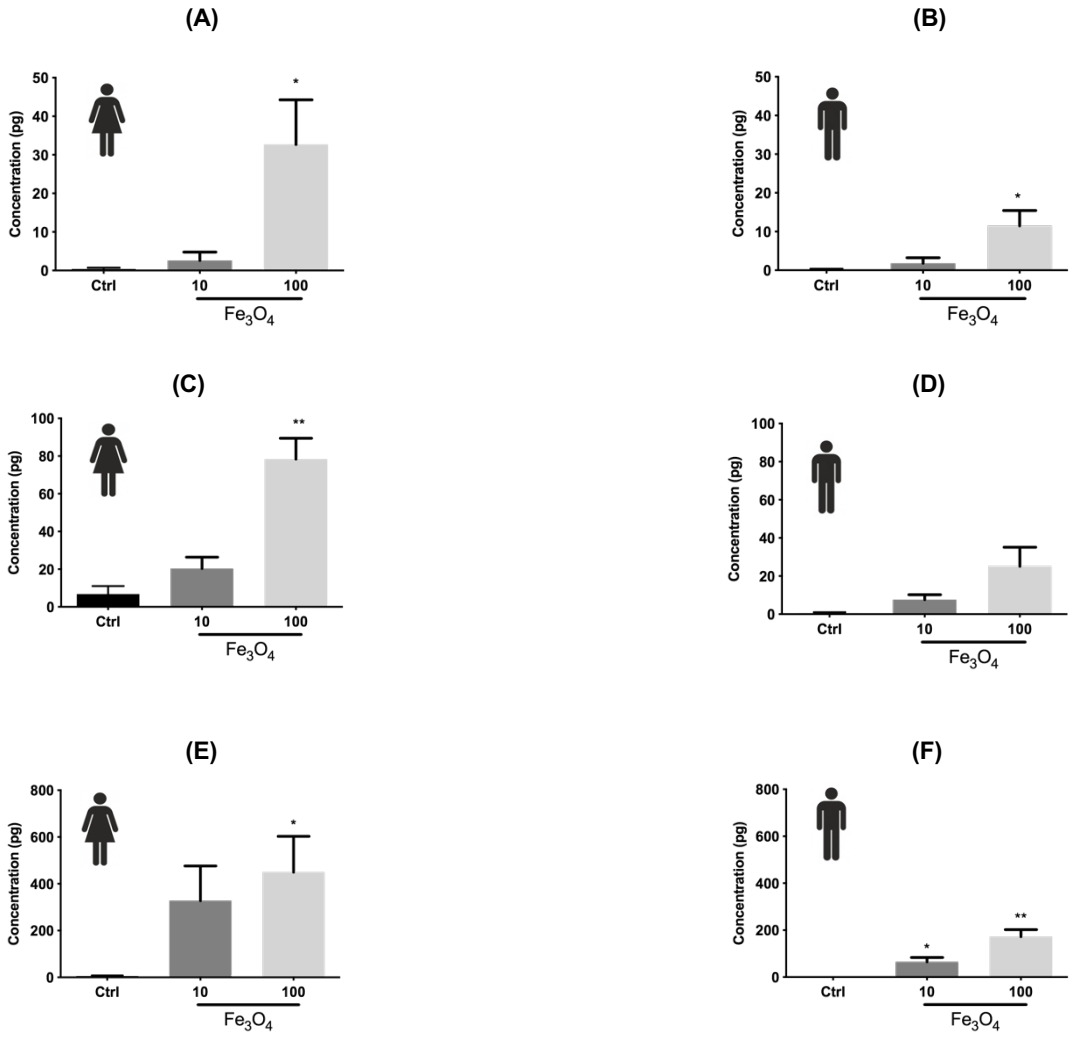


Figure 21: Fe_3O_4 NP can induce the production of pro-inflammatory cytokines TNF-α , IL-1β and IL-6 in both women and men.

Isolated human neutrophils were incubated for 24 hours in the presence of a buffer (Ctrl), 10 or 100 µg of Fe_3O_4NP, then the cell culture supernatants were conserved in -20 ℃ until analysis by ELISA. (A) and (B) present the production of the pro-inflammatory cytokine TNF-α in women (n=13) and man (13) respectively. On the other side, (C) and (D) present the production of the pro-inflammatory cytokine IL-1β in women (n=7) and men (n=7) respectively; and (E) and (F) present the production of the pro-inflammatory cytokine IL-6 in women (n=8) and men (n=8) respectively. Statistical analysis was performed using Tukey's test, (± SEM). *, $p<0.05$ vs Ctrl.

(A)

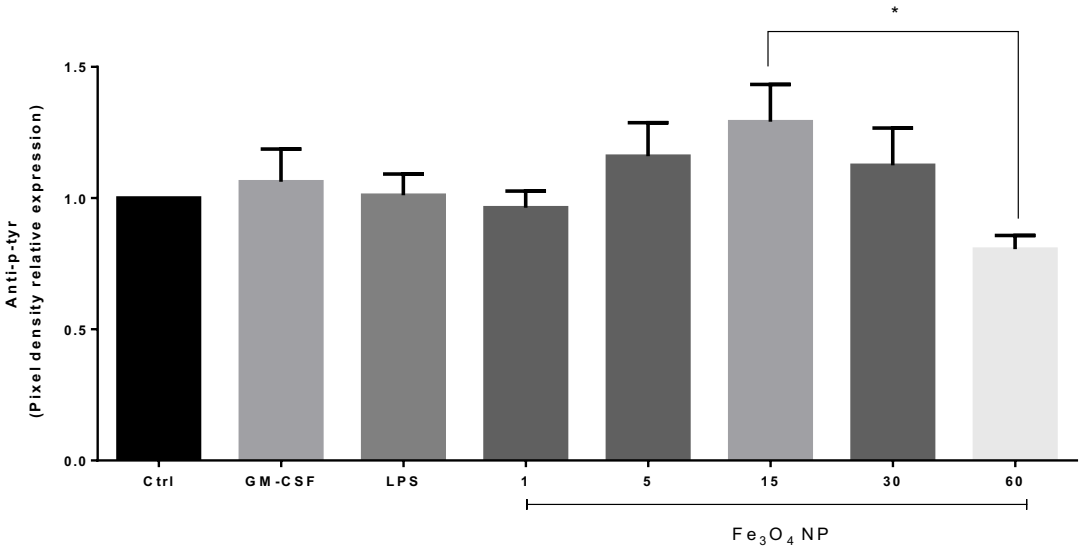
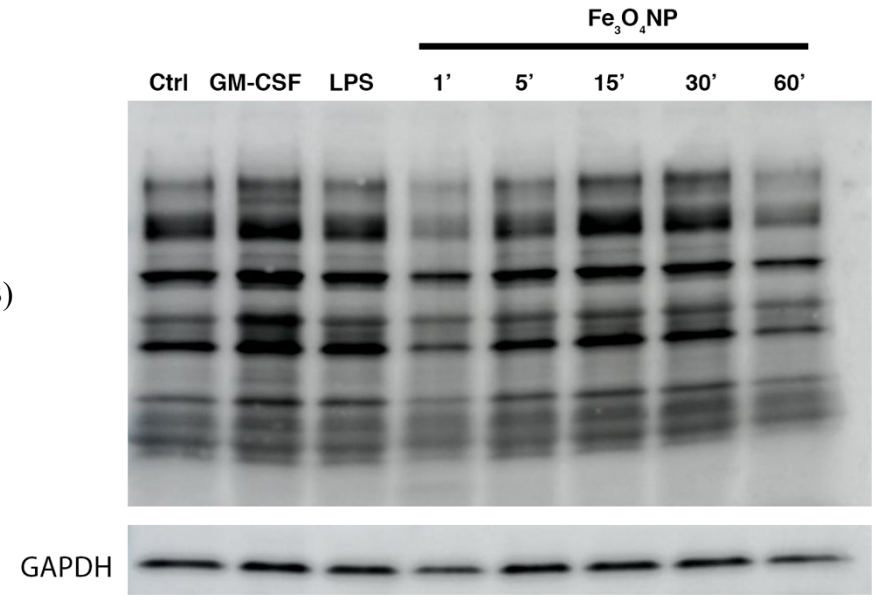


Figure 22: Fe_3O_4 NP are able to induce phosphorylation events in PMN.

Cells were pre-incubated with buffer, GM-CSF, LPS, and Fe_3O_4 NP (100 µg/mL) for 1, 5, 15, 30, and 60 min. Then from cell lysates assessment of tyrosine phosphorylation events was performed by western blot using a pan anti-phosphotyrosine antibody. Statistical analysis was conducted using Tukey's test (± SEM, n=7). *, p < 0.05 vs. Fe_3O_4 NP _15'.

(B)



(B) Representative image of one of the analyzed blot of p-tyrosine .

6.4 Methodology for assessing NETs release

The method we used was based on seeding human neutrophils (1x10⁶/well) on glass coverslips coated with poly-L-lysine. Cells were allowed to adhere for 30 min at 37°C and were subsequently incubated with buffer (Ctrl), Ag NP15 (used as a control), and Fe_3O_4 NP (100 µg/mL) for 1h. Cells were fixed directly in the plate and stained using HEMA 3 staining. NETosis was assessed by optical microscopy, observing the presence or absence of filamentous structures (Liz et al. 2015).

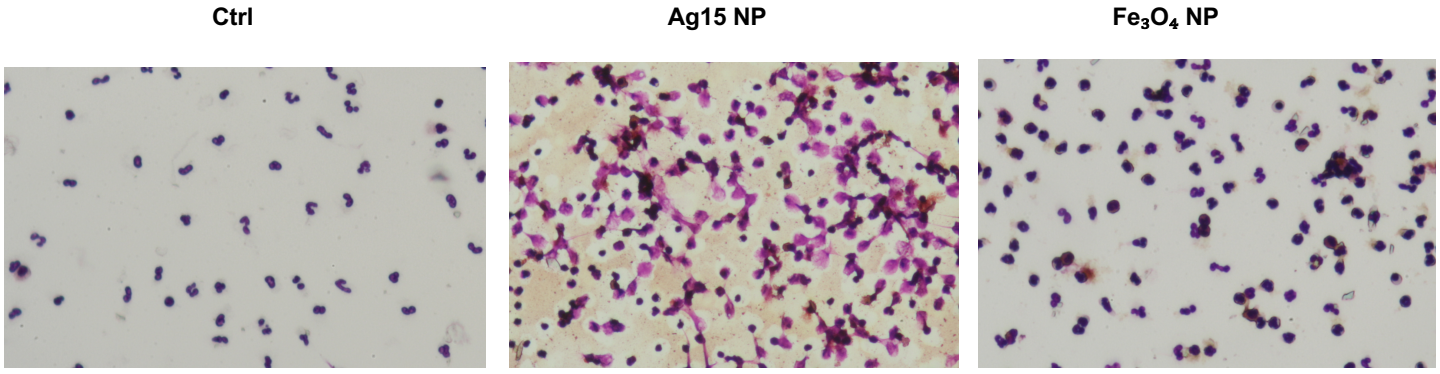


Figure 23: Fe_3O_4 NP do not induce netosis in human neutrophils.

Freshly isolated neutrophils were incubated with buffer (Ctrl), Ag15 NP or Fe_3O_4 NP for 90 minutes on Poly-L-Lysine pre-treated slides and then stained with Hema 3 staining for visualization by optical microscopy (X 100) (n=3).

6.5 Methodology for assessing degranulation (Zymography assay)

Neutrophils were treated with buffer (Ctrl), fMLP (1 µM), 10 µg/mL or 100 µg/mL Fe_3O_4 NP for 30 min and samples were then centrifuged at 2000 rpm for 10 min at 4 ℃. The pellets were discarded and the supernatants (30 µL, corresponding to 5 x 10^4 cells) were mixed with 10 µL of a non-reducing buffer (40% glycerol, Tris–HCl 1 M, pH 6.8, SDS 8%, without Beta-Mercaptoethanol) and separated on 12% acrylamide gels containing 0.2% gelatin. Then gels were washed twice for 30 min with 2.5% Triton X-100 and incubated overnight in digestion buffer (Tris–HCl 50 mM, pH 7.4,NaCl 150 mM, $CaCl_2$ 5 mM) at 37 ℃ . The gels were then stained with Coomassie blue 0.1% and destained to visualize the matrix metalloproteinase-9 (MMP-9) activities degrading the gelatin present in the gel, the densitometry of bands on the gel was analyzed using ImageJ (Hu and Beeton 2010; Durocher et al. 2017)

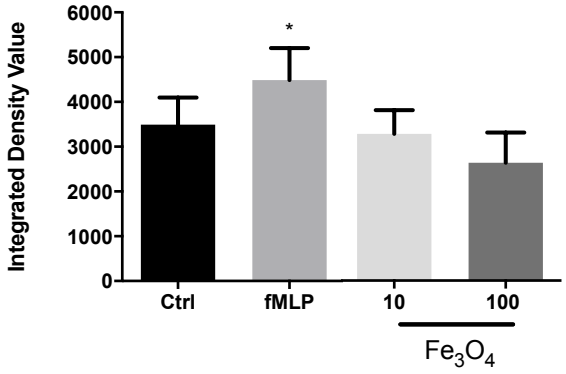


Figure 24: Fe_3O_4 NP do not induce degranulation in neutrophils.

Human neutrophils were incubated for 30 min in the presence of a buffer (Ctrl), fMLP, 10 or 100 µg/mL of Fe_3O_4 NP, and then the supernatants were collected to assess the activity of gelatinase B (MMP-9) by zymography assay. Statistical analysis was performed using Tukey's test, (± SEM). *, p<0.05 vs Ctrl (n=5).

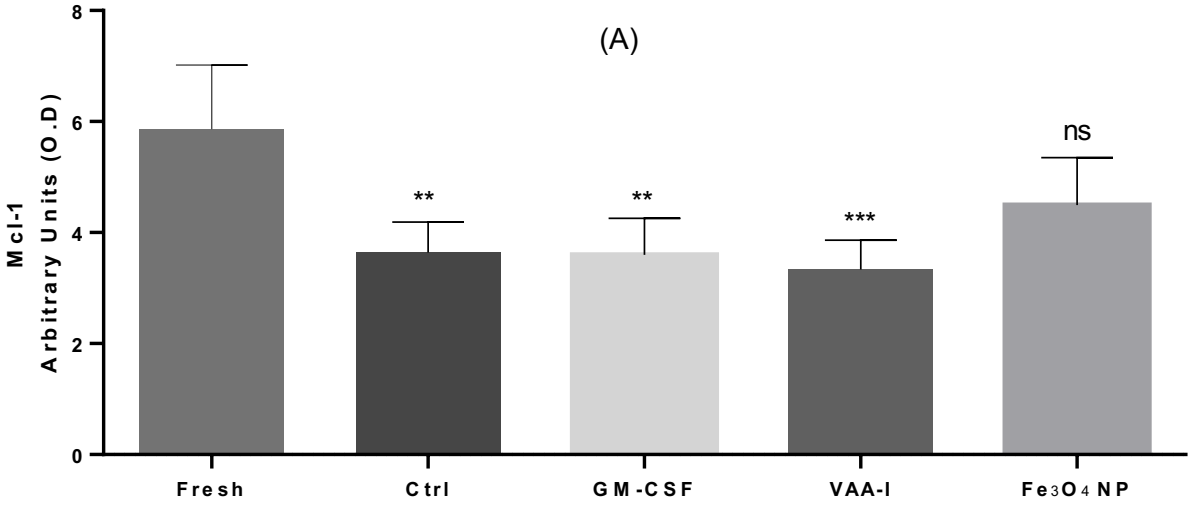
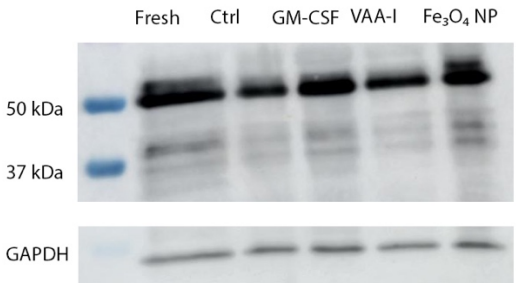


Figure 25: Fe_3O_4 NP do not decrease Mcl-1 (p40/p42) expression)in human neutrophils (A).

