Role of reactive oxygen species in rheumatoid arthritis synovial T lymphocytes

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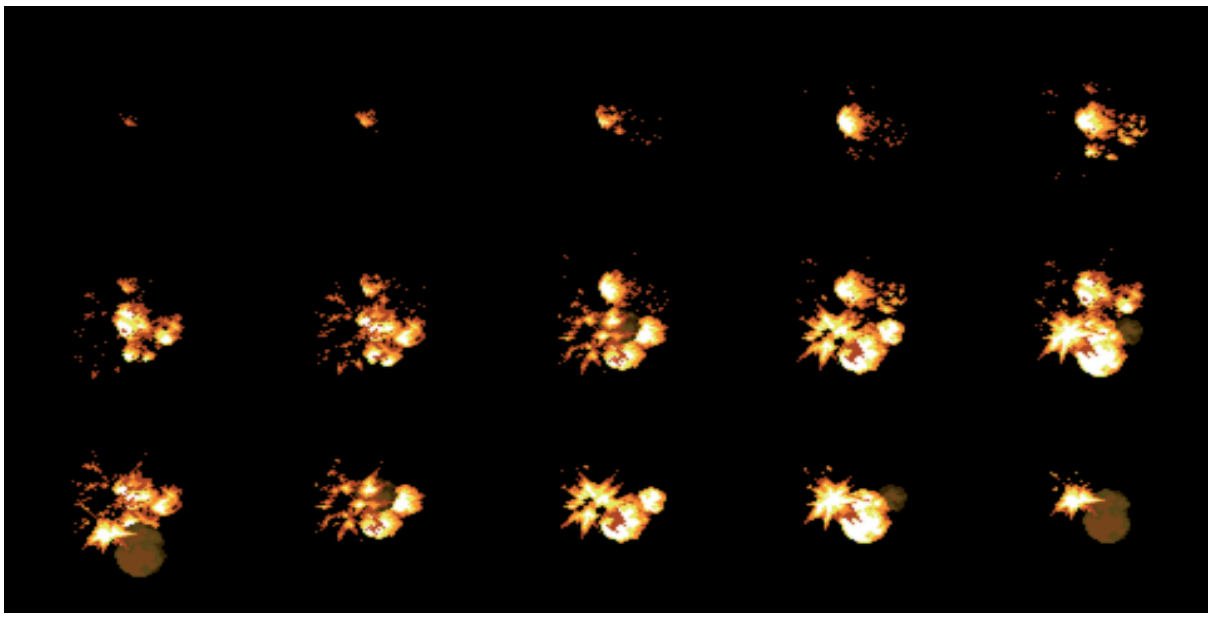

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ROLE OF REACTIVE OXYGEN SPECIES IN RHEUMATOID ARTHRITIS SYNOVIAL T LYMPHOCYTES

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Chapter1:
General Introduction

1. Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic, systemic, inflammatory joint disease affecting about 1% of the population. Although new diagnostic tools (such as detection of anti-CCP antibodies) and novel therapies (such as anti-TNFα therapy) have contributed to a greater understanding of the pathogenesis of RA, its etiology remains unknown. The inflamed synovium in patients with RA is characterised by infiltration with a diverse spectrum of leucocytes: predominantly macrophages and T lymphocytes (comprising 30-40% of the cellular infiltrate each), but also B cells and dendritic cells, each cell type playing a specific role in the chronic inflammation. In the course of the inflammation, proliferation of synovial fibroblasts is induced together with the production of proteolytic enzymes, which leads to the destruction of adjacent cartilage and bone. If left untreated, these processes lead to deformity and disability.
Recent clinical studies with abatacept® (CTLA4Ig), a selective inhibitor of T cell costimulation have demonstrated that T cells play a central role in RA (1). T lympocytes isolated from the rheumatic joint display a number of specific characteristics. While expressing markers of recent activation, including CD45RO and CD69 and proinflammatory surface receptors such as CD154 and TNF-α receptor (2,3), T cells obtained from SF exhibit low proliferative and low cytokine-responses after T cell receptor (TCR)-triggering (4). In recent years, several important aspects of the molecular basis underlying SF T cell hyporesponsiveness have been elucidated. It was recently reported that the hyporesponsive state of SF T cells was related to chronic oxidative stress in these cells (4,5). T cell hyporesponsiveness was associated with decreased intracellular levels of glutathione (GSH) (5), an intracellular nonprotein thiol with both oxidant scavenging and redox regulating capacities. In vitro replenishment with the antioxidant *N*-acetyl-L-cysteine (NAC) restored GSH equilibrium concomitant with partial rescue of SF T lymphocyte hyporesponsiveness and restoration of TCR-dependent signaling defects. In contrast, treatment of T cells from healthy volunteers with DL-buthionine (*S,R*)-sulfoximine (BSO) results in depletion of intracellular GSH and mimicked the observed hyporesponsiveness and most of the signaling defects (5,6).

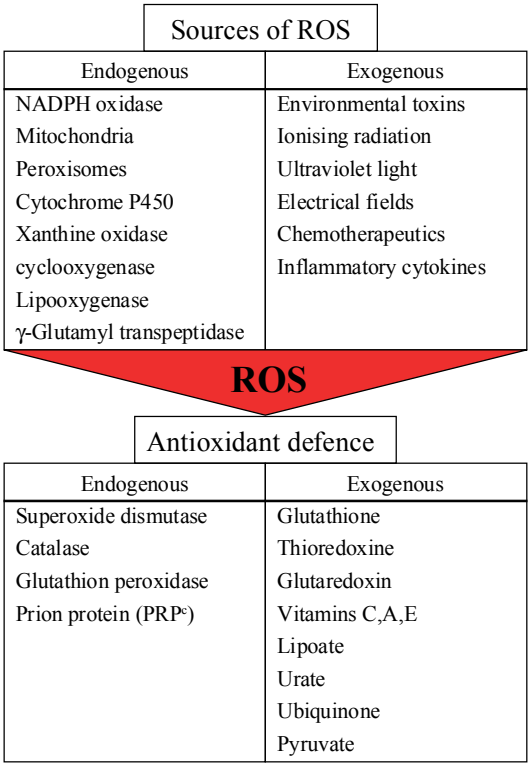
2. Oxidative stress

2.1 Introduction

Molecular oxygen (dioxygen; O_2) is essential for the survival of all aerobic organisms. In mitochondria, energy is generated by the consumption of oxygen and the reduction of oxygen to water by the transfer of four electrons without formation of intermediates. In this process of oxidative phosphorylation the oxidoreduction energy of mitochondrial electron transport is converted to the high-energy phosphate bond of ATP via a multicomponent NADH dehydrogenase enzymatic complex. Partially reduced and highly reactive metabolites of O_2 are formed during these (and other) electron transfer reactions. These O_2 metabolites include superoxide anion ($O_2\cdot$) and hydrogen peroxide (H_2O_2), formed by one- and two-electron reductions of O_2, respectively. In the presence of transition metal ions, the even more reactive hydroxyl radical ($OH\cdot$) can be formed. These partially reduced metabolites of O_2 are often

referred to as "reactive oxygen species" (ROS) due to their higher reactivities relative to molecular O_2.

ROS from mitochondria and other cellular sources have been traditionally regarded as toxic by-products of the cellular metabolism with the potential to cause damage to lipids, proteins, and DNA (7). To protect the host against the damaging effects of ROS, many antioxidative defense mechanisms have evolved. In addition to several antioxidant enzymes such as superoxide dismutase (which reduces $O_2\cdot$ to H_2O_2), catalase, and glutathione peroxidase (which reduces H_2O_2 to H_2O), a broad range of non-enzymatic scavengers exists. “Oxidative stress” may be broadly defined as an imbalance between oxidant production and the antioxidant capacity of the cell to prevent oxidative injury, and is thought to contribute to the pathogenesis of a number of human diseases including RA, atherosclerosis, pulmonary fibrosis, cancer, neurodegenerative diseases, and ageing.



During the past decade however, reduction-oxidation (redox) reactions that generate ROS (including H_2O_2, $O_2\cdot$ and $OH\cdot$) have been identified as important chemical mediators in the regulation of signal transduction processes involved in cell growth and differentiation (reviewed in 8-17). A wide range of ligand-receptor interactions has been shown to generate intracellular ROS. ROS can activate a variety of members of signalling pathways such as protein kinases, protein phosphatases and transcription factors. Although ROS are generated intracellularly by several sources, including mitochondria, the NADPH oxidases in particular have been related to receptor-mediated signalling. The rapid kinetics of activation and inactivation and the intrinsic characteristics of free radicals make them ideal intracellular messengers, that allow a tight up- and downregulation of intracellular ROS levels within the short time required for the transduction of signals from the plasma membrane to the nucleus.

From one point of view the delicate intracellular balance between oxidising and reducing equivalents reveals a complex interplay regulating cell proliferation versus cell cycle arrest or apoptosis. From another point of view it offers a potential interesting target for therapeutic intervention to module cellular function.

2.2 Chemistry of ROS.

The chemical structure of ROS influences the ability of different species to react with specific cellular substrates within the microenvironment in which they are produced. ROS-substrate reactions are likely to form the basis for our understanding of ROS specificity and their mechanisms of action. ROS are often referred to as free radicals, but this is incorrect because not all ROS are free radicals. A free radical is defined as a chemical species possessing one or more unpaired electrons in one of its molecular orbitals. Due to the unpaired electron, free radicals are very unstable and will react with any atom or molecule in their vicinity.

Free radicals can be formed via 3 mechanisms: (a) by the homolytic cleavage of a covalent bound, with each fragment retaining one of the pair of electrons; (b) by the loss of a single electron (oxidation); (c) by the addition of a single electron (reduction).

In biological systems, the most important free radicals are derivatives of oxygen.

Single-electron reduction of oxygen either by enzymatic catalysis or by "electron leaks" from various electron transfer reactions will produce superoxide:

(1) $O_2 + e^- \rightarrow O_2^{-\bullet}$

In contrast to its remarkable stability in many organic solvents, $O_2\cdot$ in aqueous solution is short-lived. This "instability" in aqueous solutions is based on the rapid dismutation of superoxide to hydrogen peroxide:

(2) $2O_2^{-\bullet} + 2H^+ \rightarrow H_2O_2 + O_2$

In mammalian cells this reaction is additionally catalysed by the enzyme superoxide dismutase (SOD). SOD speeds up this reaction almost 10^4-fold. This implies that any reaction of $O_2\cdot$ in aqueous solution will be in competition with SOD or, in its absence, with the spontaneous dismutation reaction itself. Thus in most biological systems, generation of $O_2\cdot$ usually results in the formation of H_2O_2.

Although in eukaryotic cells dismutation of $O_2\cdot$ probably accounts for much of the H_2O_2 production, H_2O_2 can also be formed by direct two-electron reduction of O_2, a reaction mechanism shared by a number of flavoprotein oxidases:

(3) $O_2 + 2e^- + 2H^+ \rightarrow H_2O_2$

Hydrogenperoxide itself is not a free radical, but because of its involvement in the production of free radicals, it falls in the category of reactive oxygen species (ROS). In the presence of transition metals (i.e. metal ions with variable oxidation numbers, for example iron as Fe^{2+}/ Fe^{3+}, or copper as Cu^+/ Cu^{2+}) hydrogen peroxide can easily break down to produce the most reactive and damaging of the oxygen free radicals, the hydroxyl radical ($^{\bullet}OH$): this equation is often referred to as the Fenton reaction.

(4) $H_2O_2 + Fe^{2+} \rightarrow {}^{\bullet}OH + OH^- + Fe^{3+}$.

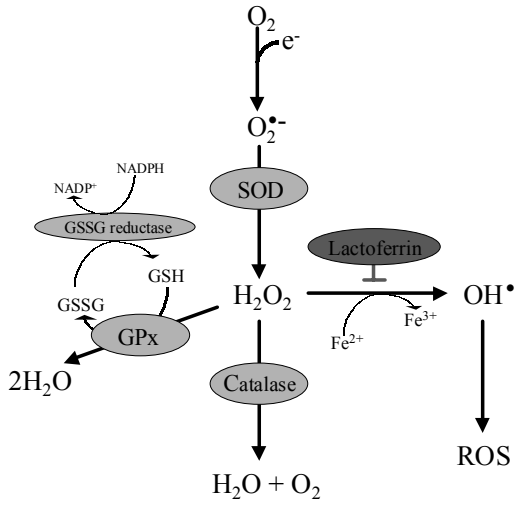
Although H_2O_2 is relatively stable and can diffuse through plasma membranes, hydrogen peroxide generally is rapidly converted into H_2O by catalase (mainly localised in peroxisomes) or glutathione peroxidase (GSH-Px; cytosolic localisation).

(5) $2H_2O_2 \rightarrow$ catalase $\rightarrow 2H_2O + O_2$

Catalase is one of the most efficient enzymes known. It is so efficient that it cannot be saturated by H2O2 at any concentration. The selenium-containing Glutathione peroxidases are also reactive towards fatty acids hydroperoxides and is dependent on the glutathione (GSH) recycling system, in which the oxidised glutathione (GSSG) is rapidly reduced back to the tripeptide GSH (γ-glutamyl-cysteinyl- glycine) by action of the enzyme GSH reductase, and through consumption of NADPH. Glutathione as such can be considered the central antioxidant in

regulating the intracellular redox state, through its critical role in the removal of peroxides and other reactive metabolic intermediates, either by directly inactivating them or by acting as a substrate for the enzymes which catalyse their removal.

Figuur 1: Schematic overview of ROS metabolism.
SOD: superoxide dismutase.
GPx: GSH peroxidase



2.3 The production of free radicals in cells.
The concept that ROS act as regulators of cell function, e.g. by acting as signalling molecules, has gained significant recognition over the past several years from studies done in laboratories worldwide. The evidence supporting this concept is based largely on the following criteria: *1*) growth factors and cytokines are capable of generating ROS in a number of different cell types, *2*) antioxidants and inhibitors of ROS-generating enzymatic systems block specific growth factor- and/or cytokine-activated signalling events or physiological effects, and *3*) exogenous addition of oxidants activates the same cytokine- and/or growth factor-mediated signalling pathway or produces the same physiological effects.
2.3.1 Ligand induced ROS production
In most cells a variety of cytokines, growth factors and hormones that bind receptors of different classes have been reported to generate intracellular ROS, following ligation to their respective receptor (Table 1).

Table 1

Ligand	Reactive Species	Enzymatic source	Cell or tissue	Functional (Or pathologic?) effects	Ref. No.
Cytokines					
TNF-α	unspecified	Mitochondria	Fibrosarcoma	NF-κB activation, IL6 induction Cytotoxicity	117
	$O_2^{\bullet}$, H_2O_2	NAD(P)H oxidase	Fibroblasts	unknown	118
	$O_2^{\bullet}$	unknown	Endothelial cells	VCAM, ICAM expression	119-121
	$O_2^{\bullet}$	NAD(P)H oxidase	Mesangial cells	MCP-1, CSF-1 expression	122
	H_2O_2	Flavoprotein oxidase	Chondrocytes	Mitogenesis	123
IL-1	$O_2^{\bullet}$, H_2O_2	NAD(P)H oxidase	Fibroblasts	unknown	124
	$O_2^{\bullet}$	NAD(P)H oxidase	Endothelial cells	MCP-1, IL-6	119
IFN-γ	$O_2^{\bullet}$	unknown	Endothelial cells	MCP-1, IL-6, VCAM	125, 126
Receptors tyrosine kinases					
PDGF	H_2O_2	unknown	BALB/3T3 cells	"competence factor" for cell growth	127
	H_2O_2	Flavoprotein oxidase	Smooth muscle cells	Mitogenesis, MAPK activation	128
	$O_2^{\bullet}$	unknown	NIH/3T3 cells	NOS expression, PGE_2 release	129
	$O_2^{\bullet}$	Flavoenzyme	Smooth muscle cells	NF-κB-dependent MCP-1 induction	130
EGF	H_2O_2	unknown	Epidermoid carcinoma	Tyrosine phosphorylation, cell growth	131
	Unspecified	unknown	HaCa T cell line	JNK activation	132
	H_2O_2	unknown	Hepatocytes	Carcinogenesis, apoptosis	133, 134
	H_2O_2	lipoxygenase	Human keratinocytes	PLA_2, MAPK activation	135
	$O_2^{\bullet}$	lipoxygenase	PC12	Cell growth	136
FGF-2	H_2O_2	Flavoprotein oxidase	Chondrocytes	Induction of c-fos	137
	$O_2^{\bullet}$	unknown	Lung fibroblasts	Mitogenesis	138
Receptor serine/threonine kinases					
TGF-β1	H_2O_2	unknown	Mouse osteoblasts	Growth inhibition; Erg1-induction	139
	H_2O_2	unknown	Endothelial cells	MAC-1	140
	H_2O_2	NADH oxidase	Lung fibroblasts	Apoptosis	141
	H_2O_2	unknown	Hepatocytes	Apoptosis	142
	H_2O_2	Suppression of AOE	Pancreatic beta cells	Apoptosis	143
G-coupled receptors					
Angiotensin II	$O_2^{\bullet}$, H_2O_2	NAD(P)H oxidase, $p22^{phox}$	Smooth muscle cells	Cell hypertrophy, p38 activation	144
	$NO^{\bullet}$	Nox4	Mesangial cells	PK B	145
	H_2O_2	PLD dependent oxidase	Smooth muscle cells	Proliferation, hypertrophy	146
	$O_2^{\bullet}$	NAD(P)H oxidase	Endothelial cells	NO induction	147

MCP-1, monocyte chemoattractant protein-1; CSF-1, colony-stimulating factor-1 HSP27, 27-kDa heat shock protein; COX-2, cyclooxygenase-2 PDGF, platelet-derived growth factor; MAPK, mitogen-activated protein kinase; NOS, nitric oxide synthase; EGF, epidermal growth factor; JNK, c-Jun NH_2-terminal kinase; PLA_2, phospholipase A_2; HB-EGF, heparin-bound EGF; FGF-2, fibroblast growth factor-2; IGF-I, insulin growth factor I; HGF, hepatocyte growth factor; NO, nitric oxide; TGF, transforming growth factor; PLD, phospholipase D; PK B, proteine kinase B

2.3.2 Cellular sources of ROS

(1) Mitochondria

As stated earlier, any electron-transferring protein or enzymatic system can result in the formation of ROS as "by-products" of electron transfer reactions. This "unintended" generation of ROS in mitochondria accounts for ~1-2% of total O_2 consumption under reducing conditions. Due to high concentrations of mitochondrial SOD, the intramitochondrial concentrations of $O_2\cdot$ are maintained at very low steady-state levels. Thus unlike H_2O_2, which is capable of diffusing across the mitochondrial membrane into the cytoplasm, mitochondria-generated $O_2\cdot$ is unlikely to escape into the cytoplasm. However, the potential for mitochondrial ROS to mediate cell signalling has gained significant attention in recent years, particularly with regard to the regulation of apoptosis (18-22). There is evidence to suggest that tumor necrosis factor (TNF-α) and interleukin (IL)-1-induced apoptosis may involve mitochondria-derived ROS (23-25). It has

also been suggested that the mitochondria may function as an "O_2 sensor" to mediate hypoxia-induced gene transcription (26,27).

(2) Endoplasmatic reticulum

Another site of electron transport is the endoplasmatic reticulum (ER) where electrons leak from the NADPH P450 reductase. Smooth ER contains enzymes that catalyse a series of reactions to detoxify lipid-soluble drugs and other harmful metabolic products. The enzyme can oxidise unsaturated fatty acids and xenobiotics and reduce molecular O_2 to produce $O_2\cdot$ and/or H_2O_2 (28). Although there does not appear to be a direct link between ER-derived oxidants and growth factor signalling, there is evidence for redox regulation of ER-related functions such as protein folding and secretion (29,30).

Nuclear membranes also contain cytochrome oxidases and electron transport systems that resemble those of the ER but the function of which is unknown. It has been postulated that electron "leaks" from these enzymatic systems may give rise to ROS that can damage cellular DNA in vivo (31).

(3) Soluble enzymes

In addition to intracellular membrane-associated oxidases, soluble enzymes such as hypoxanthine/xanthine oxidase oxidase, aldehyde oxidase, flavoprotein dehydrogenase and tryptophan dioxygenase can generate ROS during catalytic cycling. The most extensively studied of these is the $O_2\cdot$-generating xanthine oxidase, which can be formed from xanthine dehydrogenase after tissue exposure to hypoxia (32). Xanthine oxidase is widely used to generate $O_2\cdot$ in vitro to study the effect of ROS on diverse cellular processes; however, no studies have implicated a direct physiological role for endogenous xanthine oxidase in cell signalling.

(4) Lipid metabolism

In contrast to these relatively recent reports of pyridine- and flavin-linked oxidase, enzymes involved in phospholipid metabolism have been known to exist for several decades. Membrane phospholipids, in addition to their structural role in providing membrane integrity, are substrates for the action of the phospholipases (PLs) PLA_2, PLC, and PLD. Although these enzymes are important for the generation of lipid second messengers, they generally have not been associated with ROS production in nonphagocytic cells.

PLA_2 hydrolyses phospholipids to generate arachidonic acid. Arachidonic acid then forms the substrate for cyclooxygenase- and lipoxygenase (LOX)-dependent synthesis of the four major classes of eicosanoids: prostaglandins, prostacyclins, thromboxanes, and leukotrienes. These synthetic pathways encompass a series of oxidation steps that involve a number of free radical intermediates. Arachidonic acid metabolism, including the LOX pathway, which leads to leukotriene synthesis, has been reported to generate ROS (33). Recently, both phospholipase and LOX activity have also been implicated in redox-regulated signalling by ANG II (34,35), epidermal growth factor (EGF) (36), and IL-1 (37). Also, TNF-α induced apoptosis has been reported to be mediated by a LOX-dependent but mitochondrial ROS-independent mechanism (38).

(5) NADPH oxidase

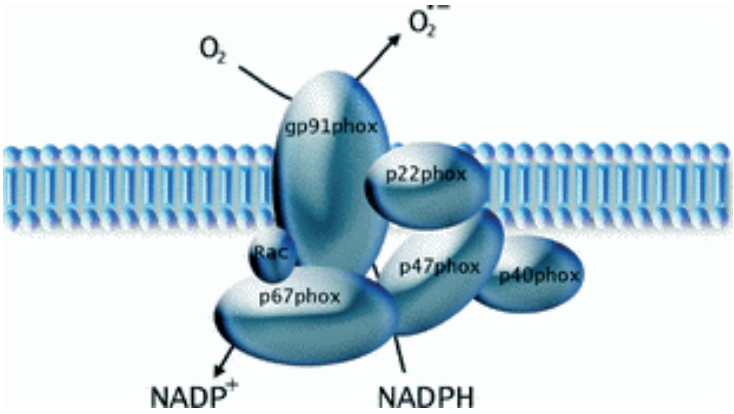
By far the most important multienzyme complex involved in the generation of ROS in signal transduction pathways is the NADPH oxidase. This plasma membrane-associated oxidase has been implicated as the source of most growth factor- and/or cytokine-stimulated oxidant production (39-44), although the precise enzymatic sources have yet to be fully characterised.

Oxidative burst in phagocytic cells

In neutrophils the membrane bound NADPH oxidase produces toxic ROS, which are used in the destruction of microbial pathogens. The transfer of electrons occurs from NADPH on the inner aspect of the plasma membrane to O_2 on the outside. During phagocytosis, the plasma membrane is internalised as the wall of the phagocytic vesicle, with what was once the outer membrane surface now facing the interior of the vesicle. This targets the delivery of $O_2\cdot$ and its reactive

metabolites internally for localised microbicidal activity (45). The neutrophil enzyme consists of 4 major subunits: a plasma membrane spanning cytochrome b-558 composed of the subunits gp91-phox and p22-phox and the 2 cytosolic components p47-phox and p67-phox. Upon stimulation, the small G protein Rac2 (Rac1 in mouse macrophages) mediates the assembly of the enzyme complex where the cytosolic components must join with the membrane bound heterodimer cytochrome b-558 for activation of the NADPH oxidase.

Figure 2: NADPH oxidase complex



The NADPH oxidase of non-phagocytic cells

A ligand-activated enzyme with NADPH/NADH oxidase activity has been shown to be present in a variety of non-phagocytic cells including endothelial cells (46,47), smooth muscle cells (48-50), chondrocytes (51, 52) and fibroblasts (53). However, whether all components of the NADPH oxidase of the phagocyte are expressed is still not clear.

In vascular smooth muscle cells angiotensin II (ANG II)-induced hypertension is mediated, at least in part, by direct interactions between $O_2\cdot$, generated by a NAD(P)H-like oxidase, and $p22^{phox}$ has been shown to be a functional component of this ANG II-stimulated oxidase (54). TNF-α also stimulates $O_2\cdot$ production in vascular smooth muscle cells by a $p22^{phox}$-based NADH oxidase and appears to upregulate $p22^{phox}$ gene expression in these cells (55). However, the smooth muscle cells appear to express $p22^{phox}$, $p47^{phox}$ but not $gp91^{phox}$ (56). More recently, Suh et al. (57) demonstrated that *Nox-1* (formerly mox-1for mitogenic oxidase), a gene encoding a homologue of the catalytic subunit of the phagocytic $gp91^{phox}$, is expressed in a number of tissues including vascular smooth muscle. In this study, *Mox-1* expression in NIH/3T3 cells was associated with increased $O_2\cdot$ production, serum-stimulated cell growth, and a transformed phenotype. This study suggests that an $O_2\cdot$-generating oxidase similar (but not identical) to the phagocytic NADPH oxidase is present in some nonphagocytic cells where it functions primarily as a regulator of cellular growth responses.

2.3.3 Signalling pathways leading to ROS production in non phagocytic cells

Although a number of growth factors and cytokines binding to different classes of receptors have been shown to raise intracellular ROS, little is known about intracellular pathways leading to ROS generation.

ROS generation in response to cytokine receptor stimulation has been reported for TNF-α, IL-1, and interferon-γ and has been mainly related to the induction of apoptotic pathways resulting in cell death. There is no consensus on the specific species produced, the enzymatic source, or the site of generation of ROS for these cytokines. In the case of TNF, recent reports (58-62) suggest that a mitochondrial source of ROS is required for activation of the transcription factor nuclear factor (NF)-B and NF-B-dependent gene transcription.

In contrast to growth factors, which elicit mitogenic stimuli by the generation of ROS through NADPH-oxidase-like enzymes, it is suggested that TNF-α generates ROS through the mitochondrial respiratory chain during activation of mitochondrial apoptotic pathways (23-25).

ROS production via an NADPH oxidase generally is associated with mitogenesis and differentiation. As mentioned, activation of the oxidase, however, requires the additional participation of Rac2 for assembly of the multi-complex enzyme. During activation, Rac2 binds GTP and migrates to the plasma membrane along with the cytosolic components to form the active oxidase complex. A requirement for Rac1 in the activation of the mitogenic oxidase has been demonstrated in nonphagocytic cells (63,64). The insert region in Rac1 (residues 124-135) appears to be essential for $O_2\cdot$ production and stimulation of mitogenesis in quiescent fibroblasts but not for Rac1-induced cytoskeletal changes or activation of Jun kinase (64).

Another GTP-binding protein, p21Ras, appears to function upstream from Rac1 in oxidant-dependent mitogenic signalling (65,66). Stable transfection of the same Ras plasmid ($H\text{-}Ras^{V12}$) in fibroblasts induced cellular transformation and constitutive production of large amounts of $O_2\cdot$ (65). Sundaresan et al. (66) showed that expression of dominant negative Rac1 not only inhibited the growth factor- and/or cytokine-generated rise in intracellular ROS in NIH/3T3 cells but also ROS production in cells that overexpress a constitutively active isoform of Ras ($H\text{-}Ras^{V12}$).

2.3.4 Molecular Targets of ROS

Although ROS have been demonstrated to be involved in many signal transduction pathways the molecular targets are not yet precisely defined. There is growing evidence, however, that redox regulation might occur at multiple levels in the signalling pathways from receptor to nucleus.

Protein Tyrosine Kinases and Phosphatases.

Although binding of growth factors to their receptors and the subsequent activation of their receptor tyrosine kinase (RTK) are at the top of signalling pathways and are generally upstream from intracellular generated ROS, receptor kinases and phosphatases themselves may also be targets of oxidative stress. Oxidants such as H_2O_2, vanadate and their reaction product pervanadate have been shown to induce phosphorylation of the epidermal growth factor (EGF) receptor and the platelet derived growth facto (PDGF) (67-69). Also, lysophatidic acid-induced activation of the EGF receptor appears to be mediated by the intermediate formation of ROS (70). A study by Knebel et al. (71) suggests that the mechanism of these effects may be related to ROS-mediated inhibition of the dephosphorylation of RTKs by inactivation of membrane-bound protein tyrosine phosphatase(s).

Not only RTK's, but also many other PTK such as non-RTKs belonging to the Src family (Src kinases) are activated by free radicals. H_2O_2, pervanadate and UV light have been shown to induce tyrosine phosphorylation and the activation of many PTK's including Lck (72,73), Fyn (74) and ζ-associated polypeptide of 70kDa (ZAP-70)(75) in T cells. Although oxidative stress apparently stimulates many PTK's in cells, there is no evidence so far that PTK's are direct targets of H_2O_2. The study by Knebel et al. (71) suggests that the mechanism of these effects may be related to ROS-mediated inhibition of the dephosphorylation of RTKs by inactivation of membrane-bound protein tyrosine phosphatase(s)'s PTP's.

The reports on the activation of RTK's and PTK's relate primarily to the effects of exogenously added oxidants. The significance of such actions in specific growth factor- or cytokine-mediated signalling is less clear, although this does not exclude the possibility that, under certain (pathological?) conditions associated with oxidative stress, ROS may directly activate cell surface receptors.

Lipid Metabolism

PLCγ produces inositol 1,4,5-triphosphate [Ins(1,4,5)P_3] which mobilises Ca^{2+} from the intracellular pool to the cytosol, and Diacylglycerol (DAG) which activates PKC.

H_2O_2 induces the phosphorylation of PLCγ together with the production of inositol phosphates (76-78), and oxidants also have been shown to activate phospholipase A2 (PLA2) (79,80) and phospholipase D (PLD)(81,82). Whether phospholipases are direct target of free radicals or act in concert with activated PTK's remains unclear. Activated RTK's can stimulate PI3-kinase, which in turn activates several protein kinases such as Akt , PKCη and PKCε. Furthermore, oxidant mediated Ca^{2+} mobilisation (mediated by Ins(1,4,5)P_3) is blocked by herbimucin A, a PTK inhibitor (83) and partly suppressed in mutant cells that do not express Syk or Lyn(84). This suggests that the Ins(1,4,5)P_3 is activated through the activation of upstream RTK's.

Ca^{2+} signalling

Ins(1,4,5)P_3 generated by PLCγ binds to the Ins(1,4,5)P_3 receptor/ Ca^{2+} channel on the endoplasmatic reticulum, thereby elevating the cytosolic Ca^{2+} level. After the mobilisation of Ca^{2+} from the intracellular pool, there is an influx from the extracellular space.

Oxidative stress increases the cellular Ca^{2+} levels consistent with the production of inositol phosphates. Additionally, both $O_2\cdot$ (85-887) and H_2O_2 (88) have been shown to inhibit the activity of ATP-dependent Ca^{2+} of the SR, which would result in passive diffusion of SR Ca2+ into the cytosol. These effects may be more important in oxidative stress responses (89) than in receptor-mediated signalling by growth factors and/or cytokines.

