MISSION-T2D

Multiscale Immune System SImulator for the Onset of Type 2 Diabetes integrating genetic, metabolic and nutritional data

Work Package 6

Deliverable 6.1

Agent-based customization for inflammation modelling

Document Information

Grant Agreement	**Nº**	600803	**Acronym**	MISSION-T2D
Full Title	Multiscale Immune System SImulator for the Onset of Type 2 Diabetes integrating genetic, metabolic and nutritional data			
Project URL	http://www.mission-t2d.eu			
EU Project Officer	**Name**	Dr. Adina Ratoi		

Deliverable	**No**	6.1	**Title**	Report on agent-based customization for inflammation modelling
Work package	**No**	6	**Title**	

Date of delivery	**Contractual**		**28.02.2014**		**Actual**		**20.06.2014**		
Status	**Version 1.7**				**Final**				
Nature	**Prototype**		**Report**	**X**	**Dissemination**		**Other**		

Dissemination level	Consortium+EU	
	Public	X

Target Group (If Public)		Society (in general)	
Specialized research communities	X	Health care enterprises	
Health care professionals		Citizens and Public Authorities	

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Version Log

Issue Date	Version	Author (Name)	Partner
12.02.2014	1.0	Teresa Colombo	CNR
14.02.2014	1.1	Filippo Castiglione	CNR
17.02.2014	1.2	Paolo Garagnani	UniBO

19.02.2014	1.3	Paolo Tieri	CNR
25.02.2014	1.4	Teresa Colombo	CNR
26.02.2014	1.5	Filippo Castiglione	CNR
03.03.2014	1.6	Paolo Tieri	CNR
19.06.2014	1.7	Paolo Tieri	CNR

Executive Summary	In this deliverable we describe the work done in task 6.1 (PM1-PM12). After brief introduction about diabetes and inflammation, we provide details on the identification and definition of the immunological knowledge necessary to model inflammation in diabetes-related tissues such as the adipose tissue. We extensively report about the contribution to adipose tissue inflammation from different types of immune cells. We also report the first-stage implementation of such knowledge into the agent-based simulator. Finally, we give account for the integrative task regarding models from other WPs.
Keywords	Diabetes, inflammation, adipose tissue, immune cells, agent-based immune system simulator

Contents

1 Deliverable Description

This deliverable describes the work done in identifying and defining the immunological knowledge that needs to be taken into account in order to customize the general-purpose immune system simulator that we employ so to have an accurate representation of the phenomena underpinning the innate immune activation (e.g., damage signals from hypertrophic adipocytes and from a high-glucose environment) and onset of the inflammatory process (e.g., macrophage chemotaxis and migration to adipose tissues).

2 Background

2.1 Background on diabetes

Diabetes i*s a growing epidemic worldwide*, with the number of people living with diabetes currently standing at over 350 million and showing a rising trend forecast for all types of diabetes, particularly type 2 diabetes (T2D), in the next years (International Diabetes Federation, 2013). This translates in an enormous burden in terms of human costs as well as financial costs. In fact diabetes is estimated to be responsible for one in ten deaths among adults in Europe (European Policy Action Network on Diabetes, 2014) and to represent 11% of the total healthcare spent worldwide (International Diabetes Federation, 2013).

Obesity is now recognized as a primary risk factor for the development of additional health problems, including but not limited to insulin resistance and T2D (Hotamisligil, 2006). Hallmark of obesity is a state of systemic low-grade chronic inflammation leaded by metabolic tissues, such as adipose tissue (hereinafter AT), liver and muscle (Gregor and Hotamisligil, 2011; Faloia et al, 2012; Rodríguez-Hernández et al., 2013) and accompanied by elevated levels of circulating markers of inflammation, such as pro-inflammatory cytokines and chemokines. This is endorsed by seventeen clinical trials using anti-inflammatory approaches to treat T2D or pre-diabetic states. Of these trials, several have already reached conclusive results confirming the role of inflammation in the T2D pathogenesis (table 2 of Donath et al. 2011).

2.2 Immunological priming

The immune system defends the host from infection with layered defences of increasing specificity. Briefly, physical barriers (skin, mucosa, etc.) avoid pathogens such as bacteria and viruses from entering the organism. If a pathogen breaks these barriers, the innate immune system provides a fast, non-specific response (e.g., by means of general-purpose antimicrobial proteins). If pathogens successfully escape the innate response, vertebrates use a second layer of protection, the adaptive immune system, which is triggered by the innate response. In the adaptive response, the immune system adjusts its reaction during an infection to improve the recognition of the pathogen and the efficacy of the immune action. Such improved, pathogen-specific response is then preserved after the pathogen has been eliminated in the form of an immunological memory, and allows the adaptive immune system to mount faster and stronger attacks each time this specific pathogen is encountered again (Murphy et al., 2008).

Immune cells are broadly categorized into innate and adaptive immunity cells. Innate immune cells (such as neutrophils, macrophages and dendritic cells) are always present and ready to mobilize and constitute the first line of surveillance against tissue injuries and the presence of invaders. Cells of the innate immune system also play critical roles as antigen-presenting cells (APCs) in activating adaptive immunity. The adaptive immune response may take days to develop and is mediated by adaptive immune cells (such as T and B lymphocytes). To distinguish the different subpopulations of lymphocytes, immunologists take advantage of particular combinations of an array of proteins exposed at the cell surface ("cell markers") that typify each subpopulation - such as cluster of the differentiation marker 4 (CD4, characterising the so called helper T-cells) and 8 (CD8, cytotoxic T-cells). Immune cells of the innate and adaptive system communicate by means of various soluble mediators and chemical messengers - such as pro-inflammatory cytokines and anti-inflammatory cytokines (Murphy et al., 2008).

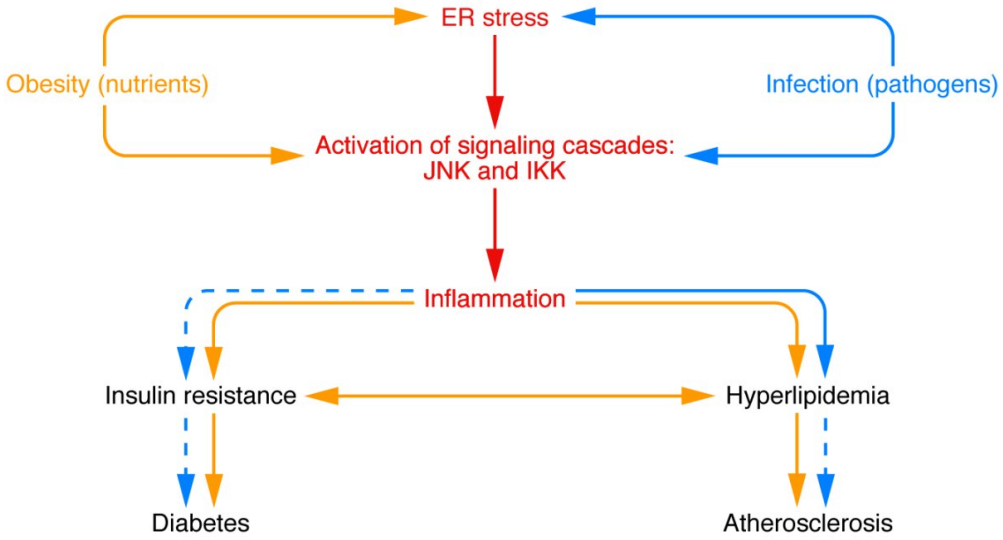

Figure 1 Growing evidence supports the envision of type 2 diabetes as an inflammatory disease to the development of which obesity-induced low-grade chronic inflammation constitutes a primary risk factor (figure credits: Wellen and Hotamisligil, 2005). ER=endoplasmic reticulum.