Freshly isolated human neutrophils were incubated for 24h in the presence of a buffer (Ctrl), GM-CSF, or Fe_3O_4 NP (100 µg/mL). Statistical analysis was performed using Tukey's test (± SEM, n=7). *, p<0.05 vs Ctrl

(B)



(B) Representative image of one of the analyzed blot of Mcl-1 (40 kDa).

7 GENERAL DISCUSSION

The toxicity of NP towards the immune system is an issue that should be addressed in order to understand the interaction between these tiny entities and immune cells, to ensure the implementation of appropriate protective measures during their production, use, and elimination in occupational and/or clinical settings. In this thesis, we have specifically studied the response of human neutrophils (PMN) to iron oxide nanoparticles (Fe_3O_4 NP) to assess their pro-inflammatory potential and to better understand their mode of action.

Spherical Fe_3O_4 NP without coating, with a size of approximately 10 nm as determined by TEM, have been found to modulate the biological functions of PMN. These particles were found to inhibit PMN constitutive apoptosis, which is a mechanism that regulates their number in circulation to maintain homeostasis. Interestingly, this interference is concentration-dependent, with the most significant effect observed at the highest concentration of 100 µg/mL. Furthermore, no difference has been observed in the modulatory effect between men and women, indicating that sexual dimorphism is not involved.

This observation confirms the capacity of NP to modulate PMN apoptosis, as it has already been documented in many other studies. For instance, zinc oxide (ZnO) NP have been shown to interfere with PMN spontaneous apoptosis, resulting in increased cell viability (Goncalves and Girard 2014). However, other NP, such as silver (Ag) NP and gold (Au) NP, have been shown to accentuate (i.e., accelerate) this process in PMN (Poirier et al. 2014; Durocher et al. 2017). It is worth noting that the highest concentration used in this study is roughly comparable to the concentration that a patient can receive clinically from a single-dose vial of Feraheme®, which is a coated Fe_3O_4 NP-based drug. Based on a formula published in a previous study (Dobrovolskaia and McNeil 2013), it has been estimated that the concentration of Feraheme® a patient receives *in vivo* is 102 µg/mL, given that the concentration of a single-dose vial of Feraheme® is 510 mg (Datum obtained from https://www.feraheme.com/dosing-administration/). Additionally, a concentration of 100 µg/mL is commonly used in *in vitro* nanosafety assessment studies (Kwemo et al. 2020; Goncalves and Girard 2014; Gonçalves, Chiasson, and Girard 2010; Babin et al. 2013b; Grosse, Stenvik, and Nilsen 2016; Couto, Sousa, et al. 2015; Couto et al. 2014).

Interestingly, the interference with PMN constitutive apoptosis by Fe_3O_4 NP shows a similar trend as GM-CSF, a well-known anti-apoptotic agent of PMN required for their differentiation during granulopoiesis (Fossati et al. 1998). Importantly, the interference with PMN apoptosis can be reversed by inhibiting *de novo* protein synthesis mechanism with cycloheximide, indicating the involvement of this process, as demonstrated previously for ZnO NP (Goncalves and Girard 2014). It is noteworthy that this response is directly attributed to Fe_3O_4 NP, rather than other artifacts such as endotoxins, as these particles were assessed using the LAL assay, a well-known assay for LPS detection (Mangini et al. 2021), and were found to be relatively free of endotoxin contamination. Furthermore, the Fe_3O_4 NP solution incubated in agar media, as used in previous studies (Bilyy et al. 2018a; Goncalves and Girard 2014), did not exhibit any bacterial growth, as no bacterial colonies were detected after 72h of post-incubation, confirming the sterility of these particles. Additionally, TEM analysis revealed that PMN are able to efficiently engulf Fe_3O_4 NP in phagosome-like structures, indicating a direct interaction between these particles and cells. These observations strongly suggest that Fe_3O_4 NP are responsible for the observed modulatory effects.

Although the exact mechanism of Fe_3O_4 NP internalization is not clear, the negative charge of Fe_3O_4 NP determined by DLS suggests that an active mechanism such as phagocytosis may be involved. In fact, negatively charged NP are less likely to be incorporated on the cell surface compared to positively charged ones, which have higher rates of non-specific internalization (Oh et al. 2010). Studies have shown that the Syk pathway associated with Fcγ receptors is a key pathway used by phagocytic cells to internalize opsonized particles (Kiefer et al. 1998). Western blot (WB) analysis of phosphorylated Syk in our study has demonstrated its activation (Refer to SUPPORTING DATA, Figure 15.D). Interestingly, it was observed that Fe_3O_4 NP enhance phagocytosis in a similar way to GM-CSF (Fleischmann et al. 1986). Treatment with Piceatannol, a specific inhibitor of Syk, resulted in a decrease in the phagocytic capacity of SRBCs by PMN, confirming to some extent the involvement of this pathway in response to Fe_3O_4 NP (Refer to SUPPORTING DATA, Figure 16). On the other hand, Src kinase protein, which is upstream of Syk, showed its upregulation in the presence of Fe_3O_4 NP, as determined by WB and the human kinase array (Refer to SUPPORTING DATA Figure 17 and 18). Most importantly, the inhibition of Src by a specific pharmacological inhibitor known as PP1 has been shown to reverse the internalization of SRBCs in PMN pre-treated with Fe_3O_4 NP (Refer to SUPPORTING DATA, Figure 19). It is unusual to mention the involvement of phagocytosis for particles that are under 100 nm, but our findings suggest that phagocytosis could be the main mechanism of internalization, primarily due to the fact that Fe_3O_4 NP have been observed by TEM to aggregate

and form particles with a diameter larger than 1 µm. It is well-known that larger particles in the micrometer scale (> 5 µm) are taken up by cells of the mononuclear phagocyte system through phagocytosis (Boraschi et al. 2017). Moreover, this can be supported by previous studies demonstrating the capacity of TiO_2, CeO_2, and ZnO to enhance phagocytosis of sheep red blood cells (SRBCs) through a Syk-dependent mechanism (Babin, Goncalves, and Girard 2015). Nevertheless, we did not study the mechanistic aspects of this process with Fe_3O_4 NP. As a result, the use of the term 'endocytosis,' which englobes pinocytosis, receptor-mediated endocytosis, and phagocytosis is recommended (Xiaofeng Ding 2018; Francia et al. 2019; Akinc and Battaglia 2013).

Moreover, it is not clear whether Syk activation is associated with absorbed proteins on the surface of Fe_3O_4 NP (referred to as biocorona) or with the particles themselves. Nonetheless, PMN that internalize Fe_3O_4 NP still retain their FcγR-dependent phagocytic functions towards SRBCs. This has been demonstrated through TEM analysis, which has shown that Fe_3O_4 NP can be found freely in the cytoplasm or enclosed within phagosome-like structures separate from the phagosome where SRBCs are enclosed in (Refer to SUPPORTING DATA, Figure 20).

The effects of Fe_3O_4 NP on PMN go beyond apoptosis and phagocytosis, as they also impact adhesion, ROS, and cytokine production, all of which are crucial mechanisms during the inflammatory response. Notably, the activation of PMN is associated with the modulation of pro-inflammatory cytokine production. Indeed, Fe_3O_4 NP promote the release of IL-8, MIP-1α/β, and CXCL-1, as confirmed by the human cytokine array (Figure 11.B). It is important to recognize that the generation of IL-8 might not be solely attributed to the presence of NP but could also result from NP being contaminated with endotoxins or the immune system of the blood donor being affected by an asymptomatic infection. Hence, the evaluation of other pro-inflammatory cytokines is essential (Riediker et al. 2019). In our study, the use of human cytokine array (HCA) was a practical approach to detect a wide range of pro-inflammatory and anti-inflammatory cytokines. However, it is worth noting that the sensitivity of HCA is relatively weak compared to the ELISA assay, as it requires a larger volume of cell culture supernatants (Anloague 2019). As a result, IL-6, IL-1β and TNF-α were not detected by HCA, unlike ELISA, which confirms the lower sensitivity of HCA in comparison to ELISA. Indeed, by ELISA, it was confirmed that Fe_3O_4 NP can induce the production of TNF-α, IL-1β, IL-6, and IL-8 in both men and women donors (Refer to SUPPORTING DATA, Figure 21). Interestingly, a previous study has shown that exposure of monocytes to Fe_3O_4 NP also induces the production of both TNF-α and IL-1β (Wu, Miao, et al.

2018). Eventually, cytokine production induced by Fe_3O_4 NP ultimately alters the biological functions of PMN, as evidenced by the observed increase in cell adhesion capacity of PMN onto the human umbilical vein cell line EA.hy926 following pre-incubation with Fe_3O_4 NP. This increase in adhesion appears to correlate with the production of TNF-α, which is known to act in an autocrine mode to upregulate the surface expression of adhesion molecules such as Mac-1 (CD11b/CD18) and LFA-1 (CD11a/CD18) on the surface of PMN, thereby promoting cell adhesion (Silveira et al. 2018).

Regarding oxidative stress, Fe_3O_4 NP do not induce the canonical mechanism of ROS machinery activation, known as oxidative burst, suggesting that these particles activate PMN through a different mechanism than the one used against microorganisms. Previous literature has shown that ROS production is correlated with PMN apoptosis (Pérez-Figueroa et al. 2021), which implies that the observed anti-apoptotic effect of Fe_3O_4 NP could be related to their incapacity to promote ROS production and oxidative stress. This observation is consistent with a study on uncoated Fe_3O_4 NP of approximately 10 nm, which also showed similar findings (Couto et al. 2014). Moreover, another study demonstrated that Fe_3O_4 NP, when incubated with PMN, significantly enhanced the viability of *Staphylococcus aureus* in a concentration-dependent manner. This suggests that the presence of Fe_3O_4 NP interferes with the machinery of ROS production in cells, leading to enhanced survival of bacteria (Świętek et al. 2022).

It is possible that some intrinsic proteins play a role in this impairment effect. Indeed, PMN express iron metabolism-related proteins, such as transferrin (iron uptake), ferritin (iron storage), and ferroportin (iron release), as well as iron scavenger proteins like lipocalin-2, neutrophil gelatinase-associated lipocalin, calprotectin, and lactoferrin in their granules. These proteins are used to tackle bacterial infection (Ni et al. 2022; Cronin et al. 2019). Particularly, lactoferrin (Lfr), which is prominently expressed by PMN at approximately 3 µg per 10^6 cells (Masson , Heremans , and Schonne 1969), equips these cells to effectively handle iron overload. The abundance of this iron chelator in PMN may confer persistence against potential cytotoxic effects of Fe_3O_4 NP. This notion is supported by the fact that iron overload is known to be associated with the Fenton reaction, where ferrous ions (Fe^{2+}) interact with hydrogen peroxide (H_2O_2) to generate hydroxyl radicals (•OH) that induce oxidative stress through lipid peroxidation (Stockwell 2022). Interestingly, a study has demonstrated that Lfr, in addition to its iron-binding activity, also possesses scavenger properties against •OH (Ogasawara et al. 2014). This suggests that Lfr may

serves as a cytoprotective element against Fe_3O_4 NP by neutralizing the reaction that leads to the production of •OH.

Mechanistically, Fe_3O_4 NP were found to induce phosphorylation events in PMN (Refer to SUPPORTING DATA, Figure 22) as has already been established for ZnO NP (Goncalves and Girard 2014). Specifically, the Akt pathway is well-known for its involvement in inhibiting spontaneous apoptosis of PMN (Zhu et al. 2006). However, according to our recent data, Fe_3O_4 NP do not induce activation of Akt (Refer to SUPPORTING DATA , Figure 15.C). Nevertheless, they are still able to interfere with the constitutive apoptosis of PMN, indicating the involvement of other pathways.

Furthermore, Akt is also necessary for NADPH activation, which in turn leads to ROS production and netosis (Douda et al. 2014). This is consistent with the observation that Fe_3O_4 NP do not induce Akt phosphorylation and, therefore, do not activate ROS production and netosis, a mechanism that is primarily associated with ROS release (Refer to SUPPORTING DATA, Figure 23). Moreover, netosis has been found to be associated with degranulation. Interestingly, it is noted that Fe_3O_4 NP, upon incubation with PMN, do not induce the release of MMP-9 (Refer to SUPPORTING DATA, Figure 24), which is stored in the tertiary granules, as confirmed by the zymography assay (Hu and Beeton 2010). This may indicate a potential impairment of this mechanism, but it is difficult to ascertain whether this was caused by the internalization of the particles or by the activation of specific pathways that promote inhibition. However, the absence of netosis also interferes with the release of MPO and elastase, which are proteases stored in the primary granules (Papayannopoulos et al. 2010).

All these observations suggest that the release of both the primary and tertiary granules might be compromised in the presence of Fe_3O_4 NP. Notably, degranulation can involve the release of granule contents into phagosomes. As a result, in the case of Fe_3O_4 NP, it is possible that the granules fuse with vacuole-like structures where the Fe_3O_4 NP have been internalized, rather than being released extracellularly. This is in contrast to previous findings which show that TiO_2 NP, CeO_2 NP, and ZnO NP promote degranulation in PMN (Babin et al. 2013b). Nevertheless, in our study, it remains unclear whether Fe_3O_4 NP target all granules or not, as a previous research study has shown that the activation of Hck and Fgr, members of the Src family tyrosine kinases, is associated with the release of primary and secondary granules, respectively (Mócsai et al. 1999).

Interestingly, in our study, the upregulation of Src was observed (Refer to SUPPORTING DATA, Figure 17), but it is not clear why such activation does not promote granule exocytosis. It is important to note that our study was conducted with cells in suspension, whereas the previous study that reported activation of Hck and Fgr in relation to degranulation was specifically conducted on PMN adherent to fibrinogen (Mócsai et al. 1999). Additionally, downstream signaling pathways also play a critical role in promoting degranulation. For instance, the activation of the p38 MAPK pathway has been observed in PMN stimulated with fMLP, which specifically induces the release of secondary and tertiary granules. In contrast, ERK 1/2 activation is not involved in granule exocytosis (Mócsai et al. 2000). In our study, Fe_3O_4 NP do not significantly activate p38 MAPK or ERK 1/2 MAPK (Refer to SUPPORTING DATA, Figure 15.A and B), unlike GM-CSF, indicating a potential correlation between the lack of phosphorylation of p38 and ERK 1/2 and the absence of degranulation. However, while degranulation may not be evident for MMP-9, it cannot be entirely ruled out for other granules. In fact, the increased adhesion function of PMN by Fe_3O_4 NP might be correlated with TNF-α release, which may act in an autocrine manner to promote the release of secretory vesicles containing adhesion molecules. To assess the existence of such specific modulation, the study of human serum albumin (a marker of secretory vesicles) release would be useful, which unfortunately has not been done in this study (Mócsai et al. 2000).

The analysis of phosphorylated proteins using a human kinase array (HKA), which is capable of detecting phosphorylation sites of 37 different signaling (pathway) proteins, has been a useful approach for understanding the signaling mechanisms by which Fe_3O_4 NP modulate PMN. Interestingly, the presence of Fe_3O_4 NP induced a significant increase in β-Catenin and p53 transcription factors (Refer to SUPPORTING DATA, Figure 18). Notably, β-Catenin has been found to be involved in regulating the canonical Wingless/Integrated (Wnt) signaling pathway in hematopoietic stem cells, from which all blood cells originate. This involves promoting the expression and increasing the number of granulocyte-colony stimulating factor receptors (G-CSF-R), which are crucial for the proper differentiation of myeloid cells into granulocytes (i.e., PMN) (Danek et al. 2020). Although research on this pathway in PMN is very limited (Haseeb et al. 2019), the observed ability of Fe_3O_4 NP to enhance the release of G-CSF by PMN, as determined by human cytokine array (Refer to Figure 11.B), may suggest a potential effect of Fe_3O_4 NP in upregulating both G-CSF and its receptor (via β-Catenin).

