Published in final edited form as:

Sci Transl Med. 2016 March 23; 8(331): 331ra38. doi:10.1126/scitranslmed.aad7151.

Restoring oxidant signaling suppresses pro-arthritis T-cell effector functions in rheumatoid arthritis

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Abstract

To promote their pathology, CD4 T-cells from patients with rheumatoid arthritis (RA) have to clonally expand and differentiate into cytokine-producing effector cells. In contrast to healthy T-cells, naïve RA T-cells have a defect in glycolytic flux due to upregulation of glucose-6-phosphate dehydrogenase (G6PD). Excess G6PD shunts glucose into the pentose phosphate pathway (PPP), resulting in NADPH accumulation and ROS consumption. With surplus reductive equivalents, RA T-cells insufficiently activate the redox-sensitive kinase ATM; bypass the G2/M cell cycle checkpoint and hyperproliferate. Insufficient ATM activation biases T-cell differentiation towards the Th1 and Th17 lineages, imposing a hyper-inflammatory phenotype. We have identified several interventions that replenishing intracellular ROS, correct the abnormal proliferative behavior of RA T-cells and successfully suppress synovial inflammation. Rebalancing glucose utilization and restoring oxidant signaling may provide a novel therapeutic strategy to prevent autoimmunity in RA.

Introduction

The autoimmune disease Rheumatoid Arthritis (RA) damages tendons, cartilage and bone and shortens life expectancy through acceleration of cardiovascular disease (1, 2). CD4 T-cells in RA patients sustain synovitis, promote autoantibody formation, facilitate osteoclast differentiation and impose endothelial dysfunction (3). When activated, RA CD4 T-cells insufficiently upregulate the glycolytic enzyme PFKFB3 and generate less ATP and lactate

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One Sentence Summary: ROS deficiency promotes pro-inflammatory T-cells in RA

Author contributions: ZY, YS and HO planned and performed experiments. ELM recruited patients. ZY and LT analyzed data. CMW and JJG conceived of the study, designed experiments, analyzed data and wrote the manuscript.

Competing interests: No competing financial interests.

(4). It is currently unknown, whether and how metabolic abnormalities are mechanistically connected to their pro-inflammatory functions.

The cardinal feature of naïve CD4 T-cells is the ability to massively proliferate when encountering antigen. When transitioning from naïve to effector status, T-cells expand 40-100 fold within days (5), making them highly dependent on energy and biosynthetic precursors (6). Resting lymphocytes rely on oxidative phosphorylation and fatty acid breakdown, but upon activation switch to aerobic glycolysis and tricarboxylic acid flux, designating glucose as the primary source for ATP generation in activated lymphocyte. Anabolic metabolism of glucose not only provides energy, but also macromolecular building blocks for the exponentially expanding biomass, typically by shunting glucose into the pentose phosphate pathway (PPP) (7). In the first rate-limiting step of the PPP, G6PD oxidizes G6P to 6-phosphogluconolactone to generate 5-carbon sugars (pentoses), ribose 5-phosphate, a precursor for nucleotide synthesis and NADPH, one of the cell's principal reductants. As an electron carrier NADPH provides reducing equivalents for biosynthetic reactions and by regenerating reduced glutathione protects against reactive oxygen species (ROS) toxicity. Cytoplasmic NADPH is an absolute requirement to convert oxidized glutathione (GSSG) to its reduced form (GSH), which is converted back when hydrogen peroxide is reduced to water.

Oxidative stress results from the action of ROS, short-lived oxygen-containing molecules with high chemical reactivity towards lipids, proteins, and nucleic acids. Until recently ROS were regarded as merely damaging agents, but are now recognized as second messengers that regulate cellular function through oxidant signaling (8, 9). Cells can produce ROS in several of their organelles and possess specialized enzymes, such as the family of NADPH oxidases (NOX), to supply fast and controlled access. Quantitatively, mitochondria stand out as persistent ROS suppliers with the respiratory chain complexes I and III releasing superoxide into the mitochondrial matrix and the intermembrane space (9, 10). It is incompletely understood how redox signaling affects T-cell proliferation and differentiation and how cell-internal ROS relate to pathogenic T-cell functions.

The current study has investigated functional implications of metabolic and redox dysregulation in RA T-cells. We find that RA T-cells fail to properly balance mitochondrial ROS production and the cellular anti-oxidant machinery. Molecular studies place excessive activity of G6PD at the pinnacle of abnormal T-cell regulation in RA and provide a new paradigm for the connection between metabolic activities, abnormal proliferative behavior and pro-inflammatory effector functions. Mechanistically, PPP hyperactivity oversupplies RA T-cells with reducing equivalents, increasing NADPH and depleting ROS. This insufficient oxidative signaling prevents sufficient activation of the cell cycle kinase ATM and allows RA T-cells to bypass the G2/M cell cycle checkpoint. ATM deficiency shifts differentiation of naïve CD4 T-cells towards the Th1 and Th17 lineage, creating an inflammation-prone T-cell pool. Several metabolic interventions are able to rebalance glucose utilization away from the PPP towards glycolytic breakdown, easing reductive stress and preventing hyperproliferation and maldifferentiation of RA T-cells. Such interventions represent possible drug candidates for a novel strategy in anti-inflammatory therapy.

Results

Disproportionate PPP activation in RA T-cells

CD4⁺CD45RO⁻ T-cells from RA patients have reduced glycolytic flux, generating lower ATP and lactate concentrations (4), while proliferating vigorously (11), suggesting intactness of metabolic outputs that support biomass generation. To examine competence of the PPP, we quantified gene and protein expression of the rate-limiting enzyme G6PD (Fig. 1A-B). Compared to controls, RA T-cells expressed higher G6PD transcript and protein levels and G6PD enzyme activity was 30% increased (Fig.1C); compatible with preferential PPP shunting in patient-derived T-cells. The response of G6PD to T-cell receptor triggering was prompt and sustained (Fig.S1) and RA T-cells were distinguishable from control T-cells over the entire post-stimulation period. The defect was disease-specific and was not present in T-cells from patients with psoriatic arthritis (PsA).