Inflammation is a term describing a series of non-specific responses of tissues to diverse stimuli or insults, such as injury or infection (Larsen, 1983). These beneficial inflammatory responses aimed to restore tissue homeostasis are essential for survival and usually resolve over a short time period ranging from hours to several days.

However, long-lasting and unresolved inflammatory state ("chronic inflammation") can be harmful for the body and has been recently associated to the pathophysiology of a wide range of diseases, including T2D (Figure 1) (Gregor and Hotamisligil, 2011).

In both cases (i.e., acute and chronic inflammation states), inflammatory responses require intervention of different classes of immune cells and coordination of their actions by production and release of cytokines (Table 2).

The list of abbreviations of cells and signalling molecules can be found in the appendix.

3 Modelling the immune system inflammation

The model of the immune system response employed in the project MISSION-T2D is derived from an earlier work (Bernaschi and Castiglione, 2001). It is a polyclonal model that makes use of bit strings to represent the "binding site" of cells and molecules (e.g., lymphocytes receptors, B-cell receptors, T-cell receptors, Major Histocompatibility Complexes (MHC), antigen peptides and epitopes, immunocomplexes, etc.).

The fundamental of this computer model is an agent-based model (ABM) in that all entities are individually represented (Wolfram, 2002; Castiglione, 2006; Castiglione,

2009). It includes the major classes of cells of the lymphoid lineage (T helper lymphocytes or Th, cytotoxic T lymphocytes or CTLs, B lymphocytes, antibody-producer plasma cells, PLB and natural killer cells, NK) and some of the myeloid lineage i.e., macrophages (MA, divided in M1 and M2), and dendritic cells. Helper T-cells are further divided in the following phenotypes: Th0, Th1, Th2, Th17 and T regulatory cells (Tregs). B and plasma B-cells are also divided into two phenotypes B-1, B-2 according to their antibody isotype IgM or IgG respectively. B-2 cells are further subdivided into those producing IgG1 or IgG2. All these entities interact each other following a set of rules describing the different phases of the recognition and response of the immune system against a pathogen. In particular, the model takes into account phagocytosis, antigen presentation, cytokine release, cell activation from inactive or anergic states to active states, cytotoxicity, and antibody secretion.

The model simulates the innate immunity and an elaborate form of adaptive immunity (including both humoral and cytotoxic immune responses). For example in the case of endogenous “danger signal” (e.g., coming from apoptotic cells) the innate immune response is modelled using the following rules acting consequentially:

- NK cells release IFN-γ in the presence of “danger signals” released by apoptotic cells (Zhang and Mosser, 2008). The interferon gamma (IFN-γ) is released when a cytotoxic respsonse is required, that is, when the danger signal is released by dying cells (by lysis or by direct killing by CTLs);
- Macrophage activation is mediated by IFN-γ;
- Dendritic cells activation is mediated by TNF-α released by macrophages and/or by danger signals released by necrotic cells.

In the case of innate immune response by “exogenous signal” (e.g., Pathogen-Associated Molecular Pattern, PAMP or PAMP agonist, used for specific adjuvants) the activation sequence will begin with antigen presenting cells (Dendritic cells, DCs or Macrophages, MAs) stimulation. This PAMP or PAMP agonist simulates the innate way of immunity, which can also remodel the adaptive immune response in case of antigen recognition and presentation.

The model of adaptive immunity follows the widely accepted Clonal Selection Theory of Burnet that states that the immune response is based on specific clones of B and T lymphocytes that are selected for destruction of the antigens invading the body (burnet, 1959). The humoral response consists in B-cell maturation into plasma cells secreting antibodies (IgM, IgG1 and IgG2 isotypes only). Cell-mediated immunity in the form of

cytotoxic T-cell activity is modelled through the cytotoxicity of CD8+ T-cells on malignant or infected cells.

In the model, a single human lymph node (or a portion of it) is mapped onto a three-dimensional ellipsoid Cartesian lattice. The primary lymphoid organs thymus and bone marrow are modelled apart: the thymus (Castiglione et al., 2011) is implicitly represented by the positive and negative selection of immature thymocytes before they enter into the lymphatic system, while the bone marrow generates already mature B lymphocytes. Hence, only immunocompetent lymphocytes are modelled on the lattice.

This simulation tool should be considered as a collection of immunological models since it incorporates several working assumptions or theories, most of which are regarded as established immunological mechanisms including:

- The clonal selection theory of Burnet (Burnet, 1959);
- The clonal deletion theory (i.e., thymus education of T lymphocytes) (Lederberg, 1959);
- The hypermutation of antibodies (Brenner and Milstein, 1966);
- The replicative senescence of T-cells, or the Hayflick limit (i.e., a limit in the number of cell divisions) (Hayflick and Moorhead, 1961);
- Anergy:
    - T-cell anergy (Schwartz, 2003);
    - Ag-dose induced tolerance in B-cells (Nossal and Pike Berverley, 1980);
- The danger theory (Matzinger, 1994);
- The idiotypic network theory (Jerne, 1974).

As in most computer models, these can be toggled on or off. Moreover, other biological processes can be added with relatively small code changes. For example, other permutations of the basic model have been used to simulate different phenomena ranging from viral infection (e.g., Human Immunodeficiency Virus, Epstein-Barr Virus) to cancer immunoprevention and type I hypersensitivity. This characteristic makes the model suited to perform “what-if” analyses to elucidate relationships between different immune response phenomena and to aid in the validation or rejection of working hypotheses.

Each time step of the simulation corresponds to eight hours. The interactions among the cells determine their functional behaviour. Interactions are coded as probabilistic rules defining the transition of each cell entity from one state to another. Each interaction requires cell entities to be in a specific state choosing in a set of possible states (e.g., naïve, active, resting, duplicating) that is dependent on the cell type. Once this condition is fulfilled, the interaction probability is directly related to the effective level of binding between ligands and receptors.

Unlike the many immunological models, the present one not only simulates the cellular level of the inter-cellular interactions but also the intra-cellular processes of antigen uptake and presentation. Both the cytosolic and endocytic pathways are modelled. In the model, endogenous antigen is fragmented and combined with MHC class I molecules for presentation on the cell surface to CTLs' receptors, whereas the exogenous antigen is degraded into smaller parts (i.e., peptides), which are then bound to MHC class II molecules for presentation to the T helpers' receptors.

At variance with classical cellular automata models, there is no correlation among entities residing on different sites at a fixed time step, and the deterministic character of automata dynamics is replaced by a stochastic behaviour. However, at the end of each time step entities diffuse from site to site introducing spatial correlations.

While the influenza virus and antibodies are uniquely represented (i.e., they are considered as agents as are the cells) with lower molecular weight molecules, such as interleukins or chemokines, only their spatial concentration is represented. The corresponding dynamics is modelled by the following parabolic partial differential equation that describes a uniform diffusion process with the addition of a degradation term that takes into account the finite half-life of molecules: $\partial c/\partial t = D\nabla^2 c - \lambda c + s(x,t)$ where $c = c(x, t)$ is the concentration of chemokines, $s(x, t)$ is the source term (e.g., macrophages), D is the diffusion coefficient and λ is proportional to the half-life. We assume $D = 3000$ μm2/min and $\lambda = 3$ hrs (Francis and Palsson, 1997; Segovia-Juarez et al., 2004).

Differences in cell mobility are also taken into account. Th cells are the fastest with an average velocity of 11 μm/min, followed by B-cells with 6 μm/min and DCs with a velocity of 3 μm/min (Segovia-Juarez et al., 2004).