Small GTP-ases

The superfamily of the Ras-like small GTPases comprises a large family of 20-25kDa proteins, which bind guanine nucleotides very closely and cycle between an inactive GDP-bound state and the active GTP-bound configuration. An upstream signal stimulates the exchange of GDP for GTP and is catalysed by Guanosine exchange factors (GEFs). The GTP-bound form is converted back to the GDP bound form due to intrinsic GTPase activity and is catalysed by GTPase activating proteins (GAPs). Based on sequence and apparent functional homology, the superfamily of Ras-related proteins can be subdivided into the Ras, Rac/Rho, Rab, Arf, Sar, Ran and Rad subfamilies. The Ras subfamily consists of Ras (with 3 isoforms H-, K- and N-Ras), R-Ras, TC21 and Rap (4 isoforms: Rap1A,B and Rap2A,B). Ras is the archetype of the family of small GTPase (for review cfr Bos 90,91), and was originally found as the oncogene in animal tumour viruses. Subsequently 15-30% of all human tumours have been found to contain an activated allele of the Ras gene. Although Ras is situated upstream is signalling pathways leading to intracellular ROS production [constutive active Ras generates free radicals in fibroblasts whereas dominant negative Ras inhibits receptor induced ROS production (64)], Ras itself was also shown to be activated by oxidative stress (92).

Serine/Threonine Kinases and Phosphatases.

The MAPKs comprise a large family of PKs that include ERK1 (p44MAPK)/ERK2 (p42MAPK), JNKs (also known as the stress-activated PKs), and p38 MAPKs. Because the MAPK pathways mediate both mitogen- and stress-activated signals, there has been significant interest in the redox regulation of these pathways. A number of groups (93-98) have

demonstrated the ability of exogenous oxidants to activate the ERK MAPK pathway. The mechanism(s) for this effect is unclear, and the precise molecular target(s) is unknown. Some studies suggest that ROS-mediated ERK activation may be an upstream event at the level of growth factor receptors (95), Src kinases (99), and/or p21Ras (100). Another potential mechanism for this effect may be oxidant-induced inactivation of protein tyrosine phosphatases (PTPs) (101). Most reports imply JNK and p38, but the mitogenic ERK MAPK pathway and other members of the MAPK family have also been implicated as potential targets of ROS. Big MAPK-1 (BMK-1) appears to be much more sensitive than ERK1/ERK2 to H_2O_2 in several cell lines tested and suggests a potentially important role for BMK-1 as a redox-sensitive kinase (102). Although not directly related to the MAPK family, p66shc, a splice variant of the p52shc/p46shc protein involved in mitogenic signalling from activated receptors to p21Ras, was found to be a target of H_2O_2 and responsible for mediating stress apoptotic responses (103).

2.3.5 Redox regulation of Trancription Factors

Oxidative radical stress induces the expression of many genes such as c-fos, c-jun, c-myc and a gene for haeme oxygenase. Such expression is mediated by transcription factors in response to the activation of upstream cellular signalling pathways. Indeed, treatment of cells with H2O2 induces the activation of the transcription factor activator protein-1 (AP-1) and nuclear factor κB (NF-κB) in some cell types. However, it should be noted that many transcription factors are directly regulated by redox in the opposite manner.

NF-κB

NF-κB is a transcription factor that regulates the expression of a number of genes involved in immune and inflammatory responses and has long been considered oxidant responsive (104,105). Free radicals activate NF-κB in response to the activation of upstream cellular signalling pathways. However, to be functional, for NF-κB the DNA binding region need to be kept in a reduced state. The DNA-binding activity of NF-κB is inactivated by treatment with NEM and diamide. Conversely, DTT and β-mercaptoethananol enhance its DNA binding. Moreover, a critical step in NF-B activation is the phosphorylation of IB. The upstream IB kinases that phosphorylate IB are also subject to oxidative stress downmodulating NF-κB activation. Li and Karin (106) found that this redox-regulated effect is observed to occur downstream from the IB kinases, at the level of ubiquitination and/or degradation of IB.

Recent reviews (107, 108) on this subject have therefore emphasised the dual regulation of NF-κB by oxidative stress and the importance of the recognition that a redox-dependent activation of NF-B is cell and stimulus specific as opposed to the concept that oxidative stress is a common mediator of diverse NF-B activators.

AP-1

The oncogene products Jun and Fos form AP-1 by hetero (Jun/Fos) or homo (Jun/Jun) dimerisation, through their leucine-zipper structure located at the C terminal region. However, unlike NF-B, however, AP-1 appears also to be activated by antioxidants (109, 110). In vitro experiments suggest that a single cysteine residue in the highly conserved tri-amino acid sequence Lys-Cys-Arg of the DNA binding domain of Fos and Jun proteins confers redox sensitivity to AP-1 (111). The reduced state of this cysteine residue is essential for its DNA-binding and transformation activity. The modification of this residue by a sulphydryl-modifying agent such as N-ethylmaleimide (NEM) reduces its activity, whereas treatment with reductants such as dithiothreitol (DTT) enhances DNA binding (111). So oxidative stress implicates downregulatation of AP-1 activity. Further evidence suggests that the redox regulation of AP-1 DNA binding is facilitated by the reducing activity of redox factor-1 (Ref-1) protein that may act directly on this critical cysteine residue (112).

Other transcription Factors

Analogous to AP-1 the conformation of the DNA binding of Myb (113), Sp-1 (114) and egr-1 (115) seem to be regulated by similar redox mechanisms as AP-1 with a cystein residue in a region of the helix-turn-helix of which the reduced state is essential.

The tumor suppressor p53 receives much more complicated redox regulation through many cysteine residues (116). This factor binds to Zn through many cysteine residues and act as a transcription factor inducing cell cycle arrest or apoptosis. While the activity of p53 is modulated by copper through a redox mechanism, treatment with NEM or diamide disrupts wild-type p53 conformation and inhibits its DNA binding activity.

Table 2: Signalling molecules regulated by redox in the cells

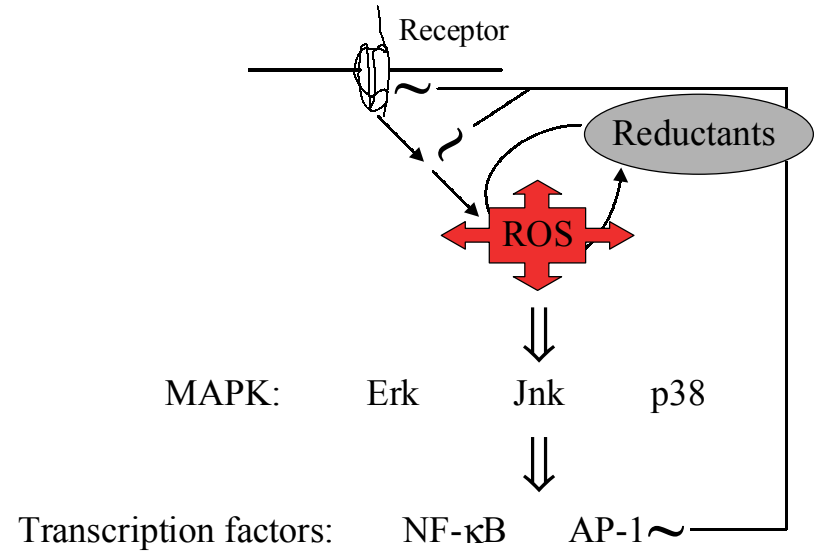
Signalling molecule	Effect of oxidants
1. Prot Tyrosine kinase EGF receptor, insulin receptor, PDGF receptor, Src, Lck, Fyn, ZAP-70, Syk, Lyn, Fgr, Hck, Btk, Ltk	Activation
2. Prot Tyrosine phosphatase	Inactivation
3. Lipid signalling PLC, PLD, PLA_2,PI 3 kinase	Activation
4. Ca2+ signalling Ins(1,4,5) receptor, Ca^{2+}ATPase	Activation
5. small G protein Ras	Activation
6. Protein serine/threonine kinase MAPK, JNK, p38, BMK1, Akt, S6 kinase, PKC	Activation Activation/ inactivation
7. Protein serine/threonine phosphatase PP1, PP2A, calcineurin	Inactivation
8. Transcription factors AP-1(c-fos,c-jun), NFκB, rel, USF,TTF-1, GR, BPV1E2, NFI, Myb, NF-Y, p53, PEBP2/AML, Oct2, Egr-1, BZLF1, Ets, GABP, ATF, CREB, TTF, Ku	Activation/ inactivation

2.4 Conclusion

The information obtained over the last decade has shown that (*1*) a large number of growth factors and cytokines are capable of generating ROS in a number of different cell types, (*2)* inhibitors of ROS-generating enzymatic systems block specific growth factor- and/or cytokine-activated signalling events or physiological effects, and *3*/ exogenous oxidants activate the same cytokine- and/or growth factor-mediated signalling pathway or produces the same physiological effects. This has led to the hypothesis that ROS act as vital mediators in signal transduction. Moreover, the intrinsic characteristics of free radicals make them ideal intracellular messengers.

Anti-oxidant systems from their part are crucial for maintaining a reduced state of most signalling proteins and transcription factors, critical for optimal functioning. Depletion of anti-oxidant systems will therefore lead to cell arrest or apoptosis. On the other hand supplementation of anti-oxidants will scavenge intracellular ROS, thereby counteracting their function.

Figure3: proposed role for ROS as mediator in cellular signaling



3. Oxidative stress in RA

3.1 Evidence of oxidative stress in RA.

It has long been appreciated that oxidative stress plays an important role in RA. RA patients have low concentrations of protective antioxidants: vitamins A, E, C and activities of GSH-Px, and SOD (148,149). Additionally, high levels of the metabolic products of ROS in blood and synovial tissue. RA patients were shown to have high synovial fluid and serum levels of the lipid peroxidation (148-150). Indirect evidence for a role of ROS in cartilage degradation comes from the presence of cartilage-derived peroxidation products (151), a nitrous type II collagen peptide (152), modified low-density lipoprotein (LDL) (153) and oxidized IgG (154) in the serum and urine of patients with arthritis. Since nitrotyrosine, nitrated proteins and oxidized LDL (ox-LDL) have been found to be accumulated in cartilage of arthritic patients, a direct implication of ROS in chronic arthritis has been suggested. Furthermore, a low antioxidant status was shown to be a risk factor for developing RA, according to a case control study within a Finnish cohort of 1419 adult men and women (155). A prospective case-control study within blood donors also showed significant lower β-carotene in patients who developed RA (156).

Treatment with antioxidants was shown to have beneficial effects in animal models of arthritis. High dosis of vitamin C (157), vitamin E (158) and N-acetylcysteine (159) had anti-arthritic effects related to their anti-oxidative potential. Other candidate antioxidants have been identified with potential benefit including selenium, zinc, niacinamine, manganese, β-carotene and bioflavonoids. Nevertheless, clinical trials with RA patients have been disappointing. A randomized, double-blind comparisement of high-dose vitamin E, the most powerfull naturally

occurring lipid-soluble antioxidant, did not show any anti-inflammatory effect as compared to placebo (160). Two placebo-controlled trials with selenium-enriched yeast did not show any clinical benefit (161,162).

3.2 Functional implications of ROS in synovial T lymphocytes

T lymphocytes derived from the synovial fluid from RA patients were shown to suffer from severe oxidative stress, with depletion of intracellular glutathione (5) and upregulation of thioredoxin (163). These findings were thought to underly the hyporesponsive state of SF T cells as demonstrated by the hyporesponsiveness to mitogenic stimuli of SF T cells as well as defective signalling events (e.g. the perturbed membrane localisation of LAT, thus preventing TCR-induced LAT phosphorylation and subsequent recruitment of phospholipase C-γ1 into signalling complexes (5,6). In vitro replenishment with the anti-oxidant NAC restored GSH equilibrium, concomittant with recovery of TCR-dependent LAT phosphorylation and partial rescue of SF T cell hyporesponsiveness, while treatment of T cells from healthy volunteers with DL-buthione(S,R)-sulfoximine (BSO) resulted in depletion of intracellular GSH and mimicked the observed signalling defects (5,6).

3.3 Sources of ROS

Several sources of ROS in the synovial joint that could lead to the disturbed redox homeostasis in SF T lymphocytes have been proposed. These include exposure to free radicals liberated by activated phagocytic cells at the site of inflammation (164), ischemia/reperfusion compromised oxygen radical tension in the inflamed joint (165), and generation of hydroxyl radicals by Fe^{2+} released from dying cells (166).

Extracellular ROS

The major cause of disturbed redox homeostasis in RA has been attributed to chronic exposure of the SF T lymphocytes to oxygen radicals liberated by activated phagocytic cells at the site of inflammation (164). Activated leukocytes can rapidly consume extra oxygen (oxidative burst) with the production of free radicals at their plasma membrane, which are released in the environment. Besides the activated macrophages that may function as a source of free radicals, additional mechanisms have been postulated to contribute to the increased production of ROS in inflamed joints.

Ischemia/reperfusion

During movement, there is an increase of intra-articular pressure (which is more severe in inflamed joints) and the synovial blood flow is compromised, generating a fall in synovial O_2-tension (167,168). This relative synovial hypoxia decelerates the mitochondrial oxidative phosphorylation, and gives rise to the concentration of ATP metabolites such as adenosine, inosine and hypoxanthine in the SF, which are substrates of hypoxanthine dehydrogenase. But under ischemic conditions hypoxanthine dehydrogenase is also converted to hypoxanthine oxidase (XO) by –SH oxidation and partial proteolysis. After exercise, when oxygen becomes available through reperfusion, XO oxidises the accumulated hypoxanthine to uric acid with the production of ROS (169).

Catalytic iron

In the rheumatoid synovium high levels of iron are present. This 'catalytic' iron is probably derived from dead cells in part from traumatic microbleeding. In the presence of Fe^{2+}, superoxide and H_2O_2 lead to the formation of the more toxic hydroxyl radical. The possible contribution of iron to the inflammatory process was shown by the exacerbation of arthritis after iron-dextran infusion, which was associated with increased oxidative damage to lipids and proteins (166)

3.3 Consequences of oxidative stress

Chronic oxidative stress leads to diminished T cell response upon antigenic stimulation as was demonstrated not only in synovial T cells (5,6), but also in T lymphocytes isolated from patients

with AIDS (170) and in ageing T lymphocytes(171). Moreover, as reviewed above, free radicals themselves can serve as intracellular second messengers. In particular, the pro-inflammatory transcription factor NF-κB is activated following treatment with H_2O_2 (172). This results in pro-inflammatory gene expression, induction of TNF-α, IL-1, NO-synthetase, and upregulation of ICAM and VCAM, all of which will contribute to the rheumatic inflammation (173, 174).

4. Scope of this thesis

The studies presented in this thesis were designed to elucidate the mechanisms that lead to oxidative stress in synovial T cells in RA, and to investigate whether altering the redox balance through anti-oxidants can modify the course of the disease. **In chapter 2**, we describe a new method for immunohistochemical detection of free radicals on fixed frozen tissues. We show that T lymphocytes that reside within the synovial tissue are characterised by the abundant production of intracellular ROS, suggesting that the oxidative stress of SF T cells originate from intracellular rather then extracellular sources. In **chapter 3** we focus on the molecular basis of the oxidative stress: we delineate a signaling pathway in T cells downstream from Ras, leading to the generation of ROS, while Rap1 (another small GTPase) signaling is required for downregulating agonist and Ras-induced ROS production. Moreover in SF T cells from patients with RA we find constitutive activation of Ras and a block of Rap1, consistent with activation of the signaling pathway leading to chronic intracellular ROS production. As we show in **chapter 4**, the inhibition of Rap1 block in T cells is induced through cell-cell contact with synovial macrophages. Blockade of synovial monocyte signaling to T cell CD28 with CTLA-4-Ig fusion protein relieved Rap1 inhibition and subsequent ROS production. Additionally, introduction of active RapV12 into T cells prevented SF monocyte-induced T cell ROS production. Also, coincubation of T cells with stimulating anti-CD28 antibody and inflammatory cytokines leads to a synergistic increase in T cell ROS production.
In **chapter 5 and 6** we investigate whether supplementation of oxidants can alter the course of rheumatoid arthritis. In a large double-blind placebo-controlled trial, we find no beneficial effect of a nutrient supplement containing most anti-oxidant substances (**chapter 5**). The clinical effects of intravenously administered NAC are described in **chapter 6**, as well as the effects on the functional properties of peripheral blood and synovial fluid T cells.

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CHAPTER 2:

Intracellular free radical production in synovial T lymphocytes of patients with rheumatoid arthritis.

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Abstract

Objective: To investigate the cellular and molecular sources of oxidative stress in patients with rheumatoid arthritis (RA) through analysis of reactive oxygen species (ROS) production in synovium.

Materials: Cytochemical procedures based on the 3,3'-diaminobenzidine (DAB)-Mn^{2+} deposition technique were applied on unfixed cryostat sections of synovium from RA patients and disease controls. For immunophenotyping, sections were fixed after incubation, followed by immunofluorescence staining with FITC-labeled antibodies. FACS analysis of the ROS-reactive dye DCF was used to measure intracellular ROS in T lymphocytes from peripheral blood and synovial fluid. To determine which enzymes produced ROS, different inhibitors were tested.

Results: Large quantities of DAB precipitated in the majority of RA synovial T lymphocytes, indicative of intracellular ROS production. These ROS producing T lymphocytes were observed throughout the synovium. To a lesser extent polymerization of DAB was also observed in other forms of chronic arthritis but was absent in osteoarthritis. DAB staining on cytospins of purified synovial fluid T cells from RA patients confirmed the presence of ROS-producing cells. One of the ROS involved appeared to be hydrogen peroxide, as catalase suppressed intracellular ROS production. Superoxide dismutase, which uses superoxide as a substrate to form H_2O_2, DPI (an inhibitor of NADP(H) oxidase), L-NMMA (an inhibitor of NO synthesis), NDGA (an inhihitor of lipoxygenase) and rotenone (which inhibits mitochondrial ROS production), failed to suppress ROS production.

Conclusion: The present study shows that chronic oxidative stress observed in synovial T lymphocytes is not secondary to exposure to environmental free radicals, but originates from intracellularly-produced ROS. Additionally, our data suggest that one of the intracellularly-generated ROS is H_2O_2, although the oxidase(s) involved in its generation remains to be determined.

Introduction

Reactive oxygen species (ROS) play an important role in a variety of pathological conditions, such as ischemia-reperfusion, carcinogenesis, AIDS and aging. In rheumatoid arthritis (RA), oxidative stress has been described as an important mechanism underlying destructive proliferative synovitis (1). In addition, oxidative stress was found to influence functional characteristics of synovial T lymphocytes with critical implications on proximal and distal T cell receptor (TCR) signaling events (2-5). Chronic oxidative stress in synovial fluid (SF) T lymphocytes inhibits TCR-dependent phosphorylation of pivotal signaling molecules required for efficient T cell proliferation, thus contributing to severe hyporesponsiveness of these cells to antigenic stimulation. Oxidative stress in RA SF lymphocytes also plays a role in NF-κB-dependent gene transcription, e.g. resulting in upregulation of TNF-α and IL-1 (6).

Several sources of ROS in the synovial joint that could lead to the disturbed redox homeostasis in SF T lymphocytes have been proposed. These include exposure to free radicals liberated by activated phagocytic cells at the site of inflammation (7), ischemia/reperfusion compromised oxygen radical tension in the inflamed joint (8), and generation of hydroxyl radicals by Fe^{2+} released from dying cells (9). Recent evidence however, suggests that T lymphocyte oxidative stress originates from intracellular enzyme activity controlled by the small GTPases Ras and Rap1 (10).

The specific detection of free radicals is hampered by several methodological problems due to high reactivity and short life of free radicals. Ongoing oxidative stress is therefore generally

analyzed by measurement of secondary products such as oxidized proteins, peroxidized lipids and their breakdown products, or oxidized DNA. These methods give only limited information however on the cellular source(s) of ROS production *in situ*. Therefore, we attempted to identify the source(s) of synovial oxidative stress using a cytochemical technique based on the principles described for the histochemical localization of ROS production in polymorphonuclear leukocytes (developed by Karnovsky 1994), using 3,3' diaminobenzidine (DAB) and manganese ions (11). Free radicals directly react with DAB, forming an insoluble DAB polymer in a reaction catalyzed by the presence of Mn^{2+}. This technique has been successfully used to identify ROS producing sites (12,13,14).

Methods

Reagents

KCN, NaN_3 and $CoCl_2$ were obtained from Merck (Darmstadt, Germany); polyvinylalcohol (weight average Mr 70 000-100 000), menadione, catalase, diphenyleneiodonium (DPI), rotenone and $MnCl_2 \cdot 4\ H_2O$ were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) was obtained from PGM Chemicals (New Germany, RSA); 3,3'-diaminobenzidine tetrahydrochloride (DAB) from Fluka Chemika (Buchs, Switzerland), Cu/Zn superoxide dismutse (SOD), nordihydroguaiaretic acid (NDGA) from Alexis Biochemicals Benelux (Breda, the Netherlands) and N^G-methyl-L-arginine, acetate salt (L-NMMA) from Molecular Probes Europe (Leiden, the Netherlands).

Patients and tissue processing.

Synovial tissue samples were obtained from patients during knee arthroscopy performed under local anaesthesia. These included 30 patients with established RA (mean disease duration 14 (SD ± 11 years), 3 RA patients with early, DMARD-naive disease (joint symptoms 3 months or less), and 25 control patients (8 psoriatic arthritis, 7 reactive arthritis, and 10 osteoarthritis (OA)). Tissue fragments were snap frozen by immersion in methylbutane (-80°C) and stored in liquid nitrogen until further use.

Peripheral blood (PB) and synovial fluid (SF) T cells from 5 RA patients (mean disease duration of 6 (SD ± 16) years) were purified from mononuclear cells using a negative isolation procedure (T Cell Negative Isolation Kit, Dynal Biotech Norway), which resulted in a > 90% $CD3^+$ cell population. Purified T cells were subsequently spun onto slides using a cytospin centrifuge (Shandon) for histochemical ROS staining or used for ROS detection on FACScan.

Histochemical procedures

Cryostat sections (8μm thick) were freshly cut on a cryostat at a cabinet temperature of -25°C. The sections were placed on Star Frost® adhesive slides (Optic Labor, Friedrichsdorf, Germany) and immediately used for staining. Before use, sections were air dried for 3 min at room temperature. The incubation medium contained 10% w/v polyvinyl alcohol (PVA), dissolved in 100 mM Tris-maleate buffer (pH 8.0). Sodium azide (5mM) was added to inhibit endogenous myeloperoxidase activity. The following components were added shortly before incubation of the cryostat sections: 0-12.5 mM DAB, 0- 6.5 mM $MnCl_2$ and 0-100mM CoCl2 (1). All the compounds were added in strict order and thoroughly mixed from stock solutions to the PVA-containing medium. Incubation lasted for 60 min at 37 °C. After incubation, sections were washed in distilled water to stop the reaction, and mounted in glycerol for light microscopical inspection.

To further characterize DAB^+ cells, an immunohistochemical staining was performed using a double-staining immunofluorescence procedure following incubation in DAB-Mn^{2+} solution.

The synovial cryostat sections were subsequently washed, air-dried and fixed with 4% paraformaldehyde. They were incubated for 30 min at 4°C with unconjugated mouse anti-human monoclonal antibodies against CD3, CD15, CD19 and CD68 (CLB, Amsterdam, the Netherlands), or the appropriate isotype control antibodies. FITC-labeled sheep anti-mouse secondary antibodies (BD 1:1000) were used for visualization. Tissue sections were scored independently by 2 observers for the presence of DAB precipitate in 50-200 FITC-positive cells. Data were expressed as mean value ± SD.

ROS detection on FACScan

Purified T cells were resuspended at 5×10^6 cells/ml in phenol red-free DMEM medium and loaded with 28 μM 6-carboxy-2', 7'-dichlorodihydrofluorescein diacetate - di(acetoxymethyl ester) (6-carboxy-DCF) (Molecular Probes) for 20 min at 37°C. Cells were analyzed on a FACScan (Becton Dickinson) for the mean fluorescence intensity (MFI) of oxidated 6-carboxy-DCF in the FL1 channel at 3 different time points (0, 10 and 20 minutes). The inhibitors of the different enzymes SOD (1000U/ml), catalase (1000U/ml), DPI (5 μM), NMMA (5 μM) and NDGA (10 μM) were added 30 min before DCF. Rotenone (5 μM) was added 60 min before ROS measurement.

Results

Production of free radicals in rheumatoid synovium.

Diaminobenzidine (DAB) precipitates in the presence of free radicals to form an insoluble final reaction product, visible as a brown DAB polymer. Manganese ions (Mn^{2+}) can be added to catalyze DAB polymerization, as Mn^{2+} is oxidized to Mn^{3+} by $O_2^{\bullet-}$ and enabling Mn^{3+} to oxidize DAB. In synovial tissue from 30/30 RA patients, a large number of DAB^+ cells were detectable perivascularly, in leukocyte aggregrates, and in the intimal and subintimal synovial lining (Figure 1). The presence of DAB in the incubation medium already led to strong formation of the insoluble final reaction product (Figure 1A). Addition of Mn^{2+} (0.5 – 5 mM) only marginally enhanced the amount of DAB precipitate, without affecting the localization pattern of the precipitate (Figure 1B). The highest amounts of final reaction product were found when the incubation medium contained 12.5mM DAB and 2.5mM $MnCl_2$. This composition was then used for all further experiments. Addition of cobalt ions (Co^{2+}) to DAB or DAB/ Mn^{2+}-containing media led to the formation of a blue final reaction product, but also did not affect the amount nor the localization pattern of the precipitate (Figure 1C).

Strong staining intensity did not allow recognition of the shape of the nuclei when counterstained with haematoxylin or Giemsa (data not shown) for differentiation between polymorphonuclear cells and mononuclear cells. To further characterize the cellular sources of ROS, cryostat sections of synovium were incubated with Mab against CD3, CD15, CD19 and CD68. Within the synovial $CD15^+$ population (neutrophils), 36% (mean, SD ± 19) of the cells contained intracellular DAB final reaction product. Additionally, 67% (mean, SD ± 22) of $CD3^+$ cells (T lymphocytes) also contained high intracellular DAB precipitate (Figure 2). No DAB precipitate was found in B-cells (CD19) or macrophages (CD68) (data not shown).

Figure 1

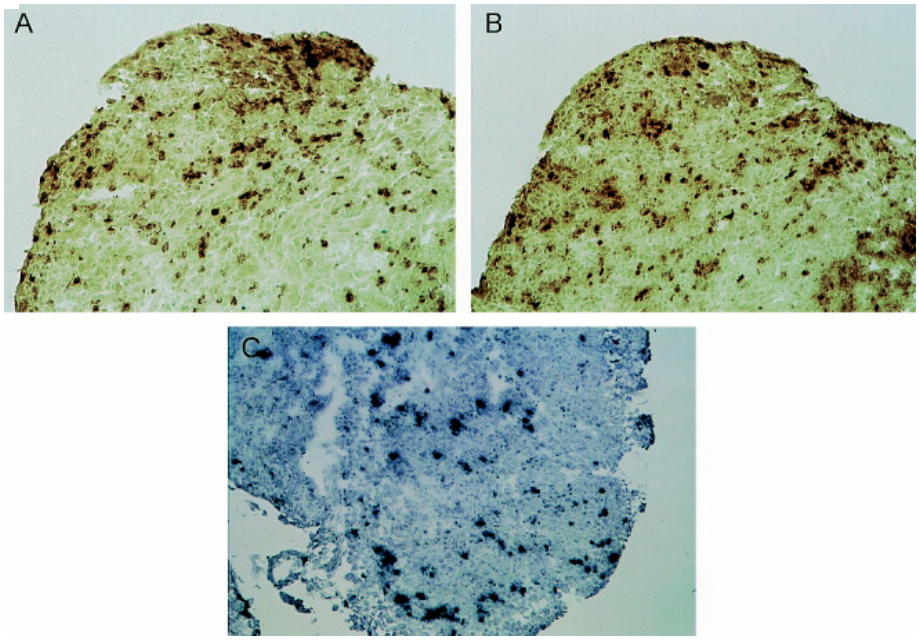

FIGURE 1: DAB staining of RA synovial tissue. DAB precipitates in the presence of free radicals to form an insoluble final reaction product, visible as a brown DAB polymer. A large number of DAB-positive cells can be identified (1A). Addition of Mn^{2+} only marginally enhances the amount of DAB precipitate, without affecting the localization pattern of the precipitate (1B). Addition of cobalt ions (Co^{2+}) to DAB or DAB/ Mn^{2+}-containing media leads to the formation of a blue final reaction product (1C).

Figure 2

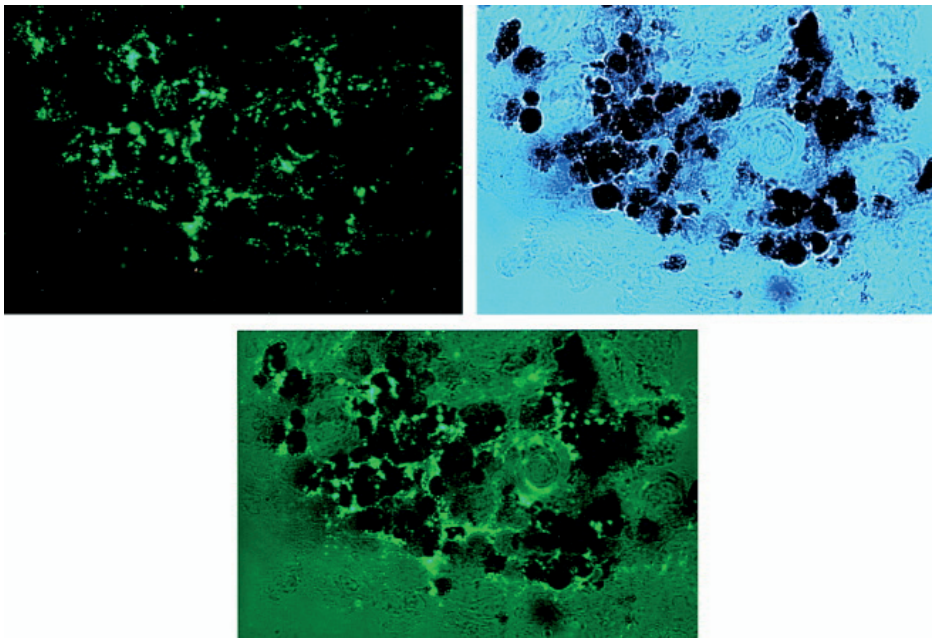

FIGURE 2: Combined DAB and CD3 immunofluorescence of RA synovial tissue. CD3-positive cells contained high intracellular DAB precipitate. Fluorescence microscopy (left panel) and corresponding light microscopy image (middle panel). Combined fluorescence and bright field imaging is shown in lower panel (X400).

To eliminate possible effects of disease modifying anti-rheumatic drugs (DMARD), and to investigate whether intracellular oxidation of DAB in T cells also occurs in early arthritis, we performed the DAB staining on sections of 3 patients with recent onset, DMARD-naive RA. ROS staining in cryostat sections from these patients was indistinguishable from the other RA patients (Figure 3, right panel). To examine the disease specificity of ROS production by synovial T lymphocytes, cryostat sections of RA and non-RA synovial tissue were compared for DAB precipitation products. No polymerization of DAB was observed in any of the T cells in OA tissue samples (n=10) (Figure 4, upper panels). A minimum of 50 $CD3^+$ DAB^- cells were detected in each of the OA tissue samples. Rare DAB^+ cells were identified as $CD15^+$ neutrophils (Figure 4, middle panels). In 1/8 patients with reactive arthritis and 3/7 patients with psoriatic arthritis DAB precipitation was observed in 73% (mean, SD ± 3) and 48% (mean, SD ± 12) of the $CD3^+$ cells respectively (Figure 4, lower panels). There was no correlation between disease activity, disease duration, the presence of RF, disease pattern or erosiveness on the one hand and DAB precipitation on the other hand.

