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Tartrate resistant acid phosphatase in the immune and nervous system

Distribution and pathophysiological implications

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ABSTRACT

Tartrate resistant acid phosphatase (TRAP) belongs to the family of purple acid phosphatases (PAP). It is a glycoprotein synthesized as a monomer with low enzyme activity containing a redox active diiron centre in the active site. Post-translational proteolytic processing of this monomer into a dimeric protein increases the enzyme activity. Traditionally, TRAP has been used as a marker for bone resorbing cells but the biological function of TRAP is still not fully elucidated. However, some studies suggested that also cells outside the skeleton express TRAP and consequently the distribution, characterization and possible functions of TRAP outside the skeleton were addressed in this thesis.

Our results show that resident and inflammatory macrophages, certain neurons and epithelial cells express TRAP. Biochemical characterization of extra skeletal TRAPs showed that they contain a redox active di-iron centre, and that proteolytic processing seems to be important for the reduction of this centre. Monomeric TRAP tended to be more highly expressed in epithelial cells compared to cells of the myeloid lineage, but, subpopulations of macrophages i.e. alveolar macrophages and adipose tissue macrophages isolated from hyperplastic obesity also seems to express high amounts of monomeric TRAP. With respect to possible physiological functions, monomeric TRAP was shown stimulate proliferation and differentiation of adipocytes. High expression of monomeric TRAP in human obese adipose tissue macrophages suggests a role of macrophage-derived monomeric TRAP in the development of hyperplastic obesity associated with normal insulin sensitivity and normal lipid- and carbohydrate-metabolism in adipocytes. In macrophages, Th1 cytokines and lipopolysaccaride (LPS) up regulated TRAP expression. Induction of TRAP expression in the early stages of experimental DSS-induced colitis in rats suggests that TRAP could be utilized as a cellular marker of Th1-dependent macrophage activation in inflammatory bowel diseases.

In summary, macrophages, certain epithelial cells and neurons express TRAP. Taking into account organ size, the largest contributors of TRAP are bone, spleen and liver. The differential expression of monomeric and proteolytically processed TRAP in a macrophage cell population could influence the biological effects of TRAP to act either as a growth factor or as a modulator of innate immune responses in certain inflammatory conditions.

LIST OF PUBLICATIONS

This thesis is based on the following publications

1. Pernilla Lång, Marianne Schultzberg, Göran Andersson.
   Expression and distribution of tartrate-resistant purple acid phosphatase in the rat nervous system.
   Journal of Histochemistry and Cytochemistry 49(3):379-96, 2001

2. Pernilla Lång, Göran Andersson.
   Differential expression of monomeric and proteolytically processed forms of tartrate-resistant acid phosphatase in rat tissues.
   Cellular and Molecular Life Science 62(7-8):905-18, 2005

3. Pernilla Lång, Stefan Lange, Dick Delbro and Göran Andersson
   Th1 response in dextran sulphate sodium-induced colitis in rats is associated with increased expression of tartrate-resistant acid phosphatase
   Submitted Manuscript

4. Pernilla Lång, Vanessa van Harmelen, Mikael Rydén, Maria Kaaman, Paolo Parini, Claes Carneheim, A Ian Cassady, David A Hume, Göran Andersson, Peter Arner.
   Monomeric Tartrate-Resistant Acid Phosphatase Induces Insulin Sensitive Obesity
   Submitted Manuscript

ABBREVIATIONS

Acp5	acid phosphatase 5
Cat K	cathepsin K
CARD15	caspase recruitment domain family member 15
CD	Crohns disease
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
cTRAP	proteolytically processed tartrate resistant acid phosphatase
CHO	Chinese hamster ovary
Da	Dalton
DC	dendritic cell
DSS	dextran sulphate sodium
DTT	dithiothreitol
EC	enzyme commission
Eta-1	early T cell antigen 1 also known as osteopontin
FSD	functional secretory domain
HCL	hairy cell leukemia
Hsp	heat shock protein
IBD	inflammatory bowel diseases
IL	interleukin
IRF-E	interferon regulatory factor-element
LPS	lipopolysaccaride
Mϕ	macrophage
MHC	major histocompatability complex
MiTF	microthalmia transcription factor
MMP	matrix metallo-proteinases
mRNA	messenger ribonucleic acid
mTRAP	monomeric tartrate resistant acid phosphatase
NF-kB	nuclear factor – κβ
NK	natural killer
NOD	nucleotide oligmerization domain
OPN	osteopontin
PAP	purple acid phosphatase
PAP/TRAP	purple/tartrate resistant acid phosphatase
PCR	polymerase chain reaction
pOPN	phosphorylated osteopontin
PP1	protein phosphatase 1
PP2A	protein phosphatase 2A
PP2B	protein phosphatase 2B
RANKL	receptor activator for nuclear factor κ B ligand

ROS	reactive oxygen species
SCID	severe combined immunodeficiency disease
Sf	Spodoptera furgiperda
TFE	transcription factor E
TLR	toll like receptor
TCR	T cell receptor
TGF	transforming growth factor
TNF	tumor necrosis factor
TRAP	tartrate resistant acid phosphatase
TRAP+	tartrate resistant acid phosphatase over expressing mouse
Uf	uteroferrin
USF	upstream stimulatory factor
UC	ulcerative colitis
uOPN	unphosphorylated osteopontin
WT	wild type

INTRODUCTION

"No one can really feel at home in the modern world and judge the nature of its problems – and the possible solutions to those problems – unless one has some intelligent notion of what science is up to."
Isaac Asimov, New Guide to Science

Tartrate resistant acid phosphatase

The enzyme tartrate resistant acid phosphatase (TRAP; EC 3.1.3.2) belongs to the family of purple acid phosphatases (PAP)[2, 3], a subclass of acid phosphatases[4]. Mammalian TRAP is referred to by multiple names i.e. purple acid phosphatase (PAP), tartrate resistant acid phosphatase (TRAP), purple/tartrate resistant acid phosphatase (PAP/TRAP), type 5 acid phosphatase (Acp5) or uteroferrin (Uf).

TRAP is a glycoprotein synthesized as a monomer, i.e. a polypeptide of 35-37 kDa, with low enzyme activity[5, 6] and contains a redox-active diiron centre in the active site[7]. During post-translational proteolytic processing, this monomer can be converted into a dimeric protein with high enzyme activity [6, 8-10]. Traditionally, TRAP has been used as a marker for bone resorbing cells, osteoclasts[11-14] and as a disease marker for hairy cell leukemia (HCL)[15].

The biological functions of TRAP are still not fully elucidated although several functions have been proposed. Among these are regulation of immune responses[16], adhesion and migration of cells by dephosphorylation of osteopontin (OPN)[17-19], function as a growth factor [20-22], degradation of phagocytosed material by formation of reactive oxygen species (ROS)[23] and transport of iron[24-26]. Recently, it has also been suggested that TRAP might influence the intracellular transport of certain vesicles in osteoclasts[27].

The TRAP gene and mRNA transcripts

TRAP corresponds to a single gene mapped to chromosome 19p13.2 – 13.3[28, 29] in humans and to the corresponding chromosome 9 in mouse[30]. Mammalian TRAP contains five exons and four introns[31, 32] (Figure 1) and the exon – intron organisation between the species is well conserved [31] as well as the sequence homology between TRAP mRNA transcripts from humans, mice and rats.

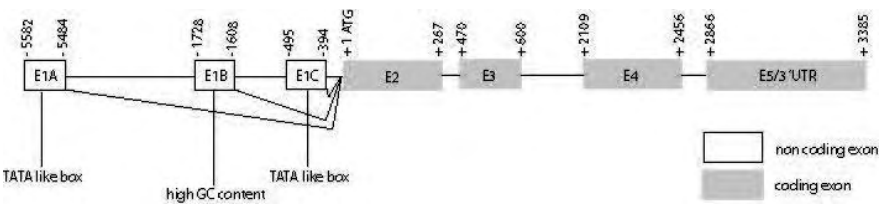

Figure 1. Structure of the mouse TRAP gene. The TRAP gene consists of five exons and four introns. Exon 1 consists of three alternative exon 1.

Promoter regions

Several investigations has been performed on the 5' region of the mouse TRAP gene to elucidate promoter regions, transcription start sites and transcription binding factors sites[33, 34]. Reddy and colleagues found evidence of two different promoter regions P1 and P2[33]. Transcripts originating from P1 were abundant in all tissues tested whereas P2 transcripts were expressed in bone, spleen and thymus.

Recently, Walsh and colleagues demonstrated the presence of three alternative exon 1 mRNA transcripts 1A, 1B and 1C[34] (Figure 1). 1C transcripts are likely to correspond to transcripts originating from the P1promotor region. 1C transcripts are thought to originate from osteoclasts and macrophages, while 1B transcripts are thought to be expressed mainly in cells of non-hematopoetic origin. The promoters of 1B and C both contain a non-classical TATA box while the 1A promoter is characterised by high GC content.

Transcriptional regulation of TRAP expression

The 1C promoter has several binding sites for transcriptional factors both upstream and downstream of the non-classical TATA box: USF1/2, SP1, PU.1 (myeloid specific factor), M-box (binding site for the microthalmia transcription factor; MiTF) and IRF-E. It has been established by several groups that MiTF has an impact on the transcription from exon 1C[35-39]. In addition to this, the osteoclast differentiating protein RANKL has been shown to activate both MiTF and USF1/2 dependent 1C transcription[37, 40]. The 1B promoter has two SP1 sites up-stream and the 1A promoter has one SP1 and one c-rel sites upstream of these promoters [34]. Additionally, is has been shown that iron can induce TRAP expression[33, 41, 42].