Regarding p53, its expression is known to be upregulated in response to cellular stress, leading to its phosphorylation via MAPK, particularly p38 and JNK (Ai and Udalova 2020). Interestingly, activated p53 is found to promote the expression of Mcl-1, a critical anti-apoptotic protein in PMN (Murphy and Caraher 2015). In our study, we observed a tendency for a decrease in Mcl-1 degradation in the presence of Fe_3O_4 NP (Refer to SUPPORTING DATA, Figure 25), which correlates well with the up-regulation of p53 observed through HKA. On the other hand, a separate study has demonstrated that Fe_3O_4 NP coated with polyacrylic acid were able to upregulate p53 in PMN. However, in contrast to our study, these particles were found to induce apoptosis (Couto et al. 2014).

Moreover, in relation to Lfr, this protein has been found to induce the activation and expression of the p53 gene through the stimulation of the inhibitor of NF-κB kinase activity (Oh et al. 2004). Additionally, Lfr has been shown to downregulate cytokine expression in LPS-stimulated THP-1 (macrophage cell line) by interfering with NF-κB (Legrand et al. 2005). In our study, it is not evident which pathway is involved in activating NF-κB to promote the release of cytokines TNF-α, IL-1β, IL-8, and IL-6. Interestingly, a study on monocytes exposed to dextran-coated Fe_3O_4 NP has shown that they release TNF-α and IL-1β by inducing the activation of JNK and ERK1/2 signaling pathways, which are known to be involved in promoting NF-κB activation (Chen et al. 2020). It is worth mentioning that the activation of JNK is also involved in ROS production and netosis, specifically in LPS-treated PMN (Khan et al. 2017).

Based on our findings, we hypothesize that Fe_3O_4 NP promote p53 expression in PMN, which, in turn, activates multiple signaling mechanisms that enhance the availability of Lfr. This prevents Mcl-1 degradation, leading to an increase in the viability of PMN and their ability to persist against iron overload induced by Fe_3O_4 NP.

In summary, Fe_3O_4 NP have been shown to modulate the response of PMN in both an immunostimulatory (e.g., pro-inflammatory cytokine release) and immunosuppressive (e.g., impairment of oxidative burst) manner. Indeed, their interference with PMN apoptosis suggests that these particles may promote PMN persistence at the inflammatory site. This should not be seen as merely a pitfall; in fact, the ability of Fe_3O_4 NP to activate PMN could be utilized in promising applications such as vaccine adjuvant development, as NP have been shown to significantly increase antibody production compared to conventional alum adjuvants [141]. Additionally, the capacity of Fe_3O_4 NP to enhance phagocytosis could be employed as an

innovative strategy to boost the phagocytic function of PMN in patients with recurrent infections, such as those with immune deficiencies. However, further investigations are needed before pursuing such promising applications. Indeed, it is not evident what role other factors, such as protein corona, play in mediating the observed modulatory effects of Fe_3O_4 NP in PMN. While it is clear that Fe_3O_4 NP would interact with serum and different molecules in the medium, the extent to which this "new particle identity" is involved warrants independent investigation.

8 CONCLUSION

The development and utilization of Fe_3O_4 NP have been driven by their magnetic properties, making them highly sought-after in nanomedicine applications. However, our research has raised concerns about their safety. We have demonstrated that Fe_3O_4 NP with a size of approximately 10 nm are significantly internalized by the body first line of defense cells known as PMN. Our findings have shown that Fe_3O_4 NP can modulate PMN biological functions, particularly by interfering with their spontaneous apoptosis, promoting pro-inflammatory cytokine production, and activating critical signaling pathways such as the Syk pathway involved in phagocytosis. It is important to note that inflammation, in which PMN play a critical role, is a physiological defensive response that may not initially be considered pathological in the presence of Fe_3O_4 NP. However, prolonged exposure to Fe_3O_4 NP in chronic scenarios, such as workplace environments where they are produced in large quantities, may lead to persistent pathological immune activation, which can be detrimental to workers health, quality of life, and productivity, resulting in financial burdens on the economy (e.g., a high rate of absenteeism) and the healthcare system. Therefore, precautionary strategies, such as safe-by-design and control banding approaches, should be implemented before the production, use, or disposal of Fe_3O_4 NP or other particles, to carefully assess their fate in the human body/environment and ensure their safe use in various contexts.

In summary, the assessment of Fe_3O_4 NP should remain a priority even after their release in the market, as any changes in particle size and shape would necessitate a new risk assessment, even though these materials originate from iron, a common element that is naively perceived as biocompatible.

8.1 Study limitations

There are certain constraints that need to be taken into consideration when interpreting the results of this study/thesis. First, the Fe_3O_4 NP were purchased from a biotech company and were suspended in water. However, the loss of their stability in cell culture media is evident, as they are not coated, which results in their tendency to form aggregates that are in the micrometer scale rather than in the nanoscale. As a result, cells would be exposed to microparticles (>1000 nm) rather than nanoparticles (<100 nm), which could result in different outcomes. Indeed, TEM micrographs clearly show that engulfed Fe_3O_4 NP are present in both individual free particle form

and aggregate form in cells cytoplasm. Secondly, the assessment of iron-free ions has not been conducted as they could potentially be generated following Fe_3O_4 NP internalization. These ions might be accountable for the observed effects instead of the intact NP themselves. In fact, it is known that metallic NP can undergo dissolution in lysosomes after being taken up by cells (Stark 2011). Thirdly, our experiments have not been conducted hand-in-hand with any standardized experimental methods for nanosafety assessment, such as the standard operating procedures based on the guidelines provided by the Organisation for Economic Co-operation and Development (OECD) or the harmonized guidelines of the International Organization for Standardization (ISO). This is primarily due to the lack of such standardized protocols, particularly regarding the toxicity assessment of NP in the immune system. As a result, sharing valuable data can be impaired without the need to waste resources on establishing similar studies in the future. Lastly, but not least, immune cells intraspecific variation in donor responses is an issue encountered in different assays (Brodin and Davis 2017), which depends on uncontrollable factors (e.g., the ratio of PMN to eosinophils after isolation). This necessitates a careful interpretation of data and an increase in the number of analyzed samples. However, achieving a larger sample size in the donor recruitment process poses a challenge.

8.2 Perspectives

As perspectives, further exploration of other mechanisms will enhance our understanding of the observed immunomodulatory effect. Some potential perspectives for future research could include:

I. Evaluating the role of biocorona in the response of PMN to Fe_3O_4 NP.
II. Assessing the role of Lfr in counterbalancing the effects of Fe_3O_4 NP in PMN.
III. Determining the proteins induced by the *de novo* synthesis mechanism in presence of Fe_3O_4 NP.
IV. Assessing uncoated Fe_3O_4 NP with smaller sizes or different shapes (e.g., 5 nm; cubic) to determine whether shape, size, or composition plays the most critical role in PMN immunomodulation.
V. Evaluating the biological functions of PMN derived from workers exposed to Fe_3O_4 NP in occupational settings.

VI. Exploring the potential of Fe_3O_4 NP to be used as a new adjuvant system in vaccine development by assessing their modulatory effects on other immune cell types like eosinophils, macrophages, dendritic cells, and lymphocytes.

VII. Evaluating the impact of Fe_3O_4 NP internalization on efferocytosis by macrophages to understand the extent to which these particles may interfere with inflammatory resolution.

9 APPENDIX I (REVIEW ARTICLE)

Interaction between iron oxide nanoparticles (IONs) and primary human immune cells: an up-to-date review of the literature.

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9.1 Résumé

La nanotechnologie prend de plus en plus d'ampleur ces dernières décennies, et l'utilisation des nanomatériaux, y compris les nanoparticules (NP), continue de croître dans divers secteurs d'activité. Parmi les NP qui ont suscité un intérêt croissant de la part de la communauté scientifique et de l'industrie, on trouve les nanoparticules d'oxyde de fer (IONs), en raison de leurs propriétés superparamagnétiques permettant leur utilisation dans de nombreux domaines, y compris la médecine. Cependant, certains effets indésirables des IONs et les risques potentiels pour la santé humaine associés à leur utilisation font l'objet d'avertissements de plus en plus fréquents dans plusieurs études. Bien que de nombreuses études *in vivo* aient rapporté que les IONs induisent une immunotoxicité dans différents modèles animaux, il n'est pas clair comment les IONs peuvent altérer la biologie des cellules immunitaires humaines primaires. Dans cette revue, nous présenterons les travaux qui ont été réalisés concernant l'interaction entre les IONs et les cellules immunitaires primaires. Cette revue met également en évidence l'avantage de l'utilisation de cellules immunitaires primaires dans l'évaluation des risques liés aux NP en tant que stratégie fiable pour encourager les approches d'études non animales, afin de déterminer les risques susceptibles d'affecter le système immunitaire humain suite à l'exposition aux NP. Dans l'ensemble, les observations rapportées dans cette revue contribuent à obtenir une idée plus complète de la manière dont les IONs modulent le système immunitaire humain, en particulier le fait que l'inflammation, impliquant plusieurs types de cellules immunitaires, est fréquemment signalée comme un effet indésirable associé aux IONs.

9.2 Abstract

Nanotechnology has been gaining more and more momentum lately and the potential use of nanomaterials such as nanoparticles (NPs) continues to grow in a variety of activity sectors. Among the NPs, iron oxide nanoparticles (IONs) have retained an increasing interest from the scientific community and industrials due to their superparamagnetic properties allowing their use in many fields, including medicine. However, some undesired effects of IONs and potential risk for human health are becoming increasingly reported in several studies. Although many *in vivo* studies reported that IONs induce immunotoxicity in different animal models, it is not clear how IONs can alter the biology of primary human immune cells. In this article, we will review the works that have been done regarding the interaction between IONs and primary immune cells. This review also outlines the importance of using primary immune cells in risk assessment of NPs as a reliable strategy for encouraging non-animal studies approaches, to determine risks that might affect the human immune system following different exposure scenarios. Taken all together, the reported observations help to get a more global picture on how IONs alter the human immune system especially the fact that inflammation, known to involve several immune cell types, is frequently reported as an undesired effect of IONs.

Abbreviations

A-PVA, amino-polyvinyl alcohol; CCK-8, cell counting kit-8; CeO_2, cerium dioxide; DCs, dendritic cells; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular signal-regulated kinase; FCS, fetal calf serum; fMLP, N-formyl-methionyl-leucyl-phenylalanine; GM-CSF, granulocyte-macrophage colony-stimulating factor; HMDMs, human monocyte-derived macrophages; IFNγ, interferon gamma; IL, interleukin; IONs, iron oxide nanoparticles; JNK, c-Jun N-terminal kinase; LAL, limulus amebocyte lysate; LDH, lactate dehydrogenase; MDDCs, monocytes-derived dendritic cells; MF, magnetic field; MRI, magnetic resonance imaging; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; NADPH, nicotinamide-adenine dinucleotide phosphate; NPs, nanoparticles; NETs, neutrophil extracellular traps; NK, natural killer; PAA, polyacrylic acid; PBS, phosphate-buffered saline; PI, propidium iodide; PMA, phorbol-12-myristate-13-acetate; PMNs, polymorphonuclear neutrophil cells; ROS, reactive oxygen species; RPMI, Roswell Park Memorial Institute; SSC, side scatter; TiO_2, titanium dioxide; TEM, transmission electronic microscopy; TNF-α, tumor necrosis factor-alpha; ZnO, zinc oxide.

10 Introduction

The biological activities of a variety of nanoparticles (NPs) with different composition, shape, and size in cells of the immune system is increasingly documented in the scientific literature. Indeed, the effect of NPs on the biology of cells of both innate and adaptive (or acquired) immune responses, including cellular uptake of NPs, cytotoxicity, cell division, reactive oxygen species (ROS) and cytokine production, etc., have been the object of several studies (Abrikossova et al. 2012; Aldossari et al. 2015; Abbas A. K. 2007; Babin et al. 2013a; Baumann et al. 2012; Haase, Fahmi, and Mahltig 2014). The innate immune response which is nonspecific and rapid to any foreign entities principally involve neutrophils, dendritic cells (DCs), and monocytes and macrophages. The adaptive immune response which is more sophisticated in terms of specificity is mainly induced by T and B cells. Several data favoring the use of NPs as a new therapy for the treatment of cancers and several diseases characterized by an inflammatory status have emerged in the literature (Liu et al. 2022; Almeida, Figueroa, and Drezek 2014; Hosseini et al. 2015; Talamini et al. 2021).

Among the great variety of NPs, iron oxide nanoparticles (IONs) certainly represent one of the most attractive NPs that could be used to deliver active substances (conventional drugs, antibodies, etc.). Moreover, both chemical forms of ION magnetite (Fe_3O_4) and maghemite (Fe_2O_3) are the most widely studied magnetic materials for biomedical applications (Dadfar et al. 2019). In particular, these NPs are of interest for cancer treatment principally due to their magnetic properties, biocompatibility and easiness to be functionalized with different types of molecules (Montiel Schneider et al. 2022).

IONs are known for their theragnostic properties but can be used for both diagnostic and treatment purposes. The use of IONs in hyperthermia is also a promising area. However, the efficacy of this intervention depends mainly to their forms, size, and superficial charge. For example, some studies demonstrated that cubic iron IONs are more effective in hyperthermia than spherical ones (Suciu et al. 2020). Some of the biomedical formulations of IONs are already in the market, mostly for the treatment of anemia with Feraheme® (ferumoxytol injection), but serious side effects could appear as a clear warning of risk for serious hypersensitivity/anaphylaxis reactions are specified with this medication. Although the passage from the bench to bedside is certainly promising with IONs, the above warning and the fact that other safety concerns including decrease in cell viability, cytoskeleton disruption, alterations in cell motility, plasmatic membrane disruption, mitochondrial

alterations, oxidative damage, and cell death have been raised regarding their use (Malhotra et al. 2020; Vakili-Ghartavol et al. 2020; Laffon et al. 2018), several works need to be conducted in this area.

Many risk assessment studies of IONs and other types of NPs have been performed *in vitro* using immortalized immune cell lines, often of non-human origin. Although this brings important new information, it also raises many questions on the reliability in their applicability in a real human context. Indeed, several studies reported that primary cells can respond differently to NPs than cell lines (Verdon et al. 2021; Ekstrand-Hammarström et al. 2012; Shahhoseini et al. 2021; Bregoli et al. 2009) revealing the complexity through which safety assessment of NPs could be established in addition to the fact that NPs could have different shapes, sizes, charges, coated or not. This is the main reason why risk assessment must be performed on individual types of NPs. Figure 26 illustrates different factors that could influence and complicate the interpretation and comparisons of results within studies.

The aim of this review is to bring an up-to-date synthesis of the literature regarding the biological effect of IONs specifically on primary human immune cells, an area of research that must be better considered as a major step for determining the nanosafety of a given NP.