In a cohort of 31 patients and 32 age/gender-matched controls, G6PD overexpression coincided with PFKFB3 deficiency and in individual patients the ratio of G6PD/PFKFB3 was clearly shifted towards G6PD (Fig.1D). To evaluate the relationship between shifted metabolic enzymes and RA inflammatory activity, transcript levels in stimulated CD4⁺CD45RO⁻ T-cells were correlated with the composite disease activity measure DAS28. DAS28 scores were strongly correlated with the G6PD/ PFKFB3 ratio ($R=0.6$, $P<0.001$, Fig.1E). To assess the impact of immunosuppressive therapy on G6PD induction, we compared G6PD transcript levels in untreated patients and patients on different types of medications (Fig.S2). G6PD transcript levels were elevated in untreated patients and enzyme expression was similar in T-cells from patients on different types of medications.

To test whether RA T-cells redirect glucose into the PPP and prefer NADPH production over glycolytic breakdown, we quantified NADPH levels and assessed the cells' redox status via quantification of reduced glutathione (mBCI) and ROS (CellRox). RA T-cells had higher NADPH levels, outperforming their healthy counterparts and T-cells from PsA patients by 40% (Fig. 1F). RA T-cells, but not PsA T-cells, contained significantly more reduced glutathione (Fig. 1G-I). Excess GSH generation was maintained in naïve T-cells that converted to the memory phenotype (Fig.1F), but the bias towards reductive species in RA T-cells became visible only after T-cell stimulation and was not present in resting cells (Fig.S3).

Following TCR stimulation, intracellular ROS levels displayed a characteristic kinetic with baseline levels maintained over 24 hours, a steep increase to >12-fold higher levels over the subsequent 48 hours and a gradual decline between days 3 and 6. Thus, while transitioning from naïve to effector cell, T-cells enter a period of oxidative stress (Fig.1J). RA T-cells followed similar kinetics early after stimulation, but ROS levels increased only 8-fold to peak on day 3, suggesting surplus reducing equivalents. Between day 3 and 6 ROS levels in RA T-cells were consistently lower. Compared to control and PsA, ROS levels in RA T-cells were significantly reduced (Fig.1K). Kinetics of ROS generation mirrored glycolytic activity, which also peaked after 72 h (4).

In essence, RA patients' naïve CD4 T-cells express an altered pattern of glucose metabolizing enzymes, resulting in slowed glycolytic breakdown and increased PPP shunting. The defect is specific for a diagnosis of RA and not present in PsA. Due to disproportionate gain in NADPH and reduced glutathione, the cells consume ROS and are under reductive stress.

ROS reduction in RA T-cells results in hyperproliferation and G2/M checkpoint bypassing

The PPP supplies reducing equivalents for macromolecule synthesis, the building blocks for new cells, rendering naïve CD4 T-cells particularly sensitive to changes in proliferative metabolism. To examine whether excessive G6PD activity affects T-cell proliferation, RA T-cells were treated with the G6PD inhibitor 6-aminonicotinamide (6-AN). Preventing glucose entry into the PPP profoundly reduced cellular proliferation (Fig.2A) and also changed intracellular ROS levels (Fig.2B). Upon 6-AN treatment, ROS levels doubled and, in parallel, proliferative activity decreased. G6PD inhibition corrected the spontaneously elevated division indices of RA T-cells (Fig.2A). G6PD's critical role in regulating T-cell proliferation was confirmed by gene-specific RNA interference. Transfection of two distinct siRNAs significantly reduced G6PD protein expression (Fig.S4). G6PD knockdown in RA T-cells reduced intracellular NADPH and GSH concentrations, increased ROS levels and normalized division indices (Fig.2C).

ROS reduction in RA T-cells was associated with hyperproliferation (Fig.2A) and increased IL-2 production (Fig.2D). On day 3, intracellular IL-2 was more than doubled in RA compared to wild-type T-cells. Higher division rates in RA T-cells became functionally relevant in the conversion of naïve into memory T-cells (Fig.2E). At the end of a 6-day stimulation period, 29% of control T-cells, but only 18% of RA T-cells retained the naïve phenotype, demonstrating that RA T-cells converted to the memory phenotype at a more rapid pace.

To test the conversion of naïve into memory T-cells in vivo, we employed a human-SCID mouse chimeric model (12). Human CD45RO⁺ PBMCs take residence in the spleen of reconstituted NSG mice and form organized T-cell-B cell aggregates (Fig.S5A-B). Transfer of 10 million CD45RO⁺ PBMCs into the murine host prompted T-cell proliferation and naïve-to-memory conversion within 7-10 d (Fig.S5C). Human naïve T-cells required co-transfer of human APC, specifically monocytes, for optimal engraftment and expansion (Fig.S6A-B). Thus, all reconstitution experiments employed T-memory-cell-depleted PBMC composed of naïve T-cells, B cells and monocytes. Transfer of such populations from RA patients and control donors permitted the direct comparison of how rapidly naïve cells acquired a memory phenotype. RA-derived cells had a higher division index (Fig.2F), and converted to a memory phenotype at a faster rate, as indicated by higher frequencies of CD4⁺CD95⁺ and CD8⁺CD95⁺ cells (Fig.2G); confirming that naïve RA T-cells are prone to faster cell cycle progression and fail to maintain naivety.