Figure 3

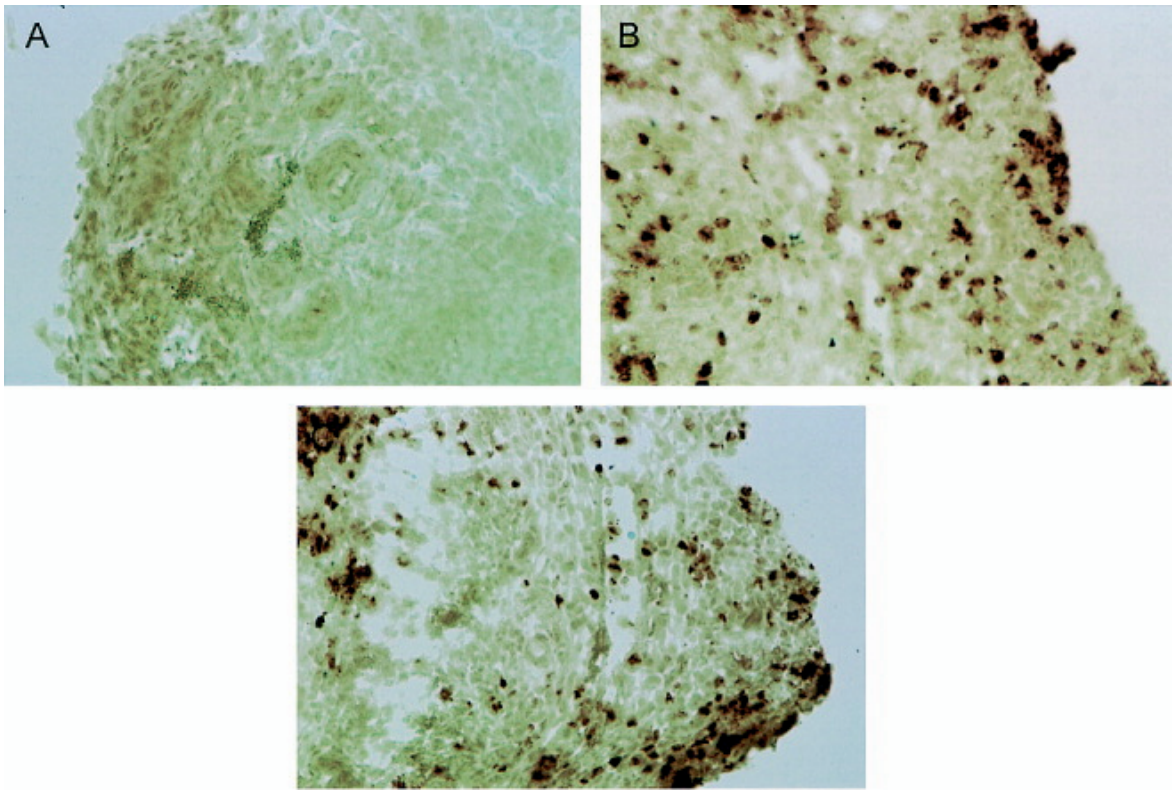

FIGURE 3: DAB staining of sections of synovium from patients with early RA and patients with long standing RA. Tissue section from patients with early RA (Fig 3A) and long standing RA (Fig 3B) both stained equally positive for DAB (X200).

Figure 4

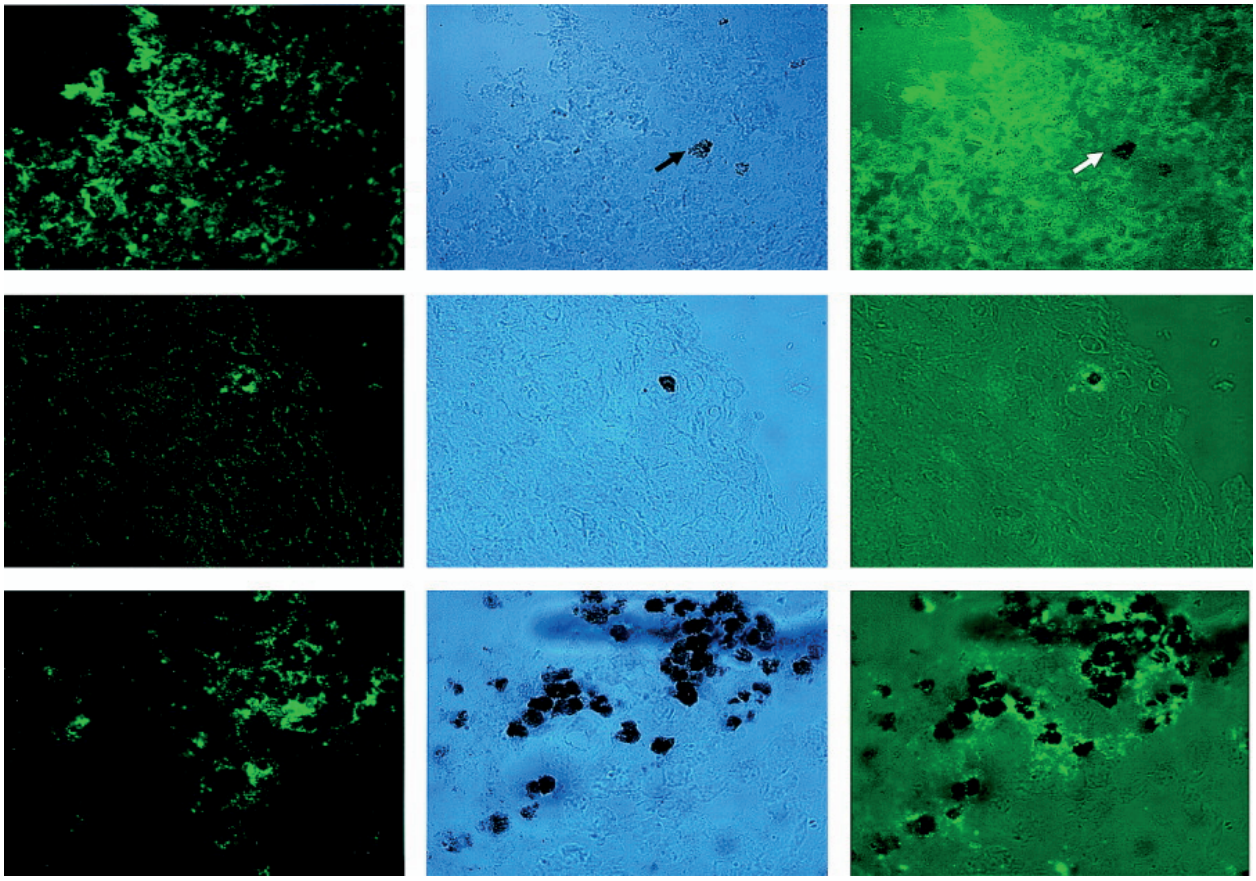

FIGURE 4 : In OA tissue sections DAB precipitate was observed rarely and only in $CD15^+$ neutrophils. Although a minimum of 50 $CD3^+$ cells per patient were counted, no DAB^+ T lymphocytes were found in OA patients. The upper panels show OA tissue with a significant number of $CD3^+$ lymphocytes and corresponding light microscopy image (middle panel). Combined fluorescence and bright field imaging is shown in right panels (X400). All DAB positive cells corresponded with $CD15^+$ neutrophils (middle lanes). $CD3^+DAB^+$ lymphocytes in a psoriatic arthritis patient are shown in lower panels.

ROS production in RA synovial fluid T cells

When DAB staining was performed on cytospins of purified paired PB and SF T cells, DAB precipitate was not found in any of the PB T lymphocytes (Figure 5, upper panels), but was easily detectable in T cells from SF (Figure 5, middle and lower panels). The formation of ROS-dependent DAB polymerization in synovial T lymphocytes is likely due to intracellular enzymatic activity. First, DAB polymerization was not affected by pre-incubation of cytospins or cryostat sections in aqueous medium at 37 °C, but was completely inhibited by preincubation for 10 min at 100 °C. Second, prefixation with paraformaldehyde 4% also abolished formation of final reaction product, as did overnight airdrying at room temperature. Third, the amount of DAB polymerization increased with increasing incubation times from 15 to 60 minutes. On cytospins, the initial DAB precipitate (15 min) in T lymphocytes was detected in the cytoplasm, but not the nucleus of the SF T cells. With increasing incubation periods, DAB precipitate could be detected throughout the cell.

Catalase-sensitive generation of hydrogen peroxide in synovial T lymphocytes

Because it is difficult to quantitate DAB precipitation, we measured intracellular ROS production in purified SF and PB T cells, using FACS analysis of the ROS-reactive dye DCF. SF T cells displayed a higher basal rate of ROS production than PB T cells (Figure 6A). In order to determine which oxidants and enzymes are responsible for the free radical production, inhibitors of the different enzyme systems proposed to regulate ROS production in T lymphocytes were tested (Figure 6B).

The addition of exogenous catalase, which uses H_2O_2 as substrate clearly diminished the DAB polymerization in synovial T cells, reducing basal ROS production in SF T cells to levels observed in PB T cells. A majority of intracellular H_2O_2 originates from the dismutation of $O_2^{\bullet-}$ by superoxide dismutases (SOD). However, when SOD was added to the incubation medium, no significant inhibition of ROS production was observed. Also, addition of DPI (an NADPH oxidase inhibitor), L-NMMA (an NO synthetase inhibitor) or NDGA (a lipoxygenase inhibitor) did not result in significant inhibition of intracellular ROS production. Addition of rotenone (the mitochondrial complex I inhibitor) also did not inhibit increased ROS production in SF T cells.

Figure 5

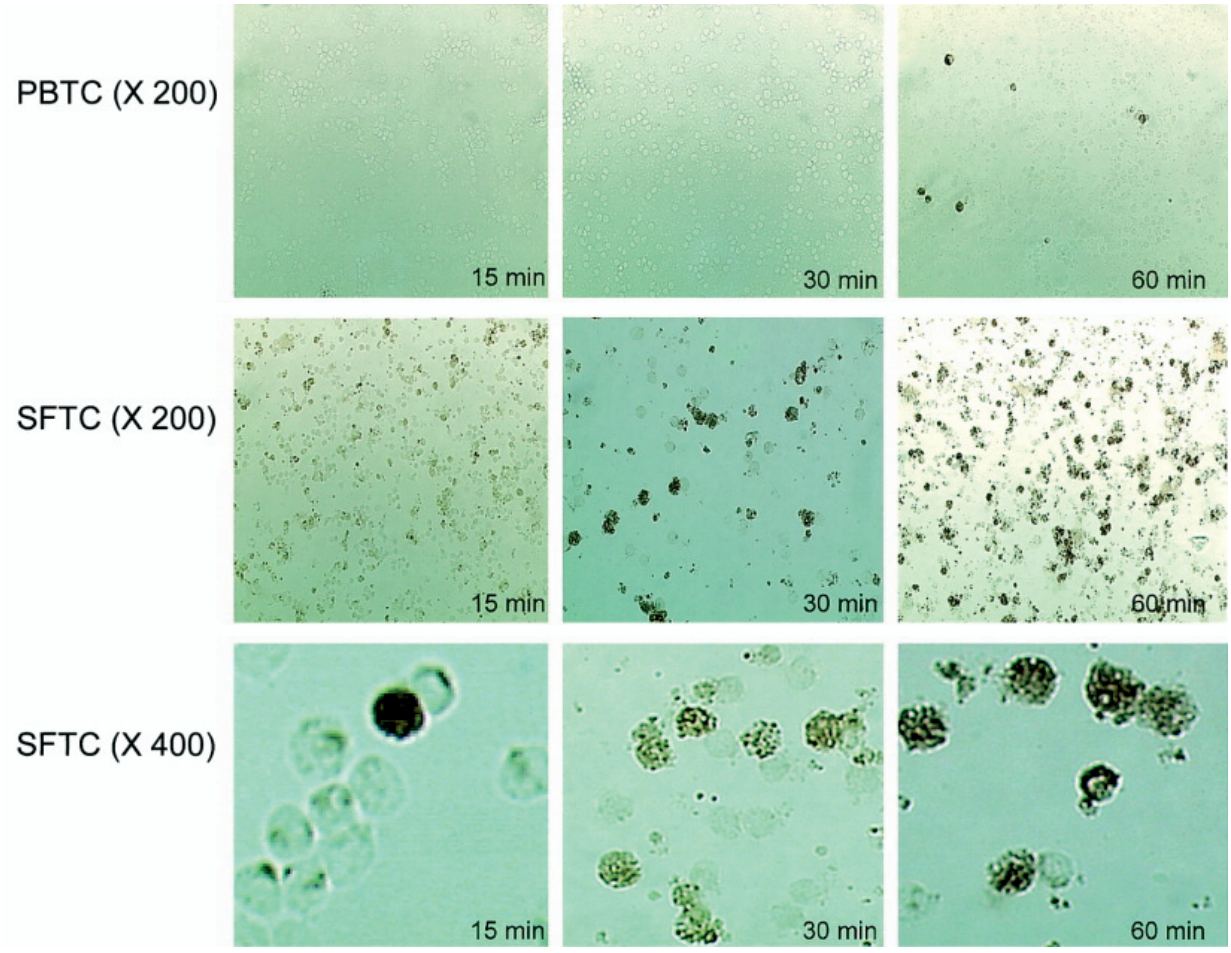

FIGURE 5: DAB staining on cytospins of purified PB TC (Upper panels) and SF TC (middle panels x 200; lower panels: x 400) after different incubation periods. DAB precipitation in SF T cells originates in the cytoplasm of SF T cells (15 min, left panels). Longer incubation period shows a time-dependent increase in intracellular DAB final reaction product (30-60 min, middle and right panels).

Figure 6

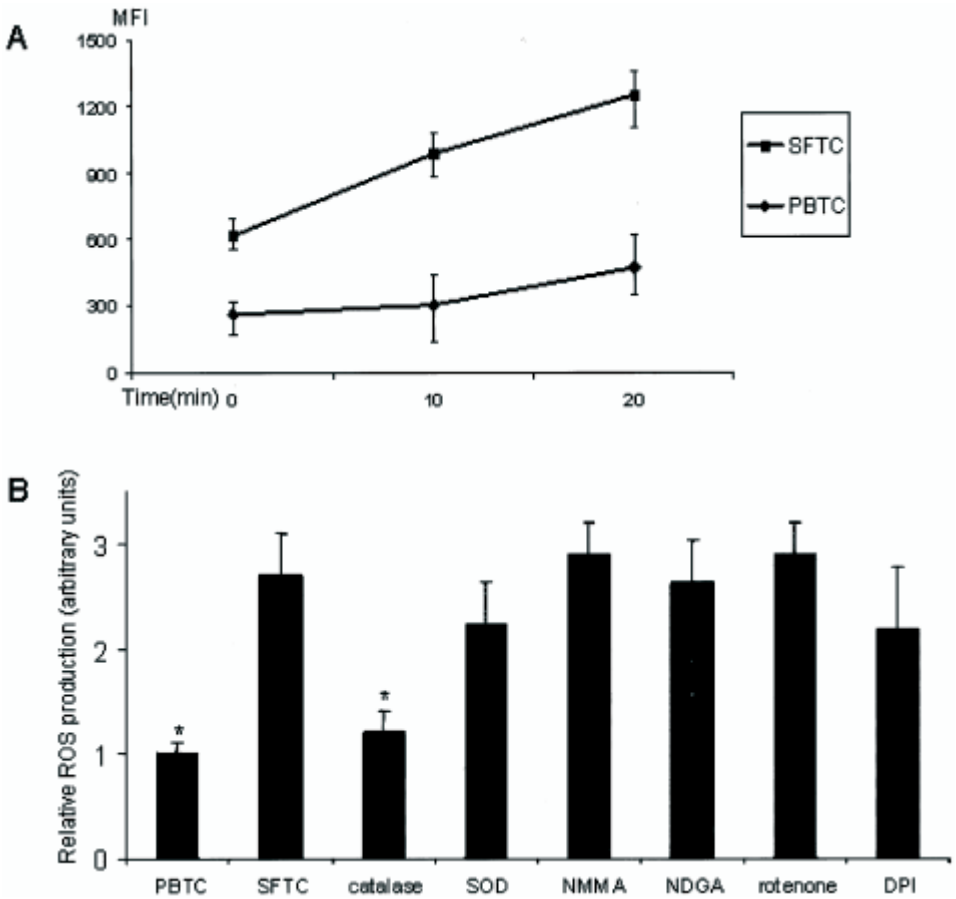

FIGURE 6: A: ROS production is constitutively upregulated in SF T cells, when compared to PB T cells. Mean fluorescence (MFI) of DCF in PB and SF T cells. After 20 min pre-incubation at 37 °C with DCF, MFI was measured at three different timepoints. The data shown are the mean (±SD) values of three independent experiments.
B: Catalase reduced basal ROS production in SF T cells to levels observed in PB T cells. No significant inhibition of intracellular ROS production was seen after pre-incubation with DPI, NMMA, NDGA, and rotenone.
The ROS generation is expressed as the increased ROS production in the different conditions compared to a basal level of ROS production in PBTC =1. The data shown are the mean values of three independent experiments.

Discussion

T lymphocytes are regarded to play a key role in the pathogenesis and the perpetuation of rheumatoid arthritis. Synovial T lymphocytes display features of severe oxidative stress, which results in a number of proliferative and signaling abnormalities (2-5). While there is general consensus about the presence of oxidative stress in synovial T cells, the origin of this oxidative stress is unknown.

Our present data demonstrate that chronic oxidative stress observed in synovial T cells mainly originates from free radicals generated by intracellular sources. This conclusion is at odds with suggestions that exposure of synovial T cells to environmental free radicals, or proximity to ROS-producing neutrophils and/or macrophages is responsible for oxidative stress in synovial T cells. Moreover, perivascular T cells, which have recently entered the synovial milieu, as well as T cells distributed in the intima, subintima and in lymphocyte clusters all produced ROS. This suggests that acquisition of intracellular ROS production is a very early event following extravascularization of T lymphocytes into synovial tissue, and again indicates that in synovial T cells free radicals originate from intracellular sources, rather than environmental free radicals. Although DAB^+ cells were clearly identified as T lymphocytes, not all synovial T lymphocytes contained DAB precipitate, suggesting only a specific subpopulation of synovial T cells are under oxidative stress. The presence of DAB precipitate in over 90% of SF T cells on cytospin versus 68% on tissue sections could be due to a difference in CD3 subset composition between synovial fluid and synovial tissue, or technical issues. Further analysis of specific T cell subpopulations within synovial fluid and tissue should provide insight into these possibilities.
The presence of ROS producing T cells was not entirely specific for rheumatoid arthritis as synovial tissue specimens from a proportion (5 out of 15) of patients with other forms of chronic arthritis also contained DAB^+ synovial T cells. No DAB precipitate was found in synovial tissue from OA patients.
During the past decade, reduction-oxidation (redox) reactions that generate ROS have been identified as important chemical mediators in the regulation of signal transduction processes (14). In particular ROS appears to have a central role in the balance between cell growth, survival and apoptosis. The specific cellular response is dependent on the species of oxidant(s) produced, their subcellular source and localization, the kinetics of production, and the quantities produced. Therefore, the identification of intracellular free radicals in synovial T cells may not only provide an explanation for the altered behavior of synovial T cells, but also prove a pivotal hallmark in understanding underlying pathophysiological mechanisms in rheumatoid arthritis. Jackson at all recently identified 3 different ROS producing events following T cell receptor activation (15): first a rapid H_2O_2 production independent of Fas or NADPH oxidase; second a sustained H_2O_2 production dependent on both Fas and NADPH oxidase; and third a delayed superoxide production that was dependent on Fas ligand and Fas, yet independent of NADPH oxidase. Our results favor the first oxidase as the primary source in synovial T lymphocytes since the intracellular ROS production was catalase-dependent and DPI-independent. Under physiological conditions however, intracellular ROS production is a transient phenomenon, occurring only up to 15 minutes after T cell stimulation. Therefore, the sustained intracellular ROS production in SF T cells might be a pathognomonic hallmark for chronic arthritis, which is not found in PB T cells isolated from RA patients or healthy controls. Identifying the oxidase responsible for the intracellular ROS in SF T cells, and the proteins that regulate the oxidase, could provide new therapeutic targets in RA.

Conclusion

There is a growing body of evidence demonstrating the critical role of ROS in the regulation of mitogenesis, differentiation and apoptosis in physiological and pathophysiological conditions. Our present results indicate that the chronic oxidative stress observed in synovial T lymphocytes from RA patients originates from intracellular generated free radicals, rather then environmental influences.

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CHAPTER 3

Rap1 signaling is required for suppression of Ras-generated reactive oxygen species and protection against oxidative stress in T lymphocytes.

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Abstract

Transient production of reactive oxygen species (ROS) plays an important role in optimizing transcriptional and proliferative responses to TCR signaling in T lymphocytes. Conversely, chronic oxidative stress leads to decreased proliferative responses and enhanced transcription of inflammatory gene products, and is thought to underlie the altered pathogenic behavior of T lymphocytes in some human diseases, such as rheumatoid arthritis (RA). Although the signaling mechanisms regulating ROS production in T lymphocytes has not been identified, activation of the small GTPase Ras has been shown to couple agonist stimulation to ROS production in other cell types. We find that Ras signaling via Ral stimulates ROS production in human T lymphocytes, and is required for TCR and phorbol ester-induced ROS production. The related small GTPase Rap1 suppresses agonist, Ras and Ral–dependent ROS production through a PI3K–dependent pathway, identifying a novel mechanism by which Rap1 can distally antagonize Ras signaling pathways. In synovial fluid T lymphocytes from RA patients we observed a high rate of endogenous ROS production, correlating with constitutive Ras activation and inhibition of Rap1 activation. Introduction of dominant-negative Ras into synovial fluid T cells restored redox balance, providing evidence that deregulated Ras and Rap1 signaling underlies oxidative stress and consequent altered T cell function observed in RA.

Introduction

Two members of the Ras family of GTPases, Ras and Rap1, play critical roles in regulating T cell proliferative responses. Ras plays an essential role in transmitting signals from the TCR to activation of the Raf-1/ERK signaling cascade, which is required for T cell proliferation, IL-2 production, and thymic maturation (1) The closely related Rap1, which is also activated in T lymphocytes following TCR stimulation, was identified as a suppressor of Ras-dependent transformation (2), and was proposed to act by sequestering Ras effector targets (Raf kinases, PI3K, and Ral GTPase, guanine nucleotide exchange factors (GEF)) away from Ras in an inactive complex (3-6). In overexpression studies, constitutive activation or inactivation of Rap1 signaling can profoundly modulate TCR-induced, Ras-dependent transcriptional events (6-9), and constitutive activation of Rap1 in anergic T lymphocytes has been suggested to contribute to the hyporesponsiveness of these cells to antigenic rechallenge (6). Although these studies have suggested that Rap1 mediates these effects through proximal antagonism of Ras signaling to the Raf/ERK pathway in T cells (6-10), no direct data support that endogenous Rap1 (5,11), or ectopically expressed active Rap1 (7,8,10), can directly interfere with Ras-dependent activation of Raf in T lymphocytes. This conclusion is most strongly supported by overexpression studies in T cell lines and analysis of transgenic mice expressing activated Rap1 in T lymphocytes at levels comparable to endogenous Rap1. In this study, active Rap1 induces integrin activation and enhances integrin-dependent activation of T cells without inhibiting TCR-dependent positive selection, proliferation or ERK activation (12-14). Thus, Rap1 modulation of Ras-dependent transcriptional activity in T cells likely occurs via Ras-distal rather than Ras-proximal signaling pathways.

In addition to the Raf/ERK pathway, Ras also signals through Ral family GTPases and PI3K (3,15). Each of these defined pathways, including the Raf/ERK pathway, is required for Ras-induced cellular transformation (15). Ras-dependent production of reactive oxygen species (ROS) is also required to induce cellular transformation (16). Of these signaling pathways, only the Raf/ERK pathway has been extensively studied in T lymphocytes (1). ROS, such as hydrogen peroxide, are proposed to act as important second messengers in TCR and phorbol ester-induced T cell activation. Both of these stimuli result in the transient production of ROS (17). Scavenging of intracellular ROS with antioxidants (e.g., *N*-acetylcysteine (NAc)) suppresses TCR-induced NF-κB, AP-1, and IL-2 promoter transcription (18,19). Conversely, acute exposure of T cells to

ROS can stimulate transcription from these promoters, and enhance TCR-induced transcription (20). Chronic oxidative stress, however, can lead to T cell hyporesponsiveness to proliferative stimuli and constitutive activation of NF-κB-dependent inflammatory gene products. In a number of human diseases, including rheumatoid arthritis (RA) (21,22) and HIV infection (23), chronic oxidative stress is thought to underlie altered, pathogenic T cell behavior.

RA is a chronic systemic disease characterized by inflammation of synovial tissue and destruction of cartilage and bone, marked by infiltration of the synovial joint by B and T lymphocytes, neutrophils, and macrophages (24). T lymphocyte interactions with synovial macrophages and fibroblast-like synoviocytes are regarded to play key roles in the pathogenesis and perpetuation of the disease (25,26). T lymphocytes isolated from rheumatoid joints display a number of signaling and proliferative abnormalities. They exhibit severe hyporesponsiveness to proliferative stimuli compared with autologous peripheral blood (PB) T cells, and produce little IL-2, IFN-γ, IL-4, TGF-β, or TNF-α in vitro or in situ (27). Despite this, RA synovial fluid (SF) T cells express markers of recent activation, such as CD69 and CD40 ligand (CD154), proinflammatory receptors for TNF-α and IL-1, and secrete matrix metalloproteinases, correlating with constitutive activation of NF-κB, a key ROS-dependent transcriptional regulator of these genes (25,28,29). Expression of these proteins by synovial T lymphocytes leads to activation of synovial macrophages and fibroblast-like synoviocytes, perpetuating inflammation, synovial hyperplasia, and joint destruction (25,26).

The hyporesponsiveness of SF T cells correlates strongly with oxidative stress, and in vitro replenishment with NAc restores reduced glutathione (GSH) equilibrium concomitant with recovery of TCR signaling defects and partial rescue of both SF T lymphocyte proliferation and IL-2 production (22). Conversely, depletion of intracellular GSH in peripheral T cells, or chronic exposure to TNF-α, mimicks signaling defects observed in SF T cells (30-32). Because ROS production has been described as a critical component of Ras-dependent transformation in fibroblasts, and Rap1 was first described as a suppressor of Ras-dependent transformation, we examined the possibility that Ras and Rap1 signaling may regulate ROS production and oxidative stress in normal and RA T lymphocytes.

Materials and methods

cDNA and transient transfections

Empty pMT2-hemagglutinin (HA), and pMT2 plasmid encoding HA-tagged RasV12, RasN17, Rap1V12, Rap1N17, RapGTPase-activating protein (RapGAP), RalV23, RalN17, Rlf-CAAX, and exchange protein directly activated by cAMP (EPAC)-CAT have been previously described (12,33). Empty pSG5 and pSG5 encoding RasV12, RasV12/G37, RasV12/E38, and RasV12/C40 have been previously described (33). Wild-type and catalytically inactive phospholipase C (PLC)ε in pCDNA were generously provided by Dr. M. Schmidt (University Hospital Essen, Essen, Germany) (34). Rap1V12 effector mutants V12/G37, V12/E38, and V12/C40, and Rap1V12-ΔCAAX in pMT2HA were generated using the Stratagene Quick-Change site-directed mutagenesis kit (La Jolla, CA) and two oligonucleotide primers containing the desired mutations, and the sequence integrity of the constructs was established by sequencing. For transient expression of cDNA in Jurkat, 6 x 10^6 cells were transfected by electroporation (250 V, 950 μF) with 15–30 μg of matching empty vector or cDNA expression vectors as indicated in figures, plus pCMV-CD20 expression vector (provided by Dr. R. Medema, Netherlands Cancer Institute, Amsterdam, The Netherlands), 10 μg of pEF-*myc*ERK2 (a kind gift of D. Cantrell, University of Dundee, Dundee, U.K.), or 5 μg of *Renilla* luciferase expression construct regulated by the thymidine kinase promoter to differentiate between transfected and untransfected cells.

Cells and FACS-based ROS detection assay

Jurkat T cells were maintained as previously described (31). Human PB T lymphocytes from healthy donors and PB and SF T lymphocytes from human RA patients were purified and maintained as previously described (30,31). Patient material was obtained following informed consent from patients according to regulations at each of the participating institutes.

For detection of ROS production, Jurkat cells were isolated 48 h posttransfection cells and stained with CyChrome-conjugated anti-CD20 mAbs (BD PharMingen, San Diego, CA), resuspended at 5 x 10^6 cells/ml in phenol red-free DMEM medium and loaded with 28 μM 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate-di(acetoxymethyl ester) (DCF; Molecular Probes, Eugene, OR) for 20 min at 37°C before stimulation with anti-CD3 Ab (1XE; Central Laboratory of the Netherlands, Red Cross Blood Transfusion Service, Amsterdam, The Netherlands) or 100 ng/ml PMA (Sigma-Aldrich, St. Louis, MO) plus 1 μg/ml ionomycin (PMA/I; Sigma-Aldrich) and ROS detection. CD20-positive cells were analyzed on a FACScan (BD Biosciences) for the mean fluorescence intensity (MFI) of oxidated DCF in the FL1 channel at different time points after stimulation. Because DCF loading continues after the first 20 min, the data are presented as ΔROS generation, whereby the measured MFI at each time point is reduced by the measured MFI at corresponding time points in the unstimulated or unstimulated mock-transfected control group.

Detection of activated GTPases, immunoprecipitations and immunoblotting

PB T lymphocytes from healthy controls and RA patient PB and SF T cells were left unstimulated or stimulated for 5 min with anti-CD3 Ab (0.5 μg/ml), 100 ng/ml PMA/I or 500 μM H_2O_2 (Merck, West Point PA). In indicated experiments, cells were pretreated with LY294002 (1 μM, 24 h), NAc (5 mM, 48 h) or L-buthionine-(*S,R*)-sulfoximine (BSO, 200 μM, 72 h). Active GTP-bound Ral, Rap1, and Ras proteins from 5 x 10^6 T cells were precipitated with RLIP-Ras binding domain (RBD), Ral guanine nucleotide stimulator protein (GDS)-RBD, and Raf-RBD GST fusion proteins, respectively, resolved by SDS-PAGE, and detected by immunoblotting with anti-Ral, anti-Rap1, or anti-Ras Abs (BD Transduction Laboratories, Lexington, KY) as previously described (11,12). Briefly, cells were lysed in buffer containing 10% glycerol, 1% Nonidet P-40, 50 mM Tris (pH 7.6), 150 mM NaCl, 10 mM $MgCl_2$, 2 mM $NaVO_4$, 10 mM NaF, and 2 mM PMSF. Clarified lysates were incubated with ~5 μg RBD-GST fusion protein precoupled to glutathione-agarose and washed with lysis buffer before elution in Laemmli's sample buffer. Proteins were resolved on 13.5% SDS-PAGE gels, transferred to polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA), and proteins detected by immunoblotting and ECL. Total Rap1 and Ras was detected in lysates to confirm equal amounts of GTPases. For detection of ERK activation, transfected *myc*-tagged ERK2 was immunoprecipitated with anti-*myc* Abs (Santa Cruz Biotechnology, Santa Crus, CA), followed by resolution by SDS-PAGE, transfer to PVDF, and detection with anti-phospho-ERK1/2 Abs (New England Biolabs, Beverly, MA). Equivalent *myc*-ERK2 expression between independent transfection samples was confirmed by immunoblotting with anti-*myc* Abs. In all other transfection experiments, equivalent expression of constructs was confirmed by immunoblotting of cell lysates with anti-Ras, anti-FLAG (Sigma-Aldrich), or anti-HA Abs (Santa Cruz Biotechnology).