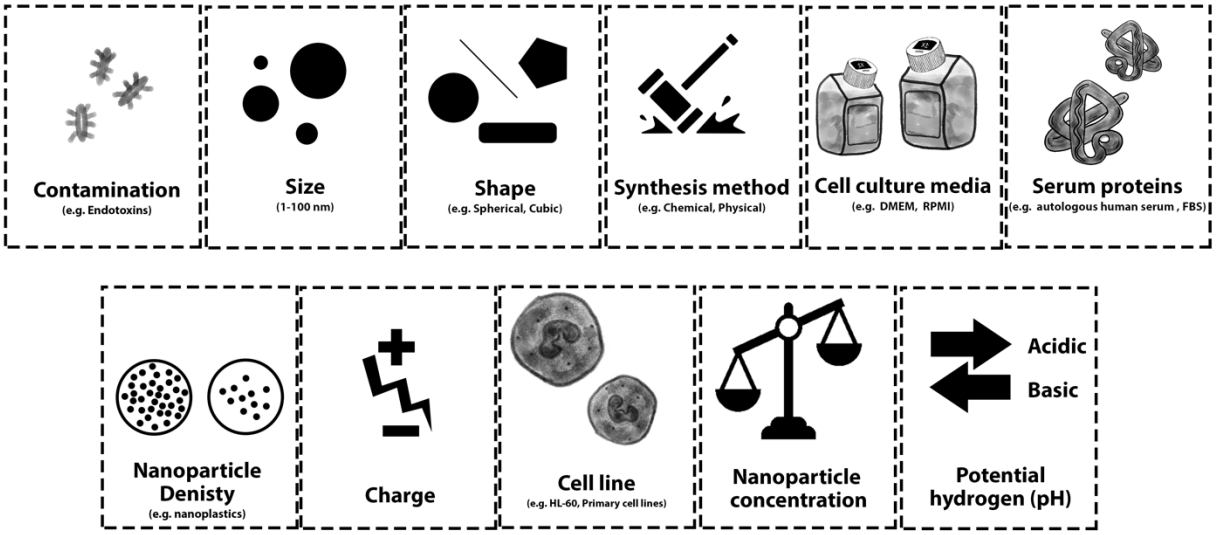

Figure 26: Potential factors that could influence assessment of IONs *in vitro.*

Different factors that could influence and complicate the interpretation and comparisons of results within studies performed with IONs are illustrated in this figure. These, although not limited to, include, contamination

by endotoxins that are pro-inflammatory by themselves, the size, shape, charge, types of coating or not, the density, the concentration used, and the way IONs are synthesized, the types of culture media in which they are suspended (known to modify their properties), the presence or not of serum that could lead to the formation of a corona. The interference that IONs could have on different methods requiring for example the measurement of a signal intensity is not always reported and the use of different immortalized cell lines used in studies further complicated inter-laboratories results.

10.1 Immune cells in nanosafety studies

Immortalized cell lines are routinely used in toxicology, nanotoxicology, and other related scientific disciplines. This is probably because such cells present more homogeneity, greater stability, are easy to use, less expensive and easier to obtain than primary human cells. However, many concerns have been issued regarding lack of protocol standardization using these cells, including a variety of cell culture media, the number of cell passages used, the cell density, the presence of serum of different origin, etc. Also, immortalized cell lines may hamper the clinical translation of the results as they do not necessarily mimic the human complexity of primary cells and, clearly, they also possess different sensitivities to NPs than primary cells probably due to their enhanced proliferative and metabolic activities. As a result, many difficulties may arise in obtaining consistent and reproducible results among different cell lines. Indeed, different results can be obtained from one cell line to the other in response to a given NP.

Moreover, many researchers are encouraged to perform risk assessment of NPs from a 3R's (replace, reduce, and refine) and NAMs (new approach methodologies) perspective, to minimize the use of animal models, and as a part of applying this strategy, the use of primary cell lines is highly recommended (Domingues et al. 2022). Many studies have already noticed differences in the response of immune immortalized cell lines with primary human immune cells in nanosafety assessment. For example, Verdon et al. (Verdon et al. 2021) have compared the response of immortalized neutrophil-like HL-60 cell line and primary human polymorphonuclear neutrophil cells (PMNs) directly isolated from fresh human blood to zinc oxide NPs (ZnO-NPs). They observed that ZnO-NPs were able to induce the activation of HL-60 cells but did not appear to activate PMNs. Moreover, HL-60 cells showed a limit phagocytic capacity towards 100 nm carboxylated fluorescent NPs than PMNs. The authors of the study suggested that cell lines such as HL-60 could be recommended as an initial screening of toxicity but that a more in-depth assessment of NP toxicity (e.g., induction of cytokine production) should be done using primary

human cells. These latter are more sensitive to the effects of exposure, meaning that they can respond to a lower concentration than cell lines do. This property offers a more realistic endpoint in risk assessment of NPs (Verdon et al. 2021).

Shah et al. (Shah et al. 2018) reported that treatment of primary human T cells to Feraheme®, an FDA approved ION formulation for anemia treatment, induced mitochondrial oxidative stress. However, this was not observed in Mo-T, Loucy, and A3 leukemia T cell lines. In other studies, a paradoxical observation regarding ZnO-NP was observed with primary human eosinophils and the AML14.3D10 eosinophilic-like cell line (Silva and Girard 2016; Vallières et al. 2016). Indeed, ZnO-NPs were found to induce a pro-apoptotic response in AML14.3D10 cells, however, using the same NPs, an anti-apoptotic effect in primary human eosinophils was observed. Of Interest, ZnO-NPs are known to induce the production of the pro-inflammatory cytokine interleukin (IL)-8, in both cells. Taken together, those observations indicate that primary immune cells can respond differently to NPs than immortalized cell lines, and that this is probably due to the increased sensitivity of primary human immune cells compared with immortalized cell lines. Although we cannot generalize on the sensitivity, the latter are normally known to be more dynamic and to proliferate more aggressively than primary cells. Moreover, for example, the HL-60 cell line is frequently used as a model of PMNs, but these latter cells are known to be mature and to not divide *in vitro* (Verdon et al. 2021). Thus, the use of primary cells might closely reflect the *de facto* responses generated *in vivo*, as they are closer to the *in vivo* condition than immortalized cell lines, in accordance with the will to use free animal testing systems in risk assessment of NPs.

10.2 Interaction between IONs and primary human immune cells

10.2.1 The effects of IONs in PMNs

PMNs are important cells of the innate immunity. They are the first cells recruited during an immune inflammatory response. PMNs are produced in the bone marrow by the effects of different growth factors like granulocyte-macrophage colony-stimulating factor (GM-CSF) on myeloblasts. During the granulopoiesis process, myeloblasts follow maturation stages from promyelocytes, myelocytes, metamyelocytes, band cells, and mature neutrophils which are characterized by their polylobed nucleus. These mature cells leave the bone marrow to enter the blood circulation where they will represent more than about 65% of total leukocytes in humans. It takes about 14 days to

obtain fully mature PMNs from the CD34+ precursor cells and one must realize that more than 50% of the bone marrow is dedicated to the generation of PMNs. The huge number of PMNs released from the bone marrow into the blood circulation has been estimated at ~5 × 10^{10} cells daily in a normal adult, represents one of the fastest-cell turnovers in the human body (Edwards and Watson 1995; Ward et al. 1999). Therefore, neutrophil cell turnover must be tightly regulated to prevent diseases. Fortunately, the number of PMNs remains relatively constant in healthy individuals, and this is due to the fact that these cells are known to undergo constitutive or spontaneous apoptosis, an important first step for regulating cell number, followed by the ability of professional phagocytes like macrophages to phagocytose apoptotic PMNs, a process known as efferocytosis (Duffin et al. 2010; Hart, Dransfield, and Rossi 2008).

PMNs have an array of defense mechanisms, including phagocytosis, ROS production, degranulation and the formation of neutrophil extracellular traps (NETs) used to fight invading pathogens. The fact that PMNs are the most abundant leukocytes in the blood circulation, and among the first cells to respond to invaders and to migrate to inflammatory sites, the presence of NPs in their environment could interfere with their biology. This has been reported in different studies with NPs other than IONs. For example, titanium dioxide (TiO_2), ZnO, and cerium dioxide (CeO_2) were found to affect phagocytosis and degranulation in human PMNs (Babin, Goncalves, and Girard 2015; Babin et al. 2013a) and silver NPs with a primary size of 20 nm were found to induce PMN apoptosis whereas those with a size of 70 nm rather delay this process (Poirier et al. 2014; Poirier, Simard, and Girard 2015).

Bilyy and collaborators (Bilyy et al. 2018b) studied how different coating of IONs can exert effects during NETosis, the process when PMN release NETs (Brinkmann et al. 2004). They used IONs coated with lauric acid, human serum albumin, or dextran. Before testing, and to prevent the unintentional activation of cells with undesired lipopolysaccharide (LPS, endotoxin), the investigators have monitored potential bacterial contamination and endotoxin levels of these NPs by incubating them in agar plates to analyze the presence of bacterial colonies and by using the EndoZyme endotoxin test, respectively. Results indicated that the tested IONs were free from bacterial contamination and the endotoxin level of 50 µg/mL of the tested sample was under 0.025 EU/mL (0.5 EU/mg *0.05 mg/mL) which is considered to be largely under the maximum limit value of 0.5 EU/ml established by FDA for approved products that come directly or indirectly with the cardiovascular system and lymphatic system (Himly et al. 2020). The presence of serum plays a role in enhancing the stability of IONs, and, in this study, they demonstrated that incubation of

IONs coated with lauric acid in the presence of plasma or 10% of serum in cell culture medium increased their stability. This was explained by the formation of a protein corona. Of interest, incubation of the same NPs in phosphate-buffered saline (PBS) decreased their stability as suggested by the observation of agglomerates. Also, the incubation of PMNs with these NPs was found to increase NETs as assessed by immunohistochemistry using specific antibodies to neutrophil elastase and citrullinated histone H3. However, NETs were not observed when the NPs were incubated in plasma or medium containing 10% serum. The evaluation of cell viability by flow cytometry based principally on forward and side scattering analyses showed a decrease in cell viability for PMNs incubated with IONs coated with lauric acid in phosphate-buffered saline (PBS). However, cell viability was unaffected when IONs were coated with dextran or human serum albumin. Nevertheless, the cell viability was intact when different tested IONs were tested in plasma. The exposition of cells incubated in a medium with 10% of serum to an external magnetic field (MF) change radically the stability of coated IONs with lauric acid. These NPs tend to aggregate and to induce NETosis as observed in PBS medium. Of interest, NPs coated with dextran and human serum albumin do not show any tendency to induce NETs even when they were exposed to an EMF. This study underlines the importance of coating in increasing biocompatibility of IONs (Bilyy et al. 2018a; Nguyen and Lee 2017).

Polyacrylic acid (PAA)-coated and uncoated IONs, with a size distribution of about 10 nm, as determined with transmission electronic microscopy (TEM), were found to affect PMNs differently (Couto et al. 2014). Both NPs tend to form agglomerates when tested in RPMI-1640 medium supplemented with 10% fetal bovine serum. Determination of ROS production using the dihydrorhodamine 123 probe in response to IONs at 4 µg/mL, 20 µg/mL and 100 µg/mL indicated that uncoated IONs induce a higher level of ROS in PMNs. Of interest, the maximal level of ROS production was observed at a concentration of 20 µg/mL. Similarly, the coated IONs induce ROS production in a concentration-dependent manner. The use of diphenyleneiodonium chloride, a specific inhibitor of intracellular ROS production induced by the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system (Buck et al. 2019), confirmed that both coated and uncoated NPs induce ROS production by a dependent NADPH oxidase activation mechanism. In both conditions, IONs do not appear to be necrotic for PMNs as determined by trypan blue exclusion assay and by lactate dehydrogenase leakage assay. In addition, the authors demonstrate that uncoated IONs did not induce apoptosis as assessed by cytology, a method routinely used by others where apoptotic PMNs are easily identifiable by the loss of the characteristic polylobed nucleus (Gonçalves, Chiasson, and Girard 2010). However, coated IONs were found to

significantly increase apoptosis after 16h and 24h post-incubation with 100 µg/mL. This observation was also confirmed by flow cytometry analysis using annexin-V/propidium iodide (PI) mixture. Therefore, it seems that the uncoated IONs have a cytoprotective effect towards PMNs. Then, it was demonstrated that uncoated IONs significantly reduce the spontaneous apoptosis of PMN by inhibiting caspase-3 and -9 activities, as demonstrated by using a colorimetric caspase assay. Of interest, the assessment of the pro-apoptotic signaling pathway p-53, known to promote apoptosis, did not show any influence of its activity using uncoated IONs (Couto et al. 2014).

Garcia et al. (Garcia et al. 2020) studied the effect of IONs (Feraheme®) on pre-activated human PMNs. They show that this nanoparticle formulation inhibits the degranulation process and reduce ROS production of PMN pretreated with *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), a well-known PMN activator. The immunosuppressive response of Feraheme® was attributed to its capacity to accelerate cytosolic calcium clearance which impedes cell signaling pathways that are implicated in immune response in which ROS production and degranulation are among the principal mechanisms of PMN response in inflammatory site. Moreover, some observation regarding the clinical use of Feraheme®, had already reported hypersensitivity reaction in some patients but the mechanism underlying this response is not fully unveiled (Shah and Dobrovolskaia 2018).

Recently, our group studied how IONs (Fe_3O_4 NPs or magnetite) can directly alter the biology of human PMNs (Saafane and Girard 2022). The IONs used in this study were spherical with a size of ~10 nm as determined by TEM. The Fe_3O_4 NP suspension reveals a level of endotoxin under the detection limit of 0.01 EU/mL. Since some NPs could interfere with classical Limulus amebocyte lysate assay for determining endotoxin level (Dobrovolskaia and McNeil 2007), the NP suspension (100 µg/mL) was incubated in lysogeny broth agar plates for 72h where no colonies were observed, confirming sterility. The cellular uptake of these IONs by PMNs was determined by TEM. The Fe_3O_4 NPs were accumulated into vacuole-like structures or randomly distributed in the cytosol or close to the nucleus after 1h or 24h post-incubation. Using the ROS sensitive dye, H_2DCFDA, in kinetic experiments, it was determined that unlike the positive control fMLP, no ROS production was induced by Fe_3O_4 NPs. However, these NPs were found to increase the ability of PMNs to adhere onto human endothelial cells and to enhance the phagocytosis of opsonized sheep red blood cells. Using an antibody array assay, we show that Fe_3O_4 NPs induce the production of chemokines, including CXCL8 (IL-8), CXCL1 (GRO-α) and macrophage inflammatory protein 1-alpha and beta (MIP-1α/MIP1β). In addition, Fe_3O_4 NPs were

found to delay spontaneous or constitutive PMN apoptosis. By incorporating a sex-based analysis, we reported that these NPs delay apoptosis regardless of sex of the donor. This was performed since sexual dimorphism has already been known to play a role in the innate immune response (Jaillon, Berthenet, and Garlanda 2019) and since, recently, other NPs were found to act differently in neutrophils or eosinophils isolated from male and female blood donors (Vanharen et al. 2022). A pharmacological approach reveals that Fe_3O_4 NPs delay apoptosis by a *de novo* protein synthesis-dependent mechanism and via different cell signaling pathways. Therefore, Fe_3O_4 NPs can alter the biology of human PMNs and can possess some pro-inflammatory effects, particularly based on their capacity to delay apoptosis and to induce the production of IL-8 and other chemokines by themselves (Saafane and Girard 2022). These observations appear to be contradictory to those from others (Garcia et al. 2020), however, herein the effects of IONs were determined in a direct manner in PMNs vs. an indirect fashion as previously reported (Garcia et al. 2020) where the authors pretreated PMNs with some agents before adding IONs. Thus, both studies indicate that inflammation can be regulated by IONs, especially by targeting PMNs directly and/or indirectly.

In a recent study, the antioxidant role of IONs in eliminating ROS produced by PMNs was investigated (Świętek et al. 2022). They tested the effect of maghemite (Fe_2O_3) which can be produced by oxidation of magnetite particles (Fe_3O_4) in PMNs. Of interest, both maghemite and magnetite are characterized by a similar structure. These IONs were coated with a layer of heparin-chitosan and a phenolic compound (gallic acid or phloroglucinol). The assessment of IONs to scavenge ROS production in PMNs was assessed by two techniques. First, by determining the viability of *Staphylococcus aureus* incubated with PMNs in presence of IONs at 100 and 500 µg/mL. Second, by measuring ROS production using the oxidant-sensing dye H_2DCFDA. The viability of bacteria decreased significantly (76%) in the negative control condition in which PMNs without IONs were incubated with bacteria. However, co-incubation of IONs with PMNs was found to significantly enhance the viability of *S. aureus* in a concentration-dependent manner, suggesting that the presence of IONs impede the ROS machinery production in PMNs, the NADPH oxidase complex necessary for bacteria elimination. Furthermore, the same was observed for the IONs coated with chitosan and the antioxidant gallic acid. However, IONs coated only with heparin significantly decreased the bacteria proliferation. Regarding the scavenger capacity of IONs, it was observed that they possess a more powerful suppression activity of oxidative burst induced by PMA at the concentration of 100 µg/mL vs. 500 µg/mL. All the tested IONs showed a capacity to decrease ROS production. However, the IONs coated with chitosan

and the antioxidant gallic acid were more potent followed by uncoated IONs. It was concluded that, the antioxidant property of IONs is attributed to the core of the IONs rather than the coating, as the use of heparin coating demonstrated to act as a physical barrier and limits this property (Świętek et al. 2023).