To understand functional consequences of ROS reduction for T-cell proliferation, we suppressed ROS through the cell-permeable superoxide dismutase mimetic, 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl (Tempol) (13) (Fig.2H-I). TCR activation essentially moved all cells into the cell cycle. Six days poststimulation, two-thirds of the T-

cells had completed 3-4 doublings. Tempol accelerated proliferation and increased the proportion of T-cells with >5 generations from 15 to 26%. In RA T-cells, proliferation was spontaneously higher and further increased by ROS scavenging, from 22 to 32% of cells. Proliferative rates in Tempol-treated wild type T-cells were similar to rates in untreated RA T-cells.

Cell cycle analysis revealed that Tempol treatment selectively fastened G2/M transit, progression through the G1 and S phase was unaffected (Fig.2J). RA T-cells effectively bypassed the G2/M checkpoint, even without treatment. Following stimulation, 8% of control T-cells were in the G2/M phase. Tempol reduced G2/M-retained T-cells to 5%, similar to only 4% in RA T-cells. Both RA origin and ROS scavenging reduced the fraction of G2/M-specific phospho-histone H3 positive cells (Fig.2K), confirming the role of ROS in regulating G2/M transition.

Taken together, the activation-induced elevation of intracellular ROS regulates cell cycle progression, proliferative efficiency and naïve-to-memory conversion. ROS-deficient RA T-cells hyperproliferate, fail to maintain the naïve phenotype and bypass the G2/M cell cycle checkpoint.

ATM insufficiency in RA T-cells

The cell cycle checkpoint leading to G2/M arrest is activated by the protein kinase ATM (14). In healthy CD4+CD45RO⁻ T-cells, TCR stimulation induced a 4-5fold rise in ATM transcripts. In RA T-cells, ATM transcripts responded slower, increasing only 2-3fold (Fig. 3A). ATM protein concentrations followed a similar kinetic, with both ATM monomers and dimers rising post-stimulation (Fig.3B). By 72 h most of the protein was in the active dimeric state. ATM protein was much less abundant in RA T-cells, particularly the dimeric form (Fig.3B-C). TCR stimulation resulted in ATM phosphorylation at serine 1981 (Fig.3D), with maximal pATM concentrations recorded on day 3 (Fig.3E). In contrast, pATM was barely detectable in RA T-cells (Fig. 3D). pATM kinetics paralleled cellular ROS dynamics, raising the question whether ATM activation deficiency was related to the ROS^{low} status of RA T-cells.

Nuclear ATM is mostly activated by DNA fragments, dimer assembly of cytoplasmic ATM is a redox sensitive process (15). H₂O₂ rapidly induced dimer formation in CD4+CD45RO⁻ T-cells (Fig.3E) with visible effects starting at doses as low as 10 μM and plateauing at 20 μM. ROS-induced ATM dimerization promoted ATM Ser1981 phosphorylation (Fig.3E). Pharmacologic inhibition of ATM revealed its role in T-cell proliferation and naïve-to-memory conversion. KU-55933-treated T-cells expanded more vigorously (Fig.3F) and ATM impairment accelerated the generation of effector-memory and terminally-differentiated memory cells at the expense of naïve cells (Fig.3G). Thus, ATM inhibition in healthy T-cells reproduced the abnormal proliferation behavior of RA T-cells, implicating the kinase in clonal T-cell expansion.

We examined whether replenishing ROS can restore ATM activation in RA T-cells. Treatment with menadione, an analog of 1,4-naphthoquinone that generates intracellular ROS via redox cycling (16), increased ROS in RA T-cells (Fig.3H). Concomitantly,

menadione-exposed T-cells formed ATM dimers and accumulated pATM. ATM activation could be blocked by KU-55933, known to interfere with ATM phosphorylation at Ser1981 (15). KU-55933 effectively prevented menadione-induced ATM phosphorylation (Fig.3H), indicating that the ROS-inducing naphthoquinone analog restored physiologic ATM activation.

Taken together, RA T-cells fail to properly activate ATM, explaining the shortened G2/M phase as well as the DNA damage accumulation (17). Insufficient ATM signaling is correctable by replenishing intracellular ROS, mechanistically connecting G6PD overactivity, NADPH excess, reductive stress and malregulation of T-cell proliferation.

The redox balance regulates RA T-cell differentiation

Protective and pathogenic T-cell functions are closely linked to cytokine production and thus to T-cell differentiation. We examined the impact of ROS scavenging on differentiating T-cells (Fig.4A-B). 18% of healthy T-cells and 32% of patient-derived T-cells committed to IFN- γ production. ROS scavenging via Tempol further enhanced the commitment to the Th1 lineage, to 33% and 40%, respectively. Under cytokine-polarizing conditions, 1.8% of control T-cells and 4.2% of RA T-cells stained positive for intracellular IL-17. ROS quenching with Tempol increased Th17 cells to 5% and 6%, respectively. There was a trend for lower IL-4 and FoxP3 expression in the presence of Tempol. Overall, ROS reduction swayed T-cells to mature into pro-inflammatory Th1 or Th17 cells.

To test whether ROS bias T-cells towards pro-inflammatory effector functions in- vivo, we used the human-SCID chimera model (Fig.S6). Mice reconstituted with human T-cells were injected daily with Tempol or vehicle to examine the in vivo behavior of ROS-depleted T-cells. ROS scavenging resulted in a significant increase in Th1-committed T-cells, whereas IL-4 producers remained unchanged (Fig.4C).

Maldifferentiation and arthritogenic potential of RA T-cells

Considering the effects of ROS depletion on T-cell differentiation, we asked whether ROS^{low} RA T-cells are spontaneously biased to develop into IFN- γ ⁺ and IL-17⁺ effector cells (Fig. 5A). Without lineage-inducing cytokine cocktails 10% of healthy naïve CD4 T-cells differentiated into Th1 cells and 0.8% into Th17 cells. Corresponding frequencies in RA samples were 14% Th1 cells and 1.7% Th17 cells.