Interactions	Activations
B phagocytosis of antigen Macrophage phagocytosis of antigen DC phagocytosis of antigen B presentation to Th Macrophages presentation to Th DC presentation to Th Formation of immuno-complexes (IC) Macrophage phagocytosis of IC Infection of EP cells Cytotoxicity of infected cells by TC	Activation of Macrophages B-cells anergy Th cells anergy Priming of Th cells TC cells anergy Activation of TC cells Isotype switching from IgM to IgG
Antigen ingestion and presentation	**Other procedures**
B exogenous pathway Macrophage exogenous pathway DC exogenous pathway EP endogenous pathway	Clone divisions Haematopoiesis Plasma secretion of immunoglobulins Entity movement Hypermutation of antibody

Table 1 Biological rules coding for interactions between cells or among cells and molecules and other specific mechanisms of the immune system. Each of the entries of this list corresponds to an algorithm implementing a specific activity of the immune cells. Legend: B=B-cell, EP=Epithelial cells, DC=Dendritic cell, TC=cytotoxic CD8+ T-cell, Th=CD4+ T-cell.

The rules listed in Table 1 are executed for each time step. The stochastic execution of these rules, as in a Monte Carlo method, produces a logical causal/effect sequence of events culminating in the immune response and development of immunological memory. The starting point of this series of events is the injection of antigen (the priming) at time step t0. This may take place anytime after the simulation starts. In general the system is designed to maintain a steady state of the glob-al population of cells if no infection is applied (homeostasis). Initially the system is “naïve” in the sense that there are neither T and B memory cells nor plasma cells and antibodies. The various steps of the simulated immune response depends on what is actually injected, i.e., recombinant virus or bacteria. For example, in the specific case of protein vaccine, the various steps of the dynamic evolution are:

(1) t0: injection of antigens (the host has been infected);

(2) t1 = t0 + δ1: if the antigenic molecule contains B epitopes, then B-cells bind the antigen; also antigen presenting cells (i.e., macrophages and dendritic cells) non-specifically bind the antigen molecules;

(3) t2 = t1 + δ2: B-cells and APC process the antigen (MHCs bind antigen-peptides if any) => they expose the MHC/peptide groove on the surface;

(4) t3 = t2 + δ3: T-cells bind APC and/or B-cells which expose the MHC-complex; => both B and T-cells get stimulated;

(5) t4 = t3 + δ4: stimulated T-cells start to clone; part of the daughter cells be-come memory cells; stimulated B-cells divide into B memory and plasma cells;

(6) t5 = t4 + δ5: plasma cells secrete antibodies;

(7) t6 = t5 + δ6: antibodies bind the antigen to create immune complexes.

This sequence of events models the humoral response with production of specific antibodies. A similar sequence of events driven by infected cells presenting viral peptides together with class I HLAs on their surface triggers the cytotoxic activation of CD8+ T-cells, the components of the cellular arm of the immune sys-tem.

In the description above, the role of signalling cytokines such as interleukin-2 (IL-2), IL-12, IFN-γ, IL-4 and tumour necrosis factor alpha (TNF-α) were not included. These cytokines have an impact on macrophage activation, lymphocyte's division and participate to the isotype switch, etc. (Murphy et al., 2008).

4 Modelling adipose tissue inflammation for the agent-based customization

4.1 Immune system cells in obesity-induced AT inflammation

The adipose tissue is primarily composed of adipocytes (i.e., cells specialized in storing energy as fat), but it also includes to a lesser extent other types of cells relevant for the tissue homeostasis and function, such as pre-adipocytes, endothelial cells and immune cells. Besides its recognized role as a metabolic tissue, the AT is increasingly regarded as an important immune organ, which actively participates in the crosstalk between metabolism and immunity by modulation of the relative abundances of its subpopulations of immune cells and by secreting a wide variety of pro- and anti-inflammatory cytokines (Caspar-Bauguil et al., 2005; Makki et al., 2013; Cao, 2014). In the onset of obesity, AT undergoes major expansion and remodelling, with the critical involvement of changes in the balance of pro-inflammatory versus anti-inflammatory

cytokines and progressive infiltration of immune cells. This obesity-associated pattern of events taking place in the AT ultimately results in the establishment of a chronic state of low-grade inflammation, also referred to as metabolically triggered inflammation or “metaflammation” (Hotamisligil, 2006) (Figure 2).

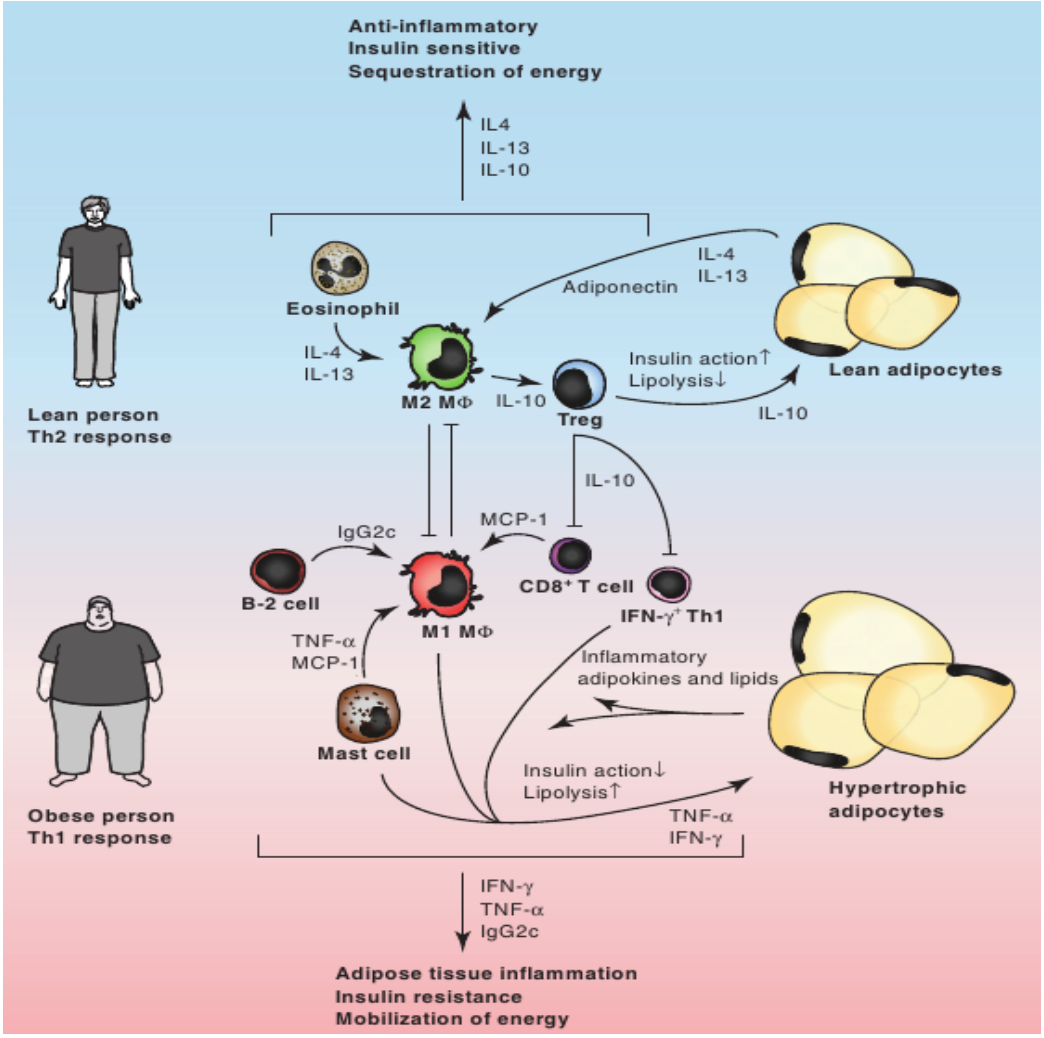

Figure 2 Obesity induces inflammation of the AT, which is associated with increased levels of pro-inflammatory cytokines and unbalanced proportion of pro-inflammatory versus anti-inflammatory immune system cells [figure credits: Schipper et al., 2012]