Adhesion assays

Adhesion assays were as previously described (12). Briefly, Jurkat cells were transfected with 10 μg of thymidine kinase-luciferase expression plasmid (to detect transfected cells) and 30 μg of empty plasmid or the indicated Rap1 construct. After 48 h, cells were washed and resuspended in

TSM medium (20 mM Tris, pH 8, 150 mM NaCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$). Costar Maxisorp 96-well plates were coated overnight at 4°C with goat anti-human IgG Abs (4 μg/ml; Jackson ImmunoResearch Laboratories, West Grove, PA) in TSM, washed with TSM, blocked for 30 min at 37°C in 0.5% BSA/TSM, and incubated with 50 ng/ml recombinant ICAM-1 (generously provided by C. Figdor, Nijmegen University, Nijmegen, The Netherlands) in BSA/TSM. Cells were allowed to adhere for 45 min at 37°C, nonadherent cells washed away with warm BSA/TSM, and adherent cells lysed and subjected to luciferase assays as previously described (12). Percentage of specific adhesion was calculated by dividing detected luciferase counts by counts obtained from lysates from total input cells.

Incubation of T lymphocytes with cell-permeable Tat fusion peptides

Tat-β-galactosidase (β-Gal) peptide was a generous gift of Dr. D. Hall (University of Wisconsin, Madison, WI) (35). Generation, purification, and characterization of Tat-RasN17 peptides have been previously described (36). Freshly isolated human PB and SF T lymphocytes were incubated at 4 x 10^6 cells/ml in culture medium alone, or medium containing 25 μg/ml Tat-β-Gal or Tat-RasN17 peptide for 16 h at 37°C. Following extensive washing in PBS, cells were left unstimulated, or stimulated for 5 min with medium, anti-CD3/CD28 Abs or PMA/I as previously described before lysis and detection of Ras, phospho-ERK and ERK protein by immunoblotting. Alternatively, ROS production was measured as previously described.

Results

Ras signaling via the GTPase Ral is both necessary and sufficient for ROS production in T lymphocytes

We first examined the role of Ras signaling in the regulation of ROS production in T cells. Treatment of Jurkat cells with either anti-CD3 Abs or PMA/I led to a rapid and transient generation of intracellular ROS that was maximal 2–5 min poststimulation (Fig. 1*A*). No further changes in rate of ROS production were observed up to 1 h poststimulation, the longest time point tested. Jurkat cells transiently expressing an active Ras mutant, RasV12, demonstrated a constitutively high basal level of intracellular free radical production that could not be further augmented by stimulation with anti-CD3 Abs or PMA/I. Conversely, expression of dominant-negative RasN17 completely blocked CD3 and PMA/I-dependent ROS production, demonstrating Ras activation is necessary and sufficient for ROS generation in T lymphocytes.

The signaling pathways coupling Ras activation to ROS production have not been identified, so we first used RasV12 effector domain mutants that selectively signal via members of the Ral (GEF) family and PLCε (RasV12/G37), Raf kinases (RasV12/E38), or PI3K (RasV12/C40) (15,37) (Fig. 1*A*). Of these three effector domain mutants, only RasV12/G37, but not RasV12/E38 or RasV12/C40, mimicked RasV12 in stimulating ROS generation in Jurkat cells. This finding suggested that Ras signaling to either the small GTPase Ral or PLCε was responsible for stimulating ROS production.

In initial experiments, we detected protein expression of two RalGEFs, RalGDS, and Rlf in T cell lysates by immunoblotting (data not shown), supporting the possibility that Ras could couple to Ral activation in T cells. Most importantly, CD3/CD28 stimulation of PB T lymphocytes led to activation of Ral, as determined using an activation-specific pulldown assay to detect GTP-bound Ral (Fig. 1*B*). To directly test whether Ral activation could stimulate ROS production in T cells, we transiently transfected Jurkat with a constitutively active mutant of the Ral-specific GEF Rlf, Rlf-CAAX (activating endogenous Ral), or an active Ral mutant, RalV23. Expression of either protein in Jurkat cells led to constitutive ROS production (Fig. 1*C*). Conversely,

expression of dominant-negative RalN blocked both PMA/I- and Ras-induced ROS production clearly indicating the relevance of the Ral signaling pathway in Ras-dependent ROS production. In the presence of dominant-negative Ras, active RalV23 still stimulated persistent generation of ROS, placing Ral downstream of Ras.

Figure 1

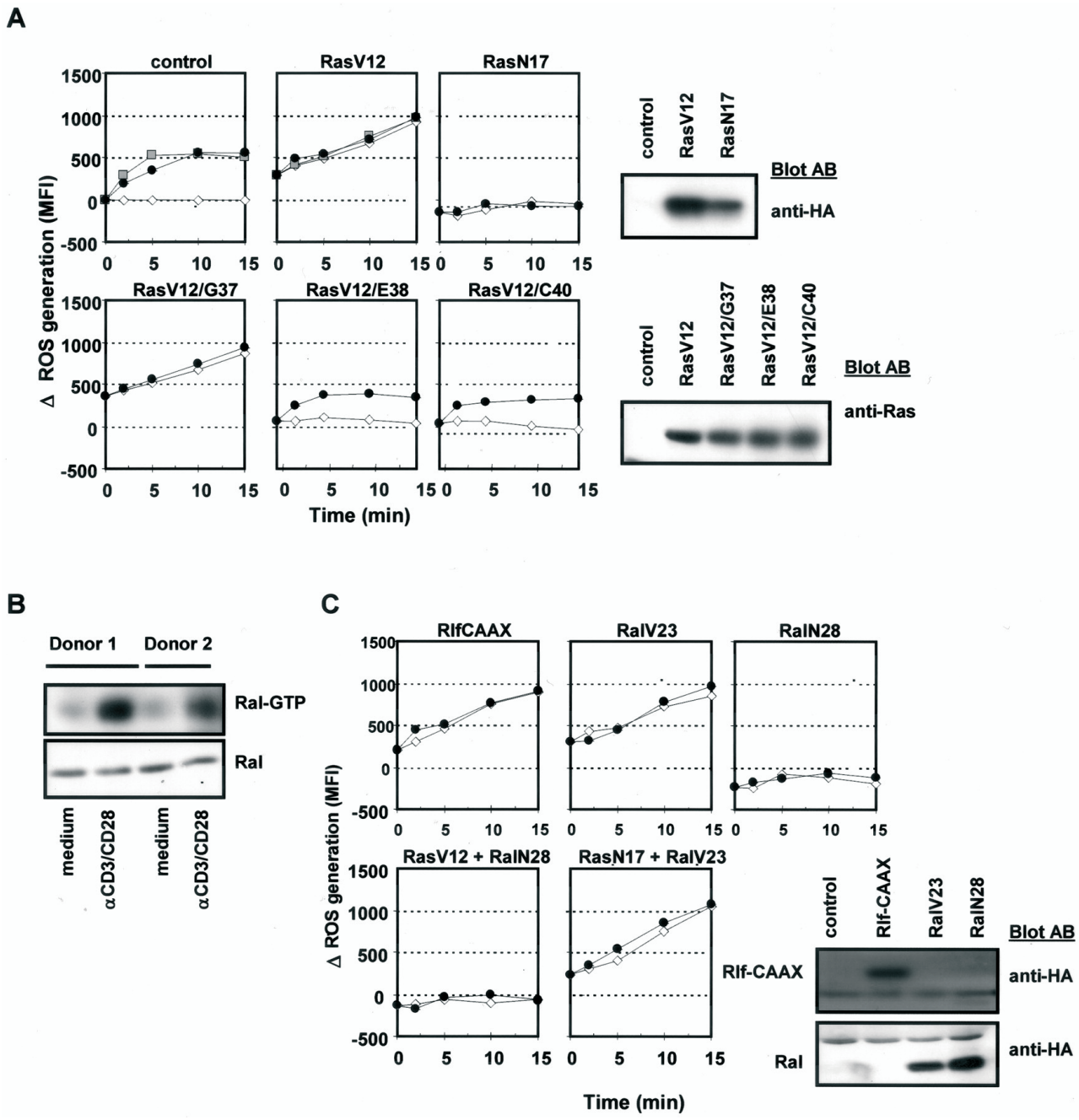


FIGURE 1. Ras signaling via a Ral-dependent pathway is sufficient and required for ROS generation in T cells. *A*, Ras is sufficient and required for ROS generation in T lymphocytes. A total of 6 x 10^6 Jurkat cells were transfected by electroporation (250 V, 950 μF) with pCMV-CD20, to differentiate between transfected and untransfected cells, and empty pMT2HA expression plasmid (control) or cDNA encoding Ras signaling constructs indicated in each panel. At 48 h posttransfection, cells were stained with CyChrome-conjugated anti-CD20 mAbs. Cells were resuspended at 5 x 10^6 cells/ml in phenol red-free DMEM medium and loaded with 28 μM DCF for 20 min at 37°C before stimulation and ROS detection. Cells were stimulated with anti-CD3 (▫) or PMA/I (•) or left unstimulated (◇) and analyzed on a FACScan for the mean fluorescence intensity (MFI) of oxidated 6-carboxy-DCF in the FL1-channel, as a measurement for the presence of intracellular ROS, of CD20-expressing cells at different time points after stimulation. The ΔROS generation is obtained by reducing the measured MFI with the measured MFI at corresponding time points in the unstimulated, control-transfected Jurkat cells. The data shown are the mean values of three or more independent experiments. Expression of all constructs was confirmed by immunoblotting of cell lysates with anti-HA (for HA-RasV12 and HA-RasN17) or anti-Ras Abs (for RasV12, RasV12/G37, RasV12/E38, and RasV12/C40). *B*, Ral participates in TCR signaling. A total of 5 x 10^6 PB T lymphocytes from two healthy donors were stimulated for 5 min with either medium or anti-CD3 and anti–CD28 Abs (α CD3/CD28). GTP-bound Ral was precipitated, resolved by SDS-PAGE and visualized by immunoblotting with anti-Ral Abs and ECL (*top*). Total Ral expression was assessed by immunoblotting of whole cell lysates (*bottom*). *C*, Ral is sufficient and required for ROS generation downstream of Ras. Jurkat T cells were transfected with cDNA encoding Rlf, Ral, and Ras signaling mutants and assessed for ROS production as in *A*. Expression of Rlf-CAAX, RalV23, and RalN28 was assessed by immunoblotting of whole cell lysates with anti-HA Abs. Constructs used in *A* and *B* were assessed in the same experiments, but are split into two sections for clarity of presentation.

Although our data clearly demonstrated that activation of endogenous Ral was required and sufficient for Ras-induced ROS generation, RasV12/G37 can also interact with PLCε (37), and overexpression of PLCε mimics RasV12/G37-induced cytokine transcription in T cells (38). We therefore examined whether PLCε might also mediate Ras-dependent ROS production. Jurkat cells were transfected with RasV12/G37 alone, or in combination with wild-type or previously characterized dominant-negative, catalytically inactive PLCε (34). If PLCε acted downstream of Ras, we would expect wild-type PLCε to enhance Ras-dependent ROS production, whereas inactive PLCε should be inhibitory. Instead, we found that cotransfection of either PLCε construct led to a small but reproducible decrease in Ras-induced ROS production (data not shown), suggesting that although PLCε might interact with Ras in this overexpression model, Ral, not PLCε, is the Ras effector responsible for ROS production in T lymphocytes. Modulation of ROS generation represents a novel function for Ral and is the first description of Ral participation in T cell signaling.

Rap1 is required for suppression of Ras-dependent ROS production

Because Rap1 can antagonize distal Ras-dependent proliferative and activation signals in T lymphocytes, we next examined whether Rap1 signaling could also influence ROS homeostasis in T lymphocytes. Transfection of Jurkat cells with an inactive mutant of Rap1, RapN17, led to a slight increase in basal ROS levels (Fig. 2*A*, *middle panel*). Strikingly, following PMA/I stimulation, persistent rather than transient ROS generation was observed. To verify that this was not a nonspecific effect of overexpressed RapN17, we used the Rap1-specific RapGAP to inactivate endogenous Rap1 (Fig. 2*A*, *right panel*). Inactivation of endogenous Rap1 by RapGAP also led to persistent ROS production in Jurkat T cells following PMA/I stimulation. Thus,

although inactivation of Rap1 signaling is not sufficient to generate ROS, endogenous Rap1 signaling is required for the down-regulation of agonist-induced ROS generation.

This interpretation was further strengthened by the finding that transient overexpression of active RapV12 completely blocked TCR-, PMA/I-, and RasV12-mediated ROS generation (Fig. 2*C*). Activation of endogenous Rap1 alone with constitutively active Rap1 GEF EPAC (Epac-CAT; Fig. 2*C*) and C3G (data not shown) also blocked ROS production by these stimuli. To determine whether Rap1 was antagonizing ROS production proximally to Ras, or further downstream, we cotransfected Jurkat cells with active RalV23 and RapV12. Active Rap1 suppressed Ral-induced ROS production (Fig. 2*C*), indicating Rap1 was interfering with Ras signaling indirectly.

Rap1 suppresses ROS production via a PI3K-dependent pathway

To gain more insight into how Rap1 might suppress ROS production in T cells, we generated RapV12 effector domain mutants analogous to those made for Ras (RapV12/G37, RapV12/E38, RapV12/C40), and examined their effect on agonist- and Ras-induced ROS generation. We also generated a RapV12 mutant lacking the carboxyl-terminal motif required for posttranslational modifications needed for membrane localization, RapV12-ΔCAAX. The analogous mutant of Ras, RasV12-ΔCAAX, acts as a dominant-negative (RasN17-like) mutant (39). Each of the mutants was transiently expressed at levels comparable to RapV12 in Jurkat cells (Fig. 3*A*). Of the three effector domain mutants only RapV12/C40, but not V12/G37 or V12/E38, abrogated ROS generation (Fig. 3*B*). If analogous to Ras, this mutant would be predicted to signal only via PI3K. Intriguingly, the RapV12-ΔCAAX mutant not only failed to suppress PMA/I–induced ROS production, but also prevented subsequent down-regulation of ROS production. This was the same phenotype observed in cells expressing RapN17 (Fig. 2*A*). These data indicate that membrane localization of Rap1 or its effector is required to suppress ROS production, and that the active Rap-ΔCAAX mutant may sequester the effector away from this cellular compartment.

To examine whether each of the Rap1V12 mutants retained biological activity, we examined their ability to induce integrin-dependent adhesion in Jurkat (Fig. 3*C*). In contrast to their differential effects on ROS production, each of the RapV12 effector mutants, as well as RapV12-ΔCAAX, supported LFA-1-dependent adhesion to immobilized ICAM as efficientlyas RapV12. Similarly, no differences were observed in the induction of VLA-4-dependent adhesion to fibronectin (data not shown), providing evidence that Rap regulates ROS production by a signaling pathway distinct from integrin regulation. If analogous to Ras, our observation that RapV12/C40 selectively blocked ROS production might suggest that a PI3K isoform was the downstream target of Rap1. However, in coimmunoprecipitation studies with transfected active Rap1, we have been unable to demonstrate a physical interaction between Rap1 and PI3K (data not shown). In separate studies we examined whether Rap-dependent suppression of ROS production was PI3K–dependent using the PI3K inhibitor LY294002. In control, mock-transfected cells LY294002 inhibited TCR-induced ROS production (Fig. 3*D*). This was likely due to inhibitory effects of the LY294002 compound on TCR-dependent Ras activation, as we observed that LY294002 inhibited Ras activation upon TCR activation (Fig. 3*E*). These findings were consistent with previous reports, which suggested the requirement of PI3K in coupling the TCR to ERK activation (40).

Use of RasV12 effector domain mutants in earlier experiments suggested that PI3K activity was not required for Ras-induced ROS generation (Fig. 1*A*). This was confirmed pharmacologically, as LY294002 had no effect on RasV12-induced ROS production (Fig. 3*D*). However, pretreatment of cells with LY294002 completely relieved the inhibition of Ras-dependent ROS generation by RapV12 (Fig. 3*F*). LY294002 has no effect on Rap-induced integrin adhesion

(Refs. 13,41 and data not shown), again indicating that Rap1 regulates integrin function and ROS production by distinct pathways. Together, these data indicate that a PI3K-dependent target acts downstream of or in parallel to Rap1 in the down-regulation of ROS production.

Figure 2

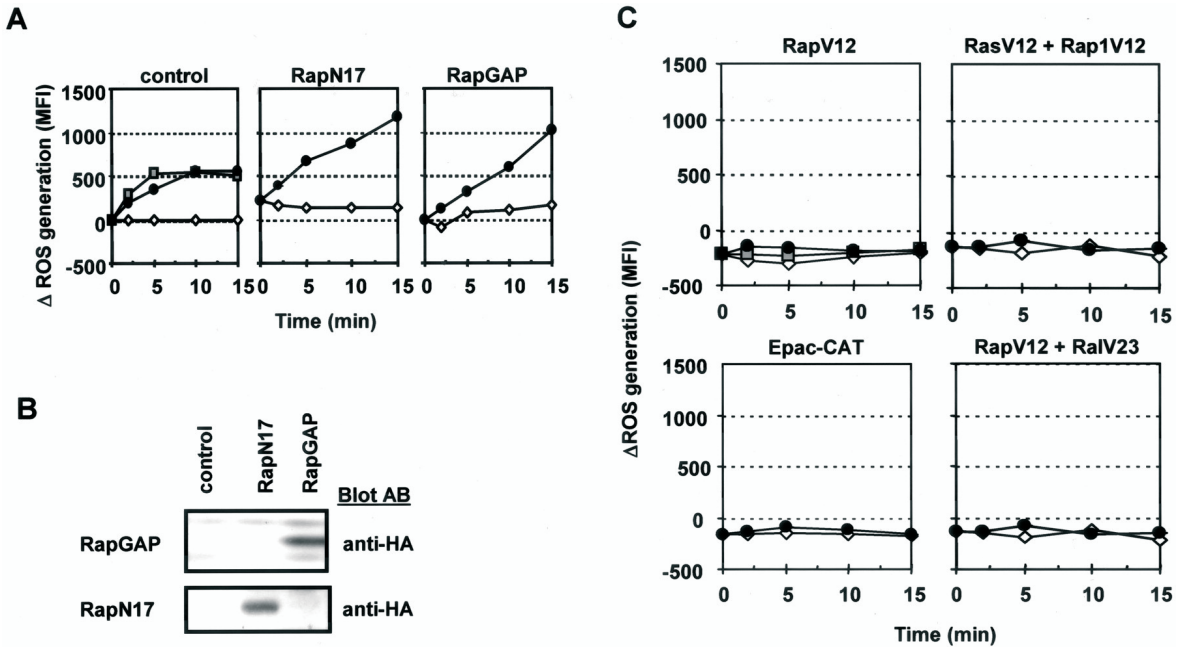
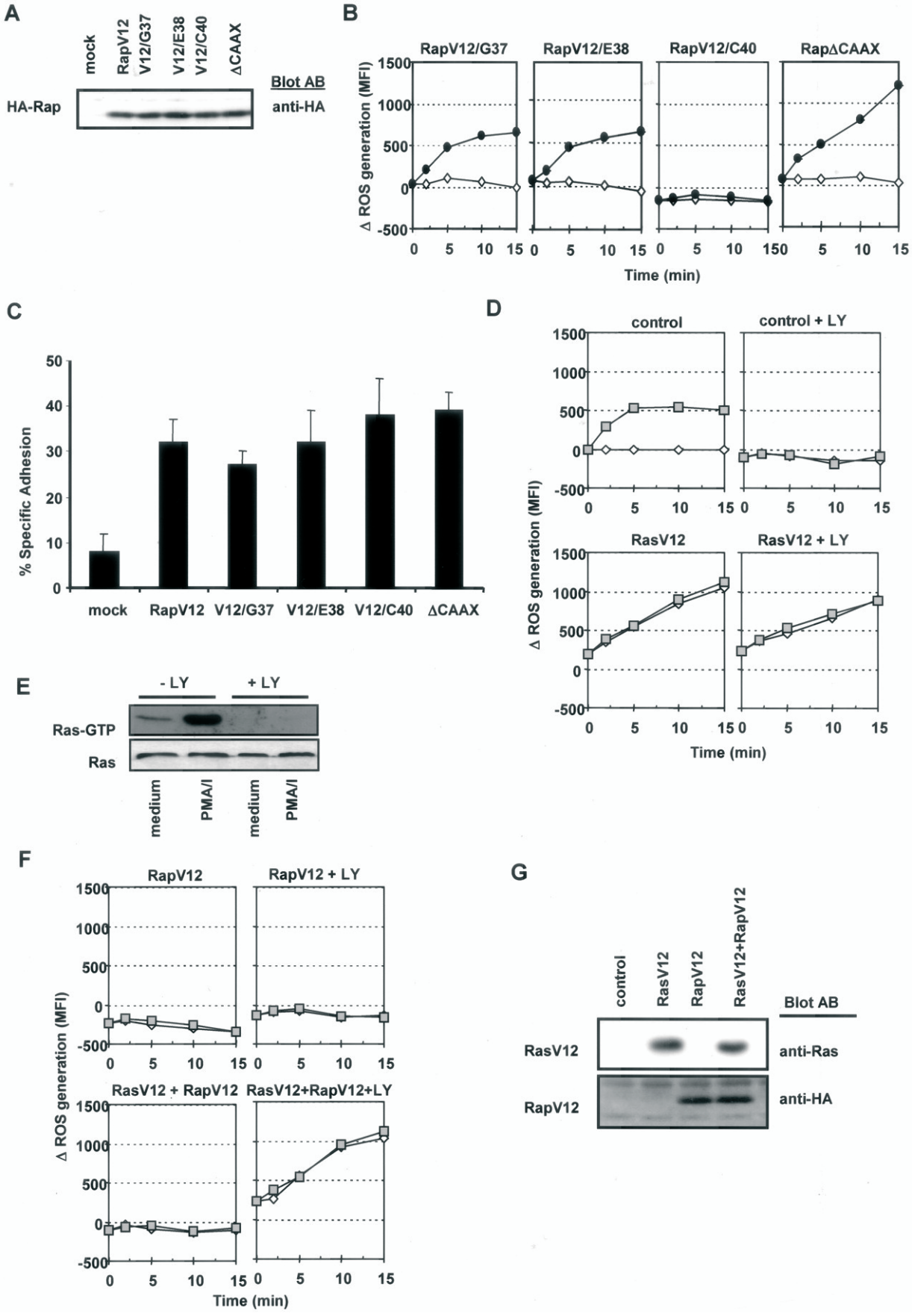


FIGURE 2. ROS generation in T cells is down-regulated through a Rap1-dependent pathway. *A*, Rap1 signaling is required for down-regulation of agonist-induced ROS production. Jurkat cells were transfected with CD20 cDNA alone (control) or in combination with RapGAP or inactive RapN17 cDNA in pMT2HA vector as indicated and processed for analysis of intracellular ROS as in Fig. 1. *B*, Expression of HA-tagged RapN17 and RapGAP in *A* were determined by immunoblotting of whole cell lysates with anti-HA Abs. *C*, Activation of Rap1 suppresses agonist-, Ras-, and Ral-induced ROS production. Jurkat cells were transfected with cDNA encoding CD20 and active RapV12 or the active catalytic domain of the Rap1 GEF EPAC (Epac-Cat), alone, or in combination with active Ras or Ral mutants in pMT2HA vector and analyzed for intracellular ROS production as in Fig. 1. In all sections, cells were left unstimulated (◇) or stimulated with anti-CD3 Abs (gray diamond) or PMA+I (•). Constructs used in *A* and *C* were analyzed in the same experiments, but are sectioned into two for clarity of presentation.

Figure 3


A
mock
RapV12
V12/G37
V12/E38
V12/C40
ΔCAAX
HA-Rap
Blot AB
anti-HA
B
RapV12/G37
RapV12/E38
RapV12/C40
RapΔCAAX
Δ ROS generation (MFI)
Time (min)
C
% Specific Adhesion
mock
RapV12
V12/G37
V12/E38
V12/C40
ΔCAAX
D
control
control + LY
RasV12
RasV12 + LY
Δ ROS generation (MFI)
Time (min)
E
- LY
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Ras-GTP
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medium
PMA/I
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F
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RapV12 + LY
RasV12 + RapV12
RasV12+RapV12+LY
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control
RasV12
RapV12
RasV12+RapV12
Blot AB
RasV12
anti-Ras
RapV12
anti-HA

FIGURE 3. Rap1 suppresses ROS production by a PI3K-dependent signaling pathway distinct from regulation of integrin function. *A*, Expression of RapV12 effector domain mutants. Jurkat cells were transfected with empty pMT2HA vector, or vector encoding HA-tagged RapV12, RapV12/G37,RapV12/E38, RapV12/C40, or RapV12/ΔCAAX. Whole cell lysates were resolved by SDS-PAGE, transferred to PVDF, and proteins detected by immunoblotting with anti-HA Abs and ECL. *B*, Selective suppression of ROS production by RapV12 effector domain mutants. Jurkat cells were transfected with CD20 cDNA and RapV12/G37, RapV12/E38, or RapV12/C40 effector domain mutants in pMT2HA as indicated and analyzed for intracellular ROS production as in Fig. 1. In all panels, cells were stimulated with either anti-CD3 (▨), PMA+I (•) or left unstimulated (◇). The data shown are the mean values of three or more independent experiments. *C*, Stimulation of integrin-dependent adhesion by RapV12 mutants. Jurkat T cells were transiently transfected with 10 μg of pG3-TK luciferase reporter plasmid and 30 μg of empty pMT2HA vector (mock) or pMT2HA vector encoding RapV12, RapV12/G37, RapV12/E38, RapV12/C40, or RapV12/Δ CAAX mutants as indicated. Cells were allowed to adhere for 45 min on immobilized ICAM, washed, lysed, and adherent cells quantitated by luciferase assay. Error bars represent the average mean binding + SE of the percentage of bound cells from four independent experiments performed in quadruplicate. Expression of constructs was confirmed by anti-HA immunoblotting of whole cell lysates of transfected cells (data not shown). *D*, CD3-induced ROS production requires PI3K signaling upstream of Ras. Jurkat cells were transfected with cDNA encoding CD20 and either empty vector (control) or RasV12 in pMT2HA. Where indicated, cells were pretreated with 1 μM LY294002 (LY) for 24 h before loading with 6-carboxy-DCF, and stimulated with anti-CD3 (▨) or left unstimulated (◇). Intracellular ROS production was measured as in Fig. 1. *E*, PI3K activity is required for TCR-dependent Ras activation. Healthy donor PBLs were incubated for 24 in the absence or presence of with 1 μM LY294002 (LY) before stimulation with medium or anti-CD3 Ab or PMA+I, as indicated. Ras-GTP was precipitated from cell lysates with GST-RafRBD fusion protein, resolved by SDS-PAGE, and detected by immunoblotting with anti-Ras Ab. F Suppression of Ras-induced ROS production by Rap1 requires PI3-kinase activity. Jurkat cells were transfected with CD20 and RapV12 cDNA, in the absence or presence of cotransfected RasV12 cDNA. Where indicated, cells were incubated with 1 μM LY294002 (LY) for 24 h before anti-CD3 stimulation and detection of intracellular ROS, as in Fig. 1. Constructs and conditions in *C* and *E* were assessed in the same experiment, but are shown as two sections for clarity of presentation. *G*, Expression of RasV12 and RapV12 constructs in *D* and *F* was detected by resolution of whole cell lysates by SDS-PAGE, transfer to PVDF, immunoblotting with anti-Ras and anti-HA Abs, respectively, and ECL.

Constitutive ROS production in RA SF T lymphocytes correlates with deregulated Ras and Rap1 activation

Because our previous experiments indicated that ROS production in Jurkat T cells was highly sensitive to Ras and Rap1 signaling pathways, we next examined whether chronic oxidative stress observed in RA SF T lymphocytes might reflect altered signaling by these GTPases. Several potential sources of ROS in the synovial joint have been proposed to contribute to disturbed redox homeostasis in SF T lymphocytes, including activated macrophages and neutrophils, ischemia/reperfusion compromised oxygen radical tension in the inflamed joint, and generation of hydroxyl radicals by $Fe2^{+}$ released from dying cells (42). However, because TCR or phorbol ester stimulation of T cells also induces ROS production, this raised the possibility that chronic oxidative stress in RA SF T cells results from endogenous ROS production. Therefore, we first compared intracellular ROS production in RA PB and SF T lymphocytes. PB and SF T cells were loaded with the ROS-reactive dye DCF, and left unstimulated or stimulated with anti-CD3 Abs or PMA/I, followed by FACS analysis of DCF reactivity with ROS. Resting PB T cells displayed a low basal rate of ROS production (Fig. 4, *left panel*), which transiently increased for 5–10 min following PMA/I (Fig. 4) or TCR (data not shown) stimulation. No further changes in the rate of ROS production were observed up to 1 h poststimulation. However, in contrast to PB T cells, RA SF T cells displayed a high basal rate of ROS production that could not be further elevated (Fig. 4, *right panel*). Basal and PMA/I (Fig. 4) or TCR-induced ROS levels were consistently lower in human PB T cells than in Jurkat T cells. It is unclear whether

this reflects differences in cell size or efficiency of ROS regulation between primary human T cells and the leukemic cell line. The same forward light-side light scatter gates were used in analyzing PB and SF T lymphocytes, eliminating the trivial possibility that observed differences in ROS production were due to differences in cell size. These results indicated that intracellular ROS production alone was sufficient to explain disturbed redox homeostasis in SF T cells.

Figure 4

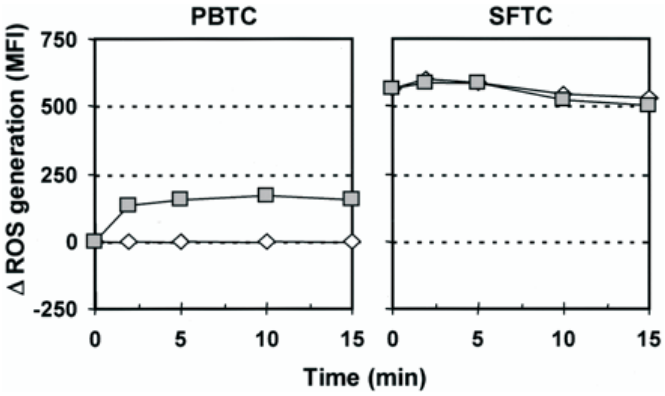

FIGURE 4. ROS is produced transiently in PB T lymphocytes and constitutively in RA SF T lymphocytes. Purified PB and SF T lymphocytes from RA patients were resuspended at 5 x 10^6 cells/ml in phenol red-free DMEM medium and loaded with DCF, stimulated with anti-CD3 (▣) or left unstimulated (◇) and analyzed for ROS production as in Fig. 1. The ΔROS generation is obtained by reducing the measured MFI with the measured MFI at corresponding time points in the unstimulated PB T lymphocyte group. The data shown are the mean values of three or more independent experiments.