To investigate the disease relevance of T-cell maldifferentiation T-cell, we tested the cells' arthritogenic potential in a human synovium-NSG chimera model. Naïve CD4 T-cells from either healthy subjects or RA patients were adoptively transferred into human-synovium engrafted NSG mice. After 10 days, the synovial graft was analyzed for T-cell infiltration and lineage commitment (TCR, T-bet, GATA-3, FoxP3, ROR γ , IFN- γ , IL-17, and IL-4) (Fig.5B). Inflammatory activity of synovial macrophages and fibroblasts was assessed by profiling inflammation-associated genes (TNF- α , IL-1 β , IL-6, CD16a/b, CD68, MMP3, RANKL, vimentin, cadherin 11) and by immunohistochemically analysis (Fig.5C). RA T-cells were prone to migrate into the synovial tissue and commit to the Th1 and Th17 differentiation program (Fig.5B-C). Synovial membranes infiltrated by RA T-cells contained significant amounts of IFN- γ and IL-17. Conversely, much lower numbers of healthy CD4

T-cells were retained in the tissue and typically expressed GATA-3 and IL-4. Inflammatory cytokines were abundant in the RA T-cell-populated synovia. Evidence for synovial fibroblast activation came from the robust induction of vimentin and cadherin 11 (18). CD3-specific staining of tissue sections confirmed that RA T-cells, but not healthy T-cells differentiate into tissue-infiltrating effector cells in vivo (Fig.5C-D). In a Transwell assay system, RA T-cells were spontaneously hypermigratory, even in the absence of chemokine signals (Fig.5E).

ATM insufficiency favors T-cell maldifferentiation

We examined whether tissue invasiveness and pro-inflammatory effector functions of RA T-cells are mechanistically linked to ROS deficiency and ATM insufficiency. Under non-polarizing conditions, 9% of CD4 T-cells committed to IFN- γ production. Increasing doses of the ATM inhibitor KU-55933 shifted T-cell differentiation towards Th1ness and IFN- γ +CD4 T-cells more than doubled to as high as 20.0% (Fig.6A), whereas IL-4+CD4 T-cells were unchanged. Th1 or Th2 polarizing conditions drove 25% of cells towards Th1 differentiation and 13.5% to IL-4 production. ATM insufficiency markedly increased IFN- γ producing T-cells, but left IL-4 production unaffected (Fig.6B).

Inhibiting ATM function by shRNA-mediated silencing confirmed that ATM regulates Th1 lineage commitment (Fig.6C-D). After transfection with pSuper-gfp/neo-shATM plasmids frequencies of IFN- γ -producing T-cells in Th1-polarizing cultures were doubled from 14.7 to 32.4%. Effects of ATM insufficiency on T-cell function in vivo was explored in the human-SCID chimeric model (Fig.S2). Mice were reconstituted with CD45RO- PBMC from healthy donors; chimeras were injected daily with the ATM inhibitor KU-55933 or vehicle. Human T-cells from the chimeric spleen were analyzed for intracellular IFN- γ , IL-4, IL-17 and FoxP3 expression (Fig.6E). Suppressing ATM activity significantly increased Th1- and Th17-committed T-cells, whereas IL-4 producers and FoxP3+ cells remained unchanged. To assess ATM's effect on lineage-specific transcription factors, T-cells were cultured under Th1-, Th2-, Th17- and Treg-skewing conditions with and without the ATM inhibitor KU-55933. Early during the differentiation process expression of both T-bet and ROR γ was higher in ATM-insufficient T-cells (Fig.6F), suggesting that ATM-dependent signaling selectively regulates lineage-defining transcription factors.

To better understand the impact of ATM deficiency in arthritis development, human-synovium-NSG mice were treated with the ATM inhibitor KU-55933 following the adoptive transfer of either control or RA CD45RO- PBMC. Inhibiting ATM kinase activity intensified synovial inflammation induced by healthy and RA T-cells (Fig.6G). ATM-impaired healthy T-cells almost matched the pro-inflammatory potential of RA T-cells. ATM insufficiency further increased inflammatory effector functions of RA T-cells. KU-55933 treatment strongly upregulated IL-17, ROR γ , TNF- α , IL-1 β and IL-6 and expression of the osteoclastogenic ligand RANKL increased multifold (Fig.6G). RANKL protein expression in tissue sections confirmed upregulation of this osteoclastogenic ligand under ATM-deficient conditions (Fig.6H).

Inhibiting pro-inflammatory T-cell differentiation by correcting reductive stress

Excessive G6PD activity supplies undifferentiated RA T-cells with reductive elements (Fig. 1). ROS shortage causes insufficient ATM activation and RA T-cells prematurely exit the G2/M checkpoint (Fig.2-3), shifting their lineage commitment towards preferential differentiation of Th1 and Th17 cells (Fig.4-6). To mechanistically link PPP hyperactivity with inflammatory maldifferentiation, we identified two pharmacologic interventions that restore the redox balance in naïve RA T-cells and correct the overrepresentation of IFN- γ + T-cells.

First, we evaluated the synthetic naphthoquinone, menadione, which is reduced into an unstable semiquinone and generates ROS when formed into a quinone. Treatment of T-cells with menadione increased cellular ROS levels (Fig.3) and resulted in ATM dimerization and pATM formation (Fig.7A-B). Combination of the ATM inhibitor KU-55933 with menadione treatment did not prevent ATM dimer assembly (Fig.7B), but as expected (15), blocked ATM phosphorylation (Fig.7B). Menadione-induced restoration of ATM activation enabled pChk2 accumulation; this effect was disrupted when ATM phosphorylation was inhibited (Fig.7B).