4.1.1 Macrophages

The role of adipose tissue macrophages in the context of obesity has been intensively studied since the publication of two seminal papers (Xu et al., 2003; Weisberg et al., 2003) reporting their accumulation in AT of obese individuals and mice. Excluding the adipocytes fraction, macrophages constitute the main component (40-60%) of the remaining tissue fraction and contribute the majority of cytokines in obesity (Weisberg et al., 2003). AT macrophages have been implicated as significant contributors in

obesity-induced inflammation and insulin resistance since both conditions ameliorate following AT macrophages depletion (Weisberg et al., 2006). The vast majority of macrophages in the AT of obese individuals surround necrotic adipocytes forming so-called 'crown-like' structures (Cinti et al., 2005). Although tissue macrophages are likely to constitute a more complex population *in vivo*, they are often categorized by the binary phenotypic distinction in M1 (pro-inflammatory) and M2 (anti-infiammatory) based on different combinations of mediators (pro-inflammatory or anti-inflammatory, respectively) able to induce their activation *in vitro* (Lumeng et al., 2007) (Figure 3). M1 or "classically activated" macrophages are induced *in vitro* by exposure to lipopolysaccharide (LPS) and interferon gamma (IFN-γ) and produce pro-inflammatory cytokines, such as tumour necrosis factor alpha (TNF-α), interleukin 6 (IL-6) and interleukin-12 (IL-12), and reactive oxygen species, including nitric oxide via activation of the inducible nitric oxide synthase. M2 or "alternatively activated" macrophages are instead induced *in vitro* by exposure to interleukin 4 (IL-4) and interleukin-13 (IL-13) and produce high levels of anti-inflammatory cytokines, such as interleukin-10 (IL-10), and express arginase, an enzyme which inhibits the nitric oxide synthase by competing for their common substrate L-arginine (Strapkova et al., 2011). In mice, several works support the prevalence of M1 (pro-inflammatory) macrophages in the obese AT (Lumeng et al., 2007; Nguyen et al., 2007; Fujisaka et al., 2009; Nishimura et al., 2009; Winer et al., 2009). However, human AT macrophages that accumulate with fat mass development appear to differ from the mouse model and rather exhibit a particular M2 remodelling phenotype, associated with a mixed expression of pro-inflammatory (TNFα, IL-6) and anti-inflammatory (IL-10, TGF-β) cytokines (Zeyda et al., 2007; Bourlier et al., 2008; Haase et al., 2014). Importantly, warnings have been issued concerning differences in the biology and function of human and mice macrophages (Schneemann and Schoeden, 2007) and the exact extent to which findings in mouse model can be relevant to human biology of obese AT inflammation deserves more thorough investigation.

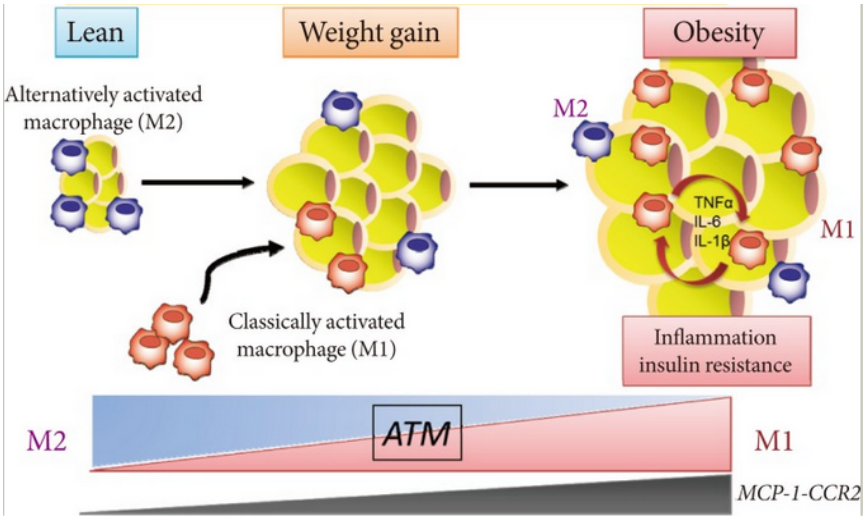

Figure 3 Phenotypic switch in AT macrophage polarization induced by obesity. [Figure credits: modified from Ota, 2013]

4.1.2 Dendritic Cells

Dendritic cells (DCs) are considered to be the preeminent antigen-presenting cells of the immune system and as such play a key role in the transition from innate immunity to adaptive immunity. Several lines of evidence suggest that DCs may be relevant regulators of obesity-induced inflammation and insulin resistance. Specifically, it has been shown that the number of DCs increases in the obese AT (Bertola et al., 2012; Stefanovic-Racic et al., 2012) and that depletion of CD11+ (i.e., the classical cell surface marker of DCs) cells improves obesity-induced insulin resistance and lowers the amounts of pro-inflammatory cytokines in AT and muscle (Patsouris et al., 2008).

4.1.3 Neutrophils

Neutrophils move with much ease and egress from the bone marrow as mature cells. These characteristics make them often the first cells to migrate at sites of infection and good markers of inflammation. Yet, the role of neutrophils in AT inflammation has been poorly investigated thus far. Few reports showed that plasma levels of neutrophils are increased in obese individuals (Nijhuis et al., 2009) and rise early in the AT of mice fed a high-fat diet (Elgazar-Carmon et al., 2008; Talukdar et al., 2012), even if the number of neutrophils remains much smaller (> 30 fold smaller) than that of AT macrophages (Lee and Lee, 2013). The observed increase may be promoted by leptin, whose levels increase in obesity and which have been reported to indirectly influence activation of neutrophils via induction of TNF-α secretion by monocytes (Zarkesh-Esfahani et al., 2004). Neutrophils can produce large amounts of cytokines and chemokines, including TNF-α, interleukin 1 beta (IL-1β), interleukin 8 (IL-8) and the macrophage inflammatory

protein-1 alpha (MIP-1α).

4.1.4 Mast cells

Mast cells in AT are increased in obesity both in humans and mouse models (Liu et al., 2009). Knockout mice studies suggest that mast cells mainly regulate metabolism and affect obesity-induced insulin resistance via production of interleukin 6 (IL-6) and interferon gamma (IFN-γ) rather than directly participating in regulating obesity-induced inflammation (Lee and Lee, 2013).