10.2.2 The effects of iron oxide nanoparticles in monocytes-macrophages

Monocytes are blood circulating immune cells which constitute approximately 10% of the total leukocytes in human blood circulation (Auffray, Sieweke, and Geissmann 2009).These cells take origin from myeloid progenitors in the bone marrow and their homeostatic control is under the influence of several soluble mediators like GM-CSF, CSF-1, and IL-34. Monocytes population is heterogenous, and classical monocytes which is the major monocytes population have high expression of CD14 but not CD16 surface markers. The classical monocytes are inflammatory effector cells that participate in the effector phase of inflammation and play a major role in sustaining resident macrophages during inflammation by joining inflammatory sites and being differentiated into monocytes-derived macrophages. They can perform several functions like cytokine production and phagocytosis. Resident macrophages are able to ensure antigen processing and presentation to naïve T cells, that is fundamental in orchestrating the immune response to a specific antigen that has a rapid clearance features upon a subsequent exposure to a pathogen (Hume 2008).

Additionally, resident macrophages have an auto-renewal capacity, and they are also a heterogenous population. Depending on their tissue location, they take different names for example osteoclasts in the bone, alveolar macrophages in the lung, and Kupffer cells in the liver. These cells can be considered as unique classes of macrophages. Macrophages can display a different spectrum, known as M1 and M2 phenotypes, a simplistic dichotomous model division basically based on the response of macrophages to signals (e.g., cytokines) present in their tissue environment. M1 macrophages are considered as pro-inflammatory, while M2 macrophages are rather anti-inflammatory. This M1/M2 classification is just a limited paradigm to define the complexity of the mononuclear phagocytes system (Mantovani et al. 2002; Italiani and Boraschi 2014a).

Monocytes/macrophages are known to be professional phagocytes, these cells intervene to eliminate foreign invaders including NPs by mechanisms that can include the Toll-like receptors 2 and (TLR2/4) as previously demonstrated in a study of single-walled carbon nanotubes (Mukherjee et al. 2018). Furthermore, these cells are known to be active in internalizing NPs of different sizes and as they play a prominent role in immune response, they have attracted researchers. Indeed, an important feature of monocytes/macrophages, in contrast to PMNs, is their ability to maintain their viability for a longer period of time after phagocytosis of particles, which make them an attractive cell population for NP drug delivery systems within cells for treatment of diseases (Vishnevskiy et al. 2021). Although it is still not clear exactly how IONs can influence the M1/M2 polarization, it was proposed, in murine macrophages, that this as to at least consider the internalization of the process involved, leading to the activation of different transcription factors such as STAT family and c-Fos/c-Jun complex and different transcription factors related to MAPK pathways and the innate response, including the TLR-AP-1 signaling pathway (Mulens-Arias, Rojas, and Barber 2021). As a result, IONs could trigger a multifactorial transcription reprogramming of macrophages involving different cell signaling pathways.

The effect of the amino-polyvinyl alcohol coated IONs (A-PVA-IONs), with a size of 7 nm, used principally as a contrast enhancer agent for magnetic resonance imaging (MRI), was investigated in human monocyte-derived macrophages (HMDMs). A-PVA-IONs did not significantly alter cell viability when HMDMs were incubated with A-PVA-IONs at their earliest stages of differentiation from monocytes. However, an increase in cytokine production was observed in a concentration-dependent manner. The cytokines that have been significantly increased, as detected by a cytokine array, are IL-1β, IL-4, IL-6, IL-8, G-CSF, MIP-1β and RANTES. By focusing on IL-1β production in whole blood and using Brefeldin A, an inhibitor of cellular transport, the authors found that granulocytes (CD15 positive) and monocytes (CD14 positive) are the principal cellular source for this cytokine (Strehl et al. 2015)

Grosse et al. investigated the immunomodulatory effects of spherical coated IONs in monocytes with a monolayer of oleic acid and a monolayer of amphiphilic polymers with a size of 10 and 30 nm, as determined by TEM, in the presence or absence of endotoxin (LPS) (Grosse, Stenvik, and Nilsen 2016). Absence of endotoxin contamination in the NP solutions was confirmed prior to starting the experiments with a luciferase reporter cell-based assay using human embryonic kidney (HEK293) cells, since no activation of TLR2 or TLR4 in transfected HEK293 cells was observed. Both IONs did not show any cytotoxicity as determined by alamar blue assay at a

concentration of 1, 10 and 100 µg/ mL. Also, both IONs did not induce the production of the pro-inflammatory cytokines tumor necrosis factor-alpha (TNF-α), IL-1β, and IL-6, as assessed by enzyme-linked immunosorbent assay (ELISA). Of interest, when cells were incubated with LPS, the cytokine production was suppressed. The authors also demonstrated by TEM that monocytes tended to internalize a larger number of IONs with a diameter of 10 nm vs. 30 nm. Intriguingly, the 10 nm IONs, at a concentration of 10 µg/mL and 100 µg/mL were found to inhibit the production of the pro-inflammatory cytokines induced by LPS as compared with 30 nm IONs. This was explained by their capacity to impair LPS binding to CD14/TLR4 and also by the adsorption of LPS on the surface of the IONs, limiting the amount of free LPS in the medium that could interact with cells, as confirmed by using Alexa594-labeled LPS (Grosse, Stenvik, and Nilsen 2016).

In another study performed with monocytes, the effects of dextran-coated IONs, with a size of 7 nm and a spherical shape confirmed by TEM, was investigated (Wu, Miao, et al. 2018). Using TEM, the interaction of cells with the IONs shows that monocytes internalize initially IONs in phagosome-like structures and at some points later, IONs were be found to be free in the cytosol. Analysis of the cell viability by flow cytometry was performed with a mixture of Annexin-V and PI staining, as well as with a neutral red assay, shows a cytoprotective effect of IONs towards monocytes. The analysis of cytokine release by ELISA showed a significant production of IL-1β and TNF-α in a concentration-dependent manner, two cytokines considered to be potent pro-inflammatory mediators. Of interest, dextran alone did not induce cytokine production, supporting a direct implication of the IONs by themselves in triggering cytokine release by cells. Using a pharmacological approach based on MAP kinases inhibitors and subsequent western blotting and ELISA analyses, the authors found that JNK and ERK signaling pathways were principally in charge of the cytokine production induced by IONs (Wu, Miao, et al. 2018).

The effect of Ferumoxtran-10, known under the brand name of Combidex, which is a formulation of dextran-coated IONs that are essentially used in MRI, was investigated in HMDMs (Müller et al. 2007). The interaction of these IONs with cells was confirmed using ferrozine assay, a technique that is used to determine the concentration of internalized iron by individual cells. A time-dependent increasing internalization was observed, and this was also demonstrated by Perl's Prussian staining assay. Of interest, the incubation in the presence of serum proteins did not enhance the cellular uptake of IONs. The cell viability was determined with neutral red and 3-(4,5-dimethylthiazol-2- yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assays and shown a

neglected cytotoxic effect even at the high concentration of 1 mg/mL. The long-term cytotoxicity of IONs was assessed for 14 days and did not reveal any variation when compared with corresponding negative controls in terms of cell viability. The production of IL-1β, TNF-α, IL-6, and IL-12 was not significantly increased when cells were incubated with 400 μg/mL of IONs. In addition, preincubation of cells with IONs for 4 days, followed by a treatment with LPS or PMA, did not alter the profile of cytokine production when compared with control cells indicating that IONs did not hamper the response of HMDMs to external stimuli. Treatment of cells with IONS (up to 400 μg/mL) did not increase superoxide anion production as determined by nitroblue tetrazolium assay. Also, incubation of cells with IONs and PMA did not inhibit or reverse PMA-induced superoxide production. Of interest, an increase in cellular iron concentration was observed when cells were stimulated with PMA, suggesting that activation of cells enhance internalization of IONs. In addition, the authors studied the effect of IONs on the capacity of HMDMs to exert chemotaxis. Using a chemotaxis chamber with a polycarbonate filter of 5 μm pore size, they observed a concentration-dependent decreasing capacity of cell to cross the membrane's pores when compared with cells treated with the positive control fMLP. The phagocytosis function was determined by flow cytometry using opsonized *E. coli* bacteria and the authors reported that IONs did not interfere in this biological process.

In a recent study, it was reported that IONs coated with silicone dioxide and polyethylene glycol, with an average diameter size of 10 nm, did not alter the cell viability of monocytes-macrophages at a concentration of 100 μg/mL, as was assessed by flow cytometry using SYTOX dye (Perekucha N.A. 2020). Of interest, Perl's Prussian staining assay indicates that more than 95% of cells internalized the IONs after 1h post-incubation. Moreover, the authors noted that incubation of cells in suspension (when incubated on a rotator) tend to internalize more IONs than cells incubated without shaking. In addition, it was found that the cellular uptake of IONs by macrophages was eightfold greater than that of monocytes (Perekucha N.A. 2020).

In one other study, the effect of dextran-coated IONs with a size of 5–10 nm was investigated in monocytes. Assessment of cell viability by light microscopy demonstrated an absence of cytotoxicity for the tested concentration of 10 μg/mL and 100 μg/mL. This was further confirmed by the cell counting kit-8 (CCK-8), a sensitive colorimetric assay for the determination of cell viability in cell proliferation and cytotoxicity assays. The internalization of IONs by cells was confirmed using TEM. The results indicate that IONs aggregate initially in phagosome-like structures. Using ferrozine assay, it was demonstrated that monocytes incubated with 100 μg/mL

of IONs have an increase in iron content, confirming the cellular internalization of IONs. The level of LC3II expression, which is a key marker of autophagy, was significantly increased in cells treated with IONs as evidenced by immunoblotting. The selectively autophagic substrate p62 was degraded in a concentration-dependent manner correlating with induction of autophagy. The use of wortmannin, an inhibitor of autophagy and the phosphoinositide 3-kinases (PI3-Ks) signaling pathway, reversed the autophagic response when cells were treated with IONs. Furthermore, the authors reported that co-incubation of monocytes with IONs and wortmannin decrease cell viability, suggesting that autophagy favor cell survival in response to IONs. An increased production of the pro-inflammatory cytokines IL-6, IL-1β, and TNF-α, was observed in a concentration-dependent manner in response to IONs. This indicates the potential of IONs to induce monocyte activation. Of interest, this production of cytokines was significantly increased in cells co-incubated with IONs and wortmannin, suggesting that autophagy might play a protective role in the resistance of cells to the pro-inflammatory effects induced by IONs. Phagocytosis was also studied by the authors, but IONs did not interfere with this biological function, as determined by flow cytometry by using fluorescent *E. coli* bacteria (Wu et al. 2017) Thus, taken together, autophagic activation in human monocytes may play a protective role against the cytotoxicity of IONs.

The effect of poly-acrylic acid coated iron oxide nanoparticles (PIONs), with a hydrodynamic diameter of ~12 nm, was studied in monocytes and during their differentiation to HMDMs (Giraldo-Villegas et al. 2019). The PIONs were tested at the concentration of 32 µg/mL and the viability was evaluated by flow cytometry using DIOC6 and PI probes to detect the integrity of the mitochondrial membrane potential and the cell membrane integrity, respectively. They found that PIONs did not affect cell viability, nor the integrity of the mitochondrial membrane potential as characterized by a low value of PI and a high value of DIOC6, respectively. The assessment of DNA integrity by flow cytometry using an anti-BrdU assay to detect DNA synthesis (cell proliferation), anti-H2AX to detect DNA damage, and anti-PARP1 to detect cell viability, did not show any effect of PIONS as compared to untreated cells. Of interest, the exposition of cells to a MF after incubation with PIONs had no effect on DNA integrity. The internalization of PIONs by cells was monitored by flow cytometry by exploiting the side scatter (SSC) parameter. In this experiment, the granularity of cells (monocytes, granulocytes, and lymphocytes) in whole blood sample showed a remarkable increased of SSC after treatment with PIONs. An increased in SSC parameter in flow cytometry was previously found to correlate well with the intracellular presence of NPs (Suzuki, Toyooka, and Ibuki 2007). Additionally, using atomic absorption spectrometry,

the authors confirmed that cells incubated with PIONs displayed more iron content than the non-exposed cells. They also studied cell granularity in two subsets of monocytes, intermediate monocytes (CD14 + CD16+) and classical monocytes (CD14 + CD16-) where an increased in the granularity of intermediate monocytes was observed and the iron content in macrophages derived from this subset showed a tenfold increase in iron content than the classical monocytes. This confirms that a higher uptake of PIONs occurs in the intermediate subset, considered to be the minor human monocyte population (Italiani and Boraschi 2014b). To investigate more in depth the effect of PIONs in differentiated monocytes to HMDMs, the investigators found that PIONs did not affect cell viability of differentiated cells. However, the analysis of cytokine production with a cytometric bead array shows an increased production of IL-8 and IL-6 but not for IL-10, IL-12, IL-1β, and TNF-α. Of interest, the exposition of cells with PIONs followed to a MF, was found to increase the production of IL-6 but not IL-8. Noteworthy, the differentiated monocytes pre-exposed to PIONs still preserve their capacity to stimulate T cell proliferation in presence of an antigen (Giraldo-Villegas et al. 2019).

In 2010, Lunov and colleagues evaluated the size effect of carboxydextran-coated superparamagnetic IONs (SPIO_Resovist™_ 60 nm) and ultrasmall superparamagnetic IONs (USPIO_Supravist™_ 20 nm) on HMDMs (Lunov et al. 2010). They found that the cellular uptake of SPIO was more important than the USPIO as confirmed by TEM and by intracellular measurement of iron content with an ultraviolet-visible spectrophotometer method. The analysis with the LysoTracker Red, a specific lysosome staining dye, revealed an accumulation of both NPs in lysosomes (acidic cell compartment). Of interest, the exposition of cells to both NPs for 24h did not show any cytotoxicity effect as assessed by the cell viability and proliferation XTT Assay. However, a decreasing in cell viability, also confirmed by flow cytometry using annexin–PI staining, was observed after >72h post-incubation where USPIO were more cytotoxic than SPIO. In agreement with this, activation of caspase- 3 was stronger after treatment of cells with USPIO vs. SPIO. The analysis of cytokine production, especially TNF-α, did not reveal any production even after 5 days of post-incubation for both NPs. The kinetic measurement of ROS production with the sensitive dye H_2DCFDA, indicates a capacity for both NPs to induce ROS in a time-dependent manner, with the highest values for USPIO after 2 days of incubation with cells. Western blot experiments indicate that ROS production occurs by the JNK signal transduction pathway and, intriguingly, treatment of cells with a ROS scavenger (Trolox) hamper the activation of JNK and the associated cytotoxic effects induced by these NPs (Lunov et al. 2010).

The cellular uptake mechanism of SPIO and USPIO by HMDMs was also investigated in one other study conducted by the same team (Lunov et al. 2011). TEM analysis demonstrated that cells internalized both types of NPs, SPIO being more important vs. USPIO, in agreement with the other study (Lunov et al. 2010). The authors used a pharmaceutical approach using different endocytic pathway inhibitors to determine the cellular uptake mechanism used by cells to ingest these IONs. No significant changes were observed for both NP uptake after pretreatment of cells with nystatin, cytochalasin B, and colchicine. This suggests that phagocytosis, pinocytosis, and caveolin-mediated endocytosis were not involved in the cellular uptake. However, a significant decrease in the uptake of both IONs was observed after treatment of cells with rottlerin and polyinosinic acid/monodansyl cadverine, which intercepts with macropinocytosis and scavenger receptor A (CD204)-dependent endocytosis pathway, respectively. Additionally, using a knockdown approach by treatment of cells with scavenger receptor A phosphorothioate-modified oligodeoxynucleotides (antisense RNA), they reported a significant inhibition of the cellular uptake of both NPs, confirming the central implication of scavenger receptor A in internalization of both IONs by HMDMs (Lunov et al. 2011).