Naïve CD4 T-cells from RA donors were differentiated in a polarizing cytokine cocktail in the absence and presence of either menadione or 6-AN; two interventions able to counteract the shift towards reductive elements. Menadione corrected the bias of RA T-cells to develop into IFN- γ -producers (Fig.7C). The G6PD inhibitor 6-AN provided an at least equally successful intervention to downregulate T-cell IFN- γ production (Fig.7C). Blocking G6PD activity reduced the frequency of IFN- γ -producing T-cells to less than 15%.

To evaluate the impact of ROS restoration on the arthritogenic potential of RA T-cells, we tested two ROS-inducing reagents in the human-synovium chimeras. Menadione raises ROS levels (Fig.3I) through redox cycling. Buthionine sulfoximine (BSO) inhibits gamma-glutamylcysteine synthetase (gamma-GCS), lowers tissue glutathione (GSH) concentration and consequently elevates intracellular ROS levels (Fig.S8). Synovium-engrafted NSG mice were adoptively transferred with T-cells derived from untreated or high-disease activity RA patients and mice were treated with optimized doses of either menadione or BSO. Treatment with both ROS inducers had a beneficial effect on synovitis (Fig.7D). Transcription factors (T-bet, ROR γ) driving pro-inflammatory T-cells were effectively downregulated, IFN γ and IL-17 were reduced, whereas FoxP3 was spared. RANKL expression responded to both treatments (Fig.7D-E), as did the inflammatory cytokines TNF- α , IL-1 β and IL-6. Menadione had more powerful effects than BSO. Immunohistochemical analysis of RANKL expression confirmed that tissue-infiltrating T-cells were almost all RANKL+ in the control arm, but lost RANKL expression after menadione and BSO treatment. Both menadione and BSO were able to correct the spontaneous hypermobility of RA T cells in Transwell migration assays (Fig.7F).

Overall, offsetting reductive stress in RA T-cells effectively suppressed synovial inflammation.

Discussion

CD4 effector T-cells are major drivers of abnormal immunity in RA by sustaining chronic synovitis and supporting autoantibody production. Deriving from infrequent naïve precursor cells, such pathogenic T-cells had to clonally expand and functionally differentiate. Here, we demonstrate that proliferative behavior and functional differentiation are critically determined by metabolic adaptations of the naïve precursor cells. Specifically, naïve CD4 T-cells from RA patients are metabolically reprogrammed, favoring NADPH production over ATP generation. Excess NADPH supplies the cell with excess reduced glutathione and depletes ROS; effectively exhausting the cell's ROS pool and weakening ROS-dependent signaling. Such reductive stress fastens the T-cells' cell cycle progression, as they skip the G2/M cell cycle checkpoint due to insufficient ATM activation. Constitutive ATM insufficiency in naïve RA T-cells and pharmacologic ATM insufficiency in healthy T-cells accelerates their conversion into effector memory T-cells. ROS loss and ATM insufficiency promotes T-cell maldifferentiation into IFN- γ and IL-17 effector cells. These abnormalities are reversible by replenishing the ROS pool with the naphthoquinone menadione, by disrupting synthesis of the ROS quencher glutathione or by blocking glucose shunting into the PPP. These pharmacologic interventions not only localize the pinnacle defect to excessive PPP utilization, they also provide a framework for entirely new anti-inflammatory strategies.

Effective T-cell responses require the massive expansion of low-frequent naïve T-cells into memory and effector T-cells. To fulfill the demands for energy, T-cells, like malignant cells, depend on oxidative glucose metabolism coupled with mitochondrial oxidative phosphorylation to efficiently generate ATP (19, 20). However, they need more than ATP to replicate single cells into thousands of copies. For biomass production, they require a carbon source and reducing power in the form of NADPH. With excess NADPH, RA T-cells are well prepared to replicate, as long as they have sufficient biosynthetic precursor molecules. In line with this metabolic state, RA T-cells proliferate well, in spite of telomeric features that identify them as pre-aged (11).

Current data pinpoint the source of excessive NADPH in RA T-cells; efficient shunting of glucose into the PPP (Fig.1). Gain of G6PD activity combined with loss of PFKFB3 activity generates a metabolic misbalance that promotes cellular proliferation. Biased glucose flux towards the PPP, however, generates a ROS shortage, resetting the signaling machinery that requires ROS as a second messenger. Human T-cells generate an early ROS peak within minutes of TCR crosslinking (21). Cellular ROS levels rise again 2-3 d after activation, with sustained elevation beyond day 6 (Fig.1). Traditionally, joint inflammation has been considered a consequence of excessive oxidative stress, but elegant work by Perl et al has demonstrated differential ROS production (22) and differential responsiveness to ROS scavenging therapy in lupus and RA (23). Also, positional cloning of genetic polymorphisms in arthritis models identified the NADPH oxidase NOX2 as a protective factor (24) with the oxidative burst suppressing autoimmune T-cells (25). Mechanistically, ROS-producing macrophages suppress T-cell immunity (26). NOX2 deficiency impacts arthritis susceptibility in aging-related murine arthritis (27) by shifting T-cell differentiation from Tregs towards Th1 cells. Also, N-acetyl cysteine treatment fosters Th17 cell generation by

targeting pyruvate dehydrogenase kinase1, which regulates the Th17/Treg balance (28). Further evidence for a detrimental role of ROS deficiency stems from chronic granulomatous disease, a hereditary disorder caused by NOX mutation. NOX-deficient patients have frequent infections while requiring immunosuppression for granulomatous and autoimmune disorders (29).