4.1.5 Regulatory T-cells

Under normal conditions, the percentage of regulatory T-cells (Tregs) in non-lymphoid sites is usually less than 15% of the CD4+ T lymphocytes fraction (Feuerer et al., 2009). However, in the visceral AT of normal adult male mice the fraction of resident Tregs has been found to be much higher (up to 70% of CD4+ T-cells) (Cipolletta et al., 2011; Feuerer et al., 2009). In lean mice, Tregs cells specifically accumulate after birth in visceral but not in subcutaneous adipose tissue and this dichotomy, mirroring specific association of visceral and not subcutaneous fat with insulin resistance, has been pointed out as potentially relevant (Feuerer et al., 2009). Tregs play a critical role in the appropriate control of immune responses to avert aberrant proliferation of pathogenic self-reactive T-cells and restrain excessive immune responses to infection or injury that could ultimately harm the host tissues (Josefowicz et al., 2012). Treg cells constitute a heterogeneous population whose different subtypes express distinct transcription factors and are specialized to respond to different inflammatory environments (e.g., Th1, Th2 or Th17 inflammatory response) (Chaudhry et al., 2009; Koch et al., 2009; Zheng et al., 2009). In mice, Tregs from visceral AT display phenotypical specialization, characterized by high expression of the PPRγ receptor, and restriction of the TCR repertoire that supports antigen-specific clonal expansion (Feuerer et al., 2009; Cipolletta et al., 2014). In mice, several studies have reported a decreased proportion of Tregs in the obese AT, most likely as a result of the unmatched expansion of other pro-inflammatory subpopulations of T lymphocytes (such as Th1 and CD8+ T-cells) (Feuerer et al., 2009; Nishimura et al., 2009; Winer et al., 2009). However, studies in human yielded different results and in particular reported an increase in the number of Tregs in obese AT (Zeyda et al., 2011).

4.1.6 CD8+ T-cells

The primary function of activated CD8+ T-cells is to kill their target cells (i.e.: cells expressing cognate antigen to their TCR receptor) by using perforin and granzyme and by releasing inflammatory cytokines. Obesity induces a large increase in the CD8+ T-lymphocyte subpopulation and in the expression of IFN-γ in the visceral AT of mice and human. (Rausch et al., 2008; Nishimura et al., 2009; Winer et al., 2009; Yang et al., 2010). Nishimura et al. also showed in mice that the rise of CD8+ T-cells is a primary event during obesity-induced AT inflammation that favours the subsequent infiltration by macrophages and can promote their differentiation towards a pro-inflammatory phenotype (Nishimura et al., 2009). CD8+ T-cells are only activated in response to T-cell receptor interaction with a specific antigen (becoming cytotoxic T-cells). Evidences of CD8+ T-cells expansion and T-cell receptor restriction in obese AT suggest that T-cells may be recognizing self-antigens within this tissue (Nishimura et al., 2009; Winer et al., 2009; Cipolletta et al., 2011). Antigen presentation to CD8+ T-cells on Major Histocomaptibility Complex (MHC) type I by professional APCs (such as DCs and macrophages) can occur via two processing routes: the direct and cross-presentation pathways (Basta and Alatery, 2007). The direct pathway relies on display of endogenous proteins on MHC I complexes and is chiefly involved in pinpointing infected cells to the cytotoxic action of activated CD8+ T-cells in inflammatory responses to pathogens. At variance, the cross-presentation pathway entails display of exogenous antigens acquired by APCs (especially DCs) from the extracellular environment and internally processed until loading on MHC I complexes (Albert et al., 1998; Rock and Shen, 2005). One source of exogenous antigens is provided from dying cells that release immunostimulatory 'danger' signals that promote the generation of immunity to their cellular antigens (Albert et al., 1998; Rock and Shen, 2005). Cross-presentation (also referred to as “cross-priming”) can be the relevant mechanism leading to CD8+ T-cells activation in the case of AT inflammation, which appears independent from pathogens and rather induced by stress signals released by hypertrophic adipocytes.

4.1.7 Helper T-cells

CD4+ helper T-cells (Th cells) aid in cell-mediated immunity by releasing different cytokines that can recruit immune cells and further activate cytotoxic T-cells. Th cells become activated by binding to an antigen presented by an APC on MHC II complexes, causing it to release cytokines. Th cells can be further subdivided into two main

subtypes: pro-inflammatory T-helper type 1 (Th1) and anti-inflammatory Th2 cells. The former (Th1 cells) secrete large amounts of IFN-γ and enhance macrophage pro-inflammatory functions by inducing the release of IL-1, IL-6 and TNF-a, while the latter (Th2 cells) secrete anti-inflammatory cytokines (such as IL-4 and IL-13) and favour anti-inflammatory M2 differentiation of macrophages and their secretion of IL-10 (Tiemessen et al., 2007).

In mice and humans obesity leads to a dramatic increase in Th1-polarized cells, whereas the Th2-polarized fraction is proportionally significantly reduced (Kintscher et al., 2008; Winer et al., 2009; Strissel et al., 2010). The number of Th1 cells is strikingly affected by diet-induced obesity and lymphocyte reconstitution studies in mice indicated that Th1 and Th2 cells exert an opposite effect on glucose metabolism and insulin resistance (worsening and ameliorating, respectively) and play an important role in AT inflammation (Winer et al., 2009).

Other subtypes of Th cells (such as pro-inflammatory Th17 and Th22 cells) have been less investigated mainly due to their much lower abundance and uneasy measurement but are gaining increasingly attention as evidence accumulate supporting their increase in obesity in human and mice and their potential role in metabolic dysfunctions (Fabbrini et al., 2013; Dalmas et al., 2014).

4.1.8 B lymphocytes

The role of B lymphocytes (B-cells) in obesity-induced AT inflammation and insulin resistance is less clear, despite the evidence that these cells in mice are recruited to AT shortly after the initiation of a high fat diet (Duffaut et al., 2009) and those individuals with T2D exhibit increased B-cell activation (Jagannathan et al., 2010). Knockout mice that lack B-cells show improved glucose tolerance and insulin sensitivity compared with wild type mice when challenged with high fat diet. Similarly, treatment with a B-cell-depleting antibody improves glucose metabolism and insulin sensitivity in obese mice, whereas transfer of immunoglobulins G (i.e.: soluble antibodies secreted by B-cells) from mice with diet-induced obesity induces impaired glucose metabolism and decreased insulin sensitivity (Winer et al., 2011).

Cytokine	Source cells	Target cells and effects	References
TNF-a	**M1 macrophages**, Adipocytes, Mast cells	It is a potent pyrogen. Stimulates IL-1 secretion. Inhibits signalling from the insulin receptor. Increases lipolysis and promotes increased serum FFA levels. Stimulates production of NO.	Hotamisligil et al., 1994; Zhang et al., 2002; Wu et al., 2007; Winer et al., 2009; Lee and Lee,