Post-translational modifications of TRAP

Proteolytic processing

Judging by the cDNA sequence, TRAP is synthesized as a monomer, also known as isoform 5a, of 35-37 kDa[28, 43, 44]. However, with the exception of uteroferrin[45, 46], mammalian TRAPs are usually isolated from cells and organs as a dimer of 21-24 + 16-18 kDa, known as isoform 5b, held together by a disulphide bond[8, 45, 47-50]. This led to the hypothesis that TRAP might be subject to proteolytic processing. It has since then been shown that trypsin[8, 9, 51], chymotrypsin[8], papain[9] and members of the cystein protease family i.e. cathepsins[6, 9, 10, 52] are able to proteolytically digest TRAP in an exposed loop region (Ser145 – Val161[9, 53] in rat TRAP), thereby generating a dimeric protein (Figure 2). However, enzyme activity and

substrate affinity of TRAP achieved after proteolytic processing of the loop region differs depending on the proteases involved. The highest enzyme activity and substrate affinity is obtained following cleavage with cathepsins L and K[6]. This is likely due to differential processing of the peptide sequence in the loop region by the different proteases[6]. Sequence comparison of the N- and C- terminal fragments generated after proteolytic processing of monomeric recombinant TRAP in vitro with isolated native dimeric bone TRAP revealed that it is likely that cathepsin K, and possibly cathepsin L, is involved in the proteolytic processing of TRAP in bone in vivo[6]. A role for cathepsin K in the proteolytic processing of TRAP in osteoclasts in vivo was recently corroborated by analysis of mice genetically depleted of cathepsin K[54].

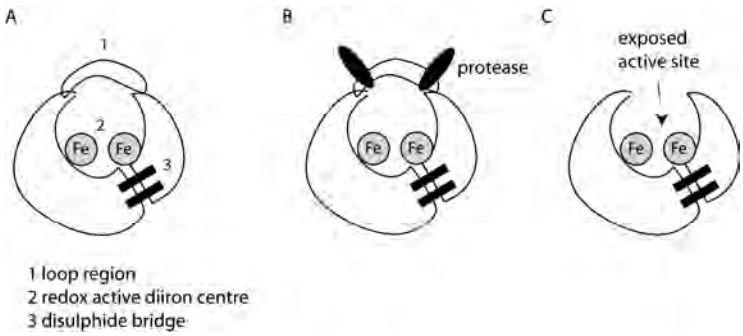

Figure 2. Proteolytic processing of TRAP. (A) TRAP is synthesized as a monomer. (B) Proteases, for example cathepsin K, process in the loop domain exposing the active site and relieving constrains on the diiron centre (C).

In osteoclasts, which express high levels of both cathepsin K and TRAP, monomeric TRAP, secreted through the ruffled border area, has been suggested to be proteolytically processed by cathepsin K in the resorption lacuna[6, 54] (Figure 3).

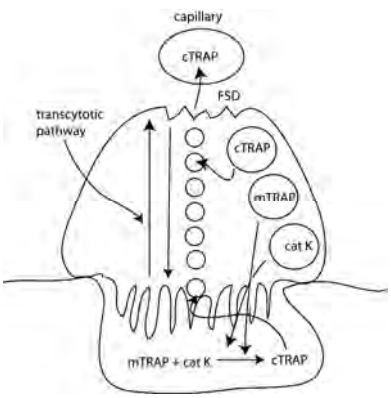

Figure 3. Hypothetical proteolytical processing of TRAP in osteoclasts. Monomeric TRAP (mTRAP) is secreted into the ruffled border together with cathepsin K (cat K) and there mTRAP is proteolytically processed to cTRAP. Part of the cTRAP is then endocytosed into the transcytotic pathway. There is also a pool of intracellular cTRAP that is partly processed in a cathepsin K independent manner and transferred to the transcytotic pathway. cTRAP is then secreted from the transcytotic pathway at the basolateral functional secretory domain (FSD) into the bloodstream.

Still, the intracellular pool of proteolytically processed TRAP is only partly dependent on cathepsin K processing[54]. This indicates that there are multiple proteases involved in the proteolytic processing of TRAP in different compartments of the cell. This hypothesis is strengthened by
results from TRAP over expressing Chinese hamster ovary (CHO) cells, in which proteolytic processing of TRAP also is largely independent of cathepsin K[55].

Regulation of the active site

The active site of TRAP consists of seven conserved amino acids together coordinating two iron atoms (in mammals) in the centre of the active site[7 2, 56, 57]. The characteristic purple colour of the enzyme originates from a charge transfer from the tyrosine ligand to the redox-inactive Fe(III) atom[7, 58, 59]. This structure of the active site shows striking similarity to the active sites of calcineurin (PP2B) [60], protein phosphatase 1 (PP1)[61] and PP2A[2].

Due to the redox-active diiron center[2, 62], mammalian TRAP can exist in two forms (Figure 4); one containing an oxidized diferric Fe(III)-Fe(III) center which is catalytically inactive or a reduced mixed-valent Fe(III)-Fe(II) center which is the catalytically active form[58, 59]. TRAP is usually isolated with an oxidized inactive FeIII-FeIII diiron center[58, 59] but addition of mild reducing agents such as β-mercaptoethanol[45, 50, 63], DTT[64-66], ascorbic acid[66], cystein[66], or divalent iron[48, 50] reduces the diiron centre to the enzymatically active Fe(III)-Fe(II) centre (Figure 4). This reduced enzymatically active Fe(III)-Fe(II) centre can then be oxidized back to the enzymatically inactive Fe(III)-Fe(III) centre by treatment with H_2O_2[67]. However, the reducers mentioned above are not likely to have a role in the reduction of TRAP in vivo. Thus, it remains to be elucidated if, and by which reductants the active centre in TRAP is reduced in vivo.

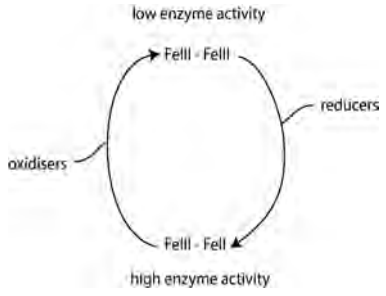

Figure 4. Reduction and oxidation of the redox active diiron center in the active site. The redox active diiron active center in TRAP can be reduced to a Fe(III)-Fe(II) centre with high enzyme activity and oxidized back to a Fe(III)-Fe(III) centre with low enzyme activity.

TRAP is also sensitive to inhibition by tetrahedral oxyanions i.e. molybdate, tungstate, arsenate and phosphate[48, 68-70]. The mechanism behind the inhibition with the small oxyanions i.e.

phosphate and arsenate seems to be competitive or a combination of competitive and non-competitive while the larger oxyanions i.e. tungstate and molybdate act in a non-competitive fashion[71].

Glycosylation

TRAP contains two putative N-glycosylation sites at Asn97 and Asn128 which are conserved in all mammalian species[72]. In the crystal structures of recombinant TRAP produced by Sf9 insect cells a carbohydrate chain was detected at Asn97, but not at Asn128[53, 73, 74]. However, it has later been shown that recombinant TRAP produced by Sf9 insect cells does contain N-linked carbohydrate chains on both Asn97 and Asn128[75], indicating that the carbohydrate at position Asn128 was present but not detected in the crystallized proteins.

The carbohydrate chains seem to have multiple functions in TRAP[75] such as increasing the stability of the protein by protecting it from proteolytic degradation and modulating enzyme activity by interacting with the loop region. Interestingly, recombinant TRAP produced in E. coli, lacking N-glycosylation, display altered conformation of the loop domain associated with low enzyme activity[76].

Cell, tissue and organ expression of TRAP

Levels of TRAP mRNA and enzyme activity

Most studies find the highest expression of TRAP mRNA or levels of enzyme activity in purified osteoclasts or bone tissue[33, 34, 72, 77, 78]. Several studies detect high levels of TRAP in spleen and liver[33, 34, 77-79], however, others do not[72, 80]. There also seems to be species differences, for example TRAP is detected in high levels in human lung[79], while expression in mouse and rat lung is lower[34, 72, 77, 78]. Generally, low levels of TRAP activity are detected in skeletal muscle, heart and brain[33, 34, 77-79], while expression in testis, kidney and small intestine varies between studies[33, 34, 72, 77-80].

Cell types expressing TRAP

During foetal development of mice, TRAP has been detected at the cellular level at several sites in the skeleton and teeth, however, expression of the enzyme was also demonstrated along epithelia of the skin, oral cavity, the gastrointestinal tract and in the thymus[81].

In adult rodents, except for expression in osteoclasts[11, 14, 82, 83], TRAP has been detected in other cell types of the myeloid lineage i.e. in different types of macrophages[13, 80, 84-87]

and in dendritic cells[79, 88]. Additionally, also certain epithelial cells have been shown to express TRAP i.e. hepatocytes[78], keratinocytes[78] and human transitional epithelium of the ureter[89]. However, there is still a debate whether or not TRAP is expressed in epithelial cells[87]. Lastly, TRAP has been detected in the parenchymal mesangial cells of the kidney[80] and acinar glandular epithelium of pancreas[80] as well as in osteoblasts[90], osteocytes[90, 91], human placenta[92] and in nerve cells of the olfactory bulb[93].

Intracellular localization

In osteoclasts, TRAP exists in intracellular vesicles[94, 95], some of them containing collagen fragments[96], and secreted to the ruffled border area of the resorption lacuna[6, 97]. In macrophages, TRAP has been partly localized to late endosomal/early lysosomal Rab7 positive vesicles and vesicles containing MHC class II and phagocytosed infectious material[78, 98]. In osteoblasts, TRAP has been located in the Golgi complex, lysosomes and secretory vesicles[99].