The effect of superparamagnetic silica-coated IONs with a size of 30 nm, 50 nm, 70 nm, and 120 nm and IONs coated with dextran (nanomag®-D-spio) with a size of 20 nm and 50 nm all with a spherical shape was studied in HMDMs (Kunzmann et al. 2011). The sterility of NPs was tested and was found to be under 50 pg/mL as determined by a LAL assay. Using a MTT assay, the authors reported that the dextran-coated IONs (DIONs) did not alter the mitochondrial function. However, a little decrease was observed for the silica-coated IONs (CSIONs), for all size tested at a concentration of 100 µg/mL after 24h or 48h of incubation. No cytotoxicity for the DIONs nor for the CSIONs of 70 nm, and 120 nm was observed using flow cytometry after staining with annexin–v and PI. However, CSIONs with a size of 30 nm and 50 nm show a significant cytotoxicity at the concentration of 100 µg/mL. Both DIONs and CSIONs did not induce TNF-α nor IL-6 release after 24h and 48h of incubation. Internalization of NPs was confirmed by TEM analysis, and they were predominantly located in membrane-enclose vesicle-like structures. Internalization was also confirmed by quantification of iron uptake using inductively coupled plasma mass spectrometry. Pretreatment of cells with cytochalasin D (an inhibitor of phagocytosis) was found to significantly decrease the cellular uptake of both CSIONs and DIONs, but internalization of DIONs was less important than CSIONs, suggesting the implication of this process in the biological response of HMDMs (Kunzmann et al. 2011).

Although a new set of SPIONs were used to efficiently label murine macrophages for magnetic resonance imaging–based cell tracking *in vivo*, the authors also performed some experiments in parallel with human macrophages (HMDMs) (Sharkey et al. 2017). Thus, the cytotoxicity of Ferumoxide (a clinically approved IONs) and coated IONs with dextran and diethylaminoethyl (IONs-DEAE) was determined in HMDMs. Ferumoxide, with a hydrodynamic diameter of 115 nm, did not show any cytotoxicity as assessed by CellTiter-Glo luminescent cell viability assay measuring ATP levels and by LDH assay. The cellular uptake of Ferumoxide was analyzed by ferrozine assay and the results indicate a concentration-dependent increase uptake after 4h and 24h. However, modified IONs-DEAE, with a hydrodynamic diameter of 68 nm, show an increased cytotoxicity at a concentration greater than 25 µg/mL for the same tested periods of time. Of interest, after 24h, a marked increase in cell death was confirmed by a drop in ATP levels already observed at 10 µg/mL (Sharkey et al. 2017).

IONs also may be employed to “re-educate” immune cells in the context of tumor immunotherapy in which metabolic reprogramming of tumor-associated macrophages from M2 (promoting tumor progression) to M1 (suppressing tumor growth) phenotypes represents an interesting therapeutic strategy against cancer (Zhang, Cao, et al. 2020). Indeed, due to the importance of the iron levels in macrophage polarization states, IONs can therefore be used to drive the tumor-associated macrophages to a tumor suppressor phenotype. This is a dynamic area of research and readers can be referred to other works on that topic (Nascimento et al. 2021)

10.2.3 The effects of iron oxide nanoparticles in dendritic cells

DCs represent 5% of the peripheral blood mononuclear cells, and they are characterized by the absence of CD14 and CD16 on their cell surface (Auffray, Sieweke, and Geissmann 2009). Like monocytes and macrophages, DCs can also be divided in different subsets. Conventional DCs are the most abundant ones as they are crucially involved in conveying environmental signals to T cells. Plasmacytoid DCs are known to produce interferons type I, principally in response to viral infection. Monocytes can be differentiated into monocytes-derived dendritic cells (MDDCs) that can share many functional aspects with DCs and macrophages. These cells take generally origin during an inflammatory response (Merad et al. 2013) and play a crucial role in presenting antigens to lymphocytes via CD80 and CD86. In addition, they are known to produce stimulatory cytokines like IL-12, which can trigger a cell-mediated immune response. Unlike macrophages, DCs are

able to migrate from the periphery of the body, like in the skin and lung into T-cell area of draining lymph nodes (Lambrecht et al. 1998). Interactions of NPs with DCs has already been reviewed in some articles (Fogli et al. 2017; Jia et al. 2018).

Kunzmann et al. have addressed the potential cytotoxic effect of different size of superparamagnetic silica-coated IONs (CSIONS_ 30 nm, 50 nm, 70 nm, and 120 nm) and commercially IONS coated with dextran (DIONs_nanomag®-D-spio 20 nm and 50 nm) by determining the apoptotic and necrotic cell levels in MDDCs (Kunzmann et al. 2011). The shape of all NPs was spherical, and the endotoxin level was below 50 pg/mL as assessed by a chromogenic LAL test method. The internalization of both types of NPs was confirmed by TEM analysis and the cell viability was determined by cytometry using Annexin-V and PI staining. DIONs were not cytotoxic, as well as CSIONs of 70 nm and 120 nm. However, a significant increase in apoptosis/necrosis was observed for smaller CSIONs of 30 nm and 50 nm. Both types of NPs did not induce the production of the pro-inflammatory cytokines TNF-α and IL-12 after 24h and 48h of treatment (Kunzmann et al. 2011).

Two different types of magnetic colloids composed of the magnetite core (Fe_3O_4) coated with a dextran shell, one with a positive charge and a diameter of 13 nm and the other with a negative charge with a diameter of 15 nm, both having a cubic-round shape, have been tested *in vitro* in MDDCs (Marcos-Campos et al. 2011). By using three different techniques for determining cell viability (trypan blue exclusion assay, MTT assay, and cytometry assay with annexin–v/PI staining), it was reported that cell viability determined by trypan blue exclusion, starts to decline after 5 days of incubation with a concentration of 50 μg/mL, but this was also observed in control cells. MTT and flow cytometry assays confirmed that this was not a cytotoxic effect induced by the NPs. The cellular uptake of the NPs was confirmed by TEM and the NPs were principally observed in endosome-like structures. Of interest, the application of a MF field on cells that have internalized the NPs was found to cause a marked decrease in their viability as assessed by the flow cytometry assay. This was observed for both positive and negative NPs. Scanning electron microscopy demonstrated that cells with retained negatively charged IONs and exposed to MF show a loss of plasma membrane integrity, a hallmark of cell necrosis. Also, a significant increase in cell death (apoptosis/necrosis) was also confirmed with flow cytometry for both types of NPs. The authors of this study suggest that this selective cell death may be induced by a disruption of the lysosome membranes by NPs resulting in uncontrolled release of their degrading enzymes that can lead to a subsequent cell apoptosis/necrosis (Marcos-Campos et al. 2011).

10.2.4 The effects of iron oxide nanoparticles in lymphocytes and natural killer cells

1.1.1.7 Lymphocytes

Lymphocytes play a critical role in the defense of the body against pathogens invaders, and they are central to the adaptive immunity. These cells can be divided principally into three subsets, the small T and B lymphocytes and natural killer (NK) cells, these latter cells being characterized as large lymphocytes known to play a role in innate immunity. T cells rely fundamentally on antigen-presenting cells like DCs and macrophages that can break down pathogenic or self-proteins into peptides and assure their presentation to T cells via the major histocompatibility complex (MHC) which interact with T-cell receptor. Although, different phenotypes of T cells express CD3 surface molecules, helper T cells express CD4 while cytotoxic T cells express CD8. The most prominent feature of lymphocytes is clonal expansion, during which individual antigen-specific cells proliferate following recognition of their cognate ligand, giving rise to a large pool of progeny that bears the same antigen specificity. Furthermore, this clonal expansion results in developing cell memory, that recognize specifically and quickly a pre-known antigen and trigger a more robust response to establish homeostasis. After appropriate activation, B cells can differentiate into plasmocytes that will secrete antibody molecules (Adams, Grassmann, and Sun 2020).

Regarding NK cells, they represent 5–20% of circulating lymphocytes and have the particularity to detect harmful cells by scrutinizing for the presence of MHC class I on their surface. Generally, tumor cells and infected cells with viruses tend to escape recognition by cytotoxic T cells which are MHC class I cell dependent by down-regulating MHC-I expression, at that point NK cells sit between the innate and adaptive immune system by acting as cytotoxic T cells without expressing T cell receptor. Indeed, the absence of MHC class I is considered a hallmark for NK cells to trigger an immune response against the targeted cells (Abel et al. 2018).

NK cells are characterized by the expression of CD3 - CD56 surface markers and by having a poor homing capacity in the tumor environment, limiting their contact with tumor cells. This represents a major challenge for their use in cancer immunotherapy. The capacity of T and NK cells to interact with NPs have attracted researchers for developing T cell- and NK cell-based immunotherapy as an innovative approach to fight against cancer. In fact, NPs can be used to

enhance the activation and expansion of T and NK cells which are extremely demanded in cell-based cancer treatment therapies (Mikelez-Alonso et al. 2021; Han et al. 2022). However, the safety of NPs should be established prior allowing a safe application of IONs in this kind of therapy. In the following lines, we summarize different studies regarding the interaction of IONs with primary human lymphocytes.

In 2016, Easo and Mohanan studied the response of T cells to dextran-coated IONs. TEM analysis demonstrated a spherical shape for these NPs with a size of ~10 nm (Easo and Mohanan 2016). Their internalization was assessed by Perl's Prussian staining assay, and they demonstrated that IONs were ingested by endocytosis as lymphocytes are not specialized phagocytic cells. The investigators have determined cell viability by flow cytometry at different concentrations (8, 40, 200, and 1000 µg/mL) where no apparent induction of apoptosis/necrosis occurred. However, using the ROS sensitive-dye H_2DCFDA, an increase in ROS production was observed in a concentration-dependent manner after incubation of cells for 4h, but a decrease was observed after 24h. Furthermore, the presence of glutathione alleviates ROS production from ~100% to ~60%. The evaluation of the genotoxic effect by studying chromosome aberration using the PBMAX TM karyotyping medium, show a non-significant increase in the proportion of aberration in metaphase versus cells treated with the positive control mitomycin c. The analysis of 84 oxidative stress genes by a PCR array technique revealed an upregulation of some gene, including myeloperoxidase, glutathione peroxidase 1, dual oxidase 2, and glutathione transferase zeta 1 which are involved in redox reaction. The measurement of intracellular antioxidant enzymes at the highest tested concentration of IONs (1000 µg/mL) show no difference in superoxide dismutase activity, but a decrease in catalase activity was observed as well as a significant increase in glutathione peroxidase (Easo and Mohanan 2016).

Lima et al. performed a study with lymphocytes aiming at determining potential genotoxicity of IONs coated with two thiol-containing hydrophilic ligands, mercaptosuccinic acid (MSA) and dimercaptosuccinic acid (DMSA) (Lima et al. 2013). To do so, the cells were incubated with 10, 100, and 500 µg/mL of these NPs for 1h and 24h, and the genotoxicity effect was assessed by the comet assay. They demonstrated that ION-MSA were not genotoxic for cells, but that ION-DMSA were able to slightly increase DNA damage. Intriguingly, the nitrosation of thiol groups of these NPs upon incubation with nitrite lead to the formation of ION S-nitroso-DMSA or ION S-nitroso-MSA which have been previously shown to have significant genotoxic effects (Lima et al. 2013).

The capacity of primary human T lymphocytes to be activated after polyclonal activation was studied after their loading with citrate coated IONs. In this study, the cytotoxic effect of IONs was determined by flow cytometry using annexin–v/PI staining at a concentration of 25, 50, 75, and 100 µg/mL. IONs did not induce cell necrosis, but after 48h post-incubation a significant increase of apoptotic cells was observed, and the viability decline from 94% to 52% at the highest tested concentration of 100 µg/mL. Flow cytometry analyses have demonstrated an increase of cell granularity in a concentration-dependent manner confirming an interaction between cells and the IONs. Additionally, when cells were stained with the fluorescent dye lucifer yellow (impermeable membrane dye), the results indicated that internalization was mainly observed after 48h post-incubation. This correlates with the known low phagocytic profile of T cells. Analysis of iron content by atomic emission spectroscopy shows a significant increase in comparison with non-treated cells. The authors have also studied the effect of IONs on cell proliferation by incubating lymphocytes with IONs for 24h at 75 µg/mL, in addition to a cocktail of CD3/CD28/CD2 and recombinant human interleukin-2 (activator mix) to stimulate cell proliferation. The results indicate that IONs did not inhibit cell proliferation after their stimulation. Of interest, they observed that loading lymphocytes with the IONs enhance T cell differentiation from a naïve subset (CCR7 + CD45RO) to a central memory T cell subset (CCR7 + CD45RO+). The production of IL-2 was decreased in the presence of IONs, but the expression of CD25, which is part of the IL-2 receptor, was not modified. In brief, taken all together, the results prompted the authors to conclude that the IONs did not impair T cell activation (Mühlberger et al. 2020).

Absence of genotoxicity in T cells was reported in one study when cells were treated with 4, 20, and 100 µg/mL polyacrylic acid (PAA)- coated and non-coated IONs, with a mean size of ~10 nm. Analysis of the cell cycle was studied by flow cytometry where none of the tested IONs was found to interfere with the progression of the cell cycle after 48h of incubation. The analysis of chromosome aberrations did not either show any genotoxic effect. Interestingly, when cells were tested with both IONs and bleomycin (clastogenic agent), no synergistic effect was observed (Couto, Sousa, et al. 2015).

The influence of coating IONs with oleate on T cell cytotoxicity and genotoxicity was studied (Magdolenova et al. 2015). The IONs, with a size between 5 and 12 nm, were with an ellipsoidal-octahedral shape structure. The authors confirmed that the presence of oleate as a coating material prevents particle-particle interactions and enhances its superparamagnetic behavior in comparison to uncoated IONs. The analysis of cytotoxicity was measured by C cytokinesis-block

proliferation index (CBPI) and by incorporation of 3H-TdR into DNA of proliferating cells. They found that uncoated IONs did not decrease the CBPI index at the concentration of 0.12, 0.6, 3, 15, and 75 μg/cm^2/mL. Furthermore, no significant change in cell proliferation activity was observed, at least for the concentrations that did not interfere with the assay (0.12, 0.6, 3 μg/cm^2). Oleate-coated IONs did not induce any change in the CBPI index, but they were found to be cytotoxic according to the 3H-TdR incorporation assay. When the cytotoxic effect of the sodium oleate by itself was studied, significant cytotoxic effect was observed only at a concentration of 108 μg/mL, corresponding to tenfold to the concentration of sodium oleate present in the oleate-coated IONs. Therefore, the authors conclude that the cytotoxicity was not due to the intrinsic toxicity of oleate. The genotoxicity was assessed by the comet assay based on detection of DNA strand breaks, and they found that induction of strand breaks was time (4h and 24h) and concentration-dependent for the coated IONs, but non-genotoxic effect was observed for the uncoated IONs. They explained the discrepancy between CBPI index and genotoxicity results by the fact that cells for the 3HTdR incorporation assay were exposed in the proliferation phase of their cell cycle, in contrast to the CBPI assay, where cells were rather exposed before in the G0 phase of their cell cycle during which cells are more resistant. The fact that only the oleate sodium-coated IONs induce genotoxicity in T cells prompted the authors to propose that the coating gives some “new features” to the NPs, like interaction with serum proteins, promoting the formation of protein corona, a complex that might be responsible for the observed toxic effects (Magdolenova et al. 2015).

The cytotoxicity of IONs (iron (III) oxide, Fe_2O_3) with a spherical shape and a primary size of 44 nm, characterized by TEM, was investigated and a significant reduction in cell viability was observed at the concentration of 50, 75, and 100 μg/mL, as assessed by MTT assay (Rajiv et al. 2016). Furthermore, a significant increase of LDH release was also observed, confirming the cytotoxic effect of these NPs. The assessment of ROS production showed a significant increase at the concentration of 75, and 100 μg/mL, but not 50 μg/mL. The assessment of antioxidant enzymes showed that IONs, at 75 and 100 μg/mL, induce a significant reduction in catalase, glutathione and superoxide dismutase level in a concentration-dependent manner after 24h of incubation. The comet assay confirmed the capacity of IONs to induce DNA breaks in a concentration-dependent manner, and, moreover, a significant increase in chromosome aberrations was observed at the concentration of 100 μg/mL (Rajiv et al. 2016).