		Induces RANTES expression in adipocytes.	2013
IFN-g	Various T-cells (Th1), NK cells populations, Mast cells	Activate macrophages. Induces M1 polarization of macrophages. Stimulates production of NO.	Rauch et al., 2013
IL-1b	**M1 macrophages**, Adipocytes, Neutrophils	Simulates B-cell maturation and proliferation, thymocyte proliferation by inducing IL-2 release, and fibroblast growth factor activity. Stimulates production of NO.	Lee and Lee, 2013
IL-6	**M1 macrophages**, Adipocytes, Mast cells	Regulates final differentiation of B-cells into Ig-secreting cells. Involved in lymphocyte and monocyte differentiation.	Lee and Lee, 2013
IL-8	Neutrophils	Attracts neutrophils, basophils, and T-cells, but not monocytes. Promotes neutrophil activation.	Lee and Lee, 2013
IL-12	Dendritic cells	Induces differentiation of naïve T-cells into Th1 T-cells.	Lee and Lee, 2013
IL-15	Dendritic cells	Promotes proliferation and activation of CD8 T-cells and NK cells	Lee and Lee, 2013
MCP-1 (a.k.a. CCL2)	Adipocytes, Pre-adipocytes, Macrophages, Mast cells	Attracts monocytes and basophils.	Weisberg et al., 2006; Keophiphath et al., 2010; Lee and Lee, 2013
MIP-1a	**M1 macrophages**, Neutrophils, Dendritic cells	Attracts pro-inflammatory cells. Activates human granulocytes (neutrophils, eosinophils and basophils). Induces synthesis and release of pro-inflammatory cytokines (such as IL-1, IL-6 and TNF-α) from fibroblasts and macrophages.	Maurer et al., 2004
CCL5 (a.k.a. RANTES)	Adipocytes, **M1 Macrophages**, T-cells	Chemoattractant for blood monocytes, memory T helper cells and eosinophils. It causes the release of histamine from basophils and activates eosinophils.	Wu et al., 2007; Keophiphath et al., 2010
IL-2	helper T-cells	Plays a dual role in inflammation: Promotes the proliferation of helper T-cells; Regulates peripheral survival and expansion of Tregs.	Almeida et al. 2006
IL-4	**M2 Macrophages, Th2 cells**	Negative regulates Th1 and Th17 inflammation. Supports differentiation and maintenance of Th2 effectors . Suppresses TNF and IL-6 production .	Lazarski et al., 2013
IL-10	**M2 macrophages,** Th2 cells, Tregs, B-cells, dendritic cells	Inhibit T-helper (Th) 1 activation and Th1cytokine production. Inhibits macrophage and dendritic cell activation and maturation. Antagonizes the expression of MHC class II and co-stimulatory molecules as well as the pro-inflammatory cytokines IL-1β, IL-6, IL-8, TNF-α, and IL-12	Winer et al., 2009; Hedrich and Bream, 2010; Josefowicz et al., 2012
IL-13	**Th2 cells**, adipocytes	Induces M2 macrophage activation.	Kang et al., 2008

Table 2 Cytokines and chemokines participating in obesity-induced AT inflammation (red: pro-inflammatory; blue: dual role (pro- and anti-inflammatory); green: anti-inflammatory; bold: main sources).

Adipokine	Source cells	Target cells and effects	References

Leptin	Adipocytes	(As a neuroendocrine mediator: modulates body weight and food intake). Promotes lymphopoiesis and myelopoiesis. Promotes the secretion of acute-phase reactants (such as IL-1 and TNF-α). Promotes Th1-cell differentiation.	La Cava and Matarese, 2004; Claycombe et al., 2008
Adiponectin	Adipocytes	(As a neuroendocrine mediator: regulates energy homeostasis and glucose and lipid metabolism). Positively regulates insulin sensitivity. Promotes M2 macrophage polarization. Reduces reactive oxygen species levels.	Yamauchi et al., 2002; Ohashi et al., 2012

Table 3 Adipokines (i.e.: cytokines secreted by adipocytes) that exert effects on AT inflammation processes. Red: pro-inflammatory; green: anti-inflammatory.

4.1.9 Model of obesity-induced AT inflammation

We modelled AT inflammation upon obesity in light of two inspiring conceptual frameworks: the Danger Model (Matzinger, 1998) and the discontinuity theory of immunity (Pradeu et al., 2013). From the latter we borrowed the idea that the signals initiating an immune responses are endogenous and originate from stressed or injured cells (Figure 4); the latter suggested us that the dynamics of appearance of a triggering stimulus (e.g., endogenous stress signal or antigen) that could better fit with the onset of a state of low-grade inflammation could be that of a weak quantitative change over time, resulting in a weak but long-lasting immune response (Figure 5).

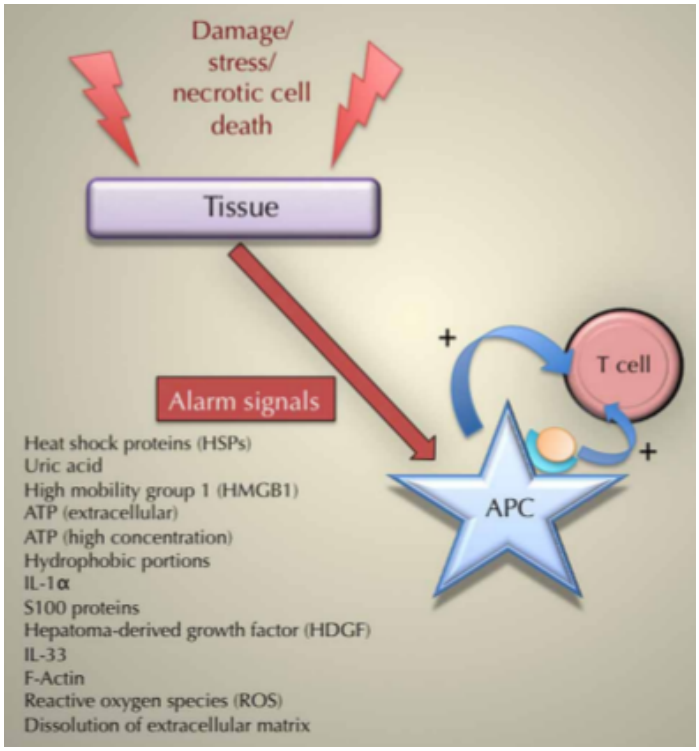

Figure 4 According to the Danger model (Matzinger, 1998), endogenous stress signals from injured tissues can initiate an immune response [Figure credits: Pradeu, 2012]

Obesity is characterized by an excessive increase in body mass due to expansion of AT via both hypertrophy and hyperplasia of adipocytes (Berry et al., 2014). Morbid expansion of the AT mass leads to local hypoxia (Trayhurn, 2013) and triggers necrosis of adipocytes (Cinti et al., 2005).

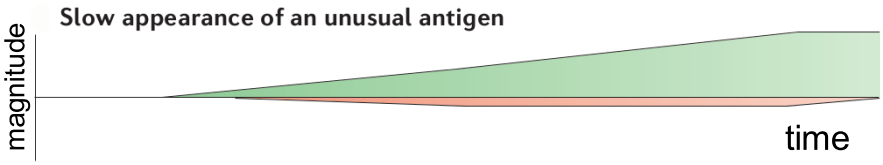

Figure 5 A chronic low-grade inflammation may be modelled as the slow and progressive appearance of an antigen (in green) that will induce a weak effector immune response (in pink) and eventually become tolerated over time. [Figure credits: Pradeu et al., 2013]

Necrotic adipocytes release stress signals - such as heath shock proteins (HSPs), uric acid, interleukin 33 (IL-33) (Giordano et al., 2013; Zeyda, 2013) - that are able to mature and activate DCs (Basu et al., 2000). At the same time, excess of nutrients and lipid storage is associated with increased secretion of leptin by the adipocytes.

Leptin is mainly produced by the AT proportionally to the body fat mass and plays the dual role of endocrine hormone - that controls food intake and systemic metabolism -

and pro-inflammatory cytokine that, among other effects, promotes: generation, maturation and survival of thymic T-cells; proliferation and interleukin-2 (IL-2) secretion by naïve T-cells; switch towards Th1 activation and anergy of Tregs (La Cava and Matarese, 2004; De Rosa et al., 2007; Matarese et al., 2010; Pucino et al., 2014).

Activated DCs migrate to local lymph nodes and activates T helper and cytotoxic T-cells by MHC-II as well as MHC-I (Albert et al., 1998; Mellman and Steinman, 2001; Rock and Shen, 2005) presentation of antigens collected in the damaged adipose tissue.