Proposed functions of TRAP

Phosphatase activity

TRAP can act as a phosphatase on both phosphotyrosine- and phosphoserine-containing proteins and therefore exhibits a broad substrate specificity[9, 100]. One potential physiological substrate might be the phosphoprotein osteopontin (OPN), also known as early T cell antigen -1 (Eta-1)[16, 17, 101]. OPN is highly dependent on the degree of phosphorylation for some of its bioactivities, and therefore hypothetically TRAP could regulate the bioactivity of OPN by dephosphorylation[16-18].

Acting as a growth factor

The pig TRAP homologue uteroferrin has been shown to expand the hematopoeitic progenitor cell pool size in pigs[20-22, 102]. In addition, it has also been suggested that TRAP induces differentiation of osteoblasts[103].

Producer of reactive oxygen species

For a protein to promote production of reactive oxygen species (ROS) through the Fenton reaction it has to match the following criteria; (1) one of the irons has to be redox-active, (2) both the Fe(III) and Fe(II) atoms must exhibit stable binding under physiological conditions and (3) one of the irons must be accessible to reducing agents[104]. It was early shown that both

uteroferrin and TRAP had the ability to produce ROS according to the Fenton chemistry[104, 105]. Since then TRAP has been shown to increase production of ROS in vitro[51, 96, 106] and TRAP over-expressing macrophages exhibit increased formation of ROS[96, 98, 107]. This ability of TRAP to form ROS has then been hypothesized to increase the capacity to degrade intracellular material[96, 107].

Involvement of TRAP in intracellular vesicular transport

Recently, TRAP has been associated with altered vesicular transport in osteoclasts. In TRAP-deficient mice, accumulation of medium-sized vesicles in the cytoplasm was observed[27]. These vesicles were hypothesized not to originate from the secretory pathway since the secretion of cathepsin K was normal, but might instead originate from the transcytotic pathway. This effect on vesicular transport might be cell-specific since no differences with respect to vesicles was noted in macrophages, that lack transcytotic pathways, derived from TRAP deficient mice[108].

Iron transport

TRAP has been implied in the transport of iron from mother to fetus in pregnant pigs[24-26, 109]. Additionally, is has been shown that iron can induce TRAP expression[33, 41, 42].

TRAP in the immune system

Generally, TRAP is expressed in macrophages and dendritic cells[78, 79] where the expression of TRAP in macrophages seems to be correlated to the activation state of the macrophage[110]. It is possible that TRAP functions as a growth factor for the hematopoeitic stem cell pool through an unknown action[20-22, 102] (Figure 5A). In support, it has been shown that mice deficient in TRAP exhibit a decreased number of elicited monocytes/macrophages in peritoneal exudates compared to WT mice[108]. It has also been demonstrated that TRAP influences the maturation of DC since TRAP deficient mice display more immature dendritic cells (DC)[111].

Contradictory results have been obtained in respect to cytokine responses in TRAP deficient mice. Their DC seem to induce a less pronounced Th1 response[111] while macrophages originating from TRAP-deficient mice produce increased levels of secreted IL-12, IL-1β and TNFα[108]. It also appears that TRAP is associated with production of NO in an unknown manner since TRAP-deficient macrophages produce more NO[108] while macrophages over-expressing TRAP produces less NO[107].

Early studies indicated that TRAP was down regulated in response to IFNγ and LPS[112], however, a later study has convincingly shown that LPS up-regulates TRAP expression[34]. Recently, TRAP was also shown to be up regulated by DNA/RNA in a TLR9 independent manner[113].

Hypothetically, one mechanism that TRAP expressed by macrophages and/or dendritic cells could modulate a Th1 response could be by dephosphorylation of the Th1 cytokine OPN[16] (Figure 5B). OPN is a Th1 cytokine which is highly dependent on its degree of phosphorylation to mount a proper immune response[114]. By dephosphorylation, TRAP could then modulate a OPN-dependent Th1 response[16, 17].

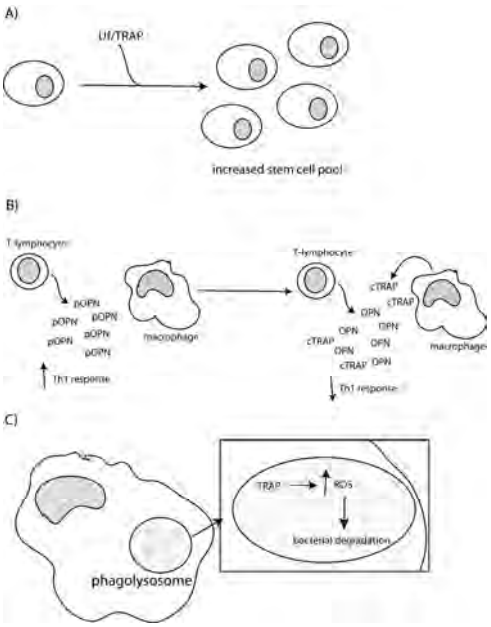

Figure 5. TRAP in the immune system. (A) Uteroferrin (Uf) has been shown to increase the hematopoeitic stem cell pool. (B) Hypothetically, TRAP could by dephosphorylation of pOPN down regulate a pOPN dependent Th1 response. (C) Intracellular TRAP has been shown to be co localized with phagocytosed bacteria. Here TRAP could participate in the degradation of bacteria by formation of ROS.

It has also been shown that TRAP is partly co-localised with phagocytosed bacteria[98], indicating a role for the enzyme in bacterial degradation (Figure 5C). Further support of this hypothesis is provided by the finding that mice deficient in TRAP demonstrate a reduced clearance of bacteria[108]. This might be due to reduced capacity of ROS production in macrophages of these mice since macrophages derived from TRAP over expressing mice display an increased level of ROS and an enhanced capacity of bacterial killing[107].

TRAP in bone metabolism

Although it has been known for a long time that TRAP is highly expressed in osteoclasts, the function of TRAP in bone metabolism in vivo is still not fully elucidated.

However, it is known that depletion of TRAP results in mild osteopetrosis as well as disorganised growth plates[27, 115]. On the contrary, over-expression of TRAP leads to increased bone formation[80].

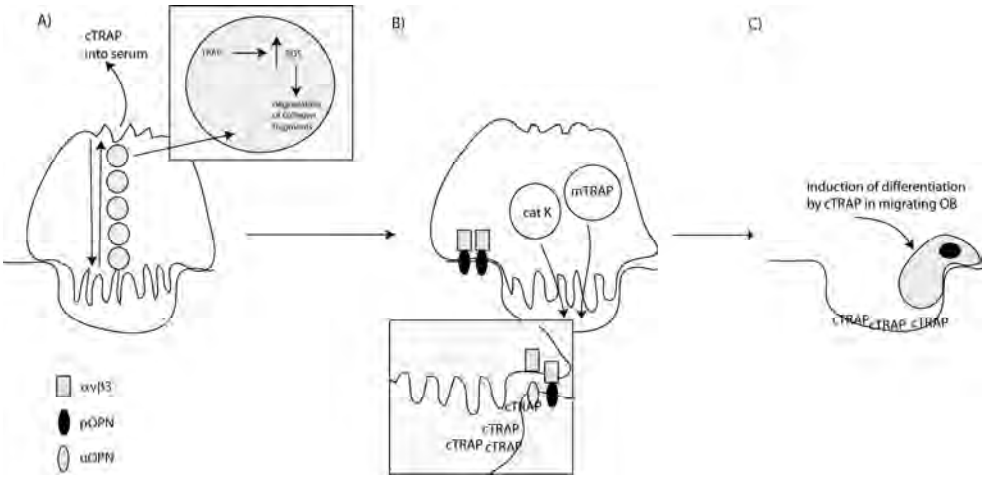

Figure 6. Possible functions of TRAP in bone metabolism. (A) proteolytically processed TRAP (cTRAP) produces ROS through the Fenton reaction in vesicles in the transcytotic pathway consequently degrading collagen fragments endocytosed from the resorption process. (B) monomeric TRAP (mTRAP) is secreted together with cathepsin K (cat K) into the resorption lacuna. Here, mTRAP is processed by cat K to cTRAP. cTRAP then dephosphorylates pOPN which subsequently can not bind to the osteoclast anchor integrin $\alpha_v\beta_3$ and migration of the osteoclasts is promoted. (C) cTRAP left on the bone surface in the resorption lacuna induces differentiation of osteoblasts (OB) thus promoting bone formation.

In osteoclasts it is hypothesised that there are two pools of TRAP (Figure 3); one intracellular pool that is proteolytically processed in a partly cathepsin K independent manner[54] and one pool of monomeric TRAP which is secreted through the ruffled border area and then proteolytically processed by cathepsin K in the resorption lacuna[43, 54]. The pool of intracellular proteolytically processed TRAP is believed to be transported into the transcytotic pathway where it might participate in degradation of resorption products such as collagen fragments by formation of ROS[96, 116] (Figure 6A). TRAP present in the transcytotic pathway is then secreted at the basolateral functional secretory domain and represent the major part of proteolytically processed TRAP found in serum[96, 117, 118] where TRAP is present in a complex with α2-macroglobulin[119, 120]. Meanwhile, the pool of TRAP that was secreted through the ruffled border and proteolytically processed in the resorption lacuna was hypothesized to

dephosphorylate OPN that anchors the osteoclast to the bone surface (Figure 6B)[17, 19]. This dephosphorylation of OPN then promote migration of the osteoclast away from the resorption lacuna[16, 18].

When the osteoclast migrates away from site of resorption, some TRAP remains on the bone surface and in the bone matrix; matrix associated TRAP could then be endocytosed by adjacent osteoblasts[121] and thereby possibly induce osteoblast differentiation[103] (Figure 6C).

TRAP in the nervous system

TRAP activity has been demonstrated in nerve cells of the rat olfactory bulb[93] and in brain macrophages from patients suffering from AIDS[122]. Interestingly, also the TRAP substrate OPN[17] has been found to be expressed in nerve tissue i.e. the olfactory bulb, brain stem and ganglie cells[123-125] implying that OPN could be a potential substrate for TRAP in nerve tissue. Another possibility is that TRAP participate in the metabolism of ROS in nerve tissue through the redox-active diiron center[104, 105].