ROS act as obligate second messengers by regulating kinases and phosphatases. In human T-cells, ATM appears to be a critical target (Fig.3), with an oxidation-sensitive site mapped to Cys2991 (15). RA T-cells express low ATM levels, causing insufficient DNA damage repair (30). Deficient DNA repair may be related to bypassing the G2/M cell cycle checkpoint (Fig. 2). Individuals born with mutated ATM genes suffer from ataxia telangiectasia (AT) and, like RA patients, have premature immune aging (31). AT patients and ATM-deficient mice have dysfunctional mitochondria and heightened oxidative stress (32), contrasting with the findings presented here. However, ATM insufficiency in RA T-cells is acquired, thus not affecting the developing immune system, but rather the functionality of mature, peripheral T-cells. Mice lacking germline ATM have abnormal thymic T-cell selection and repertoire formation (33, 34). Interestingly, ATM-insufficient mice demonstrate persistent immune activation in a model of DNA-damage-associated colitis (35), and effectively clear LCMV infection (36). ATM^{low} RA T-cells are highly susceptible to apoptosis (17, 30), stressing ATM's role in T-cell maintenance.

Metabolic control of T-cells impacts their development and their effector functions, with a particular regulatory role for the energy sensor AMP-activated protein kinase (AMPK) and the downstream signaling knot mTOR (6, 37, 38). Checkpoint function has been assigned to mTOR, which appears to function through metabolic selection (39). Similarly, AMPK regulates Th1 and Th17 development and primary anti-microbial T-cell responses (38). Functional intactness of these signaling networks in naïve RA T-cells needs to be examined to search for further implications of defective oxidant signaling beyond ATM. Also, the mechanistic causes underlying G6PD induction and PFKFB3 loss remain to be clarified. Correlation of the PFKFB3/G6PD ratio with RA inflammatory activity (Fig.S1) suggests a formidable role of the metabolic defect in chronic inflammation. Vice versa, ATP and biomass generation are known to be responsive to environmental conditions, such as oxygen availability. Naïve T-cells live in secondary lymphoid organs, not in peripheral inflammatory lesions, raising the intriguing question how tissue inflammation could regulate metabolic reprogramming in distant T-cells.

An important notion of the current study is the reversibility of the metabolic wiring (Fig.7), effectively preventing hyperproliferation and maldifferentiation in vitro and in vivo. ROS induction via menadione restored ATM signaling and suppressed IFN- γ induction, shifting T-cell differentiation towards an anti-inflammatory phenotype. Menadione, known as Vitamin K3, is used as a nutritional supplement (40). Large doses can cause hemolytic anemia in G6PD-deficient individuals, emphasizing the mechanistic link between PPP utilization and redox balance. Interfering with production of the ROS generator BSO proved effective in inhibiting synovial inflammation. Pharmacologic and genetic G6PD inhibition confirmed that the pinnacle defect lies in the excessive induction of this rate-limiting enzyme for the PPP. 6-AN treatment was even more effective in downregulating pro-

inflammatory cells, opening the door to targeting autoimmune T-cells by metabolic interference. Directing such intervention to naïve T-cells promises a new concept of preventing autoimmunity instead of blocking terminal inflammatory pathways.

Materials and Methods

Study design

This study explored functional implications of metabolic and redox dysregulation in RA T-cells, and identified metabolic interventions for novel anti-inflammatory therapies. Table S1 presents demographic characteristics of 181 patients and 164 healthy control subjects. All patients fulfilled the diagnostic criteria for RA and were positive for rheumatoid factor and/or anti-CCP antibodies. Individuals with cancer, uncontrolled medical disease or any other inflammatory syndrome were excluded. Healthy individuals did not have a personal or family history of autoimmune disease. The Institutional Review Board approved the study and written informed consent was obtained from all participants. Numbers of independent experiments or individual patients and control donors are defined in each figure legend. All statistical analyses were verified by Dr. Lu Tian, Department of Health Research and Policy, Stanford University.

Cell preparation, cell culture and cell transfers

CD4 naïve ($CD4^+CD45RO^-$) T-cells were purified by negative selection with anti-human CD45RO microbeads followed by positive selection with anti-human CD4 microbeads (Miltenyi Biotec, Auburn, CA). Subset purity monitored by FACS routinely exceeded 95%. $CD4^+CD45RO^-$ T-cells (1.0×10^5 /well) were stimulated with CD3/CD28-coated beads (Life Technologies AS, Oslo, Norway, ratio 1:1) and cultured for 7 d in previously described cytokine cocktails to induce T-cell lineage commitment (41). Intracellular IFN- γ , IL-4, IL-17 and FoxP3 were measured by flow cytometry after incubation with PMA/ionomycin in the presence of Brefeldin A for 6h as described (42). For cell proliferation assays, naïve CD4 T-cells were CFSE labeled and stimulated with anti-CD3/CD28 beads with or without KU-55933, 6-AN or Tempol (50 μ M). T-cell subpopulations were analyzed by surface staining for CD45RA and CD62L. For oxidative stress experiments, cells were stimulated (72 h), pretreated with the ATM inhibitor KU-55933 (30min) and incubated with increasing doses of H_2O_2 or menadione (3 μ M, 30min).

Ten million $CD45RO^-$ PBMCs were CFSE labeled and adoptively transferred into irradiated (10.0 Gy) NSG mice by intravenous injection. Spleens were collected on days 5, 7, 9 and 14 after transfer and analyzed by flow cytometry.

qPCR

RNA extraction and RT-PCR were performed as described (4). Primer sequences are listed in Table S2. Gene expression was normalized to 18S rRNA.