According to Nishimura and colleagues (Nishimura et al., 2009), infiltration of the AT by effector CD8+ T-cells is an early event in AT inflammation that precedes and promotes the well-documented recruitment of macrophages (Xu et al., 2003; Weisberg et al., 2003). The pro-inflammatory state of the AT promotes M1 (pro-inflammatory) differentiation of macrophages in mice (Lumeng et al., 2007), which in turn amplifies inflammation and production of pro-inflammatory cytokines (such as IL-1, INF-γ, TNF-α, IL-6, MCP-1). In human, a different paradigm emerges that suggests the accumulation in obese AT of a particular type of M2 macrophages exhibiting a mixed expression of pro-inflammatory (TNF-α, IL-6) and anti-inflammatory (IL-10, tumour growth factor, beta, TGF-β) cytokines (Zeyda et al., 2007; Bourlier et al., 2008; Haase et al., 2014).

Resolution of this acute phase of inflammation implies the intervention of anti-inflammatory cells triggered by the rise of anti-inflammatory cytokines. Good candidate anti-inflammatory cells are M2 macrophages and Tregs. This mandatory step between acute phase of AT inflammation and the establishment of a long lasting low-grade chronic inflammation, which characterizes obesity, has been left largely unexplored thus far. In human, where it has been reported that AT macrophages accumulating in obesity are able to secrete both pro- and anti-inflammatory cytokines, the prevalence of the latter at later stages of AT remodelling can be hypothesized. In mice, pro-inflammatory M1 macrophages may switch to anti-inflammatory M2 phenotype upon phagocytosis of apoptotic cells, with down-regulation of TNF-α release and up-regulation of TGF-β release. This hypothesis has been suggested from reports on inflammation dynamics observed in other tissues (Duffield, 2003. The work by Strissels and colleagues in mice fed with high-fat diet to induce obesity corroborates envision of a similar scenario in adipose tissue. The authors observed high rate of adipocyte cell death as an initial AT remodelling event coincident with the maximum expression of macrophage expressed pro-inflammatory cytokines and preceding a “repair” program marked by reestablishment of adipocyte number with differentiation of new small

adipocytes and increased expression of IL-10, possibly by alternatively activated macrophages (Strissels et al., 2007). In both mice and human, the local release of anti-inflammatory cytokines by M2 macrophages (such as IL-10 and TGF-β) may promote activation of Tregs. Further positive signals that may contribute to exit of Tregs from anergic state and activation to suppress immune response could be provided at advanced stages of AT remodelling from relaxation of stress signals due to clearance of necrotic cells and conceivably lower local concentration of leptin. While the latter condition would cause rescue of Tregs from anergy (De Rosa et al., 2007), the former may induce an array of different subpopulations of DCs lacking presentation of a second signal and predominantly driving activation of Tregs rather than effector T-cells (reviewed in Hubert et al., 2007).

Prolonged regimes of high-fat nutrition and scarcity of exercise fostering obesity may translate into cycles of alternative predominance of pro- and anti-inflammatory inputs especially in the visceral compartment of adipose tissue (crucial to inflammatory events of adipose tissue accompanying obesity), yielding as a net result the observed low-grade chronic state of inflammation. Cytokine and adipokine, cellular targets and actions have been recapitulated in Table 2 and Table 3, respectively.

The key events in AT inflammation modelling are listed in what follows.

1. Obese AT expands via hypertrophy and hyperplasia of adipocytes (Berry et al., 2014).
2. Hypoxia and necrosis of hypertrophic adipocytes cause release of stress signals - “Danger signals” - (e.g., HSPs, uric acid, IL-33) induce activation of DCs (presentation of the second signal). Excess nutrients and increased fat pad mass cause increased production of leptin.
3. Activated DCs induce activation and proliferation of specific Th1 and cytotoxic T-cells in local lymph nodes via antigen presentation on MHC-II as well as MHC-I (Albert et al., 1998; Mellman and Steinman, 2001; Rock and Shen, 2005). Leptin further promotes proliferation of Th1 and cytotoxic T-cells, while repressing anti-inflammatory Tregs via induction of anergy (La Cava and Matarese, 2004; De Rosa et al., 2007; Matarese et al., 2010).
4. Infiltration of the AT by effector CD8+ T-cells precedes and promotes (Nishimura et al., 2009) the well-documented recruitment of macrophages (Xu et al., 2003; Weisberg et al., 2003)

5. The pro-inflammatory state of the obese AT promotes M1 (pro-inflammatory) differentiation of macrophages (Lumeng et al., 2007), which results in amplified inflammation and production of pro-inflammatory cytokines (such as IL-1, INFgamma, TNFalfa, IL-6, MCP-1).
6. A wave of cell death of hypertrophic adipocytes takes place in visceral AT (caused by cytotoxic T-cells "armed" against antigens exposed by hypetrophic adipocytes?), followed by replenishment by newly differentiated, small adipocytes and expression of anti-infiammatory cytokines (Strissel et al., 2007). This pattern fits with other studies of models of inflammation reporting a phenotypic transition of macrophages from M1 (pro-inflammatory) to M2 (anti-inflammatory) activation following phagocytosis of apoptotic cells, accompanied by release of anti-inflammatory cytokines (reviewed in Duffield, 2003).
7. Anti-inflammatory cytokines produced by M2 macrophages together with relaxation of stress signals due to clearance of necrotic cells and conceivably lower local concentration of leptin may promote activation Tregs directly (Cipolletta, 2014; De Rosa et al., 2007) or via activation by specialized subpopulations of DCs (reviewed in Hubert, 2007).
8. Activation of Tregs will lead to resolution of acute phase inflammation in the AT via cell-cell interactions and/or cytokine mediated suppression (reviewed in Pandiyan et al., 2011).
9. Cycles of alternative predominance of pro- and anti-inflammatory inputs especially in the visceral compartment of adipose tissue (crucial to AT inflammatory events accompanying obesity), yielding as a net result the observed low-grade chronic state of inflammation.

5 Customization of immune simulator

In order to implement the above mentioned processes for the emergence of inflammation leading to a deregulation of insulin in the MISSION-T2D integrated model, a number of extensions have been performed to the ABM model of the immune system described in section 3. These are briefly listed below (more details will be given in the WP6's forthcoming deliverable).

- New entity/variables have been added (e.g., IL-6, IL-10, leptin, adipocyte, Treg, Th17, VAT-epitope)
- Implementation of the maturation and differentiation of macrophages into the M1 and M2 phenotypes.
- Implementation of the Boolean network model to differentiate CD4 T-cells into the Th1/2/17/reg subtypes.
- Specified the adipocytes growing and enlargement (cell hypertrophy or AT hyperplasia) dynamics.
- Implemented a number of rule-based interactions among entities.
- Implemented rule-based procedures for cytokines release.
- Etcetera.

5.1.1 Defining prerequisites for the integrated model

Task 6.2 is devoted to the definition of the prerequisites for the integrated model *(**CNR**, UniBO, UniCAM, UniRM, TNO, USFD):* WP6 will collaborate with other WPs to define the interfaces among the agent-based and the other sub- models. Also, the actual strategy for model integration is taken into account in this task. Moreover a data exchange format for linking the various models developed in WP2-WP5 to the integrated modeling platform will be identified and used.

This task has been carried out in cooperation with other WPs to define the interfaces among the agent-based (M1, WP6) and the other sub-models (M2 to M4), and to outline the actual strategy for whole-model integration. A preliminary data exchange format and strategy for linking the various models (WP2 to WP5) to the integrated modelling platform (WP6) has been identified as follows.

The general integration strategy relies on the exchange of the identified, critical parameters from one model with the other, such as the outputs of a model are used as inputs for the following one in a hierarchical way of operation.