As previously mentioned, it has been discussed that NPs can exert different effects in primary cells vs. cell lines, it is important to recall here that Shah and colleagues (Shah et al. 2018) reported cellular and molecular changes in T cells following their incubation with Feraheme®, an FDA approved ION formulation for anemia treatment. The mitochondrial membrane potential (MMP) integrity was measured by the MitoProbe™ JC-1 assay, and the treatment of cells showed an increase in JC-1 monomer formation (known to correlate with a loss on the MMP) in a concentration- and time-dependent manner. Interestingly, this observation also appears to be donor-specific, as each donor cells respond at different degrees of intensity, again reinforcing the fact that different responses can be observed in primary cells vs. cell lines. The mitochondrial ROS production was determined with the MitoSOX red kit able to detect superoxide, and the results confirmed an increase of ROS production following treatment with IONs. Of interest, pre-treatment of cells with a specific mitochondrial ROS inhibitor (MitoTempo) was found to reduce the formation of JC-1 monomers and ROS production. Furthermore, the measurement of glutathione did not show any changes in comparison with untreated cells, indicating a specific interaction of IONs with the mitochondria. As T lymphocytes are not recognized as phagocytic cells, the internalization of IONs was assessed by TEM and the results did not reveal any signs of the presence of IONs inside the cells. However, IONs seem to induce detrimental damages in the mitochondria, as evidenced by the presence of collapsed and fused mitochondria. This phenomenon is already known to be used by cells to remove damaged mitochondria when exposed to high levels of stress (Youle and van der Bliek 2012), induced here by the presence of IONs. Using ICP-MS and Perl's Prussian staining assay, they observed some internalization of IONs by the T cells, but they were not able to determine the exact form (nanoparticulate or ions).

To evaluate the influence of IONs on the intracellular iron homeostasis of T cells, the level of ferritin heavy chain (FTH), transferrin receptor 1 (TfR1) and ferroportin (FPN) was assessed by western blot experiments assay. They demonstrate an increase level expression of FTH, which is known to be implicated in iron storage inside the cells. No important changes in the level of expression of both TfR1 and FPN were observed. Pre-treatment of T cells with IONs and subsequent exposition to CD3-crosslinking antibody inducing cell activation (acting as mitogen) have shown a reduced capacity of T cells to proliferate as determined by the BrdU cell proliferation kit assay. Also, using a Q-Plex™ Array to determine cytokine production, the authors reported a decrease of RANTES, IFNγ, IL-4, IL-5, IL-10, and IL-17 following the treatment of cells with the known phytohemagglutinin (PHA) and concanavalin A (ConA) mitogens. This agrees with an immunosuppressive activity of IONs towards T cells. Interestingly, incubation of cells with the

mitoROS inhibitor (MitoTEMPO) restored the proliferation functionality of cells following their exposure to the activating cocktail, suggesting a role of mitoROS in altering T cells functions in response to IONs.

By comparing Feraheme (26.3 nm) with three other iron nanoparticle formulations used in the treatment of anemia (Pai 2017), Venofer (8.3 nm), Injectafer (23.1 nm), and Ferrlecit (8.6 nm); Injectafer was found to not affect the MMP of T cells. However, Venofer and Ferrlecit were found to induce a more salient effect than it was observed with Feraheme®. In addition, Perl's Prussian staining assay and TEM show an important accumulation of the iron greater than that observed in Feraheme-treated cells. Of interest, Injectafer-treated cells seemed to not internalize the NPs. It was also reported in this study that mitochondria were more damaged in cells treated with Venofer and Ferrlecit. The Injectafer formulation did not alter mitogen-triggered cytokine release from T cells, and, unlike Feraheme®, all iron-based complex formulations tested increased T-cell proliferation following their stimulation with CD3-crosslinking antibodies. This observation confirms that the coating material, particle size, and iron concentration could be a tricky issue in nanotoxicity assessment (Shah et al. 2018).

In a recent study, the biological effect of IONs with a monocrystalline spherical shape and a diameter in the range of 5–20 nm were determined in T lymphocytes obtained from whole blood samples using leukapheresis filters (Kakavoulia et al. 2022). Cytotoxicity of IONs was monitored after incubation of T cells with 50, 100, 200, 500 and 1000 µg/mL for periods of time of 1, 2, 3, and 24h. The IONs were tested in non-stimulated and LPS-induced T cells in the presence of monocytes that are required to present costimulatory surface marker B7 for T cell activation. The cell viability was evaluated by trypan blue exclusion assay and IONs did not induce a significant decrease in cell viability. Indeed, more than 95% of cells were viable after 24h of incubation in both conditions. This was confirmed by flow cytometry using the 7-aminoactinomycin D (7-AAD) dye that selectively binds to the GC regions of DNA of apoptotic cells. Also, MTT assay confirmed the same observation as the percentage of metabolically active cells was higher than 90% for both stimulated and unstimulated T cells. ROS production detected with the H_2DCFDA probe was increased in a concentration-dependent fashion in LPS-stimulated and unstimulated T cells (Kakavoulia et al. 2022).

To improve anti-tumor response of T cells by using citrate-coated superparamagnetic IONs (CIONs), internalization of the NPs after an external MF application was performed to favor the

accumulation of cells at a particular site (i.e., tumor environment) for developing future cell-based cancer therapy was investigated. The addition of 10% fetal calf serum (FCS) in the culture medium was found to increase the colloidal stability of CIONs, while the use of 2% FCS, rather induce their aggregation. An optimal iron concentration of 80 µg/mL was determined in this study for efficient loading of T cells. Flow cytometry analysis indicates a significant reduced number of T cells when incubated with IONs in the presence of 2% FCS. That has been attributed to a possible rupture of cells that were overloaded with CIONs, since the side scatter analysis of residual cells showed a significant increase in this parameter that was also confirmed by the amount of iron detected following the analysis of cell lysates with atomic emission spectroscopy (AES). An increased number of apoptotic/necrotic cells was observed in the presence of 2% FCS. However, when cells were incubated with 10% FCS, a smaller side scatter value was observed, correlating with a weaker internalization of CIONs. To favor CIONs loading, cells were stimulated with an ImmunoCult human CD3/CD28/CD2 T-cell activator mixture with 30 IU/mL of rhIL-2. CIONs had no effect on cell count after stimulation, but the cell viability was significantly reduced. The analysis of iron content with AES did not show any significant increase. However, TEM analysis demonstrated that CIONs were initially attached to the cell plasma membrane in a uniform layer at one side of the cell, and, even if T lymphocytes are not recognized as professional phagocytes, CIONs were found to be internalized in vesicle-like structures. Attraction of loaded T cells with CIONs by an EMF was confirmed using a peristaltic pump equipped with a magnet to imitate the blood flow in a physiological vascular system. The number of accumulating cells stimulated by the activator mixture was thirty-five-fold higher than the non-stimulated cells. The effect of CIONs on cytokine production was assessed, and following stimulation with the activator mixture, cells were able to produce INF-y, TNF-α, and IL-2 at the same level as the non-exposed cells used as control. The same observation was confirmed for cells that were not stimulated, indicating that CIONs did not induce cytokine production or cell activation (Boosz et al. 2021).

Assessment of effector T cells (CD4 + CD3+) and cytotoxic T cells (CD8 + CD3+) activation with Feraheme® have been studied in a proof-of concept study using whole blood samples from healthy individuals and from patients showing an allergic reaction to Feraheme® within 2h post-infusion. Cells were stained first with carboxyfluorescein succinimidyl ester and then incubated for 3 days with anti-CD28/ CD3 mixture for inducing proliferation in the presence of 15 mg/mL of Feraheme® or 100 mg/mL of plasma of the patients with allergic symptoms. The following markers, CD25, CD69, CD137, CD154 and IFNγ were monitored by flow cytometry to confirm cell activation. They found that Feraheme® did not increase T cell proliferation as the percentage of

cells exposed only to Feraheme® was identical to cells incubated with media alone (both close to 8%). However this increase to ~27% when cells were incubated in the presence of the plasma of patients, suggesting an activation that might be related to the protein corona complex adsorbed on the surface of the tested IONs (Muehe et al. 2021).

3.4.2. Natural killer cells

One study investigated the interaction between polydopamine-coated IONs of 50 and 60 nm and NK cells. The cells were expanded from peripheral blood mononuclear cells (PBMCs) in a medium enriched with 100 IU/mL of IL-2. Using TEM, these IONs were mainly observed freely in the cytosol as well as in endocytic vesicle-like structures. A small portion was also observed in the nuclear structures. Using flow cytometry (annexin-V/PI staining), the authors reported absence of apoptosis (both early and late apoptosis) at the concentrations of 50 µg/ mL and 100 µg /mL. The cell cycle was analyzed by measuring DNA content and no significant changes were observed in both ION-loaded cells versus control. The cell viability was determined with the cell counting kit-8 (CCK-8) assay, and the same proliferation profile was observed in both conditions. Further, the IONs did not induce activation of NK cells, since the measurement of CD56 and CD69 markers did not show any changes. Since NK cells are known to be able to produce cytokines, principally INFɣ and TNF-α, and having immunomodulatory activities in recruiting other immune cells like T cells and DCs (Abel et al. 2018), the authors evaluated their production by ELISA. The levels of both cytokines released in the supernatants of NK cells treated with IONs were not increased significantly when compared with control after 24h and 72h post-incubation. However, the NK capacity to lyse lung carcinoma epithelial cell lines (A549) was induced with 50 µg /mL IONs. Intriguingly, this effect was further improved following the exposition of loaded cells to an external MF. It was proposed that the application of the MF help to concentrate loaded-NK cells against tumor cells (Wu, Zhang, Wei, et al. 2018).

In 2019, Burga et al. proposed a “nanoimmunotherapy” approach in which the use of IONs attached at NK cell surface can enhance their homing for ambitious anti-tumoral applications (Burga et al. 2019). In their study, NK cells were obtained from fresh umbilical cord blood, considered as an abundant source for generating primary NK cells. The cells were expanded *ex vivo* with irradiated feeder cells and supplemented with human IL-2 and IL-15. IONs, at a dose of 50 pg/cell, did not impact the viability of NK cells, neither the exposition of the “loaded” cells to an external MF. Flow cytometry experiments demonstrated unaltered surface cell expression of

NKp44, NKG2D, NKp30, CD69, and PD1 on IONs-treated or untreated NK cells. Also, a cytotoxicity assay was used to assess the cytotoxicity of effectors NK cells treated or not with IONs against K562 and SHSY5Y cells where no major changes were observed (Burga et al. 2019).

10.2.5 The effects of iron oxide nanoparticles in eosinophils, basophils, and mast cells

The literature regarding the interactions of IONs with eosinophils, mast cells, and basophils is anemic. This may be explained by the fact that these cells, known to be involved during allergies and inflammation, are not abundant in the human blood circulation, limit largely their studies in the context of nanotoxicology. Therefore, how IONs could influence these cells is still unknown. However, some reviews have been published regarding the interaction of other types of NPs with these cells (Duguay et al. 2020; Gamazo et al. 2014; Vanharen and Girard 2020) opening the possibility in the future to investigate specifically how IONs can alter the biology of these cells. Nevertheless, some data regarding the interaction between IONs and basophils have been reported in a proof-of-concept study (Muehe et al. 2021). Although this study was not dedicated strictly on basophils, the main goal was to understand the role of protein corona in allergic reaction to Feraheme®. The authors examined the effect of Feraheme® on CD63 expression (biomarker of basophil reactivity) by flow cytometry. In healthy whole blood samples, basophils did not show any signs of activation following their incubation with IONs compared with cells that have been incubated with a polyclonal IgE antibody used as a positive control. Of interest, incubation of cells with plasma of the patients presenting an allergic reaction to Feraheme® within 2h of infusion shows an activation of basophils. However, the presence of Omalizumab (a recombinant DNA-derived humanized IgG1k monoclonal antibody known to specifically bind free human immunoglobulin E) was found to significantly decrease this response. This suggests the implication of corona-coated Feraheme® in basophil activation rather than Feraheme® alone.

10.3 Concluding remarks

The above observations show that evaluating the safety of IONs to humans is certainly a great challenge knowing that different functional alterations on immune cells could occur as it has been discussed in this review. The major reported effects that IONs could have on primary human immune cells are summarized in Figure 27 and in Table 4. The complexity of ION risk assessment is principally attributed to the size, shape, coating, and all experimental conditions used, including the cell culture media, the presence or not of serum, the cell density, and so on. Therefore, standardization methods and approaches for evaluating the nanosafety of IONs (as well as other types of NPs) are lacking. Of note, particularly because of the medical use of IONs (drug delivery, cancer therapy, and as contrast agents in different techniques such as magnetic resonance imaging), implying the use of diverse coatings, it is important to specifically study potential different effects due to the use of different coatings. As a reminder, in a study performed with human PMNs, PAA-coated IONs were found to increase the apoptotic rate whereas the non-coated IONs rather prevent this important biological process (Couto et al. 2014).

To our humble opinion, the above reported studies indicate clearly that one thing is sure, the interaction of IONs with primary human immune cells should be part of a standardization procedure in IONs risk assessment. In addition to guiding for a safer use of IONs and other NPs, such a procedure will help to better understand the underlying mechanisms by which NPs can alter the biology of immune cells. We believe that the two following situations investigating interaction with a NP and human PMNs are good examples supporting the use of primary immune cells for evaluating a safer use of NPs. First, one can imagine that if IONs or other types of NPs can decrease the ability of PMNs to exert phagocytosis, health could be seriously affected as this situation will favor opportunistic infections knowing that these cells are highly involved in host self-defense which is attributed to their presence in high numbers in human blood and their great capacity to exert phagocytosis and to produce ROS. Second, for the same reasons (high number and phagocytosis), if one would like to develop a new treatment for example based on drug delivery by NPs, that will normally be injected intravenously or administered orally, PMNs will eventually become in contact with NPs and will try to do their best to ingest and degrade NPs. Thus, this will represent a first obstacle to the development of a new drug. In addition, this will lead to the presence of a portion of free NPs in the body reinforcing the fact to investigate also in parallel the biological effect of naked NPs and not only NPs carrying a drug. These are only two conditions (others could be imagined) that, we believed, represent well the necessity of

investigating the interaction of NPs with primary human immune cells. In addition to the different characteristic of IONs may have as well as the experimental conditions, contamination with endotoxin add more challenges in risk assessment of IONs, as immune cells are highly sensitive to such contaminant. This could misinterpret the outcome results. Nevertheless, many standardized assessment methods have been proposed to circumvent this. However, to further complicate this, NPs were found to interfere with some of these assays (Neun and Dobrovolskaia 2011). Therefore, other methods, like incubation of NP suspensions in lysogeny broth agar plates for several hours could be used for testing sterility (Goncalves and Girard 2014; Himly et al. 2020). Nevertheless, we believe that more sophisticated endotoxin test needs to be developed and applied before declaring that an ION suspension is endotoxin free.

The use of primary human immune cells in nanosafety studies can be more reliable than immortalized cell lines as primary human cells exhibit a more realistic model and, as previously discussed, different, even opposed, results can be obtained with primary cells and immortalized cell lines (Verdon et al. 2021; Shah et al. 2018; Silva and Girard 2016; Vallières et al. 2016). Although we are aware about the existence of some negative points in using primary cells, most of them are related to the difficulty in sourcing (ethical aspects, cell isolation, cost, etc.) and the possible variation between donors (Boraschi, Costantino, and Italiani 2012). However, the use of primary cells such as those obtained from the human blood, allow the possibility to perform sex-based analysis using several female and male donors, as recently proposed in studies investigating the biological roles of NPs (Ray et al. 2020a) and knowing the existence of sexual dimorphism of the human immune system that is increasingly recognized (Jaillon, Berthenet, and Garlanda 2019; Lefevre et al. 2019).