Involvement of TRAP in diseases

Elevated levels of TRAP have been associated with several pathological conditions often involving either cells from the immune system, bone tissue or a combination. However, the precise role of TRAP in these conditions often remains to be elucidated.

Pathological conditions involving the immune system

TRAP was discovered in the B-cell leukemia hairy cell leukemia (HCL) in the early 1970s[126] and has been used as a marker for this disease[15]. Other leukocytic conditions with elevated TRAP levels are the lysosomal storage disease Gauchers disease [126-129], artheriosclerosis[130] and brain macrophages in AIDS patients[122]. Additionally, it was also noted that TRAP was elevated in cases of dengue hemorrhagic fever[131]. TRAP 5a has been suggested to be a marker of chronic inflammation[132] and rheumatoid arthritis[132, 133].

Pathological conditions involving bone

Elevated levels of TRAP has been reported in Pagets disease[134], hyper-parathyroidism[134], bone metastasis[134-136] and giant cell tumor[83, 137-139]. TRAP 5b have been found to be elevated in osteoporosis[140], bone metastasis[140-144] and multiple myeloma[145].

Immune responses

The immune system can be divided into; (1) the innate immune system and (2) the adaptive immune system. The innate immune system is the phylogenetically oldest and comprises the first line of defense against pathogenic microbes. It consists of epithelial barriers, phagocytes, NK cells and the complement system. The epithelial barrier is the physical barrier to infection, but it also kills microbes by secretion of anti-bacterial peptides. Phagocytes identify, phagocytose and destroy microbes, while NK-cells kill virus-infected cells and cells that have lost expression of MHC class I, and activates macrophages through IFNγ. The complement system promotes phagocytosis of microbes, stimulates inflammatory reactions and mediates cytolysis of microbes. The innate system also provides signals that together with antigen stimulate an adaptive immune response.

Cells of the innate immune system recognizes pathogen associated molecular patterns (PAMPs) through the family of pattern recognition receptors (PRRs) which mediate opsonization, activation of complement and coagulation cascades, phagocytosis, activation of proinflammatory signalling pathways and induction of apoptosis[146].

The concept that T cells produced certain subsets of cytokines, i.e. Th1 and Th2 cytokines, which can be used to distinguish different immune responses, developed in the 1980's[147, 148]. Since then it has been established that there are other types of responses as well e.g. Th17[149, 150] and that the immune response rarely is strictly Th1, Th2 or Th17 but rather a combination. Th1 responses, mediated primarily by IL12 and IFNγ, are thought to participate in defense against intracellular antigens while Th2, mediated through for example IL4, participates in parasitic infections and allergy. On the other hand, Th17 responses, mediated through TGFβ and IL23, can participate in defence against both intracellular pathogens and parasites[151].

Macrophages

Macrophages are generally defined as the mononuclear phagocyte system (MPS)[152]. The MPS consists of bone marrow cells that differentiate into blood monocytes and then homes to tissues becoming resident tissue macrophages[152]. Inside the tissue most macrophages are renewed by infiltration of new tissue macrophages, however, some populations of tissue macrophages proliferate within the tissue e.g. Kupffer cells and alveolar macrophages[153, 154].

The main function of macrophages is elimination of dying cells and pathogens[155]. Additionally, it has also been suggested that macrophages regulates the physiological function and differentiation of neighbouring cells by secretion of regulators[156-159].

Functional subpopulations – M1 and M2

The functional polarization of macrophages has been divided into four groups; M1, M2a, M2b and M2c, which represents the extremes of a continuum (Figure 7)[1, 160]. M1 macrophages promote Th1 immune responses and are in general $IL12^{high}$, $IL23^{high}$ and $IL10^{low}$. They express PAMPs that are linked to activation of NF-kB and subsequently, they express high amounts of proinflammatory cytokines, ROS and NO. They also express MHC class II and exhibit high endo- and phagocytic activity. In inflammation, M1 macrophages participates in Th1 responses, delayed-type hypersensitivity (DTH), killing of intracellular pathogens and tumour resistance.

The so called M2 macrophages ($IL12^{low}$, $IL23^{low}$ and $IL10^{high}$) are divided into three different groups; M2a, M2b and M2c where M2a corresponds to macrophages activated by IL4/IL13, M2b is activated by immune complexes and TLR ligand stimulation and M2c by IL10 stimulation. M2a and M2b macrophages display a relatively high expression of MHC class II and exhibit endo- and phagocytotic activity just as M1 macrophages. However, they promote Th2 responses instead of Th1 since they express high amounts of anti-inflammatory cytokines, although M2b macrophages also express pro-inflammatory cytokines. M2a macrophages participate in Th2 responses, type II inflammation, allergy and killing and encapsulation of parasites while M2b macrophages participate in Th2 activation and immunoregulation.

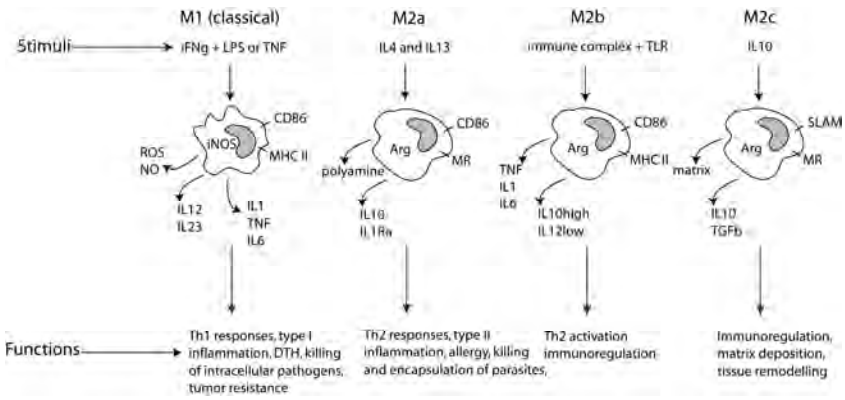

Figure 7. Functional grouping of macrophages. Macrophages can be divided into four functional groups. Keep in mind that these represent extremes of a continuum. Adapted from [1]

M2c macrophages produce high amounts of IL10 and TGFβ and participate in immunoregulation, matrix deposition and tissue remodeling. In contrast to M1 macrophages, M2 does not express PAMPs that leads to activation of NF-kB but rather non-opsonic PAMPs as the mannose receptor (MR).

Subpopulations of resident and inflammatory macrophages

Another way of characterizing macrophages is to group them according to the expression of chemokines on monocytes. In mouse blood, two main populations of monocytes can be found; (1) $CX_3CR_1^{high}$ CD16+ $CCR2^{low}$ $CCR1^{low}$ and (2) $CCR2^{high}$ $CX_3CR_1^{low}$[161-163]. CX_3CR_1+ monocytes are believed to home to non-inflamed tissues and there constitute the resident macrophage population with a relatively long half-life. CCR2 positive monocytes on the other hand homes to inflamed tissues and are thus named inflammatory macrophages.

Inflammatory Bowel Diseases

Inflammatory bowel diseases (IBD) consist of ulcerative colitis (UC), Crohns disease (CD) and non-infectious inflammations of the bowel[164]. The highest incidence rate of IBD is found in northern Europe and the US; however, in these areas rates are beginning to stabilize. On the contrary, the incidence is still rising in low-incidence areas such as southern Europe, Asia and developing countries[165].

Clinical symptoms include weight loss, diarrhea, accompanied by blood stools and abdominal pain[166]. Disease progression of IBD is accompanied by increased formation of granulomas and activated monocytes which produce significant amounts of eicosanoids and cytokines[167]. Affected sites typically reveal transmural inflammation characterized by lymphoid hyperplasia, sub-mucosal edema, ulcerative lesions and fibrosis[168].

Genetics and environment in development of IBD

There has been much debate to whether IBD is caused by genetic and/or environmental factors. The strongest evidence of a contribution of genetic factors is concordance studies in twins[169, 170]. Genome wide scans of patients with CD or UC has identified susceptible regions on 12 chromosomes, indicating that CD and UC are polygenic diseases[171]. Contribution of the environment in the development of IBD is supported by studies showing an increased incidence rate of IBD in immigrants from low-incidence regions moving to urban regions[172, 173]. Animal models also support the hypothesis that environmental factors affects genetically susceptible hosts and are responsible for the induction of IBD[174].

Example of a gene that has been linked to increased risk of developing CD is CARD 15 encoding NOD2[175, 176]. Although it is not known how mutations in NOD2 increases the susceptibility of CD, it has been shown in mice that absence of or mutations in NOD2 can result in altered TLR signalling, increased production of proinflammatory cytokines, defective activation of NF-kB and impaired production of antimicrobial proteins by intestinal epithelial cells[177].

Immunobiology of the healthy gut

The environment in the healthy gut is constantly in a state of controlled inflammation. This control is believed to be maintained by a phenomenon called oral tolerance although this process is incompletely understood[178].

The polarized single mucus-covered epithelial cell layer of the gut is the physical barrier against the outside world and its integrity is maintained by tight junction proteins[179]. It is here that the first line of defense against pathogens is mounted[180]. The mucous covering the epithelium is home to around 500 species of commensal microbes[181, 182]. These microbes affect the expression of different genes, the absorption of nutrients, xenobiotic metabolism, angiogenesis and post-natal intestinal maturation[183]. This is also the place where the first recognition and processing of luminal antigens take place[184]. This is possible because the epithelial cells expresses PRRs i.e. TLR which through NFkB activation can increase the production of inflammatory cytokines[185, 186]. When no pathogens are present in the gut, TLR interacts with commensals. Thereby they contribute to the intestinal homeostasis and maintainence of the epithelial barrier[187, 188].