Partner UNIBO (WP2) is developing a simplified gut dynamics model (M2) able to relate patient's nutritional patterns (mainly described by quantity of ingested lipids from food) and patient's enterotype as inputs to yield levels of short chain fatty acids (SCFAs) such as butyrate and pyruvate, major metabolites in colonic lumen that regulate (colonic) inflammation via the inhibition of the IFNγ/STAT1 signalling

pathways. Specific mechanistic associations will relate the presence (and quantity) of such SCFAs outputting from M2 with the production of given cytokines (such as TNF-α, IL-2, IL-6 and IL-10) by lymphocytes and other cell types in model M1.

Partner UniCAM (WP3) is developing a model for mTOR signaling in immune cells, pivotal in eliciting an inflammatory process, that has strong influence on the efficiency of pancreatic beta-cells for the production of insulin. This model (M3) will be embedded in the agent-based model M1 to drive the inflammation process from metabolic deregulation. Relevant parameters such as cytokines (e.g., TNF-α) and, possibly, genes involved in the major intra- and intercellular processes described in model M1 will be identified as the link between the two models. Binary states ("on/off") or continuous quantitative parameters, when available and relevant, related to the involved players will be taken into account.

Partner TNO (WP4) is adapting, extending and further developing a model (M4) for metabolism (described in deliverable D4.1). Dynamic mass balances and major cellular metabolic reactions describe seven tissue compartments. A number of parameters (identified in **Table 4**) are being used as inputs for the agent-based model M1 to get an integrated description of inflammation. The metabolic model M4 will be also linked to the model M5 developed by partner USFD/UniRM (WP5) regarding the influence of physical activity on specific metabolites. Feedback loops M1→M4 are foreseen.

Partners USFD and UniRM (WP5) have identified a model (M5) to introduce the physical activity (PA) in the project's global architecture. Preliminary work in this respect has been described in D5.1. This model impinges upon few variables of the metabolic model (M4). In particular the parameter "work rate" which stands for a measure of the intensity of the physical activity, influences the dynamics of the metabolic model M4. This parameter in M5 is related to the heart rate, either measured by means of suitable equipment, or calculated on the basis of patters of physical activity as declared by the user.

The integration of the models (M1+M2+M3+M4+M5) to be performed in WP6 by partner CNR will result in a model that describes the inflammation in obese individuals (but also in healthy individuals) developing over time as a function of nutritional habits, lifestyle habits, and other personal parameters like age, gender, body mass index, etc.

Model	Input / Output (→ M1 input)
M2 (gut)	Nutritional pattern Enterotype SCFAs IFNγ/STAT1 signaling, cytokine production
M3 (inflammatory pathways)	Cytokines Cytokines
M4 (metabolic)	Energy intake and expenditure Major metabolites
M5 (PA)	Physical Activity Energy expenditure Specific metabolites

Table 4 General scheme of major input (in red)/output (in green) parameters linking the various models. Model M1 will input/output from/to all other models.

6 Deliverable conclusions

6.1 Open questions

- How is the transition from open AT inflammation to obesity-associated chronic low-grade inflammatory state established? While actors and dynamics of acute phase inflammation in obese AT have been extensively investigated in mice and humans, very few studies addressed events leading to resolution of the acute phase, which is instead needed by the model in order to achive establishment of long lasting state of low-grade inflammation. This is reflected in the speculative attempt to reconcile sparse knowledge into a coherent model of AT inflammation partial resolution and establishment of persistent low grade inflammation (cfr. points 7-9 in section 4.1.9).

- What is the role of Tregs in AT inflammation and what are the sources and essence of signals that promote their activation? In fact, evidences concerning the dynamics of abundance of T regulatory cells in obese AT are controversial. Specifically, the loss of protective Tregs in obesity-induced inflammation supported specifically in mice by several studies (Winer et al., 2009; Feuerer et al., 2009; Deiuliis et al., 2011) has been challenged by different observations in

a recent study in humans, where on the contrary an increase in obese AT Tregs has been reported (Zeyda et al., 2011).

- What is the role of leptin in visceral AT inflammation? Aside from its well-known role as a metabolic hormone, leptin has been reported to act as an early acute phase pro-inflammatory reactant in several systems (La Cava and Matarese, 2004). The AT is the primary source of leptin that produces it proportionally to the body fat mass. Thus, leptin could represent an elective sensor of changes in AT mass and early mediator of AT inflammation in cases of morbid hypertrophy. In particular, local leptin concentration in the expanding AT may conceivably increase to a high extend and promote inflammation, while following AT remodelling it will likely decrease and result in the withdrawal of this massive inflammatory stimulus. Support (or confutation) for this model would need time-dependent and fat-mass-dependent curves of leptin abundance in the visceral AT as well as focused functional studies especially with regards of the interplay between leptin and the crucial anti-inflammatory Tregs, which are induced to anergy by leptin.

6.2 Perspective: integration with models from other WPs

Partner CNR (WP6) is developing a model (M1) of the immune activation and inflammation (the present deliverable).

Partner TNO (WP4) is adapting/developing a model (M2) for metabolism (deliverable D4.1). This model is an extension of the model of Kim and coworkers (Kim et al., 2007). It includes seven tissue compartments: brain, heart, liver, GI (gastrointestinal) tract, skeletal muscle, adipose tissue, and other (generic) tissues. Dynamic mass balances and major cellular metabolic reactions describe each tissue compartment. The glucagon-insulin controller is incorporated into the whole body model to predict hormonal changes during exercise. This metabolic model will eventually be integrated with the model of inflammation developed by WP6. Moreover, the metabolic model will be linked to the model developed by partner USFD/UniRM (WP5) regarding the influence of physical activity on specific metabolites.

Partner UniCAM (WP3) has developing a model for mTOR signalling inside immune cells pivotal in eliciting an inflammatory process. The latter having strong influence on the efficiency of pancreatic beta-cells for the production of insulin. This model, called

M3, will be embedded in the agent-based model M1 to drive the inflammation process from metabolic dysregulation.

Partners USFD and UniRM (WP5) have identified a model (M4) to introduce the physical activity in the project's global architecture. Preliminary work in this respect has been described in D5.1. This model impinges upon few variables of the metabolic model (M2). The integration of the models (M1+M2+M3+M4) performed in WP6 by partner CNR will result in a model that describes the inflammation in obese individuals (but also in healthy individuals) developing over time as a function of nutritional habits, lifestyle habits, and other personal parameters like age, gender, body mass index, etc.

The final simulation tool will be eventually used to create a lookup table relating user inputs to personalised outputs in terms of health/lifestyle recommendations. This relationship will be packed in a suitable data structure and embedded in the app developed by MED (WP8).

The mobile app developed in project year 2 by partner MED in WP8 will allow the user to input data and receive the output recommendation. The app will provide access to data measured through other apps (or on standalone devices), using available online platforms (including, but not necessarily limited to partner MED's own online platform). Initial discussion of this topic is provided in Deliverable 8.2.

7 Appendix: Partial list of abbreviations used

AT	Adipose Tissue
APC	Antigen-Presenting Cell (immunocompetent cell type)
CD	Cluster of Differentiation (surface markers of lymphocytes)
CTL	Cytotoxic T lymphocyte (immunocompetent cell type)
DC	Dendritic Cell (immunocompetent cell type)
HSP	Heath shock protein (signalling molecule)
IL	Interleukin (signalling molecule)
IFN-γ	Interferon gamma (signalling molecule)
LPS	Lipopolysaccharide (component of pathogen cell walls, signal of danger)
MCP-1	Monocyte Chemotactic Protein-1 (signalling molecule)
MHC	Major Histocompatibility Complex (surface protein of immunocompetent cell types)
MIP-1α	Macrophage Inflammatory Protein-1 alpha (signalling molecule)
T2D	Type 2 Diabetes

TNF-α	Tumor Necrosis Factor alpha (signalling molecule)

Table 5 List of abbreviation.

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