We next examined the possibility that Ras and Rap1 signaling may be altered in oxidatively stressed RA SF T lymphocytes. SF and PB T lymphocytes were isolated from RA patients, left unstimulated or activated with anti-CD3 Abs or PMA/I. Active GTP-bound Ras and Rap1 proteins were precipitated with Raf1-RBD and RalGDS-RBD GST fusion proteins, respectively, and detected by immunoblotting. Both anti-CD3 and PMA/I stimulation potently activated Rap1 in PB T lymphocytes from RA patients (Fig. 5*A*), in accordance with previous observations in PB T lymphocytes from healthy controls (5). In contrast, basal levels of activated Rap1 were decreased in SF T lymphocytes and could not be increased through TCR or PMA/I stimulation (Fig. 5*A*). Minimal amounts of GTP-bound Rap1 were detected in SF T cells following long overexposure of the immunoblot, but again no increase was noted following cell stimulation (data not shown). Our failure to detect activation of Rap1 in SF T cells was not due to decreased expression of Rap1 in SF T cells, as no consistent differences in total Rap1 were observed between PB and SF T cells. TCR and PMA/I–dependent Ras activation was also observed in PB T cells from RA patients. However, unlike Rap1, Ras activation in SF T cells was constitutively high and could not be further enhanced through stimulation (Fig. 5*B*). Equivalent amounts of total Ras were observed in whole cell lysates of both PB and SF T cells. Despite constitutive Ras activation, TCR and PMA/I–dependent ERK activation, as detected by phospho-specific Abs, was blocked in SF T cells (Fig. 5*B*). No differences in total ERK expression were observed. Similar results were obtained for Ras and Rap1 in SF T cells from six different RA patients, whereas ERK activation was diminished in four of five patients examined (data not shown).

Unlike previously noted defects in RA SF T lymphocyte signaling, as observed for linker for activation of T cells phosphorylation and IL-2 production (30,31), we obtained no evidence that

deregulated Ras and Rap1 activation was due to chronic oxidative stress. Acute stimulation of PB T lymphocytes from healthy controls with hydrogen peroxide transiently activated Rap1, but not Ras (Fig. 5*C*). Pretreatment of PB T lymphocytes with BSO, which results in depletion of intracellular GSH and abrogated TCR-dependent linker for activation of T cells phosphorylation (data not shown) also failed to mimic the inhibition of Rap1 activity observed in SF T lymphocytes (Fig. 5*D*). Finally, restoration of GSH equilibrium in SF T lymphocytes with the antioxidant NAC, failed to rescue Rap1 activation (Fig. 5*E*). Together these data indicated that constitutive activation of Ras and a block in agonist-induced Rap1 activation observed in RA SF T lymphocytes was not resultant from chronic SF T lymphocyte exposure to ROS. Instead, these results raised the possibility that altered Ras and Rap1 signaling was responsible for chronic oxidative stress in these cells.

Figure 5

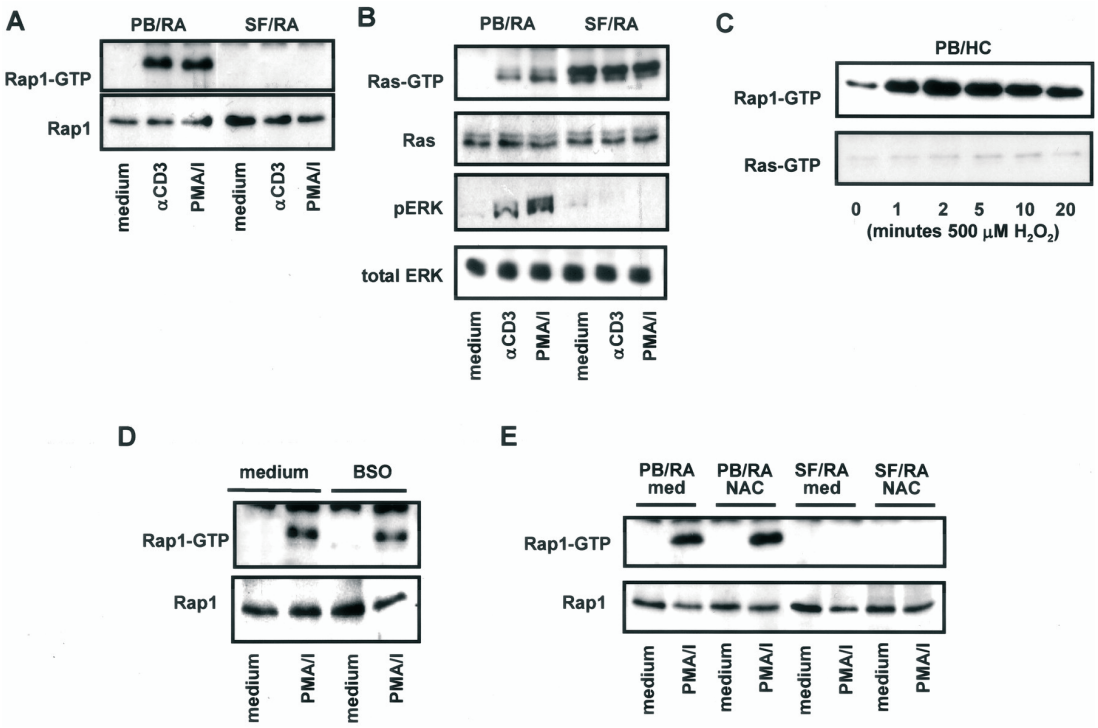

FIGURE 5. Ras and Rap1 signaling are deregulated in RA SF T lymphocytes. *A* and *B*, Blocked Rap1 activation and constitutive Ras activation in RA SF T lymphocytes. Peripheral blood (PB) and synovial fluid (SF) T lymphocytes of rheumatoid arthritis (RA) patients were isolated and stimulated for 5 min with medium, anti-CD3 Ab (0.5 μg/ml anti-CD3, αCD3) or 100 ng/ml PMA plus 1 μg/ml ionomycin (PMA/I). Active GTP-bound Rap1 (*A*) and Ras (*B*) proteins from 5 x 10^6 T cells were precipitated with RalGDS-RBD and Raf-RBD GST fusion proteins, respectively, resolved by SDS-PAGE, and detected by immunoblotting with anti-Rap1 or anti-Ras Abs. Total Rap1, Ras, phosphorylated ERK (pERK), and total ERK were detected in 35 μl whole cell lysate. *C*, Acute oxidative stress activates Rap1, but not Ras. PB T lymphocytes from healthy donors were left unstimulated or stimulated for the indicated times with 500 μM hydrogen peroxide (H_2O_2) before lysis and precipitation and detection of activated Rap1 and Ras as in *A* and *B*. *D*, Depletion of intracellular GSH levels does not affect Rap1 activation. Normal PB T lymphocytes were cultured in medium alone (medium) or medium containing 200 μM BSO for 72 h. Cells were then left unstimulated or stimulated for 5 min with PMA/I, and GTP-bound Rap1 (*upper*) and total Rap1 (*lower*) detected as in *A*. *E*, Supplementation of intracellular GSH levels does not restore Rap1 activation in SF or RA cells. PB and SF T lymphocytes from a representative RA patient were cultured in 5 mM NAc for 48 h before stimulation with medium or PMA/I and detection of GTP-bound Rap1 *A*.

Ras and Rap-1 dependent oxidative stress negatively regulates ERK activation

One important consequences of chronic oxidative stress in RA SF T lymphocytes is a decreased proliferative response to mitogenic stimuli. Therefore, we examined whether manipulation of Ras and Rap1 activation in Jurkat T cells could recapitulate altered ERK signaling observed in RA SF T lymphocytes. In initial experiments, we had noted that TCR and PMA/I–induced activation of ERK was diminished in RA SF T cells (Fig. 5*B*). Transfection of Jurkat cells with small quantities (1–3 μg) of RasV12 cDNA led to enhanced phosphorylation of cotransfected Myc epitope-tagged ERK2 (data not shown). However, higher concentrations of RasV12 cDNA used in ROS experiments failed to activate ERK2, and resulted in an ~50% inhibition of TCR-induced ERK2 phosphorylation (Fig. 6*A*). A similar reduction in TCR-stimulated ERK2 activation was observed in cells cotransfected with the active Ral exchange factor Rlf-CAAX (Fig. 6*B*). In both cases, maintenance of intracellular GSH levels by incubation in NAc restored ERK responsiveness to TCR stimulation, indicating that constitutive Ras and Ral activation inhibited ERK activation through ROS production. Cotransfection of active RapV12 with RasV12 (Fig. 6*C*) also rescued defective TCR-stimulated ERK activation. Thus, chronic oxidative stress resulting from constitutive activation of Ras and Ral diminishes TCR-proximal proliferative signals, whereas Rap1 activation protects against this inhibitory effect.

Activation of Ras is required to maintain ROS production in RA SF T lymphocytes

Finally, we directly examined whether altered Ras family GTPase signaling was responsible for oxidative stress in RA SF T lymphocytes. To test this, we incubated PB and SF T cells in medium alone or containing cell-permeable Tat peptides fused to control β-Gal (Tat-βGal) or dominant-negative RasN17 (Tat-RasN17). Introduced Ras protein was readily detected in T lymphocytes incubated with Tat-RasN17, but not control PB T lymphocyte lysates (Fig. 7*A*). As compared with cells treated with medium alone or Tat-βGal, TCR-dependent ERK activation was completely inhibited in PB T cells exposed to Tat-RasN17, demonstrating that the RasN17 peptide was internalized and functional (Fig. 7*B*). We observed no significant differences in the basal rate of ROS production between PB T cells incubated with Tat-βGal and Tat-RasN17 (Fig. 7*C*). In contrast, Tat-RasN17, but not control Tat-peptide, completely inhibited the enhanced rate of ROS production observed in SF T lymphocytes, providing direct evidence that altered GTPase signaling is responsible for oxidative stress in RA SF T lymphocytes.

Figure 6

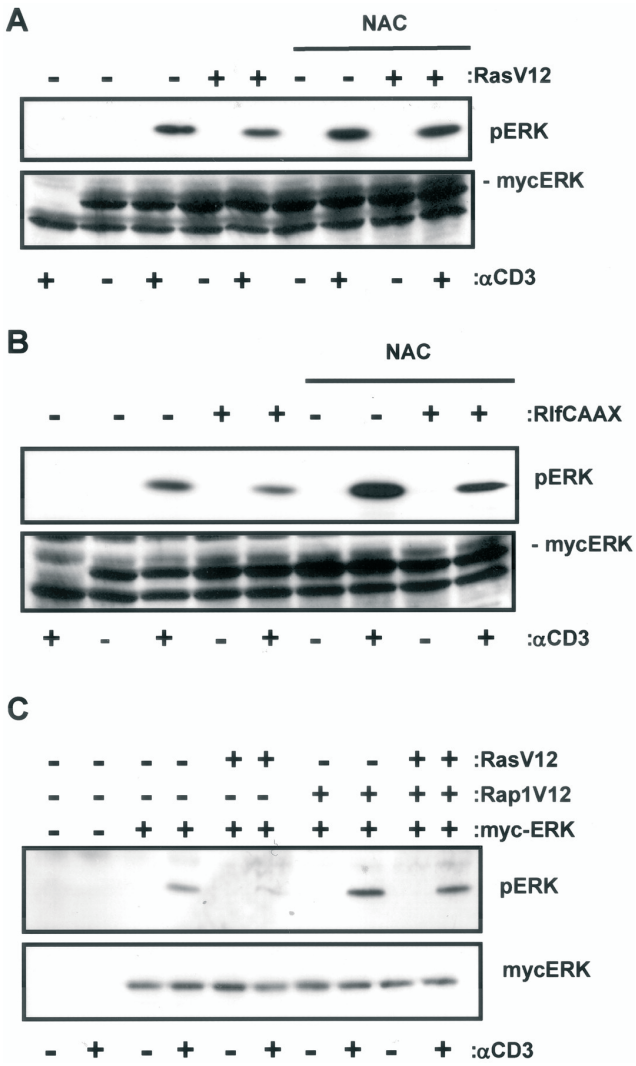

FIGURE 6. Chronic oxidative stress by constitutive Ras or Ral signaling diminishes TCR-dependent ERK phosphorylation that can be suppressed by Rap1. *A* and *B*, Constitutive Ras (*A*) or Ral (*B*) signaling inhibits TCR-dependent ERK activation via ROS production. Jurkat T cells were mock-transfected (*first lane, two columns*) or transfected with *myc*-tagged ERK, alone or in combination with Ras V12 (*A*) or Rlf-CAAX (*B*). Where indicated, cells were maintained in 5 mM NAc. At 48 h posttransfection, cells were harvested and left unstimulated, or stimulated for 5 min with anti-CD3 (α CD3) Ab. Cell lysates were immunoprecipitated with anti-*myc* Ab, and ERK activation detected by immunoblotting with phospho-specific anti-ERK1/2 Abs (pERK). *C*, Rap1 relieves inhibition of ERK activation by Ras. Jurkat T cells were mock transfected (*first lane*), or transfected with *myc*-tagged ERK alone or in combination with active RasV12 and/or Rap1V12 as indicated. Cells were activated, and ERK phosphorylation assessed as in *A*. Results are representative of two to three independent experiments. Equivalent levels of transfected *myc*-tagged ERK were detected within each experiment by resolution of whole cell lysates by SDS-PAGE, transfer to PVDF, immunoblotting

Figure 7

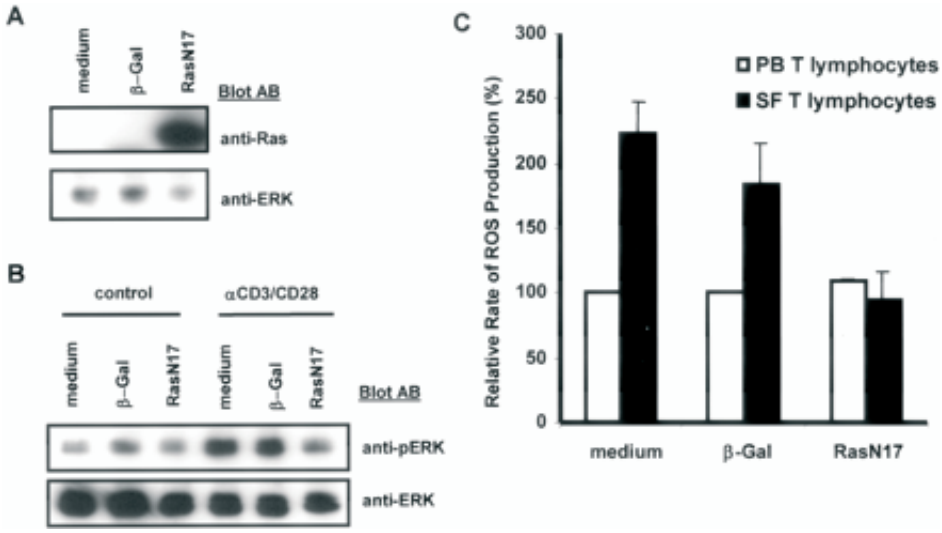

FIGURE 7. Inhibition of Ras signaling blocks oxidative stress in RA SF T lymphocytes. *A*, Expression of cell-permeable Tat-RasN17 peptide in T lymphocytes. PB T lymphocytes were cultured overnight in medium alone, or 25 μg/ml Tat-β-galactosidase (β-Gal) or Tat-RasN17 (RasN17) peptide. Cells were washed extensively, lysed, and Ras detected by SDS-PAGE resolution, immunoblotting with anti-Ras Ab and ECL (*top*). Immunoblotting with anti-ERK Ab demonstrated that equivalent amounts of protein were loaded from each sample (*bottom*). *B*, Tat-RasN17 peptide blocks TCR-dependent ERK activation in T lymphocytes. PB T cells were treated with medium, Tat-β-Gal or Tat-RasN17 peptides (*A*), and stimulated for 5 min with medium (control) or anti-CD3 and anti–CD28 Abs (αCD3/CD28), before lysis and analysis of ERK phosphorylation (pERK) and expression (ERK) by SDS-PAGE, immunoblotting and ECL. *C*, Inhibition of Ras signaling blocks ROS production in RA SF T lymphocytes. Purified RA PB (□) and SF T lymphocytes (■) were incubated with medium, Tat-β-Gal or Tat-RasN17 peptides (*A*), before measurement of basal ROS production as in Fig. 1. The data shown represent the mean and SD of three independent experiments. Within each experiment, the rate of ROS production was normalized to 100 for medium-treated PB T lymphocytes.

Discussion

Our results reveal a novel function for Ras, Ral and Rap1 GTPases: a critical role in the regulation of transient ROS production in T lymphocytes following agonist stimulation. In PB T cells, agonist-induced activation of Ras, signaling via Ral, leads to the transient production of ROS (Fig. 8, *left panel*). Concomitant activation of Rap1 serves to attenuate ROS production, distally from Ral. When both Ras and Rap1 are transientally activated, limited ROS production is used as a second messenger optimizing Ras-dependent activation of ERKs and transcription factors. Intriguingly, we find that acute stimulation of T cells with ROS activates Rap1, suggesting that Rap1 may serve as a sensor to protect against oxidative toxicity. The cellular machinery responsible for coupling Ral and Rap1 to ROS regulation in T cells remains to be identified. While this report was under review, Nagy and colleagues (43) reported a detailed pharmacological analysis of the relationship between TCR signaling, ROS production, and changes in mitochondrial integrity, an important step in T cell apoptosis. They found that TCR-dependent ROS production and calcium-release combined to increase NO production as a

requisite step in mitochondrial hyperpolarization, further ROS production, and apoptosis. Previous studies have shown that TCR stimulation results in ROS production via catalase-sensitive hydrogen peroxide production, requiring BAPTA-AM-sensitive calcium release, and diphenylene iodonium (DPI)-sensitive superoxide anion generation (44). Preliminary studies from our laboratory indicate that the primary source of ROS in SF T cells is hydrogen peroxide, but not superoxide anion. Additionally, we find that Ras-induced ROS, as well as enhanced ROS production in RA SF T cells, is also sensitive to catalase and BAPTA-AM, but not DPI. However, monitoring mitochondrial integrity with the potential-dependent J-aggregrate-forming dye 5,5',6,6'-tetrachloro-1,1'3,3'-tetraethylbenzimidazolocarbocyanine iodide (JC-1), we observed no differences in mitochondrial potential between PB and SF T cells, nor mock and RasV12-transfected Jurkat T cells. Rap1 has been shown to associate with NADPH complexes in neutrophil-differentiated human HL-60 cells (45), but many components of the NADPH oxidase are not expressed in T lymphocytes. A homologue of gp91^{phox} has been identified in T cells, but this enzyme is also DPI-sensitive (46).

Figure 8

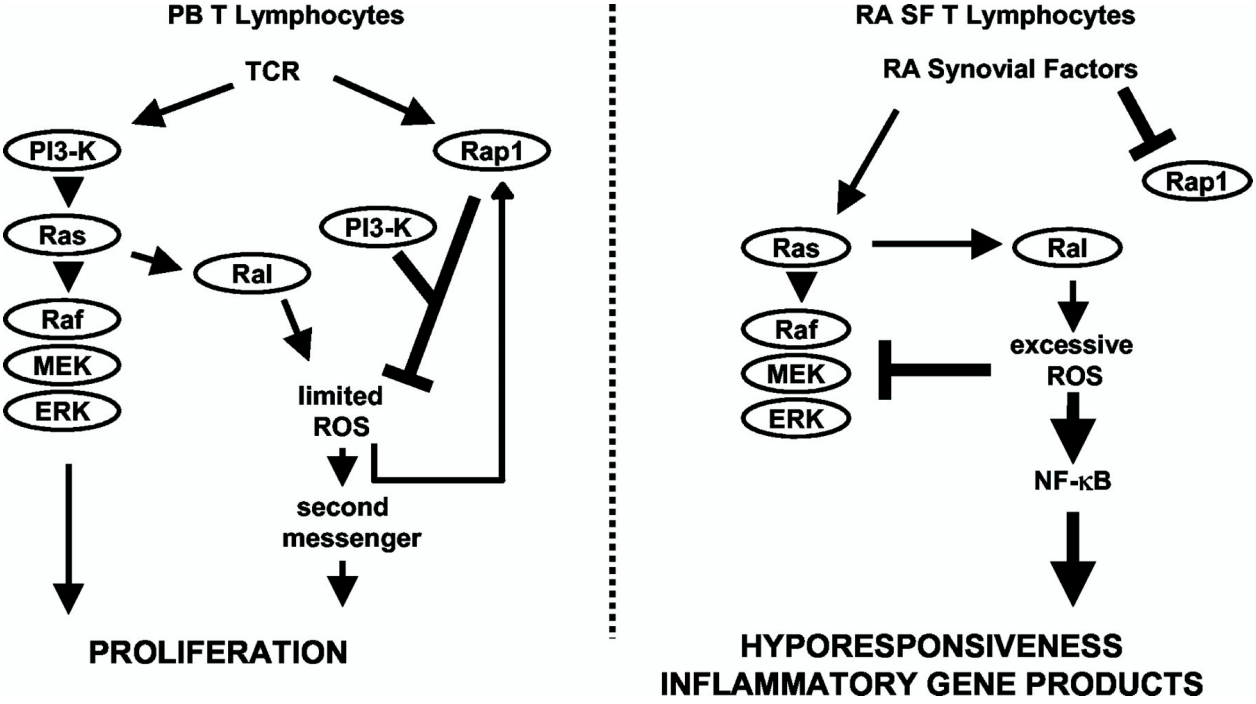

FIGURE 8. A hypothetical model for regulation of ROS production in PB and RA SF T lymphocytes by Ras family GTPases. *A*, In PB T lymphocytes, TCR stimulation leads to PI3K–dependent activation of Ras. Subsequent activation of Ral leads to production of ROS. Concomitantly, TCR stimulation activates Rap1. Rap1, signaling via a pathway dependent upon or acting in parallel to PI3K, down-regulates ROS production. Transient ROS production cooperates with the Raf/ERK pathway to optimize proliferative transcriptional events. *B*, In RA SF T lymphocytes, unidentified synovial microenvironment factors constitutively activate Ras and inhibit Rap1 signaling. In the absence of Rap1 signaling, ROS production by Ras and Ral is not down-regulated, leading to oxidative stress. Oxidative stress inhibits TCR signaling to proliferative pathways, such as Raf/ERK, causing antigenic hyporesponsiveness. Conversely, resultant oxidative stress may lead to enhanced NF-κB–dependent transcription of inflammatory gene products. Activating pathways are indicated by arrowheads and arrows, with proposed relative strength of signal correlating with arrow thickness. T bars indicate negative or suppressing signaling pathways.

Rap1 appears to regulate ROS production distally from Ras, rather than via competitive sequestration of Ras effectors by Rap1. We observe that Rap1-dependent inhibition of ROS is PI3K-dependent, whereas Ras-induced ROS generation is Ral-dependent. Moreover, RapV12 effectively inhibited Ral-induced ROS generation (Fig. 2), and Rap1 and Ral have dissimilar effector domains (3). Although our data show that Rap1 suppresses ROS production via a LY294002-sensitive pathway, we have been unable to demonstrate a direct association between Rap1 and PI3K in T cell lysates. Additionally, in a DO11 murine thymoma line stably expressing active RapV12, we detect no constitutive activation of PI3K, despite suppressed ROS production and enhanced integrin activation in these cells (data not shown). Together, this supports the idea that a PI3K-dependent effector of Rap, rather than PI3K itself, is responsible for ROS suppression in T lymphocytes.

It is clear from previous studies in T lymphocytes that constitutive Rap1 activation can profoundly influence Ras-dependent transcriptional activity, such as NFAT, the minimal IL-2 promoter, and Elk-1 (6-8). However, these are very distal readouts of Ras signaling, and little data support a model in which Rap1 activation in T lymphocytes antagonizes Ras-dependent Raf activation. In transgenic mice expressing activated Rap1 in the T cell compartment at levels equivalent to endogenous Rap1, no inhibition of TCR-dependent ERK activation is observed, despite constitutive integrin activation by Rap1 (14). Similarly, we and others (our study and Ref. 9) fail to observe inhibition of ERK activation in T cells overexpressing active Rap1. Although this strongly argues against a role for Rap1 in directly antagonizing Ras activation of the Raf/ERK pathway, it does not explain the previously observed effects on Ras-dependent transcriptional activity. Our finding that activation of Rap1 down-regulates Ras-induced ROS production in T cells provides a molecular mechanism by which Rap1 can antagonize Ras signaling pathways, distally, downstream of Ral. Reevaluation of Rap1 effects on TCR-dependent NFAT and Elk-1 activity within the context of ROS production will be important. Also, Rap1 antagonism of ROS production by Ras may represent a physiological mechanism by which Rap1 suppresses Ras-dependent oncogenic transformation. Our identification of Rap1 effector domain mutants that fail to regulate ROS production, yet support integrin-dependent adhesion, will serve useful in assessing these possibilities.

In RA SF T cells, as yet unidentified factors lead to constitutive activation of Ras and a block in Rap1 signaling (Fig. 8, *right panel*). In the presence of constitutive Ras-dependent ROS production, the resultant oxidative stress results in inhibition of TCR-proximal proliferative signals, via misfolding of linker for activation of T cells, and diminished ERK activation, possibly via redox sensitive interactions between Ras and Raf (47). Oxidative stress has also been proposed to induce constitutive NF-κB transcriptional activity in RA synovial T lymphocytes, contributing to expression of inflammatory markers such as TNF-α and IL-1 receptors that perpetuate inflammation in the synovial joint. Ral-dependent suppression of ERK signaling and activation of NF-κB have been previously reported in other cell types, but involvement of ROS was not addressed in these studies (48,49). Preliminary experiments indicate that Ras-dependent signaling via Ral also stimulates NF-κB transcriptional activity in Jurkat T cells, which can be blocked by Rap1 activation (P.H.J. Remans and K.A. Reedquist, unpublished observation). Further studies will be required to determine whether Ral and Rap1–dependent modulation of NF-κB activity is mediated by ROS.

The chronic oxidative stress observed in SF T cells can be mimicked by transient transfection of Jurkat cells with activating Ras and interfering Rap1 proteins, and inhibited in RA SF T cells with dominant-negative Ras. Therefore, we propose that deregulation of Rap and Ras are critical events leading to the disturbed intracellular redox balance underlying antigenic hyporesponsiveness and inflammatory gene transcription in RA synovial. It is likely that complex

interactions of T cells with other synovial cells and secreted stimuli contribute to deregulated Ras and Rap1 signaling in the synovial joint, and we are currently attempting to identify these factors. Many of the inflammatory stimuli encountered by T cells in the synovial joint (for example, TNF, IL-1, stromal-derived factor-1, and CD40) are known to activate Ras in T lymphocytes and other cell types. Similarly, ligation of CD28 on SF T lymphocytes and/or stimulation via $G_{\alpha i}$ subunits used by chemokine receptors might be responsible for inactivation of Rap1 (5,7,10).

The identification of Rap1 as a key regulatory protein involved in oxidative stress in SF T lymphocytes provides the first direct evidence for a role for Rap1 in human disease. In vitro, and in pharmacological and genetic studies in rodent models of arthritis, there are strong indications that ROS-dependent activation of NF-κB in T lymphocytes contributes to pathogenesis (21,50,51). Strategies aimed at restoring regulated Ras and Rap1 function in SF T lymphocytes may be therapeutic in RA. Recently, adenoviral-mediated expression of dominant-negative Ras in the joints of arthritic rats was shown to decrease inflammation and joint destruction, indicating the therapeutic potential in targeting these pathways (52). It will be of considerable interest to examine whether deregulated Ras and Rap1 signaling contributes to other human diseases in which chronic oxidative stress is thought to alter T cell function, such as systemic lupus erythematosus and HIV infection.

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CHAPTER 4 :

CTLA4Ig Suppresses Rheumatoid Arthritis T Cell Reactive Oxygen Species Production by Preventing Inactivation of Rap1

Inflammatory Cytokines and Synovial Adherent Cells Synergistically Induce Oxidative Stress in Rheumatoid Arthritis T Cells through Modulation of Ras Family GTPase Activity

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Abstract

Objective. Oxidative stress contributes to the inflammatory properties of rheumatoid arthritis (RA) synovial T lymphocytes. In this study we investigate the mechanisms leading to reactive oxygen species (ROS) production and oxidative stress in RA synovial T lymphocytes.
Methods. ROS production in healthy donor (HD) peripheral blood (PB) T lymphocytes and RA patient PB and synovial fluid (SF) T lymphocytes was measured by ROS-dependent fluorescence of 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate-di(acetoxymethyl ester). Rap1 GTPase activation was assessed by activation-specific probe precipitation. RA PB and SF T lymphocyte proliferation was assayed by ^{3}H-thymidine incorporation. In some experiments, RA PB T cells were preincubated with autologous SF or PB or SF adherent cells. Experiments were performed in the absence or presence of transwell membranes, or CTLA4Ig fusion proteins. Acute and chronic stimulations of HD PB T lymphocytes were performed with inflammatory cytokines, in the absence or presence of activating anti-CD28 antibodies.
Results. T lymphocyte ROS production and Rap1 inactivation was mediated by cell-cell contact with SF adherent cells, correlating with T cell mitogenic hyporesponsiveness. CTLA4Ig blockade of synovial adherent cell signaling to T cell CD28 relieved Rap1 inhibition and ROS production. Introduction of active RapV12 into T cells also prevented induction of ROS production. Coincubation of T cells with stimulating anti-CD28 antibody and inflammatory cytokines synergistically increased T cell ROS production.
Conclusion. Cell-cell contact between T cells and RA synovial adherent cells mediates Rap1 inactivation and subsequent ROS production in T lymphocytes following exposure to inflammatory cytokines. This process can be blocked by CTLA4Ig fusion protein.

Introduction

In vitro studies, animal disease models, and clinical studies have all provided strong evidence that T lymphocytes perpetuate inflammation and eventual joint destruction in the rheumatoid arthritis (RA) synovial joint (1-3). T lymphocytes derived from RA synovial tissue display markers of recent activation, including upregulation of HLA class II proteins and very late antigen (VLA) -4 integrins (4;5). Consistent with this activated phenotype, RA synovial T lymphocytes can stimulate monocyte TNF-α production in a cell-cell –dependent manner (6). Paradoxically, RA synovial T lymphocytes are noncycling and hyporesponsive to subsequent mitogenic stimuli, including T cell receptor ligation (7-12). Although direct evidence that T cell activation in RA synovial tissue is mediated by antigen-specific stimulation is still lacking, a pivotal pathogenic role for T lymphocytes in RA is highlighted by the recent clinical success of CTLA4Ig fusion protein (abatecept), which disrupts T cell CD28 costimulatory protein interactions with CD80/86 on antigen-presenting cells (13;14).

Reactive oxygen species (ROS)-dependent chronic oxidative stress is thought to underlie the pathophysiology of T cells in many autoimmune diseases, including RA, systemic lupus erythmatosis, and human immunodeficiency virus infection (1). In RA synovial T cells, chronic oxidative stress leads to misfolding of cysteine-rich signalling

proteins required for proliferation, including Linker for activated T cells (LAT) and the T cell receptor –associated ζ chain (15-17). This effect can be mimicked pharmacologically in normal T lymphocytes (18), and in murine T cell hybridomas chronically exposed to TNF-α (19). Restoration of redox balance, or overexpression of mutant ROS-insensitive LAT (20) and TCR-ζ (21) can partially relieve induction of mitogenic hyporesponsiveness by oxidative stress. Oxidative stress may also contribute to inflammation through enhancing NF-κB-dependent transcription of inflammatory cytokines and apoptotic and anti-apoptotic Bcl-2 family proteins, conferring resistance to apoptotic stimuli. In contrast, transiently produced hydrogen peroxide and superoxide anions act as important second messengers in TCR signaling (22). Acute stimulation of T cells with physiologically relevant concentrations of ROS can enhance MAP kinase activation, proliferation, and transcription by NF-κB, AP-1 and the IL-2 promoter. As many cellular effects of ROS can be suppressed by antioxidants, physiological ROS generation is thought to "fine-tune" T cell antigen responses (23;24).