IONs can have a defined immune interaction with immune cells and ‘undesirable’ interactions may include principally immunosuppressive or immunostimulatory effects. This response should not be seen as a drawback issue, in fact, this property could open a new innovative way for the treatment of chronic inflammatory diseases or immune deficiency diseases, respectively. Finally, the study of immune cells should not be taken in a way that each immune cell subtype can act by its own, but all cells interact in a system that has a lot of other components which play a critical role in the immune responses towards NPs as the complement system (Inturi et al. 2015). Moreover, the elucidation of the mechanisms behind the effects of IONs specifically in eosinophils, basophils and mast cells is still limited and certainly needs to be further investigated knowing these cells play a role in asthma and allergic diseases. Further, studying interactions

between IONs and these cells will help to get a more global picture, especially knowing that inflammation is a reported undesired effect of IONs, and that inflammation involves several immune (and others) cell types.

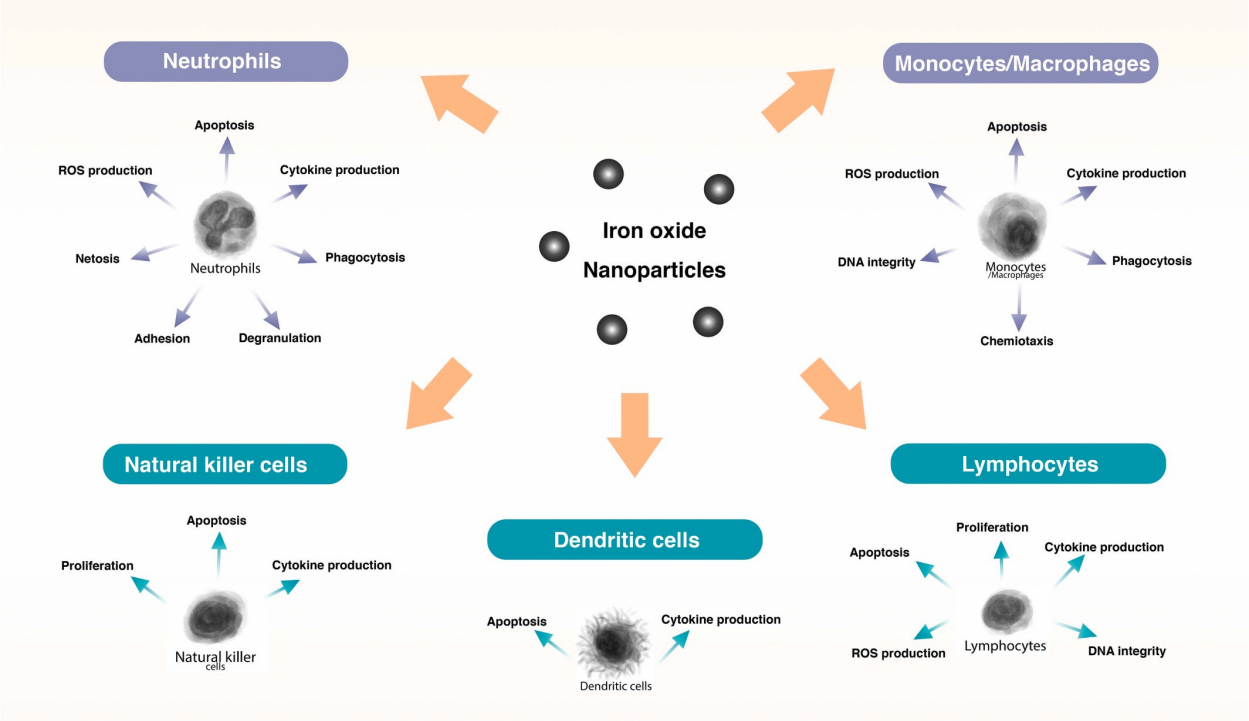

Figure 27: The main biological functions of primary immune cells modulated by iron oxide nanoparticles exposure.

This figure summarizes the major reported effects that IONs could have on primary human immune cells. Although several studies reporting how IONs could influence the biological functions in primary human immune cells are found in the scientific literature, most of them focus on cytotoxicity (mostly necrosis and apoptosis), cell proliferation, phagocytosis, cytokine and ROS production, DNA integrity. In addition, and more specifically for PMNs, NETosis, adherence, and degranulation are also reported.

Table 4: Summary of the modulatory effects of iron oxide nanoparticles in immune cells.

Neutrophils

IONs category	Coating	Shape	Hd (nm)	ZP (mV)	Endotoxin check	Cell viability	Oxidative stress	Degranulation	Chemotaxis	Phagocytosis	Adhesion	Cytokines	Reference
Non-commercial	Lauric acid	NR	55.8	−25.1	Yes	No effect	NR	NR	NR	NR	NR	NR	(Bilyy et al., 2018)
Non-commercial	Human serum albumin	NR	58.9	−11.9	Yes	No effect	NR	NR	NR	NR	NR	NR	(Bilyy et al., 2018)
Non-commercial	Dextran	NR	31	−1.7	Yes	No effect	NR	NR	NR	NR	NR	NR	(Bilyy et al., 2018)
Non-commercial	Polyacrylic acid	NR	10.1	−30	NR	↓	↑	NR	NR	NR	NR	NR	(Couto et al., 2014)
Non-commercial	Uncoated	NR	9.9	−15	NR	↑	↑	NR	NR	NR	NR	NR	(Couto et al., 2014)
Commercial	NR	NR	NR	NR	NR	No effect	↓	↓	↑ (CXCR1 and CXCR2)	NR	↓	NR	(Garcia et al., 2020)
Commercial	Uncoated	Spherical	NR	NR	Yes	↑	↓	↓	NR	↑	↑	↑ IL-8, MIP-1α/ MIP1β, GRO-α	(Saafane and Girard, 2022)
Non-commercial	Heparin-chitosan	NR	NR	NR	NR	NR	↓	NR	NR	NR	NR	NR	(Świętek et al., 2022)
Non-commercial	Gallic acid	NR	NR	NR	NR	NR	↓	NR	NR	NR	NR	NR	(Świętek et al., 2022)
Non-commercial	Phloroglucinol	NR	NR	NR	NR	NR	↓	NR	NR	NR	NR	NR	(Świętek et al., 2022)
Non-commercial	Uncoated	NR	NR	NR	NR	NR	↓	NR	NR	NR	NR	NR	(Świętek et al., 2022)

Monocytes/macrophages

IONs category	Coating	Shape	Hd (nm)	ZP (mV)	Endotoxin check	Cell viability	Oxidative stress	Chemotaxis	Phagocytosis	Cytokines	Reference
Non-commercial	Polyacrylic acid	NR	NR	−49.2	NR	No effect	NR	NR	NR	↑ IL-8 and IL-6	(Giraldo-Villegas et al., 2019)
Commercial/size 10 nm	Oleic acid /Amphiphilic polymers	Spherical	21.2	−53.3	Yes	No effect	NR	NR	NR	↓ TNF-α, IL-1β and IL-6	(Grosse et al., 2016)
Commercial/size 30 nm	Oleic acid /Amphiphilic polymers	Spherical	43	−48	Yes	No effect	NR	NR	NR	↓ TNF-α, IL-1β and IL-7	(Grosse et al., 2016)
Commercial/ size 30 nm	Silica	Spherical	44.4	−22	Yes	↓	NR	NR	NR	↓ TNF-α and IL-6	(Kunzmann et al., 2011)
Commercial/ size 50 nm	Silica	Spherical	125.6	−22	Yes	↓	NR	NR	NR	↓ TNF-α and IL-6	(Kunzmann et al., 2011)
Commercial/ size 70 nm	Silica	Spherical	140.3	−22	Yes	No effect	NR	NR	NR	↓ TNF-α and IL-6	(Kunzmann et al., 2011)
Commercial/ size 120 nm	Silica	Spherical	145.5	−22	Yes	No effect	NR	NR	NR	↓ TNF-α and IL-6	(Kunzmann et al., 2011)
Commercial/ size 20 nm	Dextran	Spherical	87.1	−1.5	Yes	No effect	NR	NR	NR	↓ TNF-α and IL-6	(Kunzmann et al., 2011)
Commercial/ size 50 nm	Dextran	Spherical	73.4	−1.5	Yes	No effect	NR	NR	NR	↓ TNF-α and IL-6	(Kunzmann et al., 2011)
Commercial/ size 60 nm -	Carboxydextran	NR	60.32	−8.02	NR	No effect	↑	NR	NR	↓ TNF-α	(Lunov et al., 2011)
Commercial/ size 30 nm	Carboxydextran	NR	20.84	−14.4	NR	No effect	↑	NR	NR	↓ TNF-α	(Lunov et al., 2011)
Commercial	NR	NR	NR	NR	NR	No effect	No effect	↓	No effect	No effect	(Müller et al., 2007)

(*continued on next page*)

Table 1 (*continued*)

Monocytes/macrophages

IONs category	Coating	Shape	Hd (nm)	ZP (mV)	Endotoxin check	Cell viability	Oxidative stress	Chemotaxis	Phagocytosis	Cytokines	Reference
Non-commercial	Silicon dioxide /polyethylene glycol	NR	190	NR	NR	No effect	NR	NR	NR	NR	(Perekucha et al., 2020)
Commercial	NR	NR	115	−6.7	NR	No effect	NR	NR	NR	NR	(Sharkey et al., 2017)
Non-commercial	Dextran / diethylaminoethyl	NR	68	16.8	NR	↓	NR	NR	NR	NR	(Sharkey et al., 2017)
Non-commercial	Amino-polyvinyl alcohol	NR	31	22	NR	No effect	NR	NR	NR	↑ IL-1β, IL-4, IL-6, IL-8, G-CSF, MIP-1β and RANTES	(Strehl et al., 2015)
Non-commercial	Dextran	NR	60	−11	NR	No effect	NR	NR	No effect	↑ IL-6, IL-1β, and TNF-α	(Wu et al., 2017)
Non-commercial	Dextran	Spherical	62.8	−11	NR	No effect	NR	NR	NR	↑ IL-1β and TNF-α	(Wu et al., 2018a, 2018b)

Lymphocytes

IONs category	Coating	Shape	Hd (nm)	ZP (mV)	Endotoxin check	Cell viability	Oxidative stress	Genotoxicity	Cytokines	Reference
Non-commercial	Citrate	NR	58	−53.7	NR	↓	NR	NR	No effect	(Boosz et al., 2021)
Commercial	Uncoated	NR	9.9	−1.91	NR	No effect	NR	No effect	NR	(Couto et al., 2015)
Commercial	Polyacrylic acid	NR	10.1	−3.02	NR	No effect	NR	No effect	NR	(Couto et al., 2015)
Non-commercial	Dextran	Spherical	25	−7.87	NR	No effect	↑	No effect	NR	(Easo and Mohanan, 2016)
Non-commercial	Uncoated	Spherical	NR	NR	NR	No effect	↑	NR	NR	(Kakavoulia et al., 2022)
Non-commercial	Thiol-containing hydrophilic ligands	NR	NR	NR	NR	NR	NR	No effect	NR	(Lima et al., 2013)
Commercial	Oleate	Ellipsoidal-octahedral	NR	−32	NR	↓	NR	↑	NR	(Magdolenova et al., 2015)
Commercial	Uncoated	Ellipsoidal-octahedral	NR	−2.8	NR	No effect	NR	No effect	NR	(Magdolenova et al., 2015)
Non-commercial	Citrate	NR	51	−41.9	NR	↓	NR	NR	↓ IL-2	(Mühlberger et al., 2020)
Commercial	Uncoated	Spherical	74.6	NR	NR	↓	↑	↑	NR	(Rajiv et al., 2016)
Commercial	Polyglucose sorbitol carboxymethylether	NR	32.4	−0.362	NR	↓	↑	NR	↓ RANTES, IFNγ, IL-4, IL-5, IL-10, and IL-17	(Shah et al., 2018)
Commercial	Sucrose	NR	12.7	−1.19	NR	↓	NR	NR	↓ RANTES, IFNγ, IL-4, IL-5, IL-10, and IL-17	(Shah et al., 2018)
Commercial	Dextran	NR	28.4	−1.75	NR	No effect	NR	NR	No effect	(Shah et al., 2018)
Commercial	Sodium gluconate	NR	19.9	−0.1	NR	↓	NR	NR	↓ RANTES, IFNγ, IL-4, IL-5, IL-10, and IL-17	(Shah et al., 2018)

Dendritic cells

IONs category	Coating	Shape	Hd (nm)	ZP (mV)	Endotoxin check	Cell viability	Cytokines	Reference
Commercial/ size 30 nm	Silica	Spherical	44.4	−22	Yes	↓	↓ TNF-α and IL-12	(Kunzmann et al., 2011)
Commercial/ size 50 nm	Silica	Spherical	125.6	−22	Yes	↓	↓ TNF-α and IL-12	(Kunzmann et al., 2011)
Commercial/ size 70 nm	Silica	Spherical	140.3	−22	Yes	No effect	↓ TNF-α and IL-12	(Kunzmann et al., 2011)
Commercial/ size 120 nm	Silica	Spherical	145.5	−22	Yes	No effect	↓ TNF-α and IL-12	(Kunzmann et al., 2011)
Commercial/ size 20 nm	Dextran	Spherical	87.1	−1.5	Yes	No effect	↓ TNF-α and IL-12	(Kunzmann et al., 2011)
Commercial/ size 50 nm	Dextran	Spherical	73.4	−1.5	Yes	No effect	↓ TNF-α and IL-12	(Kunzmann et al., 2011)
Commercial / size 13 nm	Dextran	Cubic-round	244	NR	NR	No effect	NR	(Marcos-Campos et al., 2011)
Commercial / size 15 nm	Dextran	Cubic-round	217	NR	NR	No effect	NR	(Marcos-Campos et al., 2011)

Hd = Hydrodynamic size in nanometer (nm); ZP = Zeta Potential in millivolt (mV); NR = Not Reported; ↓ Decrease; ↑ Increase.

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Data availability: Review of the literature.

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12 APPENDIX II (ETHICAL CERTIFICATE)

INRS Institut national de la recherche scientifique

Comité d'éthique en recherche avec des êtres humains - INRS
Rapport de suivi

Numéro d'identification du projet :	**CER-15-397**
Titre du projet :	Protocole standard de dons de sang pour l'obtention de cellules sanguines afin d'effectuer des expériences in vitro pour divers projets de recherche
Chercheur principal :	Denis Girard
Cochercheur(s) :	
Directeur de recherche :	
Centre :	INRS-Institut Armand-Frappier

Référence SOFE : 302806 303026, 304033, 304044

1. Au cours de la dernière année, avez-vous apporté des modifications ayant eu une incidence sur les dimensions éthiques de ce projet? ☐ Oui ☒ Non
   Dans l'affirmative, veuillez préciser la nature de ces modifications (joindre document).
2. Est-ce que des préoccupations éthiques sont apparues au cours de la recherche? ☐ Oui ☒ Non
   Dans l'affirmative, veuillez préciser la nature de ces préoccupations (joindre document).
3. Est-ce que des participants ont subi des effets indésirables importants liés à leur participation à votre étude? ☐Oui ☒ Non
   Dans l'affirmative, veuillez préciser la nature de ces effets indésirables ainsi que les mesures mises en place pour les atténuer (joindre document).

Statut du projet

Projet toujours en cours ☐ Projet complété ☐ Projet interrompu/abandonné ☐

Signature du chercheur : Denis Girard Date : 2019-08-26

Signature du directeur de recherche (le cas échéant) : _____ Date : _____

Une fois le formulaire rempli, veuillez en acheminer une copie dûment signée au secrétariat du CER, à l'attention de M^me^ Danielle Drolet, dans les trois semaines suivant sa réception. Les personnes qui ne respecteront pas cette obligation verront leur certificat d'éthique suspendu, ce qui entraînera automatiquement le gel des fonds liés au projet de recherche pour lequel le certificat a été émis.

Approbation CER

Danielle Drolet, Institut national de la recherche scientifique, 490, rue de la Couronne, Québec (Québec) G1K 9A9