In the sub-epithelial space, many types of immune cells are gathered i.e. T cells, B cells, granulocytes, mast cells, NK cells, macrophages and DC. In the healthy gut, immature DC opens tight junctions between the epithelial cells and extend dendrites through this opening out into the lumen to sample antigens from microbes[189]. They can then induce unresponsiveness probably by stimulating naïve T cell differentiation into regulatory CD4+ T cells, such as Th3 cells rather than Th1 or Th2 cells[190]. The DCs are the key cells in control against pathogens and tolerance towards commensals since they express the entire spectrum of TLR and NODs[191].

When DC senses danger, they mature, acquire an activated phenotype and induce immunity[192] . This process can involve remodelling of the cell cytoskeleton and activation of TLR[193].

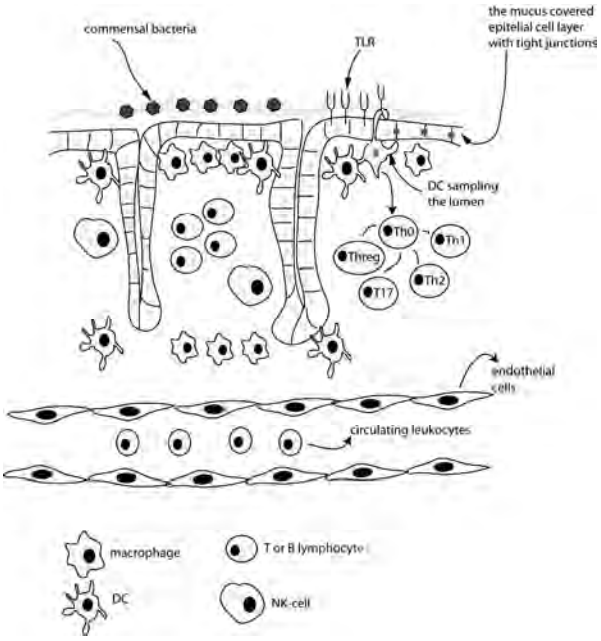

Figure 8. Simplified picture of the immunobiology in healty gut. In healty gut, the epithelial cell layer is a barrier between the lumen and the mucosa. Tight junctions maintain the structure of the epithelial cell layer. DC extends dendrites through the epithelial cell layer sampling the luminal antigens and keeps the balance between Treg and Th1, Th2 and Th3. Macrophages are found in the sub-epithelial area.

Immunobiology of IBD

Two hypotheses have been suggested for the induction of IBD: (1) defective immune tolerance against commensal bacteria or (2) an alternated microflora in the gut. Today, most studies points in the direction of a defect in the immune response against commensal bacteria[164].

As stated above, the first line of defence is the single epithelial cell layer. In mice models, it has been shown that a leaky epithelial barrier can induce an immune response towards commensal bacteria[194]. It has also been shown that patients with CD and UC have lowered epithelial resistance and increased permeability of the epithelium[195-197]. In addition, intestinal epithelial cells from patients with IBD seems to have a disturbed innate immune response i.e. dysregulated expression of TLR with an up regulation of TLR4[198], NOD2 and NF-kB[199, 200].

In IBD, the second line of defence, i.e. antigen-presenting cells DC exhibit disturbed processing and recognition of antigens[201, 202]. These atypical APCs then potentiates T cell activation[203, 204].

The T cell population has also been reported to be disturbed in patients with IBD i.e. display altered clearance of over- or auto reactive T cells[205, 206] or altered balance between regulatory and effector T cells[207, 208].

In response to the increased inflammation in IBD, there is also an increased transmigration of leukocytes into the inflamed areas of the gut[209, 210]. These new leukocytes then produce metabolites i.e. NO, prostaglandins, ROS, histamines, proteases, and matrix-metallo proteases (MMPs), causing tissue damage in the gut[211-214].

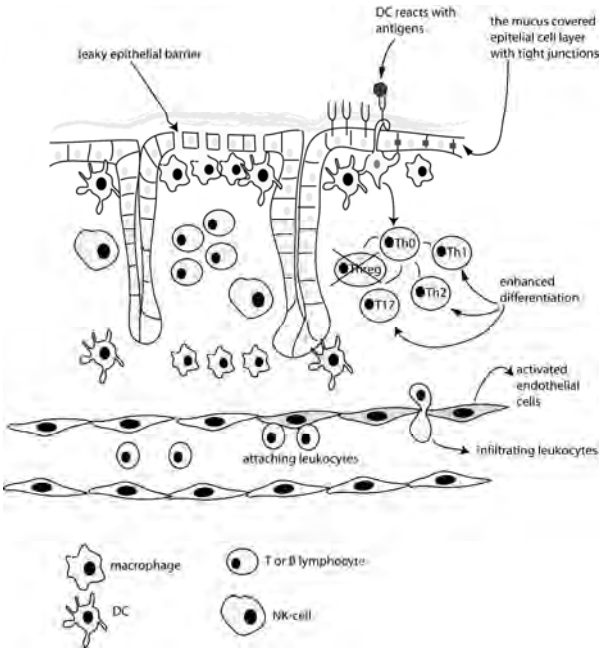

Figure 9. Simplified picture of the immunobiology in IBD. In IBD, the epithelial cell layer is leaky. DC recognises bacterial antigens and promotes differentiation of Th1, Th2 and Th3. Endothelial cells are activated and there is an increased influx of leukocytes to the mucosa.

Macrophages in normal colon tissue and in IBD

The colon is the largest reservoir of macrophages in the body[215, 216] . Colonic macrophages do not proliferate[217] but are recruited from blood monocytes[217, 218]. In normal colon, tissue macrophages do not function as APC but demonstrates high phagocytic-[219] and bactericidal activity[220]. Consequently, they exhibit low expression of CD14[221, 222] , CD80 and CD86[223] and respond poorly to chemotactic agents[217].

On the other hand, in IBD, macrophages display a different phenotype. Here they express co stimulatory molecules such as CD80 and CD86[223]. Additionally, they usually also express CD14 and TLR receptors[222, 224, 225]. This implies that macrophages in colon tissue from IBD patients can function as APC and thereby also mount a pro-inflammatory cytokine response[225] as well as activating the adaptive immune system. It is likely that these macrophages originate from an influx of new macrophages to the inflamed tissue[218, 226 227] since

resident colon macrophages do not respond to stimulating agents such as LPS, TNFα or PMA[224, 228].

DSS induced colitis – model of IBD

Today there are many models of IBD[174, 229] that have contributed to our current understanding of this condition[230, 231]. An animal used in IBD models should have a well-defined genetic background and preferably a well-characterized immune system. In an optimal model of IBD, the intestine should have morphological changes and signs of inflammation as well as symptoms. Overall, the animal should show a pathophysiology that resembles the human condition as close as possible[174] .

One commonly used model of human IBD is dextran sulphate sodium (DSS) induced colitis. The sulphated polysaccharide DSS causes an inflammatory response which is restricted to the large intestine[232] and is characterized by decreased body weight, bloody diarrhoea and a mucosal inflammation with ulcers. DSS induced colitis can be acute or chronic, and lead to dysplasia and cancer[233, 234]. The response depends on the duration and concentration of the DSS treatment as well as the species and strain used[235-237]. Acute DSS induced colitis is dependent on Mϕ[238-240] and independent of B- and T- lymphocytes, NK-cells and neutrophils[239-241]. The latter notion, as well as the dependence on Mϕ, makes this model a useful tool to study activation of innate immunity in colitis. The initial event in DSS induced colitis is thought to be an increased permeability of the epithelial cell layer[242 243, 244], and it has been shown that proliferation and apoptosis is increased in the epithelial cell layer[242-244]. There is also a shift in the bacterial population following a DSS treatment[245], although the impact of bacteria on the pathogenesis of acute DSS induced colitis is debated[246, 247]. The initial stage is followed by infiltration of PMN[238, 248-250] and a biphasic activation of NF-kB[251]. If treatment is not prolonged, this phase is followed by resolution and a healing phase. DSS induced colitis has been shown to be neither a pure Th1 nor a Th2 response but rather a mixture of Th1 and Th2 cytokines with up-regulation of IL12, IFNγ, TNFα and IL10 and down regulation of IL4[235]. Recently, it was shown that 387 genes were up-regulated or down-regulated during acute DSS induced colitis. When compared with 32 commonly affacted genes in human IBD, 15 of these were also up- or down-regulated in DSS induced colitis[252]. To conclude, most data points in the direction that DSS induced colitis is mainly a model for the initial epithelial damage and early innate immune responses rather than a model for the chronic inflammation.

Obesity

Obesity, that is pathological gain of weight in response to excessive calorie intake, has increased dramatically over the recent decades. This is alarming, since obesity is associated with an increased risk of developing insulin resistance, type 2 diabetes, fatty liver induced hepatitis and liver cirrhosis, atherosclerosis, respiratory airway diseases and certain cancers. Historically, white adipose tissue, here referred to as adipose tissue, have been considered an organ that simply stores fat in the form of triglycerides but else is an inert tissue. This view of adipose tissue was dramatically changed when the adipocyte-derived protein leptin was cloned in the 1990s[253]. Today it is acknowledged that adipose tissue is an endocrine organ that secretes factors that participates in the regulation of energy balance, insulin sensitivity, immunological responses and vascular diseases[254, 255].

Development of adipose tissue

Adipocyte differentiation is the result of transcriptional remodeling that leads to activation of a considerable number of adipocyte related genes. It has been shown that in the pre-adipocytic cell line 3T3-L1[256-258] over 2000 genes are affected during differentiation to adipocytes and that ~100 of these are uncharacterized[259]. Pre-adipocytes display an exponential growth until reaching confluence when they exhibit an initial growth arrest and become committed. Young adipocytes can then undergo mitotic clonal expansion before terminal differentiation to mature adipocytes[260-262] .