Plasmid constructs and transfection

Constructs encoding shATM were purchased from Addgene (Addgene plasmid 14581; Addgene, Cambridge, MA). An EcoRI/KpnI shATM expression cassette was subcloned into

the pSuper-gfp/neo vector for reconstructing pSuper-shATM-gfp plasmids (shATM plasmids). Naïve CD4 T-cells were transfected using AMAXA technology (4). Transfection efficiencies were monitored by measuring GFP-positive cells using flow cytometry. G6PD-targeting siRNAs were purchased from GE Healthcare (#L-008181-02-0005) and Thermo Fisher Scientific (#4390824).

Western blotting

Cellular proteins were extracted using kits from Active Motif (Carlsbad, CA). Expression levels were examined following standard Western blotting protocols as described (4). Primary antibodies used: anti-G6PD (#12263S, Cell signaling Technology), anti-ATM (#NB100-306, GeneTex), anti-phospho-ATM (#GTX70103, Novus Biologicals). HRP-conjugated anti-GAPDH (#3683, Cell signaling technology) or anti- β -actin (#5125, Cell signaling technology) served as internal controls.

NADPH measurements

CD4+CD45RO⁺ T-cells were stimulated for 72h, washed with cold PBS and NADPH levels were measured with NADPH assay kits (Abnova, Walnut, CA).

Human synovial tissue-NSG chimeras

NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ (NSG) mice (Jackson Laboratory, Bar Harbor, ME) were kept in pathogen-free facilities and used at the age of 10-12 wk as previously described (43, 44). Pieces of human synovial tissue free of inflammatory infiltrates were placed into a subcutaneous pocket. Following engraftment, mice were injected with 10 million CD45RO⁺ PBMCs from untreated RA patients or patients with highly active disease. Chimeric mice carrying the same synovial tissue were randomly assigned to treatment arms: (A) vehicle control (PBS or DMSO); (B) Treatment with 1 mg/kg/day KU-55933; or 1000 mg/kg/day Buthionine Sulfoximine (BSO); or 10 mg/kg/day Menadione. All treatments were delivered by daily ip injection over a period of 9 days. At completion, harvested synovial tissues were OCT embedded (Tissue-Tek; Sakura Finetek, Torrance, CA) for histology or shock frozen for RNA extraction. All experiments were carried out in accordance with guidelines required by the Institutional Animal Care and Use Committee.

Immunohistochemistry staining

Hematoxylin-stained sections (5 μ m) of explanted synovial tissues were examined for inflammatory infiltrates and synovial T-cells were identified by immunohistochemical staining for human CD3 as described (43). Sections were analyzed by using an Olympus BX41 microscope, and CellSense software.

Statistical analysis

All data are presented as mean $\pm$ SEM. Data were analyzed using SPSS 10.0 software. Statistical significance was assessed by ANOVA and unpaired Student's t-test as appropriate. A P-value of <0.05 was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding: Supported by the National Institutes of Health (AR042527, AI044142, AI108906, HL058000, AI108891, AG045779).

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Figure 1. Glucose shunting towards the pentose phosphate pathway results in accumulation of NADPH and reduced glutathione and loss of ROS

CD4⁺CD45RO⁺ T-cells from patients with RA, patients with PsA and age-matched controls were stimulated for 72 hours. (A) Expression of G6PD and PFKFB3 in 31 RA patients, 14 PsA patients and 32 controls quantified by RT-PCR. (B) G6PD immunoblots from 4 control and 4 RA samples. Relative band densities from 8 RA-control pairs. (C) Correlation of G6PD and PFKFB3 mRNA expression in individual patients and controls. (D) Correlation of the disease activity DAS28 score with the ratio of G6PD and PFKFB3 transcripts. (E) G6PD enzyme activities quantified in 13 RA and 13 control samples. (F) NADPH levels measured in T-cell extracts of 11 RA patients, 8 PsA patients and 14 controls. (G) Representative dot blots of monochlorobimane (mBCI) staining in control and RA T-cells. (H) Intracellular glutathione levels quantified by mBCI fluorescence. Data from 7 RA patient, 7 PsA patients and 9 controls. (I) Representative fluorescent imaging of mBCI staining in normal and RA T-cells. (J) Kinetics of intracellular ROS over 6 days following stimulation measured with the fluorogenic probe CellROX in 11 RA patients and 7 controls. (K) Intracellular ROS levels measured in T-cell extracts of 15 RA patients, 8 PsA patients and 14 controls. All data are mean $\pm$ SEM.

Figure 2. ROS-depleted T-cells hyperproliferate and bypass the G2/M cell cycle checkpoint
 (A) Proliferation of CD4⁺CD45RO[−] T-cells with and without the G6PD inhibitor 6-AN measured by CFSE dilution 72h post stimulation. Representative histogram (left) and division indices from 7 experiments. (B) Intracellular ROS in RA T-cells cultured with and without 6-AN. Representative histogram (left) and MFI from 5 experiments (right). (C) T-cells from 4 RA patients were transfected with control siRNA or two different G6PD-targeting siRNAs (si-1, si-2). NADPH levels, GSH, intracellular ROS and division indices were measured 72h later. (D) MFI of intracellular IL-2 in 4 patients and 4 controls. (E) Naïve-to-memory conversion of CD4 T-cells after TCR stimulation monitored by flow cytometry of CD45RA. Data from 4 patient-control pairs. (F) CFSE-labeled CD45RO[−] PBMC from RA patients and controls were injected i.v. into NSG mice. Left: CFSE dilution in CD4 T-cells as a measure of in vivo proliferative activity. Right: Division indices from 12 patients and 15 controls. (G) FACS analysis of NSG splenocytes to identify human CD4 and CD8 T-cells converted to the CD4⁺CD95⁺ and CD8⁺CD95⁺ memory phenotype. Results from 12 experiments. (H, I) T-cells were cultured with and without the ROS scavenger Tempol. Generational assignment was made by CFSE dilution. (H) Representative patient-control pairs. (I) Percentages of T-cells that underwent >5 doublings from 3 patient-control pairs. (J) CD4⁺CD45RO[−] T-cells cultured with and without the ROS scavenger Tempol. Assignment to the G1, S and G2/M phase of the cell cycle by propidium iodide staining.