We have recently demonstrated that oxidative stress in RA synovial fluid (SF) (25) and synovial tissue (26) T lymphocytes is a result of T cell-intrinsic intracellular ROS production. Persistent ROS production in RA SF T cells correlates with constitutive activation of the small GTPase Ras, and blocked activation of the related GTPase Rap1 (27), two signaling proteins which play central roles in integrating intracellular signaling pathways to determine functional outcomes of T cell stimulation. Ras activation is sufficient and necessary for intracellular ROS production, while activation of Rap1 can block agonist and Ras-dependent ROS production. In contrast, inhibition of Rap1, as observed in RA SF T cells, prevents downregulation of ROS production in a human model T cell line following agonist stimulation. As these studies provided evidence that altered Rap1 signaling is responsible for oxidative stress in RA synovial T cells, we here explored which factors in the synovial microenvironment may alter T cell Rap1 activation to induce T cell oxidative stress in RA.

Patients and Materials

Patient characteristics. Peripheral blood (PB) from healthy volunteers, and PB and SF from RA patients in our clinic, were obtained under protocols approved by the medical ethics committee of the Academic Medical Center, University of Amsterdam. Paired PB and SF samples were obtained from 28 RA patients (18 women and 10 men) meeting the American College of Rheumatology criteria for RA (28). The median disease duration of patients was 4.4 years (+/- 7.6 years), 21 were seropositive for rheumatoid factor, 25 were receiving disease-modifying anti-rheumatic drugs and 3 were taking prednisolone.

Isolation and culture of PB and SF cells. PB and SF mononuclear cells (MCs) were obtained by Ficoll gradient centrifugation. PB and SF T cells were purified from MCs using a negative isolation procedure, according to the manufacturer's specifications (T Cell Negative Isolation Kit, Dynal Biotech, Norway). Purified T cells were greater than 90% $CD3^+$ as assessed by FACs analysis. In some experiments, autologous PB and SF adherent cells (>70% $CD14^+$ monocytes) were obtained by allowing PBMCs and SFMCs to adhere to plastic tissue culture dishes overnight, followed by washing. PB T cells were then incubated alone or with purified adherent cells for 72 hours at a ratio of

3:1. Cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, glutamine, HEPES buffer, penicillin and streptomycin (all from Gibco).

T cell proliferation assays. In control experiments, PB and SF T lymphocytes were stimulated at a concentration of 5 x 10^5 cells/well in 96-well plates with activating anti-CD3 (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service [CLB], Amsterdam, The Netherlands) and anti-CD28 antibodies (15E5, provided by Dr. R. van Lier, our institute). Seventy-two hours post-stimulation, cells were pulsed with 1μCi/well [^{3}H]thymidine (New England Nuclear, Boston, MA) for an additional 20 hours. Cells were subsequently harvested on filter-mats (Skatron Instruments, Lier, Norway) and incorporation of radioactivity measured using a liquid scintillation counter (Skatron Instruments). Where indicated, purified PB T lymphocytes were preincubated for 72 hours at a ratio of 3:1 with autologous PB or SF adherent cells, or with 50% autologous SF, prior to repurification and stimulation. Alternatively, T cell preincubation with PB or SF adherent cells was conducted in the presence or absence of transwell membranes (Costar), or in the presence of 10 μg/ml control Ig or recombinant CTLA4Ig (kindly provided by Dr. R.A. van Lier, Division of Experimental Immunology, our institute).

Measurement of ROS production in T cells. Purified PB and SF T cells were resuspended at 5 x 10^6 cells/ml in phenol red-free DMEM medium (Gibco) and loaded for 20 minutes at 37C with 28 μM ROS-reactive dye 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate – di(acetoxymethyl ester) (DCF) (Molecular Probes, Eugene, OR). Cells were then either left unstimulated, or stimulated with anti-CD3 antibody 1XE, TNF-α (10 ng/ml), TGF-β (4 μg/ml), IL-1β (125 pg/ml), IFN-γ (100U/ml) (all cytokines from R&D) or 50% autologous RA patient SF. Cells were analyzed for ROS production on a FACScan (Becton Dickinson, San Jose, CA) for the mean fluorescence intensity (MFI) of oxidated DCF in the FL1 channel at 0, 10, and 20 minutes post-stimulation. For nucleofected PB T cells (see below), T cells were gated first by forward and side scatter to identify viable cells, and then by CD20 staining (CyChrome-conjugated anti-CD20 antibody, BD Pharmingen, San Diego, CA) to detect transfected cells. Nucleofected T cells were 51%- positive (+/-9%, n=4) for CD20 expression 24 hours post-nucleofection, and 38%- positive (+/-15%) 96 hours post-nucleofection. Viability of CD20-positive T cells was greater than 90% at both time-points as assessed by Annexin V staining.

Detection of Rap1 activation. Repurified PB and SF T cells were resuspended at 5x 10^6 cells/ml in serum-free RPMI 1640 medium, equilibrated for 10 minutes at 37°C, and then left unstimulated, or stimulated for 5 minutes with PMA (100 ng/ml) and ionomycin (1 μg/ml) (both compounds from Sigma). Cells were then lysed in lysis buffer containing 1% NP-40, 10% glycerol, 20 mM Tris (pH 7.6), 150 mM NaCl, 4 mM $MgCl_2$, 2 mM NaVO4, 10 mM NaF, and 2 mM PMSF. Insoluble cell material was removed by centrifugation. GST fusion proteins of RalGDS RBD (for precipitation of active Rap1) were precoupled to glutathione-sepharose beads (Amersham) and incubated with cellular lysates while rocking for 1 hour at 4C. Subsequently, bound activated Rap1 was precipitated, washed 5 times in lysis buffer, and eluted in Laemli's sample buffer. Proteins were separated by SDS-PAGE, and transferred to PVDF membranes (Bio-Rad, Hercules, CA). Following blocking of blots in 2% milk/TBS/T, GTPases were detected by immunoblotting overnight in anti-Rap1 antibodies (1:500 in TBS/T) (BD

Transduction Laboratories, Lexington, KY). Blots were then washed 6x in TBS/T, incubated anti-mouse-horesradish peroxidase –conjugated antibody (BioRad, 1:3000 in 2% milk/TBS/T), washed again six times, and developed in ECL Detection Solution (Amsersham).

Introduction of cDNA into human PB T lymphocytes by nucleofection. To examine the influence of active Rap1 on SF monocyte-induced ROS production in PB T cells, 5 x 10^6 purified human T lymphocytes were incubated in nucleofection buffer (Human T Cell Nucleofection Kit, Amaxa) alone, or buffer containing 3 μg pCMV-CD20 (to detect transfected cells) and 7 μg empty vector pMT2-HA or pMT2-HA-RapV12 (encoding constitutively active Rap1). Cells were nucleofected according to the manufacturer's directions using program setting U-14. Cells were allowed to rest overnight prior to stimulation and ROS detection, or incubation with PB and SF adherent cells.

Statistical analysis. Data was analyzed using SPSS 11.5.1 software for Windows (SPSS, Chicago, IL). Because the data was non-parametrical, the Wilcoxon signed rank test was performed to compare paired data from control samples versus treated samples from the same patient. P values less than 0.05 were considered significant.

Results

Regulation of T lymphocyte ROS production and Rap1 activity by synovial adherent cells

Similar to previous results (29), RA SF T cells displayed a high basal rate of intracellular ROS production as compared to autologous PB T lymphocytes ($P<0.05$) (Figure 1A). Initial experiments revealed a time-dependent decrease in intracellular ROS production in purified RA SF T lymphocytes cultured *ex vivo* (data not shown), indicating that T cell signaling pathways regulating ROS production were maintained by synovial microenvironment factors *in vivo*. To gain insight into the identity of these factors, we examined the effects of autologous synovial fluid (SF) on PB T cell ROS production. However, coincubation of PB T cells with autologous SF cells failed to induce a significant increase in ROS production (Figure 1A). Next we examined the effects of antigen presenting cells (APC) isolated from PB and SF on PB T cells. APC were obtained by allowing PBMCs and SFMCs to adhere to plastic tissue culture dishes overnight, followed by washing, and consisted of >70% monocytes as detected by CD14-staining. Coincubation of PB T cells with autologous SF adherent cells induced significant T cell ROS production, as compared to untreated PB T cells ($P<0.05$), similar to levels observed in SF T cells (Figure 1A).

We next sought to determine if synovial adherent cell-stimulated T cell ROS production was mediated by secreted products or cell-cell contact. To address this, purified PB T cells were preincubated with SF adherent cells, either together, or separated by a transwell membrane. Separation of T cells and SF adherent cells reduced T cell ROS production to levels observed in unstimulated PB T lymphocytes ($P< 0.05$) (Figure 1B).

Consistent with a proposed role for inactivation of Rap1 in mediating RA SF T cell oxidative stress (30), a block in T cell Rap1 activation was observed only following coincubation with SF adherent cells. Activated, GTP-bound Rap1 could be readily

detected in RA PB T lymphocytes following PMA/ionomycin stimulation (Figure 1C). Preincubation of T cells with autologous SF had no inhibitory effect on subsequent Rap1 activation. Maintenance of PB T cells in the presence of PB adherent cells also failed to influence Rap1 activation. However, preincubation of PB T cells with SF adherent cells, or PB adherent cells exposed to SF, led to a complete block in Rap1 activation. Again, separation of PB T cells and SF adherent cells by a transwell prevented inhibition of Rap1 signaling (Figure 1D). Together, these results demonstrated a strong relationship between T cell Rap1 inactivation and ROS production by activated SF adherent cells.

Figure 1

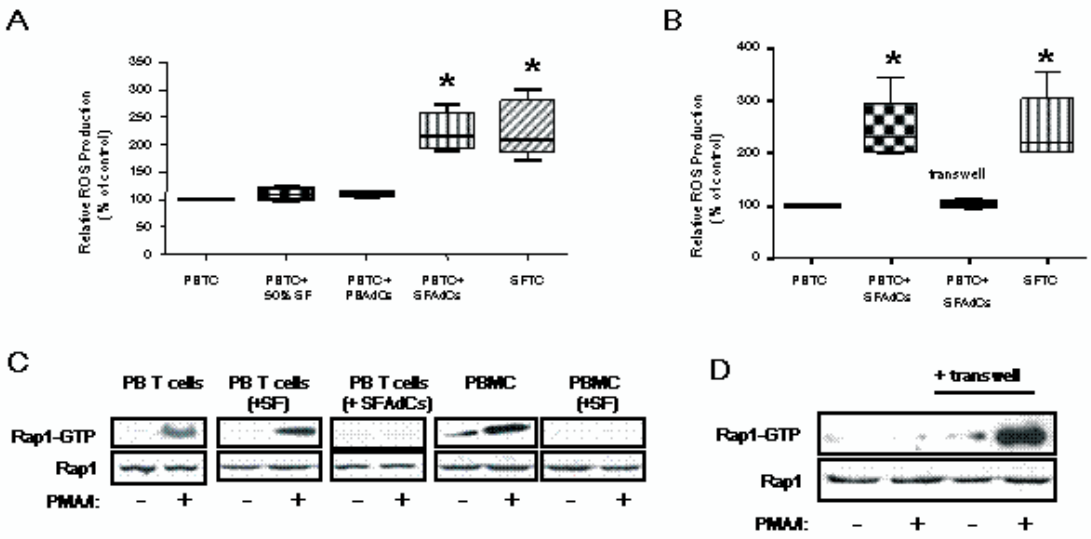

Figure 1. T cell reactive oxygen species (ROS) production and Rap1 inactivation by cell-cell contact with synovial fluid (SF) adherent cells (AdCs). **A,** SFAdCs induce ROS production in rheumatoid arthritis (RA) peripheral blood (PB) T cells (PBTC). Purified RA PB T lymphocytes were incubated overnight alone or in combination with autologous 50% SF, PBAdCs or SFAdCs. Autologous SF T cells (SFTC) were incubated in medium alone. T cells were purified, labelled with the ROS-reactive dye DCF, and ROS production assessed by FACs analysis. Data are expressed as the change in DCF reactivity over twenty minutes, normalized to 100% for PB T cells. Results represent the mean +/- SD of 5 independent experiments. Conditions displaying significant differences in ROS production as compared to PBTC alone are indicated (* $P< 0.05$). **B,** SFAdC induction of T cell ROS production requires cell-cell contact. Experiments were performed as in A. Where indicated, RA PB T cells and SFAdCs were separated during the incubation by a transwell membrane. Data are expressed as in A, and represent the mean +/- SD of five independent experiments. Conditions displaying statistically significant differences from untreated PBTC are indicated (* $P<0.05$). **C,** SFAdCs block activation of Rap1 in T lymphocytes. RA PB T lymphocytes were incubated overnight in medium alone, or in medium containing 50% autologous SF or SFAdCs. Alternatively, unfractionated PB mononuclear cells (PBMCs) were cultured overnight in the absence or presence of 50% SF. T cells were then purified, stimulated with medium alone or PMA + ionomycin (PMA/I) for 5 minutes and lysed. Active GTP-bound Rap1 was precipitated with immobilized RalGDS-RBD GST fusion protein, and analyzed by immunoblotting. One experiment representative of five independent experiments is shown. **D,** SFAdCs inactivate T cell Rap1 by cell-cell contact. RA PB T cells were coincubated overnight with SFAdCs, either together or separated by a transwell membrane. Rap1 activation was determined as in C. Results displayed are representative of three independent experiments.

Activation of Rap1 prevents induction of T cell ROS production by SF adherent cells

We next sought to determine if Rap1 signaling was sufficient to maintain redox balance in T cells exposed to SF adherent cells. We nucleofected RA PB T lymphocytes with cDNA encoding CD20 to detect nucleofected cells, and either empty vector or vector encoding HA-tagged active RapV12. Nucleofected T cells were allowed to recover 24 hours, and then coincubated a further 72 hours with autologous SF adherent cells. As compared to non-nucleofected PB T cells, T cells nucleofected with empty vector were not protected against induction of oxidative stress (Figure 2). In contrast, T cell nucleofected with active RapV12 displayed an approximately 80% decrease in intracellular ROS production ($P<0.05$). Thus, rescue of Rap1 signaling is sufficient to restore redox balance in T cells exposed to SF adherent cells.

Figure 2

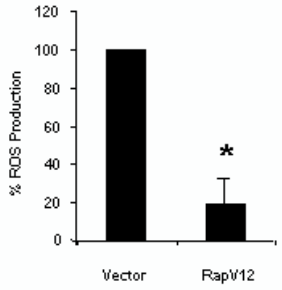

Figure 2. Rap1 activation prevents ROS production in RA PB T cells exposed to SFAdCs. Purified RA PB T cells were left untreated, or nucleofected with cDNA encoding CD20 to mark nucleofected cells and either empty control pMT2HA vector or pMT2HA encoding active RapV12. Following overnight rest, nucleofected cells were exposed overnight to autologous SFAdCs, prior to T cell purification and detection of ROS production in CD20-positive T cells as in Figure 1. Results represent the mean +/- SD of five independent experiments.

Cell-cell contact with RA synovial adherent cells induces mitogenic hyporesponsiveness in PB T lymphocytes

We next examined if induction of ROS production and inactivation of Rap1 in T cells correlated with T cell mitogenic hyporesponsiveness, a hallmark of oxidatively stressed T lymphocytes. As expected (31-34), freshly isolated SF T lymphocytes stimulated with anti-CD3/CD28 antibodies were mitogenically hyporesponsive as compared to autologous PB T lymphocytes (Figure 3A). Incubation of PB T cells with autologous SF for 72 hours prior to CD3/CD28 stimulation had no effect on T cell proliferative responses (Figure 3B). In contrast, incubation of PB T cells with SF adherent cells, but not PB adherent cells, blocked T cell proliferative responses to levels observed with purified SF T lymphocytes. Inhibition of T cell proliferation by SF adherent cells was unlikely due to secreted factors as one, SF, which contains a multitude of inflammatory cytokines, had no effect on T cell proliferation, and two, separation of PB T cells from SF adherent cells during incubation by a transwell membrane completely blocked the inhibitory effect on T cell proliferation (Figure 3C). These experiments indicated that induction of T cell mitogenic hyporesponsiveness in RA required cell-cell contact with SF adherent cells.

Figure 3

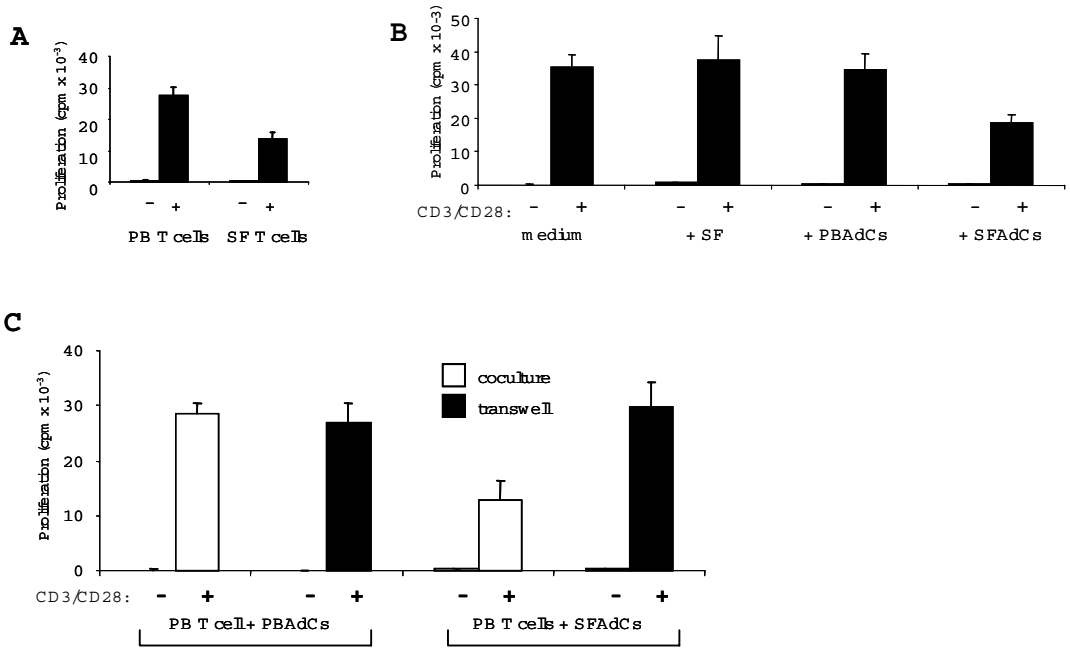

Figure 3. RA SFAdCs induce T cell mitogenic hyporesponsiveness via cell-cell contact. **A,** RA SF T cells are hyporesponsive to mitogenic stimulus. Purified RA PB and SF T lymphocytes were left unstimulated (-) or stimulated for 72 hours with anti-CD3 + anti-CD28 antibodies (+), and labelled with ^{3}[H]thymidine for 20 hours. Cells were harvested and incorporated radioactivity measured by liquid scintillation. **B,** SFAdCs induce T cell hyporesponsiveness. Purified PB T lymphocytes were preincubated with autologous 50% SF, PBAdCs or SFAdCs prior to repurification and stimulation as in **A**. **C,** SFAdCs inhibit T cell proliferation via cell-cell contact. T cell preincubation with PBAdCs and SFAdCs was carried out in the absence (unfilled bars) or presence (filled bars) of an intervening transwell membrane, prior to T cell repurification and stimulation as in **A**. Data is expressed in counts per minute (cpm) x 10^{-3} and represents the mean +/- SD of three independent experiments performed in triplicate.

Synovial adherent cells induce T cell oxidative stress via CD28-dependent inactivation of Rap1

Our above results suggested that T cell surface protein interactions with SF adherent cell surface ligands was responsible for actively suppressing Rap1 signaling. Our attention turned to a possible role for CD28 in this process, as we and others have previously demonstrated that CD28 costimulatory signaling can block T cell Rap1 activation via RapGAP I (35;36). Additionally, CD28 signaling pathways, as opposed to CD3, have been reported to be intact in RA SF T lymphocytes (37). To examine if T cell

CD28-dependent interactions with synovial adherent cells were responsible for inactivation of Rap1 and subsequent ROS induction, PB T lymphocytes were coincubated with autologous SF adherent cells in the presence of control human Ig, or chimeric CTLA4Ig recombinant fusion protein to disrupt CD28 interactions with adherent cell CD80/86. CTLA4Ig, but not control Ig protein, led to an almost complete inhibition of ROS production in T cells exposed to SF adherent cells ($P < 0.05$) (Figures 4 A and B). The effects of CTLA4Ig treatment on T cell ROS production correlated with a rescue of Rap1 signaling, as PB T cells coincubated with CTLA4Ig, but not control Ig, displayed no defects in Rap1 activation after exposure to SF adherent cells (Figure 4C).

Figure 4

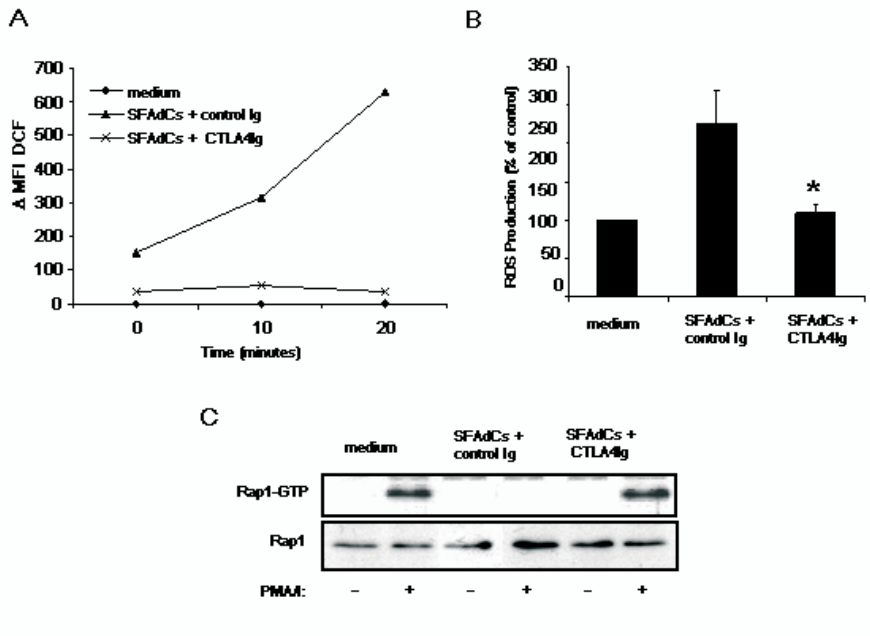

Figure 4. CTLA4Ig prevents T cell ROS production and Rap1 inactivation by SF adherent cells. **A**, Representative experiment of purified RA PB T lymphocytes preincubated for 72 hours in medium alone, or in medium containing autologus SFAdCs (T cell:SFAdC ratio 3:1) and either control IgG-Fc or CTLA4Ig (both at 10 μg/ml), prior to T cell purification, DCF loading, and ROS detection as in Figure 1. **B**, The mean +/- SD of five independent experiments performed as in **A**. Data is expressed as % ROS production compared to control, in which the rate of ROS production over 20 minutes in T lymphocytes incubated in medium alone was normalized to 100% (* - addition of CTLA4Ig results in a statistically significant reduction in ROS production as compared to incubation with control Ig, $P<0.05$). **C**, CTLA4Ig prevents inhibition of T cell Rap1 activity by SFAdCs. PB T lymphocytes were preincubated with medium alone or SFAdCs in the presence of control or CTLA4Ig as in **A**. Reisolated T cells were left unstimulated (-), or stimulated for 5 minutes with PMA/ionomycin (PMA/I) (+), and GTP-bound active Rap1 precipitated and detected by immunoblotting as in Figure 1. Results shown in **C** are representative of five independent experiments.

Inflammatory cytokines and CD28 stimulation synergistically induce ROS production in T lymphocytes

Our results indicated that CD28-dependent inactivation of Rap1 was required for SF adherent cell induction of ROS production in T lymphocytes. To test whether CD28 stimulation was sufficient to induce intracellular ROS production we stimulated PB T cells with different cytokines in the presence or absence of CD28. Acute stimulation of PB T cells isolated from healthy donors with TNF-α, TGF-β or SF, in contrast to activating CD3/CD28 antibodies, failed to induce T cell ROS production (Figure 5A). Similarly, chronic stimulation of PB T cells with TNF-α, IL-1β, IFN-γ or TGF-β for 3-7 days also failed to induce significant ROS production above basal levels (data not shown and Figure 5B). Strikingly, coincubation of PB T cells with activating anti-CD28 antibody with each of these inflammatory cytokines led to a synergistic increase in T cell ROS production ($P < 0.05$) (Figure 5B).

Figure 5

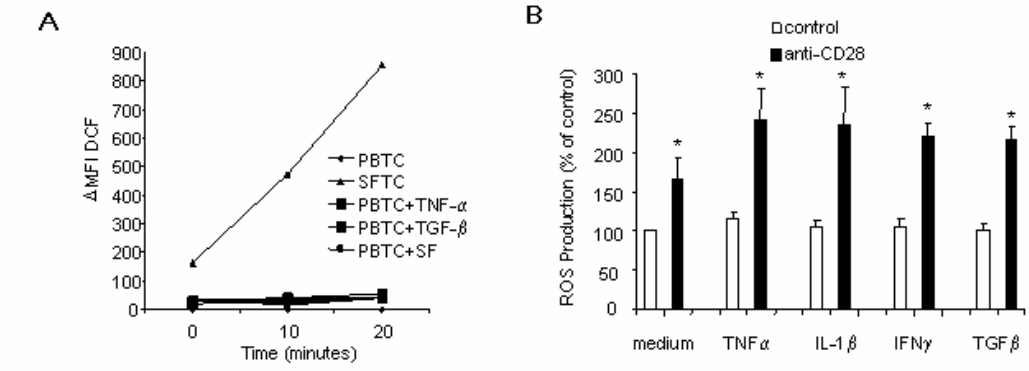

Figure 5. Inflammatory cytokines and CD28 stimulation synergistically induce ROS production in PB T lymphocytes. **A,** Inflammatory cytokines alone are insufficient to induce ROS production in RA PB T lymphocytes. Isolated PB T cells (PBTC) were incubated for 72-96 hours in medium alone or medium containing TNF-α (10 ng/ml), TGF-β (4 μg/ml), or 50% autlogous SF. SF T cells (SFTC) were maintained in 50% SF. Cells were then harvested, loaded with DCF, and ROS detection measured as in Figure 1. **B,** Costimulation of PB T lymphocytes with inflammatory cytokines and activating anti-CD28 antibodies synergistically induces ROS production. Healthy donor PB T cells were maintained for 72-96 hours in medium alone, or medium containing TNF-α (10 ng/ml), IL-1β (125 pg/ml), IFN-γ (100U/ml), or TGF-β (4 μg/ml), in the absence (white bars) or presence (black bars) of activating anti-CD28 antibody. T cells were then harvested, loaded with DCF, and ROS production measured as in Figure 1. Results represent the mean +/- SD of 3 independent experiments. For all cytokines, ROS production was enhanced significantly (* $P<0.05$) as compared to treatment with anti-CD28 alone, or to cytokine stimulation in the absence of anti-CD28 antibody.

Discussion

Recent studies from our group, utilizing FACS-based detection of intracellular ROS production in RA SF T lymphocytes (38), and ROS-dependent visualization of diamino benzadine precipitation in RA synovial tissue (39), demonstrated that endogenous, intracellular ROS production was sufficient to induce oxidative stress in RA synovial T lymphocytes. Our results provide a molecular and cellular basis for the induction of oxidative stress in RA synovial T cells, suggesting a model in which inactivation of Rap1 plays a central role in establishing oxidative stress and altered T cell behavior in RA synovial tissue. Upon arrival in synovial tissue, T cells are exposed to a mix of inflammatory cytokines and cell-cell interactions, many of which have been reported to activate Ras in T cells. In RA, engagement of CD28 by CD80/86 expressed on monocytes, B lymphocytes, dendritic cells and stromal cells will lead to prolonged Rap1 inactivation and a subsequent inability of the T cell to downregulate ROS production. Although chronic stimulation of T cells with inflammatory cytokines was not sufficient to induce T cell oxidative stress, preliminary experiments in our group have found that chronic TNF-α exposure constitutively activates Ras in T cells. T cell activation of Ras by presentation of inflammatory cytokines, in combination with CD28-dependent inactivation of Rap1 by CD80/86-expressing synovial cells, might be responsible for the high levels of intracellular ROS production observed in synovial T cells. Consistent with this idea, Cope and colleagues have established that chronic stimulation of murine T lymphocytes with TNF-α renders cells defective in TCR-dependent proliferative responses, which is in part due to T cell ROS production (40;41). We propose that deregulation of Rap and Ras are critical events leading to the disturbed intracellular redox balance underlying antigenic hyporesponsiveness and inflammatory gene transcription in RA synovial T cells. Rap1 plays a central role in integrating TCR and costimulatory signals to determine T cell immune responses (42). In T lymphocytes, studies on Rap1 have focused on its role in regulating integrin-dependent adhesion (43;44). Our results suggest that improper, or chronic inactivation of Rap1 can also influence T cell function through deregulating T cell redox balance. Consistent with our previous studies, the ability of RapV12 to prevent oxidative stress in T cells exposed to SF adherent cells was not secondary to effects on T cell integrin activity, as a RapV12/E38 mutant which does not regulate ROS production in Jurkat T cells, yet like RapV12 can stimulate integrin-dependent adhesion (45), failed to suppress T cell ROS production (data not shown). CD28-dependent inactivation of Rap1 is mediated by Lck tyrosine kinase activation of RapGAP I (46;47). Intriguingly, in mice transgenically overexpressing RapGAP I in the T cell compartment, an age-dependent accumulation of activated T lymphocytes is observed (48), although susceptibility of these mice to spontaneous or induced chronic inflammatory diseases has not been examined.

Our results underscore the importance of CD28 costimulation in the activation of T cells in the synovium of RA. In particular, CD28 stimulation upregulates intracellular ROS production. ROS regulation in RA synovial T lymphocytes may contribute to inflammation as, *in vitro*, and in pharmacological and genetic studies in rodent models of arthritis, there are strong indications that ROS-dependent activation of NF-κB in T lymphocytes contributes to pathogenesis (49-51). Conversely the resultant

oxidative stress results in inhibition of TCR-proximal proliferative signals, via misfolding of LAT and TCR-ζ (52;53).