Examples of factors promoting adipogenesis are insulin, IGF-1, MCS-F, glucocorticoids (GC) and glucose. On the other hand, Wnts, TGFβ, TNFα, IL1, IL6, IL11, IFNγ and resistin are examples of factors inhibiting adipogenesis. Examples of transcription factors promoting adipogenesis are PPARγ2, C/EBPs and FOXC2 while GATA2, GATA3 and SMADS are examples of inhibitory transcription factors[263, 264].

Expansion of adipose tissue

Adipose tissue can expand due to two different events; (1) hyperplasia i.e. differentiation of new adipocytes and (2) hypertrophy i.e. enlargement of pre-existing adipocytes due to increased storage of triglycerides. Studies on human and non-human models of obesity reveal that hypertrophy (increase in cell size) mostly seems to precede hyperplasia (increase in cell number)[265 266-269]. This hyperplastic component is associated with severe obesity and has a poor prognosis for treatment[270 269]. It has been hypothesized that there is a "maximum adipocyte cell size" that would explain why hyperplasia occurs after

hypertrophy[271, 272]. One suggestion is that when adipocytes reach a certain cell size they start to produce and secrete growth factors that stimulate formation of new adipocytes[273] (Figure 10). Today, there are evidence both supporting and contradicting this hypothesis[274-276].

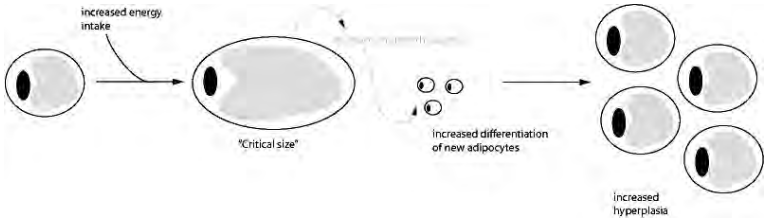

Figure 10. Expansion of adipose tissue in response to increased calorie intake. Normally sized adipocytes increases in size and become hyperthropic. When they reach the "critical adipocyte cell size" they cannot increase more in size and they may start to secrete growth factors. These then stimulate pre-adipocytes to differentiate and thereby increase the hyperplastic component in adipose tissue.

Additionally, with respect to the correlation between obesity and insulin resistance, several studies have highlighted the relationship between adipocyte size, rather than adipose tissue mass, and insulin malfunction[277-282].

Inflammation and obesity

Lately, it has also been established that obesity is associated with a low-grade inflammation[283, 284] which has been linked to the development of insulin resistance[285] . Consequently, increasing obesity activates pro-inflammatory NF-kB pathways[286-289] and display an increased level of oxidative stress markers[290].

Macrophages in obesity

Obesity is associated with an influx of macrophages into the adipose tissue[291-294] mainly originating from the bone marrow[291], although it has been speculated that adipocytes could exhibit characteristics of macrophages[295, 296]. What causes the influx of macrophages into adipose tissue is not elucidated; however, there have been several proposed mechanisms. One of the hypothesis propose that the influx is a consequence of adipocyte necrosis because of adipocyte hypertrophy [297], others point to the importance of local hypoxia[294] and increase of monocyte attractants such as MCP-1/CCL2 [298-300]. For example, mice deficient in CCR2 have a reduced infiltration of macrophages in the adipose tissue[301], still, the influx seems to be

multifactorial. Additionally, Western diet up-regulates the expression of adhesion markers in endothelial cells[302] which could lead to increased influx of macrophages (Figure 11).

The macrophage population in adipose tissue is not a homogenous population[303]. Additionally, macrophages in lean vs. obese states seems to belong to different functional subpopulations since macrophages from lean subjects express M1 markers and macrophages from obese subjects express M2 markers[304, 305]. This increase in M1 macrophages in obese subjects can be a direct consequence of the increased influx of macrophages since CCR2-/- mice display increased numbers of M2 macrophages than WT mice after diet-induced obesity[305].

One debate today is "where starts the fire" [306]? That is, what starts the low-grade inflammation seen in obesity? Is it the elevated levels of lipids that may activate circulating monocytes in a pro-inflammatory fashion[307]? Alternatively, is it the increased volume of the adipocyte in response to the increase in triglycerides that causes an altered gene expression profile in the adipocyte[308]?

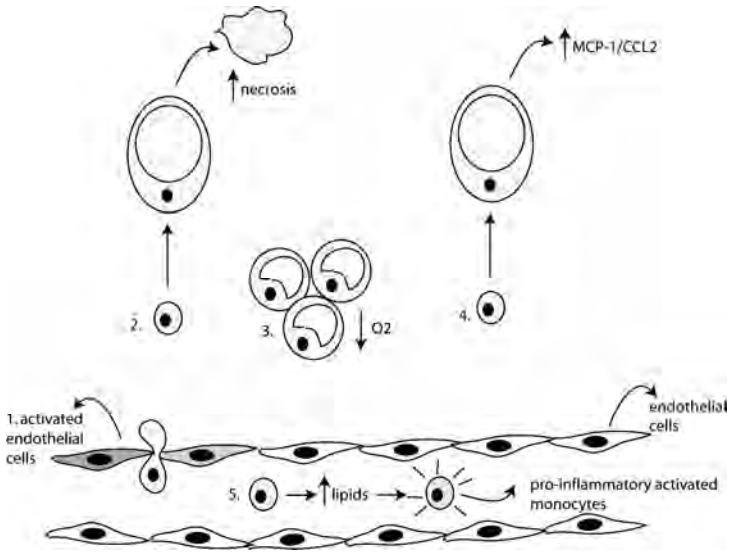

Figure 11. The hen and the egg…? What starts the low-grade inflammation in obesity? Suggested mechanisms are increased activation of endothelial cells (1), increased adipocyte necrosis due to hypertrophy (2), local hypoxia (3), altered gene expression du to hypertrophy (4) and activation of circulating monocytes (5) due to increase in lipids.

PRESENT INVESTIGATION

Aims of the investigation

TRAP has long been recognized as a marker for the bone resorbing cell, the osteoclast, and been implied in the process of bone resoption. As a result, it was hypothesized in the late 1990's that TRAP might be a target for drugs inhibiting bone resorption. However, some studies suggested that TRAP was also expressed in cells outside the skeleton and consequently the role of TRAP in non-skeletal tissues was actualized.

In light of these observations the major aim of this thesis was to characterize extra skeletal TRAP. This aim has then been divided into three sub-aims:

1. To investigate the cellular distribution of monomeric and proteolytically processed TRAP in organs in non-skeletal tissues.

2. To identify TRAP isoforms from different cellular sources.

3. To investigate expression and regulation of TRAP in inflammatory responses.

Comments on methodology

Measurement of TRAP enzyme activity

There are at least five different mammalian acid phosphatases with different characteristics. TRAP is characterized by resistance to inhibition by tartrate and sensitivity to inhibition by the tetrahedral oxyanion molybdate. Another characteristic of mammalian TRAPs is the redox active diiron centre (Figure 4). This centre can be manipulated in vitro by adding reducing agents thereby converting to the mixed-valent Fe(III) – Fe(II) centre and thus increasing the enzyme activity. On the contrary, the mixed-valent centre can be oxidized to a Fe(III) – Fe(III) centre and thus decrease the enzyme activity of TRAP.

In the present investigation, the resistance to tartrate and sensitivity to molybdate has been used to exclude involvement of other phosphatases when measuring TRAP enzyme activity in crude homogenates. When measuring TRAP enzyme activity two measurements have been performed: (1) measurement in the presence of tartrate, which should exclude activity from acid phosphatases sensitive to inhibition to tartrate, and (2) measurement in the presence of both tartrate and molybdate, which then should also exclude TRAP enzyme activity. The true TRAP enzyme activity has then been defined as the activity in the samples containing tartrate subtracted from the activity in the sample containing both tartrate and molybdate.

Additionally, when measuring TRAP enzyme activity we have chosen to add reducers to the substrate buffer to ensure that we were preferably measuring the activity from an enzyme with a fully reduced enzymatically active diiron centre i.e. a Fe(III) – Fe(II) centre.

Cellular detection of TRAP protein

Two polyclonal antibodies have been used in this investigation, directed toward TRAP to detect monomeric and total TRAP in tissue sections. The antibody directed toward total TRAP was made in rabbits against the whole monomeric TRAP protein[43]. That is, the antigen contained the loop region as well as both the C-terminal 16 kDa and N-terminal 22 kDa parts of TRAP and therefore the antibody detect both monomeric and proteolytically processed TRAP. On the other hand, the antibody used to detect monomeric TRAP was raised in rabbits against a peptide corresponding to the loop region, which is only present in the monomeric protein, and therefore this antibody should only detect monomeric TRAP.

When assessing cellular distribution of a protein in tissue sections there is always the question of the specificity of the staining, and therefore the appropriate controls are crucial. In the present investigation, two types of controls have been used; (1) omitting the primary antibody, which tells if the secondary antibody and possibly avidin-biotin complexes are un-

specifically bound and (2) pre-absorption of the primary antibody with its antigen, which tells if the primary antibody is able to recognise the antigen. However, it does not conclusively prove if your antibody specifically binds only the antigen in the tissue sections. Therefore, the antibodies used have also been validated using Western blot.

Animal models of inflammation used in this thesis

DSS induced colitis

Male adult Sprague-Dawley rats (300-350 g) were treated with 3% DSS in the drinking water for 1, 2, 3 or 7 days while controls received ordinary tap water. The animals were inspected daily with regard to their general condition and the overt appearance of their feces. The general condition of all the animals appeared normal without general signs of distress, e.g. piloerection or soiling of the fur, during the entire treatment. During days 5-7, most animals exhibited loose stools, sometimes with streaks of blood. Macroscopically, the distal colon was not altered during the first three days of treatment, while, at day 7, some of the rats displayed thickening of the wall of the distal colon. Microscopically, there were clear-cut alterations of the tissue structure only at 7 days of treatment. Thus, in a heterogeneous fashion, there were shortening of the crypts, loss of goblet cells, cryptectasiae, superficial erosions and crypt abscesses. At the end of the respective treatment, the rats were killed by an overdose of pentobarbitone followed by exsanguinations and the distal colon was dissected. One specimen was snap frozen, and one was fixed in formalin for subsequent paraffin embedment and immunohistochemistry.

The TRAP over expressing mouse

The TRAP over expressing mouse was made as previously reported[80] using the TRAP promoter itself together with a SV40 enhancer in the construct. The use of the TRAP promoter in the construct led to a over-expression of TRAP only in cells normally expressing TRAP. We used two strains of TRAP over-expressing mice, one (TRAP+) which was obese and expressed both monomeric and proteolytically processed TRAP in the adipose tissue and one (TRAP+p) which expressed mainly proteolytically processed TRAP in the adipose tissue.

Results and discussion

Distribution and biochemical characterization of TRAP

Expression and cellular distribution of TRAP in rat organs

The expression levels of TRAP was investigated both at the mRNA and enzyme activity levels in young rats and for certain organs in newborn, 2-5 days post-partum, and adult rats, approximately 12 weeks old (Paper I and II). In young, three-week old, animals, (Table I) highest expression of TRAP mRNA and enzyme activity was detected in bone followed by spleen, trigeminal ganglia, spinal cord and liver. In addition, thymus, colon and kidney expressed lower, but significant, amounts of TRAP while the expression of TRAP in brain was very low relative to bone.

Table I. Expression levels of TRAP mRNA and enzyme activity in rat organs

Organ	TRAP mRNA	TRAP enzyme activity
Bone	100	100
Spleen	85	22
Trigeminal ganglia	*	11
Spinal cord	*	4.4
Liver	9	3.2
Thymus	10	1.4
Colon	9	1.2
Lung	27	1.1
Kidney	8	0.95
Brain	*	0.42

* mRNA (cDNA) was detected using agarose gels after PCR therefore no values were obtained.

When comparing the expression of TRAP in organs at different ages (Paper II), it was apparent that two of the organs exhibited a reduction in TRAP expression over age i.e. bone and liver. On the contrary, expression levels in spleen, thymus and lung remained more or less constant over time.

TRAP mRNA was detected in neurons in the spinal cord, Purkinje cells in the cerebellum as well as in medium-sized neurons in the trigeminal ganglia (Paper I). Additionally, TRAP mRNA was also detected in mononuclear cells of the lamina propria in colon (Paper III).

Total TRAP protein was detected in cells from the myeloid lineage (Paper II, III, and IV), in epithelial cells (Paper II) and in neurons (Paper I). In spleen, total TRAP was mainly found scattered through the red pulp and the outer part of the marginal zone, while it was evenly distributed in both the medulla and cortex in thymus. Additionally, total TRAP was also detected in alveolar macrophages, Kupffer cells, and mononuclear cells in the colon and CD68 positive macrophages in human adipose tissue. In epithelial cells, total TRAP was detected in kidney tubule cells. In neurons, total TRAP was detected in aggregates in the cytoplasm of neuronal cell bodies in both the central and peripheral nervous system. TRAP was also detected in neuronal processes close to the cell soma. Notably, high expression of both TRAP mRNA and protein was detected in various ganglia and in α-motor neurons of the ventral spinal cord.

Monomeric TRAP was detected in cells derived from the myeloid lineage (Paper II, III, and IV) and in epithelial cells (Paper II). Staining of monomeric TRAP in spleen, thymus and colon showed the same distribution as total TRAP but with a weaker signal. On the other hand, alveolar macrophages exhibited a strong signal for monomeric TRAP. In epithelial cells, strong staining for monomeric TRAP was detected in bronchiolar epithelial cells resembling Clara cells and kidney tubuli cells, with a lower signal in some intestinal epithelial cells; however, no staining was detected in liver.

To conclude, TRAP is expressed by mononuclear cells i.e. macrophages as well as certain epithelial cells and neurons. The most important organ contributors of TRAP in rats are bone, spleen and liver. However, the level of TRAP expression decreases in bone and liver over age.

Biochemical properties and proteolytic processing of extra skeletal TRAP

In order to establish if the protein which enzyme activity was measured actually was PAP and if it contained a redox active Fe(III)-Fe(II) centre, the enzyme from different organs were characterized with respect to pH optimum, substrate affinity (K_m) and redox properties of the diiron centre (Paper I and II).

Table II shows a summary of the results obtained from this characterization, showing that TRAP isolated from these organs exhibited the hallmarks of the structurally defined diiron PAP enzyme[3]. That is, an acidic pH optimum similar to PAP and sensitivity to inhibition by tetrahedral oxyanions. Additionally, the results supports the hypothesis that rat TRAP contains a redox active Fe(III)-Fe(II) centre rather than a Fe(III)-Zn(II) centre since it was possible to increase the enzyme activity by treatment with reducers, and decrease the enzyme activity by treatment with oxidizing agents.

Table II. Biochemical properties of extra skeletal TRAP

Tissue	pH optimum	K_m	IC_{50} molybdate (μM)	Activation with reducers* (fold)	Activity after oxidation** (% of reduced activity)
Bone	5.8	0.23	39	28	0.3
Spleen	5.5-5.8	0.45	15	58	0.3
Trigeminal ganglia	ND	0.56	58	14	0
Spinal cord	ND	0.56	30	17	0
Liver	5.8	1.8	13	17	0.5
Thymus	5.8	0.51	19	15	1.0
Colon	5.8	1.32	28	22	0.5
Lung	5.8	1.12	25	30	1.0
Kidney	5.5-5.8	0.42	19	17	3.3
Brain	ND	0.38	10	17	0.7

* activity was measured first in the absence and then in the presence of 1mM ascorbic acid and 0.1mM $FeCl_3$

** Oxidation was achieved by adding 5mM H_2O_2 to a reduced sample.

Since TRAP either can exist a monomeric protein or as a proteolytically processed dimer, held together by a disulphide bridge, it was of interest to investigate if the proteolytically processed form was present in different organs (Paper II). Both FPLC and Western blot showed that the proteolytically processed form is present in all organs examined. The results also suggested that cathepsin L might be responsible for the proteolytic processing of TRAP in certain cell types given that TRAP and cathepsin L were co localised in spleen, thymus, colon and kidney.

As the proteolytically processed form of TRAP was present in all tissues and since proteolytic processing had been shown to increase the enzyme activity of TRAP[9, 10], we investigated if proteolytic processing as such was sufficient to generate an active enzyme (Paper II). The data obtained suggested that this was not the case. Proteolytic processing in the absence of reducers only led to a minimal reduction of the substrate affinity and no increase in enzyme activity. However, the proteolytic processing seems to be important for the reduction of the diiron centre since the enzyme activity of monomeric TRAP only is increased ~8 fold in the presence of reducers compared to ~26-90 folds for TRAP processed by cathepsin L and K.

In conclusion, TRAP s from all rat organs tested exhibit the characteristic features of PAPs. Proteolytically processed TRAP was found in all organs examined and in spleen, thymus, colon and kidney cathepsin L could possibly participate in the proteolytic processing of the enzyme. The proteolytic processing does not increase the TRAP enzyme activity but seems to enable reduction of the diiron centre.

Expression and regulation of TRAP in inflammatory responses

Regulation of TRAP in Th1 immune responses

Given the knowledge that TRAP is highly expressed in macrophages and dendritic cells[78, 79] and possibly associated with activation of macrophages[110] it was of interest to investigate regulation and expression of TRAP in a model of inflammation. We choose to use DSS induced colitis (Paper III), a model of acute gastrointestinal inflammation believed to involve macrophages[238-240].

Measurement of mRNA for known cytokines revealed that the model was characterized by a Th1 cytokine response with increased expression of IL12, IFNγ and TNFα on day 1-2. TRAP enzyme activity increased already after one day of DSS treatment and remained increased throughout the study, while TRAP mRNA increased after two days of treatment. Both cytokine and TRAP mRNA levels were normalized at day seven.

At the cellular level, the model was characterised by (1) an activation of resident macrophages in the early (day 1-3) phase and (2) an influx of macrophages, including CCR2+ macrophages, into the mucosa and sub-mucosa in the late phase (day 7). TRAP was present in a population of resident macrophages (CX_3CR_1+ and CD68+) which exhibited signs of activation, i.e. expression of MHC class II during the first days of the inflammation. Later, TRAP was also present in inflammatory leukocytes (CCR2+) and the co localisation with MHC class II and CD68 decreased with time. On the contrary, monomeric TRAP was not co localised with macrophage marker CD68 at any time point.

Since the DSS-induced colitis was shown to be mediated through Th1 cytokines it was interesting to investigate if Th1 cytokines with or without the addition of LPS would increase TRAP mRNA in an in vitro system. Using the acute monocytic leukemia cell line THP-1 it was shown that IFNγ in combination with LPS, but not alone, increased expression of TRAP. Additionally, stimulation with LPS also seemed to change the intracellular distribution of TRAP from a perinuclear compartment to vesicles.

In summary, TRAP seems to be elevated and tightly regulated in Th1 responses. At the cellular level, TRAP is expressed in both resident and inflammatory macrophages. Cells expressing monomeric TRAP could possibly represent an immature population of macrophages, possibly the precursors to the TRAP positive macrophages in the upper lamina propria.