Our model suggests CTLA4Ig therapy could block oxidative stress in synovial T cells in RA patients, and it will be of interest to determine if intracellular ROS production in RA synovial T cells may predict or correlate with clinical responses to CTLA4Ig therapy. In many animal models of arthritis, CD28 acts as a classical essential costimulatory protein in permitting TCR-dependent responses to collagen required for initiation and progression of joint inflammation (54-57). Trials with CTLA4Ig blockade of CD28 signaling in RA have been extremely promising, but the mechanism of its therapeutic activity in humans has yet to be assessed (58;59). Several mechanisms might explain how CTLA4Ig therapy exerts its clinical benefits despite rescuing the proliferative responsiveness of potentially autoreactive T lymphocytes. First, CTLA4Ig would be expected to both decrease oxidative stress-dependent NF-κB inflammatory gene transcription and block CD28 signaling critical for TCR-dependent T cell activation and proliferation. Second, restoration of TCR-dependent IL-2 production may simultaneously act to enhance regulatory T cell function, which is defective in RA (60). Finally, restoration of Rap1 function may allow integrin-dependent emigration of T cells from the synovium, independent of TCR-dependent proliferative signals.

Intriguingly, in a subset of RA patients $CD4^+CD28^-$ T lymphocyte numbers are greatly expanded in the synovium (61). $CD4^+CD28^-$ T cell clones, displaying some similarities to NK cells, are often autoreactive, sensitive to TCR triggering, and are associated with extra-articular organ involvement in RA (62-64). Lack of CD28 expression may protect these cells from induction of oxidative stress, contributing to TCR-dependent activation. Alternatively, T cell costimulatory proteins other than CD28 may redundantly regulate Rap1 function and ROS production in these cells. It will be of interest to see if synovial $CD28^-$ T cells also suffer from oxidative stress, or whether oxidative stress leads to CD28 down-regulation in these cells. The recent development of techniques to quantitatively detect ROS-producing T lymphocytes in RA synovial tissue *in situ*, in conjunction with functional analysis of T cell function following RA patient CTLAIg treatment, will allow more detailed characterization of which T cell subsets are under oxidative stress in the RA synovium, and how these T cells respond to therapeutic treatment.

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CHAPTER 5

Nutrient supplementation with poly-unsaturated fatty acids and micronutrients in rheumatoid arthritis: clinical and biochemical effects.

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Abstract

Objective: To investigate in a double blind placebo-controlled, parallel group study the effects of a nutrient supplement, containing, among other ingredients, the omega-3 fatty acids eicosapentaenoic acid (1.4 g EPA), docosahexaenoic acid (0.211 g DHA), omega-6 fatty acid gamma-linolenic acid (0.5 g GLA) and micronutrients in patients with active RA.
Design, subjects and intervention: RA patients were randomized to receive either daily liquid nutrient supplementation or placebo for 4 months. The primary endpoint was the change in tender joint count at 2 and 4 months. Other clinical variables included swollen joint count, visual analogue scales for pain and disease activity, grip strength, functionality score, and morning stiffness. Biochemical parameters included plasma concentrations of PUFA and vitamins C and E.
Setting: Outpatient university clinic.
Results: *S*ixty-six patients enrolled, 55 completed the study. No significant change from baseline in tender joint count or any of the other clinical parameters was detected in either group. Patients receiving nutrient supplementation but not those receiving placebo had significant increases in plasma concentrations of vitamin E (p=0.015), and EPA, DHA and docosapentaenoic acid (DPA) concomitant with decreases of arachidonic acid (AA)(p = 0.01). Intergroup differences for PUFA and vitamin E were significantly different (p=0.01 and 0.03 respectively).
Conclusions: This double-blind, placebo-controlled study in RA patients did not show superior clinical benefit of daily nutrient supplementation with EPA, GLA and micronutrients at the doses tested as compared to placebo. The study adds information regarding doses of omega-3 fatty acids below which anti-inflammatory effects in RA are not seen.

Introduction

Nutrient supplementation as add-on therapy in rheumatoid arthritis (RA) has witnessed a resurgence of scientific interest due to preclinical and clinical studies on supplementation with polyunsaturated fatty acids (PUFA) or micronutrients.
Initial studies, demonstrating beneficial effects of dietary fatty acids, made use of fish oils, that contains the omega-3 polyunsaturated fatty acids (PUFA) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). When supplemented these lipids compete with arachidonic acid (AA) for incorporation in cellular membranes, resulting in decreased synthesis of specific leukotrienes and prostaglandins (1). Furthermore, downmodulatory effects on pro-inflammatory cytokines have been described. Similar clinical results have been published with the omega-6 fatty acid gamma-linolenic acid (GLA), isolated from certain plant and seed oils (2-4). Administration of GLA leads to an increased incorporation of its metabolite dihomogammalinolenic acid (DGLA) in cell membranes, thus blocking the metabolism of AA and changing the balance of lipid mediators towards the production of less potent eicosanoids. Several clinical studies in RA patients have demonstrated modest but reproducable beneficial effects on joint tenderness of supplements with EPA + DHA at doses 2.3 – 7.1 g per day (5). Perhaps due to the high number of capsules needed to achieve clinical effects combined with their fishy aftertaste, however, nutritional supplements with PUFA have not received the same status as conventional antiphlogistic and analgesic drugs in the management of RA (6).
Micronutrient supplementation too has been propagated for its potential benefit in RA, based on the low antioxidant status in RA patients as compared to healthy controls, the role of chronic oxidative stress in functional hyporesponsiveness of synovial T lymphocytes, and the effects of reactive oxygen species (ROS) in cartilage degradation, inflammation and cell signaling (7-10). Additionally, dietary intake of several micronutrients has been reported to be inadequate in RA patients (11). In animal models, high doses of vitamin C, vitamin E and N-acetyl-cysteine had

anti-arthritic effects related to their anti-oxidative potential (12-14). Other candidate antioxidants have been identified with potential benefit including selenium, zinc, manganese, niacinamide, bioflavonoids and ß carotene. Nevertheless, clinical trials with anti-oxidants in RA patients have been disappointing. A randomized double-blind comparison of high-dose vitamin E (1200 mg daily), the most powerful naturally occurring lipid soluble antioxidant, did not show any anti-inflammatory effect as compared to placebo (15). Two placebo-controlled trials with selenium-enriched yeast (200µg/d and 156µg/d) did not result in detectable clinical benefit (16, 17).
Given the lack of evidence to date demonstrating anti-arthritic effects of supplementation with a single anti-oxidant, it has been suggested that combination of several such nutrients might be efficacious (18, 19). We therefore developed a liquid nutritional supplement as a vehicle to deliver supplementary concentrations of EPA, GLA and selected micronutrients to investigate its clinical and biochemical effects in a prospective, double-blind, placebo controlled, parallel group trial in patients with active rheumatoid arthritis.

Subjects and methods

Patients

Sixty-six patients with an established diagnosis of RA enrolled in a single center study (outpatient clinic LUMC) between August 2000 and September 2001. The study was approved by the Medical Ethical Committee and informed consent was obtained from all patients. All had active disease defined as 6 or more tender or swollen joints, morning stiffness > 1 hour, or erythrocyte sedimentation rate (ESR) > 25 mm/h. Concurrent treatment for RA with corticosteroids and/or disease modifying antirheumatic drugs was required to be stable for at least 2 months. Exclusion criteria were serious gastro-intestinal disease (inflammatory bowel disease, atrophic gastritis, stoma), malignancy, previously established intolerance of fish oil, or regular use of dietary supplements containing fish oil and/or antioxidants.

Study design, randomization, and supplementation

This was a placebo-controlled, randomized, double-blind, parallel group intervention trial. Patients were randomly allocated to receive either a liquid nutritional supplement containing polyunsaturated fatty acids and micronutrients or the placebo, one serving daily for the duration of 4 months. The product was specially produced for this study. The composition of the supplement is shown in **Table 1**. Placebo-drinks with same taste, odor and color were produced with water, sweetener (sodiumsaccharine/acesulpham-k), cloudifier, flavor and colorant. Both drink-feeds were provided in 200-ml tetrapacks (white) in two fruit flavors (forest and citrus fruit). The formulae were randomized and the tetrapacks were labeled with the appropriate number before delivery, and the patients were allocated a number on enrolment of the study. Patients and physicians were kept blinded to treatment assignment during the entire study. Patients were instructed to continue their dietary habits. Clinical parameters were evaluated at baseline and after 2 and 4 months by the same observer.

Sample size

The primary outcome parameter was the change of tender joint count. With alpha 0.05 (two-sided), beta 0.8 and allowing for a 20% drop-out rate a sample size of 32 patients per group was needed to detect a difference in tender joint count of 4, given a baseline tender joint count of 13 and standard deviation of 5.1.

Table 1 Contents of nutrient supplement per 200 ml

Energy (kcal)	150
Protein (whey) (kcal)	7.5 (20% of En)
Cysteine (mg)	170
Glutamin/mate (μg)	412
Carbohydrates (g)	19.4 (52% of En)
Fat (g)	4.7 (28% of En)
n-3 PUFA	
Eicosapentaenoic acid (mg)	1400
Docosahexaenoic acid (mg)	211
Docosapentaenoic acid (mg)	40
Alpha-linolenic acid (mg)	16
n-6 PUFA	
Gamma-linolenic acid (mg)	500
Linoleic acid (mg)	440
Dietary fibre (g)	3
Minerals	
Calcium (mg)	235
Phosphorus (mg)	235
Magnesium (mg)	37.5
Trace elements	
Iron (mg)	9.0
Zinc (mg)	9.0
Copper (mg)	0.75
Manganese (μg)	0.75
Selenium (μg)	150.0
Molybdenum (μg)	16.6
Chromium (μg)	11.0
Iodide (μg)	34.0
Fluoride (mg)	0.3
Vitamins	
Vit A (μg)	200
Vit D (μg)	3.75
Vit E (mg)	37.5
Vit K (μg)	12
Vit C (mg)	150
Thiamin (mg)	0.3
Riboflavin (mg)	0.75
Niacin (mg)	6.0
Pyridoxin (B_6) (mg)	1.3
Vit B_{12} (μg)	1.6
Folic acid (μg)	200
Panthothenic acid (mg)	1.2
Biotin (μg)	11.3
Others	
Choline (mg)	60
Coenzyme Q10 (mg)	2
Flavonoids (mg)	10
Carotenoids (mg)	1.5

Clinical evaluation
Patients participating in the study were evaluated at the start of the trial, and after 2 and 4 months. The clinical evaluation encompassed a 28-tender joint count and a swollen joint count (using a dichotomous scale: 0 = absent, 1= present for both counts), patient's visual analogue scale (VAS) for pain, disease activity, general health and physician's VAS for disease activity. Based on these data the 20% response rate as defined by the American College of Rheumatology (ACR20-response) criteria (20) and Disease Activity Score (DAS28, 28-joint count)(21) were calculated. Gripstrength was measured using a manometer (kPa). Duration of morning stiffness (in minutes), anthropometric (weight and height) data and concomitant drugs were recorded. Additionally, patients were asked to document their NSAID intake during the first week after initiation and the week prior to termination in a diary, and to complete a Health Assessment Questionnaire (HAQ) and Arthritis Impact Measurement Scale (AIMS).

Laboratory evaluation
All mentioned variables were performed on fasting blood samples at baseline and after 4 months. Plasma concentrations of fatty acids and vitamin C and E were measured in a subset of both groups (11 supplement + 12 placebo for fatty acids, and 20 supplement + 19 placebo for vitamins C and E). The fatty acid composition of the phospholipid fraction was determined in plasma as follows: after total lipid extraction by the method of Bligh and Dyer (22), phospholipids were separated from the total lipid fraction by column chromatography using bonded phase columns (23). Fatty acids were then methylated with boron trifluoride in methanol according to the method of Morrison and Smith (24). The methyl esters (FAME) were separated and quantified by capillary gas chromatography (Shimadzu GC-17A) with a CPSIL88 column (Chrompack; Middelburg, Netherlands).
Vitamin C and E were analyzed by HPLC as previously described (25, 26).

Data analysis
All statistical analyses were done with the statistical software package STATA 6.0 (StataCorp, College Station, TX, USA). For both treatment groups, Student's paired t-test was applied to assess whether the outcome at 2 and 4 months differed from the baseline value. Unpaired t-tests were used to test whether baseline values and/or the
changes from baseline were different between the groups. Statistical significance was accepted at a probability level of 0.05. Correlation analysis was performed in order to assess whether changes after 4 months of treatment were consistent among biochemical parameters.

Results

Sixty-six patients enrolled in the trial: 33 in the placebo group and 33 receiving the nutrient supplement. The two groups did not differ with respect to clinical and demographic variables at baseline, except for a higher age in the experimental group. Eleven subjects (17%) dropped out during the 4 months treatment period: 3 (5%) due to gastro-intestinal intolerance (2 in the experimental group, 1 in the control group), 8 (12%) due to lack of efficacy (5 in the experimental group and 3 in the control group). Baseline characteristics of completers in both groups are shown in **Table 2**.
Fifty-five patients completed the 4 months of treatment: 29 receiving placebo and 26 the supplement. Changes in clinical parameters are listed in **Table 3**. Mean tender joint count, the primary study parameter, did not change significantly between the groups nor within each group. Disease activity as measured with DAS28 deteriorated in both groups after 4 months, but this attained statistical significance only in the control group, but not in the experimental group or between the groups. Two patients receiving the supplement and 2 receiving placebo achieved an

Table 2 Baseline characteristics of patients who completed the study (data expressed as mean ± s.d.)

Variable	*Placebo (n=29)*	*Nutrient supplement (n=26)*
Sex (m:f)	7:22	3:23
Age (y)*	52.9 ± 11.2	59.5 ± 11.0
Disease duration (y)	11.7 ± 11.1	13.6 ± 11.9
DMARD users[a]	28/29	22/26
DAS 28[b]	5.14 ± 1.05	5.43 ± 0.94

*Significantly higher in supplement group ($P<0.05$).
[a]DMARD=disease-modifying antirheumatic drug. Placebo group: methotrexate 16, methotrexate + sulfasalazine + hydroxychloroquine 1, sulfasalazine 9, gold 1, d-penicillamine 1, prednisone 3; supplement group: methotrexate 16, methotrexate + sulfasalazine + hydroxychloroquine 1, sulfasalazine 4, gold 1, prednisone 1.
[b]DAS28=disease activity score, 28-joint count.

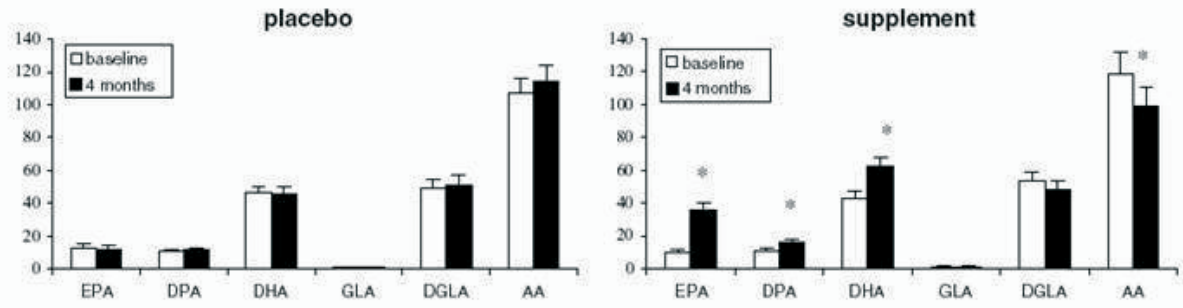
Table 3 Changes in clinical parameters (mean ± s.d.) in patients who completed the study

		Supplement			
Parameter	*Placebo (n=29)*	*(n=26)*	*Parameter*	*Placebo*	*Supplement*
Tender joint count			Morning stiffness (min)		
Baseline ± s.d.	8.7 ± 5.3	10.7 ± 4.9	Baseline ± s.d.	83 ± 100	63 ± 68
Δ Mean 2 months ± s.d.	1.0 ± 3.0	−0.8 ± 4.4	Δ Mean 2 months ± s.d.	−9 ± 67	2 ± 69
Δ Mean 4 months ± s.d.	1.0 ± 5.1	0.0 ± 4.1	Δ Mean 4 months ± s.d.	−12 ± 40	13 ± 70
Swollen joint count			Grip str. R. (kPa)		
Baseline ± s.d.	8.8 ± 3.5	9.4 ± 3.4	Baseline ± s.d.	193 ± 79	183 ± 72
Δ Mean 2 months ± s.d.	−0.3 ± 3.8	−0.6 ± 4.9	Δ Mean 2 months ± s.d.	7 ± 56	1 ± 29
Δ Mean 4 months ± s.d.	−0.7 ± 4.14	0.5 ± 4.6	Δ Mean 4 months ± s.d.	6 ± 61	−7 ± 27
DAS28[a]			Grip str. L. (kPa)		
Baseline ± s.d.	5.14 ± 1.05	5.36 ± 0.92	Baseline ± s.d.	192 ± 80	169 ± 69
Δ Mean 2 months ± s.d.	0.22 ± 0.74	−0.01 ± 0.82	Δ Mean 2 months ± s.d.	−4 ± 55	−5 ± 27
Δ Mean 4 months ± s.d.	0.21 ± 0.93	0.22 ± 0.77	Δ Mean 4 months ± s.d.	−2 ± 45	−2 ± 32
VAS[a] health (mm)			HAQ[a]		
Baseline ± s.d.	48 ± 18	47 ± 18	baseline ± s.d.	1.16 ± 0.64	1.23 ± 0.62
Δ Mean 2 months ± s.d.	−1 ± 21	4 ± 25	Δ Mean 2 months ± s.d.	ND	ND
Δ Mean 4 months ± s.d.	3 ± 9	7 ± 23	Δ Mean 4 months ± s.d.	0.06 ± 0.23	−0.06 ± 0.33
VAS[a] patient (mm)			AIMS[a]		
Baseline ± s.d.	55 ± 18	50 ± 18	Baseline ± s.d.	5.12 ± 1.40	4.83 ± 1.47
Δ Mean 2 months ± s.d.	−3 ± 21	2 ± 21	Δ Mean 2 months ± s.d.	ND	ND
Δ Mean 4 months ± s.d.	−4 ± 17	5 ± 18	Δ Mean 4 months ± s.d.	0.01 ± 1.37	0.46 ± 1.48
VAS[a] physician (mm)			Weight (kg)		
Baseline ± s.d.	35 ± 17	39 ± 18	Baseline ± s.d.	77.9 ± 15.6	71.6 ± 11.0
Δ Mean 2 months ± s.d.	2 ± 23	−2 ± 17	Δ Mean 2 months ± s.d.	0.3 ± 1.3	0.9 ± 1.3*
Δ Mean 4 months ± s.d.	3 ± 18	−1 ± 7	Δ Mean 4 months ± s.d.	0.0 ± 2.0	0.8 ± 1.5
C-reactive protein (mg/l)			BMI (kg/m^2)		
Baseline ± s.d.	18.6 ± 19.8	14.8 ± 12.4	Baseline ± s.d.	26.4 ± 4.9	25.6 ± 3.3
Δ Mean 2 months ± s.d.	0.0 ± 10.5	1.0 ± 10.6	Δ Mean 2 months ± s.d.	0.1 ± 0.4	0.31 ± 0.46
Δ Mean 4 months ± s.d.	−0.4 ± 11.2	2.6 ± 10.0	Δ Mean 4 months ± s.d.	0.0 ± 0.7	0.31 ± 0.59
ESR (mm/first hour)			NSAID (tablets/week)		
Baseline ± s.d.	29 ± 23	30 ± 21	Baseline ± s.d.	12.5 ± 11.5	12.0 ± 3.9
Δ Mean 2 months ± s.d.	4 ± 12	1 ± 10	Δ Mean 2 months ± s.d.	ND	ND
Δ Mean 4 months ± s.d.	2 ± 9	4 ± 10	Δ Mean 4 months ± s.d.	0.8 ± 3.6	0.6 ± 2.9

[a]DAS28 denotes disease activity score, VAS visual analogue scale, HAQ health-assessment questionnaire, AIMS arthritis impact measurement scale.For both treatment groups, Student's paired *t*-test was applied to assess whether the outcome at 2 and 4 months differed from the baseline value. Unpaired *t*-tests were used to test whether baseline values and/or the changes from baseline were different between the groups. With statistical significance accepted at a probability level of 0.05, none of the changes reached significance except for the change in body weight in the supplement group at 2 months ($P=0.002$).

ACR20 response at 4 months. When the individual components of the ACR20 response criteria and DAS28 outcome criteria were analyzed, no significant changes within the groups nor differences in changes between the groups were detected. Functional assessment by means of the AIMS and HAQ questionnaire also showed no changes. No differences in the taking of NSAIDs were found. A statistically significant increase in body weight was detected in the experimental group at 2 months, but the changes in weight and BMI were not significant between the groups. **Figure 1** shows the changes in plasma concentrations of the key PUFA. All patients receiving nutrient supplementation had significant increases in the omega-3 PUFA (EPA, DPA, DHA) when compared to baseline. These changes were also significantly higher when compared to changes in the placebo group. The mean concentration of omega-6 PUFA (GLA, DGLA) did not change in either group. Plasma arachidonic acid (AA) concentrations significantly decreased in the patients taking the nutrient supplement, but not in the placebo group. Changes in plasma concentrations of EPA correlated inversely with AA (r –0.66, p=0.001) and positively with DPA and DHA (r 0.83 for both, p<0.0001), while those of DPA with DHA and GLA with DGLA also significantly correlated (r 0.75, p<0.0001, r 0.59, p=0.003 respectively). Increases in serum concentrations of vitamin C (mean±SD at baseline 51.7±25.1, delta mean 6.9±28.3; p 0.74) and vitamin E (mean±SD at baseline 33.4±7.5 mM, delta mean 4.3±6.5; p=0.015) were observed in the experimental arm, and decreases in the placebo-group of vitamin C (mean±SD at baseline 42.7±21.6 mM, delta mean –3.8±15.1; p=0.98) and vitamin E (mean±SD at baseline 34.6±8.6, delta mean –0.4±6.3; p=0.76). The intergroup difference in vitamin E was significant (p = 0.03).

Figure 1



- p<0.01 compared to baseline and compared to placebo;
- EPA denotes eicosapentaenoic acid, DPA docosapentaenoic acid, DHA docosahexaenoic acid, GLA gamma-linolenic acid, DGLA dihomogammalinolenic acid, AA arachidonic acid

Discussion

This is the first double-blind, placebo-controlled study to investigate the clinical efficacy of a daily nutrient supplementation containing polyunsaturated fatty acids (PUFA) and micronutrients in RA patients. Based on preclinical data it was postulated that the combination of these nutrients would produce synergistic or additive effects without compromising safety (27, 28). An odorless liquid nutritional supplement was developed as a vehicle to enhance compliance. Compared to placebo, supplementation resulted in significant increases in serum concentrations EPA, DPA, DHA, and vitamin E and a modest increase in body weight indicating that compliance was indeed achieved. This was further corroborated by correlations between individual PUFA. However, no statistically significant difference was found in the primary outcome parameter, the change in tender joint counts (TJC), nor in the other clinical parameters measured.

Several factors may have influenced the outcome of our study. First, our trial was designed as a pragmatic trial to evaluate the add-on therapeutic effect of nutrient supplementation in RA patients with persistent disease activity despite antiphlogistic and antirheumatic medication. This may have introduced bias by selection of patients in whom marked improvement of (symptoms of) disease activity is difficult to achieve. The probability to detect significant changes in TJC (but not the other clinical parameters) may have also been compromised because baseline values were lower than anticipated. Second, the metabolism and effects of PUFA and micronutrients may have been affected by continued use of NSAIDs and dietary factors. Recent studies reported that the beneficial effects of EPA are reduced when the diet is high in essential omega-6 PUFA by the intake of margarine and polyunsaturated oil (29, 30). With an average intake of omega n-6 PUFA in a Dutch diet of 12-13 g/day (31) (approximately 10x the amount in the supplement) such interaction cannot be ruled out. On the other hand, in most published studies patient populations did not differ from ours with respect to background medication and diet. Third, the concentrations of EPA, GLA and micronutrients may have been too low, even though marked and significant effects on plasma concentrations of EPA and AA were detected in the present study. Although we did not measure membrane fatty acid levels, changes in plasma fatty acids have been extensively used as surrogate markers. Previous reports demonstrated efficacy of slightly higher doses of omega-3 PUFA, but not of lower doses than used in our study. One study comparing low and high dose omega-3 PUFA (27 mg/kg EPA + 18 mg/kg DHA versus 54 mg/kg EPA + 36 mg/kg DHA respectively) showed equal effectiveness on tender and swollen joint counts, but only effects on other variables in the high dose group (32). Another study comparing daily supplements with either 2.6 gm of omega-3 PUFA, 1.3 gm omega-3 PUFA plus 3 gm olive oil, or 6 gm olive oil in patients with active RA only found significant clinical benefit in the first group (33). GLA has been shown to be clinically effective in RA at doses higher than 0.45 g/day, but the possibility of a dose dependence of these effects could explain the lack of efficacy in our trial. Food technological constraints precluded the use of higher concentrations of PUFA in our supplement. With respect to micronutrients little is known about the doses needed to induce immunomodulation (34). Studies with single antioxidants failed to demonstrate a significant clinical effect of high doses selenium, or vitamin E in RA patients (15-17).

Although our study was primarily designed to investigate potential add-on effects of PUFA and micronutrient supplementation on symptoms and disease activity, other beneficial clinical effects cannot be excluded, cardioprotective in particular. RA patients suffer from excessive cardiovascular morbidity and mortality when compared to age- and sex-matched individuals (35). Epidemiological and interventional studies have shown protective effects of fish oil and PUFA on cardiovascular events in various populations, in part through modulation of serum lipids (36). Interestingly, a recent study showed significant effects of supplementation with fish oil-derived PUFA and GLA on circulating plasma lipids and fatty acid profiles in healthy women, estimated to result in a 43% reduction in the risk of myocardial infarction (37). Whether

these results apply to patients with a chronic inflammatory disorders remains to be proven. Extrapolating results from these studies, large-scale clinical trials would be needed to demonstrate cardioprotective effects in RA.
We conclude that nutritional supplementation with micronutrients and PUFA at the doses tested does not ameliorate signs and symptoms in RA patients. More needs to be learned on the role of anti-oxidants in chronic inflammatory conditions such as RA, possible interactions (synergistic or antagonistic) between various anti-oxidants and PUFA, and dose-response relationships.

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CHAPTER 6
High dose intravenous N-acetyl-L-cysteine (NAC) therapy in rheumatoid arthritis, results from a pilot study.

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Submitted

Abstract

Objective: to assess feasibility, safety, and efficacy of high dose NAC in patients with RA and to evaluate its effects on functional parameters of T lymphocytes.

Methods: fourteen patients with active RA were treated with NAC as an intravenous bolus of 150 mg/kg over 30 min, followed by an continuous i.v. infusion of 50 mg/kg over 4h and 100 mg/kg over 20h.. Disease activity parameters from the American College of rheumatology (ACR) core disease activity set were determined at baseline, immediately after NAC treatment and 2 weeks post NAC treatment. To assess T cell function thymidine incorporation was measured following a mitogenic stimulus. To assess oxidative stress LAT staining was performed on cytospins of purified peripheral blood (PB) and synovial fluid (SF) T lymphocytes., and NF-κ B activation was determined visualising the NF-κ B p65 subunit by confocal microscopy**.**

Results: Although there was no significant improvement of the DAS, the primary study parameter, there was a small but significant reduction in number of swollen (p=0.03) and tender joint counts (p=0.04). There was a trend (p=0.06) towards normalization of proliferative responses of synovial T lymphocytes, but we could not detect restoration of LAT dislocation nor did we detect any effect on NF-κ B activation. Intriguingly though, we found NF-κB activation in the synovial T lymphocytes from the same patients where cytoplasmatic dislocation of LAT was observed, which suggests that NF-κB activation in SF T lymphocytes could, at least in part, be regulated by oxidative stress.

Conclusion: a 24-hour treatment with high dose i.v. NAC did not significantly improve disease activity in patients with active RA, nor did it correct signaling abnormalities of synovial T lymphocytes.

Introduction

Oxidative stress due to production of reactive oxidant species (ROS) is thought to play an important role in the pathogenesis of rheumatoid arthritis (RA). ROS have been shown to act as critical mediators in inflammatory signaling cascades, e.g. by activation of transcription factors like NF-κB, resulting in pro-inflammatory gene expression, induction of TNF-α, IL-1, NO-synthetase, and upregulation of ICAM and VCAM.

Oxidation products and depleted levels of antioxidants have been demonstrated in blood and synovial tissue of RA patients(1), notably in (synovial) T lymphocytes. T lymphocytes isolated from rheumatoid joints display a number of signaling and proliferative abnormalities (for review:(2)). They exhibit severe hyporesponsiveness to proliferative stimuli compared with autologous peripheral blood (PB) T cells, and produce little IL-2, IFN-γ, IL-4, TGF-β, or TNF-α in vitro or in situ(3). These functional characteristics of synovial T cells from RA patients correlate strongly with oxidative stress(4). In vitro replenishment with *N*-acetylcysteine (NAC) restores reduced glutathione (GSH), a key intracellular antioxidant, resulting in partial recovery of TCR signalling, proliferation and IL-2 production (5).

NAC is a powerful antioxidant, which is easily deacetylated to cysteine, the precursor of cellular glutathione synthesis upregulating the cellular glutathione system. Furthermore, a reduced sulfydryl group on NAC scavenges H_2O_2 (hydrogen peroxide), $OH^{\cdot}$ (hydroxol radical), and HOCl (hypochlorous acid). In vitro, NAC was shown to inhibit H_2O_2 and TNF-α induced NF-κB activation (6), and block the production of IL-1 and TNF-α (7)**.** In animal models, high doses of NAC had anti-arthritic effects (8), and in AIDS patients, the beneficial effects of NAC have been ascribed to attenuation of NF-κB activation following chronic cytokine stimulation (9). Additionally, Paterson and co-workers found decreased NF-κB activation after NAC treatment in patients with sepsis (10). Bioavailability of oral NAC in humans is poor though, and to attain

similar concentrations of NAC in vivo as used in experimental studies, high doses of NAC need to be administered intravenously.
Given the anti-inflammatory potential of NAC, we conducted an open study to assess feasibility, safety, and efficacy of high dose i.v. NAC in patients with active RA and to evaluate its effects on functional parameters of synovial T cells.

Patients and methods

Patients

This was an open-label, single center, prospective intervention trial. The study was approved by the Medical Ethical Committee of LUMC and informed consent was obtained from all patients. Eligibility criteria included the presence of six or more tender or swollen joints, morning stiffness > 1h, or erythrocyte sedimentation rate (ESR) > 25 mm/h. Additionally, all patients were required to have arthritis of a large joint for sampling of synovial fluid pre- and post-treatment. Concurrent treatment for RA with corticosteroids and/or disease modifying antirheumatic drugs was required to be stable for at least 2 months. Exclusion criteria included pregnancy, severe liver failure or creatinine clearance < 20 ml/min.

Treatment protocol

Patients were given N-acetyl cysteine (Zambon, Vicenza, Italy) as an intravenous bolus of 150 mg/kg over 30 min, followed by an continuous i.v. infusion of 50 mg/kg over 4h and 100 mg/kg over 20h. The protocol was based on a treatment protocol for acetoaminophen-intoxication.

Assessment of efficacy

The following clinical and laboratory investigations were performed prior to NAC treatment, immediately after, and 2 weeks after NAC treatment: physical examination, 28-tender and swollen joint counts (using a dichotomous scale: 0=absent, 1=present), patient's visual analog scale (VAS) for pain, disease activity, general health, and physician's VAS for disease activity. Based on the above-mentioned data, efficacy was determined by the 4-variable Disease Activity Score (DAS) (primary study parameter). Health Assessment Questionnaires were taken before and 2 weeks after NAC treatment.