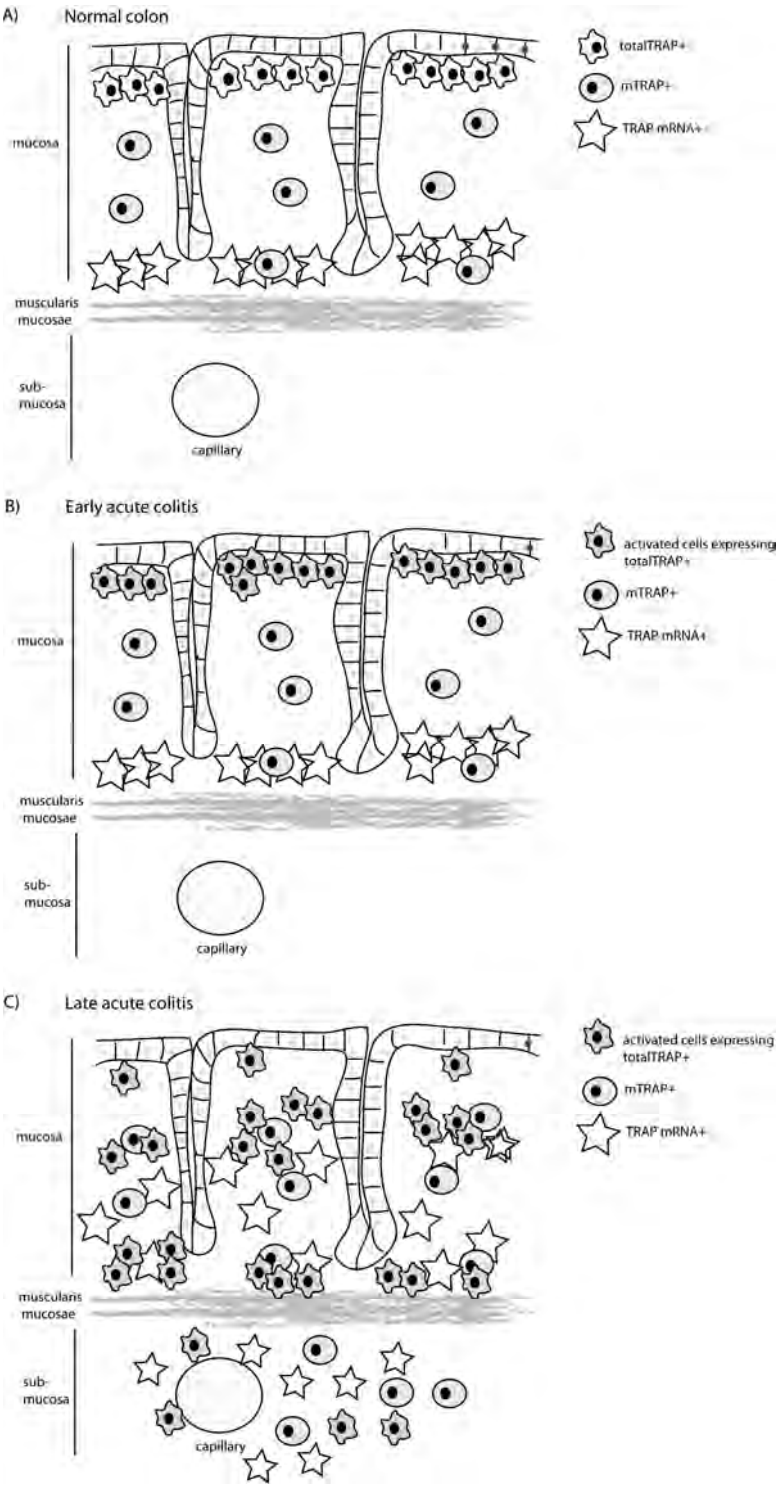

Figure 12. Regulation of TRAP in acute DSS induced colitis. (A) Normal colon is characterized by total TRAP+ resident (CX_3CR_1+) macrophages in the sub-epithelial area. Contrary, cells positive for monomeric TRAP (mTRAP) is found evenly distributed over the lamina propria. TRAP mRNA+ cells are mainly found in the lower part of the lamina propria. (B) During the early phase of acute DSS colitis, TRAP is found in activated (CX_3CR_1+MHC II+) macrophages in the sub-epithelial area. Cells positive for mTRAP are still distributed over the mucosa and TRAP mRNA+ cells found in the lower part of the mucosa. (C) During the late phase of acute DSS colitis, total TRAP positive cells starts to appear in lower parts of the mucosa while TRAP mRNA positive cells are found higher up in the lamina propria. Total TRAP+, mTRAP+ and TRAP mRNA positive cells starts to appear in the sub-mucosa.

A possible function of macrophage secreted monomeric TRAP

Since it has been demonstrated that uteroferrin can expand the haematopoietic stem cell pool[20-22] and that TRAP could induce differentiation of osteoblasts[103] it was intriguing to examine possible involvement of TRAP in adipocyte differentiation using the TRAP over expressing mouse[80], which were found to be severely obese (Paper IV).

Using dual X-ray analysis (DXA) and dissection of adipose tissue depots it was shown that the TRAP over expressing (TRAP+) mouse exhibited an increase in adipose tissue mass without apparent signs of increased adipocyte volume due to overeating. Treatment of mice and human pre adipocytic cell lines with monomeric or proteolytically processed TRAP showed that monomeric TRAP induced proliferation and differentiation of adipocytes. These data indicated that the increase in adipose tissue mass in the mouse could be due to recruitment of macrophages over-expressing monomeric TRAP, acting as a growth factor for adipocytes. In support of this idea a TRAP over expressing mouse mainly expressing proteolytically processed TRAP in the adipose tissue was lean. Additionally, human hyperplastic obesity was associated with increased levels of TRAP mRNA and monomeric TRAP, but not with proteolytically processed TRAP.

When investigating the cellular source of TRAP in the TRAP+ mouse as well as in human adipose tissue it was apparent that the expression of TRAP in adipocytes was low. However, the expression of TRAP in adipose tissue macrophages was high in both the TRAP+ mouse as well an in human adipose tissue.

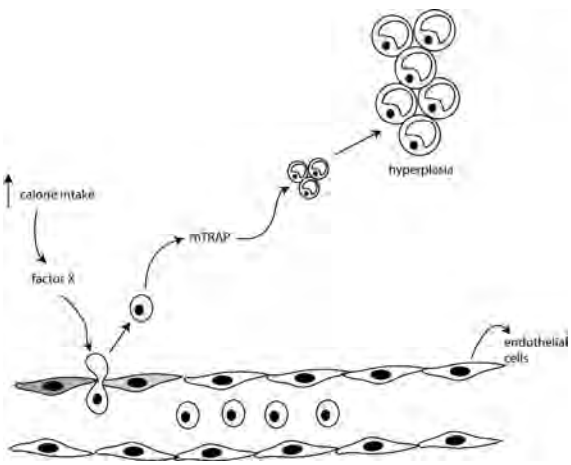

Figure 13. Suggested function of monomeric TRAP in obesity. Increased calorie intake leads to increase of factor X. This increases the influx of macrophages expressing monomeric TRAP. Secreted monomeric TRAP then induced hyperplasia by increasing the proliferation and differentiation of adipocytes.

Obesity is known to be associated with increased insulin resistance; therefore metabolic studies on adipocytes from WT and TRAP+ mice as well as measurement of insulin and glucose in serum from TRAP+ mice was performed. Nor the metabolic studies neither the

levels of glucose or insulin was changed between the WT and TRAP+ mice indicating that the TRAP+ mice in spite of being obese was not insulin resistant.

From these data we hypothesize (Figure 13) that macrophage secreted monomeric TRAP induces hyperplastic obesity by increasing the proliferation and differentiation of new adipocytes. Since adipocyte volume seems to the correlated to the degree of insulin resistance, this hyperplastic obesity is most likely not affecting the insulin sensitivity of adipocytes.

Conclusions

1. Distribution and characterisation of TRAP.
    a) TRAP is expressed in subpopulations of cells from the myeloid lineage, neurons and epithelial cells. Monomeric TRAP tends to be more highly expressed in epithelial cells compared to cells of the myeloid lineage.
    b) Taking into account organ size, the largest contributors of TRAP in rats are bone, spleen and liver.
    c) Extra skeletal TRAPs contain a redox active diiron centre.
    d) Proteolytical processing of TRAP is important for the reduction of the diiron centre and thereby for obtaining optimal enzyme activity.

2. Expression and regulation of TRAP in inflammatory responses.
    a) TRAP is expressed by both resident and inflammatory macrophages.
    b) TRAP mRNA and enzyme activity is up regulated by Th1 cytokines.
    c) IFNγ in combination with LPS alters distribution of TRAP intracellularly.
    d) Macrophage secreted monomeric TRAP can induce proliferation and differentiation of adipocytes.

Future perspectives

Thorstein Veblen rightly said "The outcome of any serious research can only be to make two questions grow where only one grew before", and consequently this thesis leaves several important questions to be answered in the future.

In this thesis, one interesting perspective put forward is that the two isoforms of TRAP, monomeric (5a) and proteolytically processed (5b) TRAP, are differently expressed and also might have dissimilar physiological functions. One increasingly important question to study then is how the proteolytic processing of TRAP is regulated in cells in response to different stimuli, for example: what dictates if a macrophage expresses and secretes monomeric or proteolytically processed TRAP, since this clearly can lead to different physiological events. Another interesting question is if TRAP regulates the bioactivity of osteopontin in cells that expresses the proteolytically processed isoform of TRAP. This could be relevant to study in peripheral ganglia cells which are known to express both TRAP and osteopontin but also certain macrophages and kidney epithelial cells. Lastly, the impact of TRAP on the proliferation and differentiation of adipocytes raises the question if there is a TRAP receptor present in certain cell types. It has previously been shown by Sheu and co-workers that TRAP can interact with the TGF-β receptor interacting protein TRIP-1[103], however, it remains to be seen if this is true also for other cell types.

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