Laboratory measurements

Laboratory measurements were performed at the aforementioned timepoints and included the erythrocyte sedimentation rate, hemoglobin, hematocrit, white blood cell count with differential, platelet count and C-reactive protein level. Synovial fluid samples were also obtained at the same time points. Peripheral blood (PB) and synovial fluid (SF) T cells were purified from mononuclear cells using a negative isolation procedure (T Cell Negative Isolation Kit, Dynal Biotech Norway), which resulted in a > 90% $CD3^+$ cell population. Purified T cells were subsequently used for proliferation assay or were mounted onto adhesive microscope slides (1 x 10^5 T lymphocytes/slide), air dried, and kept frozen until staining.

Proliferation assay

For the thymidine incorporation assay, lymphocytes were seeded in 96-well flat-bottomed plates at 5×10^5 cells per well in 200 μl of RPMI-1640 medium (Eurobio, Courtaboeuf Cedex B, France) containing 10% (v/v) fetal-calf serum. Mitogenic stimuli (50 ng/ml PMA plus 1μg/ml ionomycin) were added at the beginning of the culture. After 72 h, cells were pulsed with [^{3}H]thymidine (1 μCi per well; New England Nuclea, Boston) and incubated for a further 20 h. Cells were then harvested on filtermats (Skatron instruments, Lier, Norway) and subjected to liquid-scintillation counting (Skatron instruments).

LAT localization and NF-κB activation

For LAT staining, cells were fixed in 4% para-formaldehyde in PBS for 15 min at RT. Cells were permeabilized using 0.1% Triton X-100 in PBS for 4 min at RT, washed three times (PBS/BSA), and preblocked for 45 min at RT in PBS containing 10% FCS. Cells were then incubated with a rabbit polyclonal Ab against LAT (Upstate Biotechnology, Lake Placid, N.Y.)

PBS/5% BSA/0.5% FCS and incubated with FITC-conjugated swine anti-rabbit Ig (Dako, Glostrup, Denmark). Negative control was incubated with the secondary Ab only. Cells were imbedded in vectashield and covered with a coverslip.

NF-κB activation was assessed by nuclear translocation of NF-κB p65 subunit as visualized by confocal microscopy. Cells were fixed with ice-cold acetone 5 minutes, pre-blocked for 45 min at RT in PBS containing 10% FCS, and stained with p65 mAb (Santa Cruz, California, USA) in PBS/5% BSA/0.5% FCS followed by Alexa 594-conjugated goat-anti-rabbit IgG (Molecular Probes, Leiden, The Netherlands) in PBS/5% BSA/0.5% FCS. A negative control was incubated with the secondary Ab only. Cells were imbedded in vectashield containing DAPI and covered with a coverslip. Cells were visualized using a Leica TCS SP (Leica Microsystems, Heidelberg, Germany) confocal system, equipped with an Ar/Kr/HeNe laser combination. Images were taken using a ×40 1.25 NA objective.

Statistical analysis

Statistical analyses were done with the statistical softwarepackage STATA 6.0. Student's paired t-test was applied to assess whether the outcome at the above mentioned timepoints differed from the baseline values.

Results

Fourteen RA patients were treated with high dose i.v. NAC (Table 1A). All patients tolerated the administration well. Three patients had complaints of flushing during administration of the bolus infusion, and one patient suffered from nausea. No other side effects were noted. Although there was no significant improvement of the DAS, the primary study parameter, there was a small but significant reduction in number of swollen (p=0.03) and tender joint counts (p=0.04) (Table 1B). One patient achieved an ACR 20 response after NAC administration.

Table 1: Baseline characteristics and changes in clinical parameters. Values are expressed as the mean ± SD.

Variable	(n=14)
Sex (m:f)	8:6
Age (years) (*Mean ± SD*)	53.3 ±12.5
Erosive disease	10 (71%)
Reuma factor positive	7 (50%)
DMARD use	14 (MTX 9, sulfasalazine 3, MTX+ sulfasalazine+plaquenil 1, leflunomide1)

parameter	baseline	*Δ Mean* Day 2	*Δ Mean* Day 14	*Sign*
ESR (mm/ hr)	41.1 ±34.9	- 2.8 ± 4.6	- 3.9 ±11.2	0.33
CRP (mg/L)	44.9 ±38.8	3.3 ± 7.9	- 10.6 ±34.1	0.34
tjc	12.3 ±8.6	0.3 ± 1.7	- 1.3 ±2.4	0.04
sjc	14.7 ±8.3	-0.2 ± 1.5	- 2.9 ±4.6	0.03
VAS global health (mm)	47 ±18	-0.8 ± 10	-9 ± 10	0.62
DAS28	4.87 ±0.85	-0.09 ± 0.20	-0.40 ± 0.80	0.07

In 10/14 patients we were able to isolate sufficient synovial fluid T lymphocytes at all 3 timepoints for determination of NF-κB activation and LAT localization. Dislocation of LAT from the membrane is considered to be the result of oxidative stress and is a consequence of a conformational change interfering with the insertion of LAT into the plasma membrane (11). In 7/14 patients there were sufficient T lymphocytes to additionally measure the proliferative characteristics of the T lymphocytes. As previously reported (5)T lymphocytes isolated from the synovial fluid displayed hyporesponsiveness when compared to peripheral blood (Figure1) before NAC therapy. Administration of NAC led to a partial restoration of T cel hyporesponsiveness as measured by thymidine incorporation after a mitogenic stimulus (Figure 2), but this restoration did not reach statistical significance (p=0.06). We observed LAT dislocation to the cytoplasm in purified synovial T lymphocytes when compared to PB T lymphocytes in 8/10 patients tested. However, whereas *in vitro* incubation with 5mM NAC has been shown to restore LAT localization to the cellular membrane (5;11), we found no such restoration in the SF T lymphocytes of the patients that received NAC infusion. We also examined localization of the NF-κB p65 subunit, which translocates to the nucleus following activation. Nuclear translocation of the NF-κB p65 subunit was observed in synovial T lymphocytes from the same 8 patients displaying LAT dislocation. NF-κB p65 was localized in the cytoplasm of the synovial fluid T of the remaining 2 patients tested, and in the PB T cells of all patients. This suggests that activation of NF-κB in synovial T lymphocytes could also be modulated by oxidative stress. However, as observed for LAT localization, administration of NAC did not change NF-κB activation in the synovial T lymphocytes.

Figure1

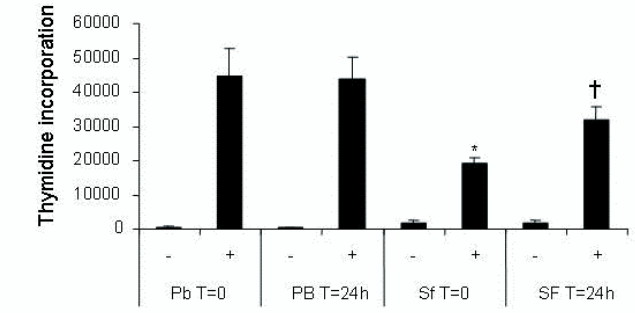

Figure 1: Thymidine incorporation of unstimulated (-) and PMA+ionomycine stimulated (+) PB and SF T lymphocytes before and 24h after NAC treatment. T lymphocytes isolated from the synovial fluid are hyporesponsiveness when compared to PB T cells blood (*: p=0.01), but administration of NAC only led to a partial restoration (†: p=0.06) of the proliferative respone SF T cell to a mitogenic stimulus.

Figure 2A

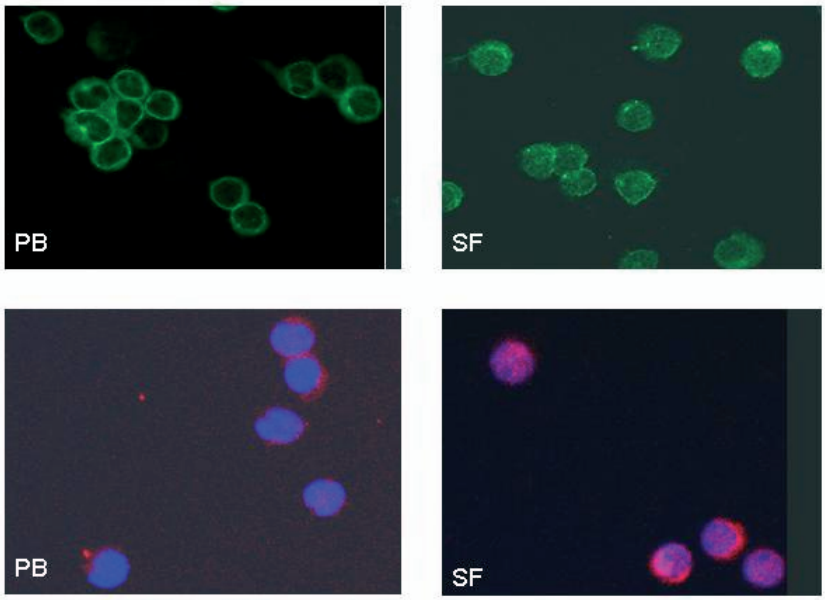

Figure 2B

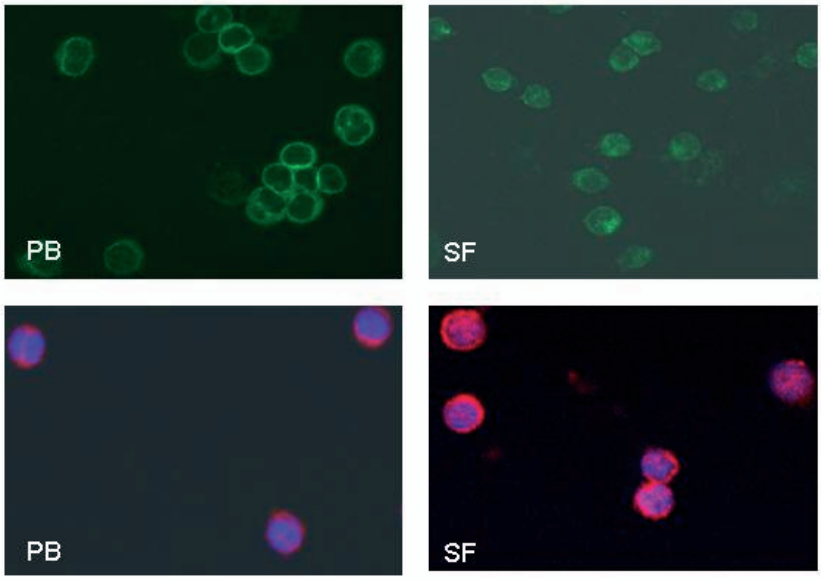

Figure 2: Representative images of intracellular LAT (upper panels) the NF-κB p65 subunit (lower panels) staining before (fig 2A) and 24h after (fig 2B) NAC treatment, as visualized by confocal microscopy. LAT was dislocated to the cytoplasm in purified SF T lymphocytes when compared to PB T lymphocytes in 8/10 patients tested (upper panels). Nuclear translocation of the NF-κB p65 subunit was observed in SF T cells from the same 8 patients displaying LAT dislocation (lower panels). NAC treatment did not restore membrane localization of LAT, nor did it inhibit NF-κB activation (fig 2B).

Discussion

There is accumulating evidence underscoring the central role of reactive oxidant species (ROS) in inflammatory processes. Patients with rheumatoid arthritis (RA) have low concentrations of protective antioxidants and high levels of the metabolic products of ROS in blood and synovial tissue (1). In vitro studies and animal studies have shown N-acetylcysteine (NAC) possesses strong anti-inflammatory characteristics due to its anti-oxidative capacity, suggesting NAC could have beneficial effects in RA. We therefore conducted an open pilot trial to study the effects of high dose i.v. NAC in patients with active RA. This treatment did not result in significant improvement of DAS, although small improvements were observed of tender and swollen joint counts. Although there was a trend towards normalization of proliferative responses of synovial T lymphocytes, we could not detect restoration of LAT dislocation in these cells after NAC admininistration. Interestingly though, in the same patients (8/10) where we detected LAT dislocation from the cellular membrane in synovial T cells as marker for severe oxidative stress, we also found activation of NF-κB. NF-κB is a key transcriptional regulator of pro-inflammatory genes (12;13) and is proposed to be ROS-dependent(7;13). Our results demonstrate that NF-κB is activated in RA synovial T cells, and are in line with the findings of Collantes who described qualitatively different DNA binding capacities of NF-κB isolated from synovial fluid or peripheral blood(13). Furthermore, since the p65 subunit was translocated to the nucleus in the same cells where we detected LAT dislocation, our results suggest that NF-κB activation in synovial T lymphocytes could, at least in part, be regulated by oxidative stress. NAC treatment, however, did not modulate the observed NF-κB activation in synovial T lymphocytes, nor did it restore the dislocation of LAT.
The modest clinical effects of NAC administration in patients with RA are consistent with recent randomised, placebo controlled studies in patients with severe sepsis. Paterson and coworkers found decreased NF-κB activation in mononuclear leukocytes which was assossiated with decreased levels of IL-8, but not IL-6 after NAC administration(10). However, this was only found in the surviving patients after 72h of continuous NAC administration. A bolus of 150 mg/kg N-acetylcysteine over 15 mins was given, followed by 50 mg/kg over 4 hrs as a loading dose, and then a maintenance infusion of 50 mg/kg over each 24-hr period. No differences in cytokine levels were found in patients with severe sepsis after NAC infusion by Emet et al (14), and Spapen et al found no effect of NAC on plasma TNF, IL-6 or IL-10, but only temporarily decreased IL-8 and soluble TNFreceptor/p55 levels(15). They did not detect significant differences between NAC treated patients and placebo in their patients with ARDS and early septic shock in gas exchange, development of ARDS or mortality.

In conclusion, a 24-hour treatment with high dose i.v. NAC did not significantly improve disease activity in patients with active RA, nor did it correct signaling abnormalities of synovial T lymphocytes. These results do not rule out the possibility that multiple dosing regimens with or without maintenance treatment are effective.

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Chapter 7
Summary and General discussion

In rheumatoid arthritis (RA), an inflammatory infiltrate accumulates and persists in the synovial membrane. The pathophysiological events that initiate and perpetuate the inflammation have still not been elucidated, although it is generally believed that multiple immulogical and genetic factors play a role. Dendritical cells, macrophages, B cells, T cells, synoviocytes, (auto-) antibodies and a wide arrange of cytokines all seem to contribute to the pathogenic events in RA. Starting from the concept of autoimmunity, many still believe T cells play a key role in the pathogenesis of RA. Synovial T cells however, display a number of particular characteristics. While displaying markers of recent activation, synovial T lymphocytes respond poorly to mitogenic stimuli and their cytokine production appears to be suppressed both in situ and in vitro. One of the critical hallmarks from synovial T cells is that they suffer from oxidative stress, as demonstrated by decreased levels of the intracellular anti-oxidant glutathione (GSH) (1). Recently it was shown that the intracellular redox disturbance has critical implications on proximal and distal TCR signaling events. Oxidative stress in synovial fluid T lymphocytes inhibits T cell receptor (TCR)-dependent phosphorylation of pivotal signaling molecules, required for efficient T cell proliferation, and contributes to severe hyporesponsiveness of these cells upon antigenic stimulation (2,3).
Chronic exposure of T lymphocytes to free radicals produced by activated phagocytic cells at the site of inflammation has been proposed to be the major cause of deregulated redox homeostasis in RA. To investigate the exact localization of endogenous ROS production in human synovium we adapted a new cytochemical technique developed by Karnovsky, using the 3,3' diaminobenzidine (DAB) probe and manganese. Free radicals directly react with DAB, forming an insoluble DAB polymer which can be visualised by microscopy. In **chapter 2** we demonstrate that the oxidative stress found in synovial T cells is not the result from exogenous sources but originates from (an) intracellular activated oxidase(s). **In chapter 3** we demonstrate that the oxidase generating ROS in SF T cells is controlled by the small GTPases Ras and Rap1. Whereas introduction of constitutive active Ras in the Jurkat T cell line generates intracellular ROS production via a Ral dependent signalling pathway, introduction of constitutive Rap1 inhibits mitogenic and Ras induced ROS production via a PI3-kinase dependent signaling pathway. Conversely inactive Rap1 increases intracellular ROS production. In SF T cells we find constitutively activated Ras and inactive Rap1. We also show that constitutive Ras activation and inhibition of Rap1 activation are not a result from oxidative stress, but the origin of intracellular free radical production, and that introduction of dominant negative Ras in synovial T cells downregulates the excess ROS production. During the last decade it has become increasingly clear that free radicals can serve as critical second messengers in a wide variety of intracellular signaling events (for review: see introduction). The specific signaling function depends on the kinetics, the localisation and the species of the produced ROS. It was shown that H_2O_2 activates the stress MAPKinases p38 and JNK and the pro-inflammatory transcription factor NF-κB(4-6). Although the exact role of the intracellular free radicals in synovial T cells remains illusive, it is tempting to speculate that intracellular free radicals in T lymphocytes contribute to NF-κB-dependent gene transcription, which in turn results in upregulation of pro-inflammatory cell surface markers such as TNF-α and IL-1 receptors. These proteins play critical roles in the activation of synovial macrophages and fibroblast-like synoviocytes, which in turn secrete cytokines and proteases perpetuating inflammation. Importantly, oxidative stress is also found in other pathological conditions besides RA, e.g. CD4 lymphocytes in patients with AIDS and in

ischemia-reperfusion laesions (7,8). It will be interesting to investigate whether similar oxidases that we found in synovial T lymphocytes are also involved in these diseases.
In **chapter 4** we show that in synovial T cells from RA patients Ras can be activated by a variety of cytokines. Rap1 inhibition is induced by direct cell-cell contact of T lymphocytes with antigen presenting cells (APC), and can be prevented by blocking the co-stimulatory T cell receptor CD28 with CTLA-4. These findings underscore the critical role of free radicals in disturbed T cell function in rheumatoid arthritis. One could speculate that increased ROS production is seen after defective (auto-)antigen presentation by APC or it is possible that in RA T cells are only activated through T cell receptor independent pathways, in casu CD28.
Based on the pro-inflammatory properties of free radicals, one might expect anti-oxidants to have anti-arthritic effects. In animal models, high doses of vitamin C, vitamin E and NAC had beneficial effects on rheumatoid inflammation, related to their antioxidative potential (9-11). Other candidate antioxidants have been identified with potential benefit, including selenium, zinc, manganese, niacinamide, bioflavonoids and β carotene. In **chapter 5**, the results of a double-blind placebo-controlled study with a daily nutrient supplement containing antioxidants and the omega-3 fatty acids eicosapentaenoic acid, docosahexaenoic acid and the omega-6 fatty acid gamma-linolenic acid are presented. We did not find superior clinical benefit at the doses tested as compared to placebo. One could postulate that the doses of the antioxidants used were insufficient. However, treatment of RA patient with high dose NAC did not result in significant clinical improvement either. As described in **chapter 6**, 14 patients with active RA were treated with high dose intravenously administered NAC, adopted from the protocol for acetaminophen intoxication. Only minimal clinical improvement was documented. Whereas in vitro treatment of T lymphocytes with NAC restores T cell defects and hyporesponsiveness, no such effects were seen in the patients that received NAC. Interestingly though, in the SF T cells of all RA patients where we found oxidative stress-dependent signaling defects, we also found NF-κB activation, again suggesting a critical role for free radicals as second messenger in the activation of NF-κB.
Many questions about the exact role of upregulated free radical production in synovial T cells remain. Do ROS aggravate inflammation through activation of NF-κB? Or do they have a benificial effect, downregulating T lymphocytes? Do they play a central role in synovial T cell signaling leading to T cell proliferation and differentiation, or are they involved in T cell apoptosis? Or are they an innocent collateral effect of other cellular processes? Are all synovial T cells suffering from oxidative stress, or is there a subgroup of T lymphocytes that do not display upregulation of intracellular free radicals? Answers to such questions remain important, before answers can be given to questions whether we should counter the oxidative stress with anti-oxidants to enhance SF T cell function, or whether we should specifically target synovial T lymphocytes suffering from oxidative stress, and try to eliminate them. Equally interesting is to examine whether in self-limiting arthritis these ROS-loaded T cells also occur. At present, no such T cells were found in any of the reactive arthritis examined nor in synovial tissue of patients with osteoarthritis.

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Samenvatting

Reumatoïde artritis (RA) is een ziekte die gekenmerkt wordt door chronische gewrichtsontsteking. Dit leidt tot gewrichtspijn en zwelling en zal, indien onbehandeld, ernstige gewrichtsschade veroorzaken. Bij patiënten met RA ontstaat een ontstekingsinfiltraat in het synovium, het weefsel dat de gewrichten omkleed. In dit ontstekingsinfiltraat wordt een grote variëteit cellen aangetroffen: dendritische cellen, T – en B lymfocyten, plasmacellen, macrofagen, fibroblastachtige synoviocyten en andere. Hoewel al deze cellen en hun cytokinen een rol spelen in het ontstaan en men name in het persisteren van de gewrichtsontsteking, wordt met name aan T lymfocyten een belangrijke rol toegeschreven.
T lymfocyten die we in het synovium terugvinden, onderscheiden zich van "normale" T lymfocyten in het bloed van reumapatiënten of gezonde mensen. Eén van de bijzondere eigenschappen van synoviale T cellen is dat ze veel minder gevoelig zijn ten aanzien van externe prikkels, ofwel "hyporesponsief" zijn. Een tweede bijzondere eigenschap is dat ze lijden aan wat "oxidatieve stress" genoemd wordt. Van oxidatieve stress wordt gesproken wanneer een cel in contact komt met meer zuurstofradicalen dan de normale cellulaire buffercapaciteit (bestaande uit alle anti-oxidanten) kan verwerken. Recent werd gevonden dat bij reumatoïde artritis de oxidatieve stress waaraan synoviale T cellen lijden, lijkt samen te hangen met hun ongevoeligheid op externe prikkels. Behandeling van synoviale T cellen in kweek met antioxidanten, deed deze ongevoeligheid afnemen, terwijl T cellen van gezonde donoren na blootstelling aan oxidatieve stress even zo ongevoelig voor externe prikkels werden als synoviale T cellen.
Algeheel wordt aangenomen dat de oxidatieve stress bij synoviale T cellen ontstaat door langdurige blootstelling aan een overmaat zuurstofradicalen uit hun directe omgeving. Zuurstofradicalen vinden echter niet noodzakelijk hun oorsprong in de directe omgeving waarin een cel zich bevindt. Een cel zelf maakt ook zuurstofradicalen aan, meestal als bijproduct van ons normale metabolisme. Zo ontstaan in de mitochondriën zuurstofradicalen als bijproduct van ons aëroob metabolisme waarin zuurstof wordt omgezet tot energie in de vorm van ATP. Bij activiteit van verscheidene enzymen die toxische stoffen afbreken zoals peroxisomen, cytochroom P450, en xanthine oxidase ontstaan eveneens zuurstofradicalen als nevenproduct. Zuurstofradicalen kunnen echter ook intracellulair ontstaan na stimulatie van specifieke cellulaire oppervlaktereceptoren. Waar alle boven vermeldde zuurstofradicalen gezien worden als schadelijke afbraakproducten, zijn er sterke aanwijzingen dat de zuurstofradicalen die intracellulair geproduceerd worden na receptorstimulatie een eigen specifieke functie hebben in het doorgeven van de extern stimulus naar specifieke intracellulaire targets. Zo wordt bijvoorbeeld NF-κB door zuurstofradicalen geactiveerd. NF-κB is een transcriptiefactor die verantwoordelijk is voor de activatie van diverse pro-inflammatoitre genen, waaronder TNF-α, die een centrale rol spelen bij RA.
In dit proefschrift wordt daarom het onderzoek beschreven naar de oorsprong en de gevolgen van de oxidatieve stress in synoviale T cellen. Tevens wordt het resultaat beschreven van therapieën, die gericht zijn op het opheffen van deze oxidatieve stress. Zowel een oraal voedingssupplement dat de meeste anti-oxidanten bevat, als intraveneuze therapie met hoge dosis N-acetyl cysteïne, het sterkste antioxidant.

Hoofdstuk 1 is een introductie hoe zuurstofradicalen ontstaan en geeft een gedetailleerd overzicht van studies naar de mogelijke rol van zuurstofradicalen als signaaltransmissie. Welke celreceptoren tot zuurstofradicaal productie lijden, welke enzymen betrokken zijn en welke de downstream effectormoleculen voor de zuurstofradicalen kunnen zijn.
In **hoofdstuk 2** beschrijven we de ontwikkeling van een nieuwe techniek die het toelaat rechtstreeks de productie van zuurstofradicalen te meten en deze productie in het synoviaal weefsel te visualiseren. Door gebruik te maken van deze techniek tonen we aan dat de oxidatieve stress in de synoviale T cellen bij RA patiënten niet veroorzaakt wordt door chronische blootstelling aan zuurstofradicalen in hun omgeving, maar dat de overmaat aan zuurstofradicalen geproduceerd wordt door intracellulaire enzymen. Bovendien kunnen we door toevoeging van verschillende enzyminhibitoren aantonen dat het geproduceerde zuurstofradicaal waarschijnlijk waterstofperoxide (H_2O_2) is.
In **hoofdstuk 3** onderzoeken we hoe het enzym dat de intracellulaire zuurstofradicalen genereert, geactiveerd wordt. We tonen aan dat er een signaalroute in T lymfocyten bestaat, dat bij activatie leidt tot intracellulaire zuurstofradicaal productie. Deze route wordt gecontroleerd door 2 vitale eiwitjes: Ras en Rap1, die beide behoren tot de groep van kleine GTPase eiwitten. Waar activatie van Ras lijdt tot productie van zuurstofradicalen, zal activatie van Rap1 lijden tot blokkade van deze zuurstofradicaal productie. In de synoviale T cellen vinden we dat het Ras eiwit constant geactiveerd is, terwijl zijn tegenpool Rap1 juist niet kan geactiveerd worden. Dit lijdt tot de continue overmatige productie van de intracellulaire zuurstofradicalen. Introductie van een dominant negatief Ras in synoviale T cellen kan deze zuurstofradicaalproductie opheffen.
Hoofdstuk 4 gaat verder in op de activatie van het Ras eiwit en de blokkade van het Rap1 eiwit. We laten zien dat activatie van Ras kan voortkomen na diverse stimuli, met name na chronische stimulatie van verschillende cytokinen waaronder TNF-α. Belangrijker echter is dat we vinden dat de inactivatie van Rap1 in synoviale T cellen ontstaat na cel-cel contact tussen de T lymfocyt en een macrofaag uit het synovium. Bovendien worden sterke aanwijzingen gevonden dat dit proces loopt via activatie van de CD28 co-receptor op het T cel membraan. Niet alleen experimenten waarin het cel-cel contact tussen macrofaag en T lymfocyt wordt verbroken lijden tot afwezigheid Rap1 inactivatie, ook toevoeging van CTLA4, de natuurlijke antagonist van de CD28 receptor, blokkeert de inactivatie van Rap1. Ook wordt in hoofdstuk 4 nogmaals aangetoond hoe cruciaal de inactivatie van Rap1 is in de productie van zuurstofradicalen. Parallel aan Rap1 inactivatie vinden we de toegenomen intracellulaire zuurstofradaalproductie, en terzelfder tijd de "hyporesponsiviteit " van T lymfocyten ontstaan.
Hoofdstuk 5 geeft de resultaten van een dubbelblind uitgevoerde trial waaraan 66 patiënten met actieve RA deelnamen en waarin het effect wordt nagegaan van een dagelijks voedingssupplement waarin naast alle genoegzame anti-oxidanten ook de ω-3 (eicosapentaeenzuur (EPA) en docosahexaeenzuur (DHA0) en ω-6 (gamma-linoleenzuur (GLA)) polyonverzadigde vetzuren zaten. Hoewel RA patiënten zich enthousiast uitten ten aanzien van voedingssupplementen en we significante toename zagen van de serumconcentraties vitamine E, EPA en DHA, werd geen klinisch significant benificieel effect gevonden ten aanzien van een placebo groep.
Hoofdstuk 6 beschrijft de resultaten van een studie waarin 14 patiënten met actieve RA werden behandeld met hoge dosis van het sterke antioxidant NAC, dat werd toegediend via een continu infuus gedurende 24 uur. Ook hier werd geen belangrijke klinische verbetering van de RA activiteit gevonden. En hoewel de synoviale T cel responsiviteit zich na het NAC infuus zich lijkt te herstellen, vonden we geen herstel van andere cruciale signaaleiwitten (zoals Linker for activation of T cells (LAT) dislocatie, of NFκB inactivatie). Tot slot werden ook in deze setting opnieuw sterke aanwijzingen gevonden dat zuurstofradicalen een cruciale rol kunnen spelen in cellulaire signaaltransductie en dat de belangrijke pro-inflammatoire transcriptiefactor NFκB gereguleerd zou kunnen worden door de productie van intracellulaire zuurstofradicalen en de

cellulaire redoxbalans: in dezelfde synoviale T cellen die de kenmerken dragen van ernstige oxidatieve stress (dislocatie van LAT uit celmembraan), vinden we activatie van het centrale pro-inflammatoire transcriptiefactor NFκB.

Samenvattend draagt dit proefschrift bij tot het bewijs dat vrije radicalen meer zijn dan alleen maar afbraakproducten van ons aëroob metabolisme of toxische stofjes uit ons milieu. Wij hebben bewezen dat in normale T lymfocyten een mechanisme bestaat dat op specifieke externe prikkels een kleine hoeveelheid zuurstofradicalen doet produceren in het cytoplasma van deze cellen. Deze zuurstofradicalen zijn essentieel voor een normale respons van de cel, en spelen een rol in de activatie van specifieke genen. Aanvullend toonden wij aan dat bij patiënten met reumatoïde artritis de productie van zuurstofradicalen in de T lymfocyten volledig ontregeld is. Waar "gezonde" T lymfocyten slechts een zeer kleine hoeveelheid zuurstofradicalen aanmaakt, produceert de T lymfocyt in ontstoken gewrichten een overmaat aan zuurstofradicalen. Hierdoor raakt de T lymfocyt volledig ontregeld. Niet allen reageert de cel verschillend op externe prikkels, de cel maakt hierdoor een overmaat aan ontstekingsbevorderende eiwitten.
Echter, behandeling met anti-oxidanten leverde bij onze patiënten met actieve reumatoïde artritis geen significante verbetering op van hun ziekteactiviteit.

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Curriculum vitae

De schrijver van dit proefschrift werd geboren op 25 december 1969 te Genk. In 1988 werd het diploma middelbaar onderwijs behaald aan het Sint Jozef college te Turnhout. In datzelfde jaar begon hij met de studie geneeskunde aan de Katholieke Universiteit te Leuven (KUL), aan welke in 1995 het diploma in genees-, heel- en verloskunde cum laude werd behaald. Tijdens de studie geneeskunde was hij lid van het presidium geneeskunde van 1991-1992. Van 1993-1995 liep hij aanvullende stage aan de KUL bij de vakgroep klinische genetica bij professor Frijns. Van 1995 tot 1998 was hij in opleiding tot internist in het Atrium Heerlen. Deze opleiding werd gevolgd door opleiding tot reumatoloog van 1998 tot 2003 in het Leids Universitair Medisch Centrum (opleider: Prof.dr. F.C. Breedveld). Dit werd gecombineerd met weteschappelijk onderzoek, waarvan het hier beschreven proefschrift het resultaat is. Sedert 2003 is hij als staflid verbonden aan de vakgroep Klinische Immunologie en Reumatologie van het Amsterdam Medisch Centrum (hoofd: Prof.dr. P.P. Tak), waar het onderzoek naar oxidatieve stress, signaaltransductie en apoptose wordt voortgezet.

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