Percentages in each cell cycle phase for 6 patients and 12 controls. (K) Representative scatter blots of cells in the G2/M phase identified with anti-phospho-Histone H3 Ab staining. Percentages of phospho-histone H3+ cells in 7 patients and 7 controls. All results are mean $\pm$ SEM.

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Figure 3. Insufficient activation of the ROS-sensitive cell cycle regulator ATM results in T-cell hyperproliferation

(A) ATM gene expression in activated CD4⁺CD45RO⁺ T-cells measured by RT-PCR in 7 controls and 6 patients. (B) Quantification of ATM monomers and dimers by Western blotting. Post stimulation dynamics of protein expression for a representative control and RA patient. (C) Relative band intensities for total ATM quantified at 72 h. Results from 8 patient-control pairs. (D) Kinetics of ATM phosphorylation on days 0, 1, 3 and 6 after T-cell stimulation. Representative immunoblots (left) and results from 4 controls and 4 patients (right). (E) Healthy stimulated T-cells were treated with H₂O₂ on day 3. Cell extracts were immunoblotted with anti-ATM and pATM(Ser1981). Results from 1 of 4 experiments are shown. (F) Cells were cultured with the ATM inhibitor KU-55933 and proliferation assessed by CFSE dilution. Frequencies of proliferating T-cells in 5 experiments. (G) Effect of the ATM inhibitor KU-55933 on naïve-to-memory conversion. KU-55933-treated T-cells were phenotyped as CD45RA⁺CD62L⁺ central memory (CM), CD45RA⁺CD62L⁺ effector memory (EM) and CD45RA⁺CD62L⁺ end-differentiated effector T-cells (TEM) by flow cytometry. Results from 6 experiments. (H) Increasing cellular ROS levels restores ATM activation. T-cells were treated with menadione (3 μ M) for 72 h. ROS were measured with the fluorogenic probe CellRox (left). ATM and pATM were quantified by Western blotting; 1 of 4 experiments is shown (right). All results are mean $\pm$ SEM.

Figure 4. ROS scavenging mimics the maldifferentiation of RA T-cells

(A, B) CD4⁺ CD45RO⁻ T-cells were cultured under Th1- and Th17-skewing conditions with or without the ROS scavenger Tempol, restimulated with PMA/ionomycin and stained for intracellular cytokines. (A) Representative dot plots. (B) Percentages of IFN- γ - (left) and IL-17-producing (right) cells from 4 experiments. (C) Healthy PBMCs depleted of CD45RO⁺ cells were adoptively transferred into NSG mice. On day 7, splenocytes were analyzed for human CD45⁺CD4⁺IFN- γ ⁺ cells by flow cytometry. Representative dot plots from 1 control-patient pair (left) and results from 4 independent experiments (right).

Figure 5. Arthritogenic effector functions in RA T-cells

(A) CD4⁺ CD45RO⁺ T-cells were stimulated for 6 hours. IFN- γ , IL-4, IL-17 and FoxP3 were detected by intracellular staining in 6 patients and 6 controls. All results are mean $\pm$ SEM. (B) NSG mice were engrafted with human synovium and CD45RO-depleted PBMC from healthy controls or RA patients were adoptively transferred into the chimeras. Synovial inflammation was assessed by RT-PCR analysis of 17 inflammation-related genes. Results from 8-16 tissue grafts are shown as a heat map. (C) Densities of synovial T-cell infiltrates analyzed by immunostaining for human CD3. (D) T-cells migrated into synovial tissue were quantified by RT-PCR and tissue-infiltrating T-cells were enumerated by anti-CD3 staining per high power field (HPF) (E) T-cell mobility was measured in Transwell migration assays. Mean $\pm$ SEM from 9 patient-control pairs.

Figure 6. The cell cycle kinase ATM regulates the lineage commitment and the arthritogenic potential of T-cells

(A) CD4⁺CD45RO⁻ T-cells were cultured with the ATM inhibitor KU-55933. Cytokine production patterns after non-polarizing conditions from 6 experiments. (B) Cytokine production patterns after culture under Th1- and Th2-skewing conditions with and without KU-55933. (C) T-cells transfected with control or shATM plasmids were cultured under Th0, Th1 and Th2-polarizing conditions. Intracellular cytokine stains from a representative experiment. (D) Frequencies of cytokine-producing cells from 5 experiments with ATM-silenced cells. (E) NSG mice were reconstituted with CD45RO-depleted PBMC and injected with KU-55933 (0.5 mg/kg i.p.) or vehicle daily. Cytokine production in splenocytes was measured by intracellular cytokine staining in human cells. Left: Representative dot plots. Right: Percentages of IFN- γ , IL-4, IL-17 and FoxP3⁺ cells from 4 independent experiments. (F) Flow cytometric analysis of lineage-defining transcription factors in T-cells cultured under Th1-, Th2-, Th17- and Treg-skewing conditions with or without KU-55933. Mean $\pm$ SEM of MFI from 3 experiments. (G) CD45RO⁺ depleted PBMC from healthy individuals or RA patients were adoptively transferred into synovium-engrafted NSG mice. Mice were treated with the ATM inhibitor KU-55933 for 9 days. Gene expression was quantified in explanted synovial tissues by RT-PCR. Mean $\pm$ SEM from 8 tissues. (H)

Immunohistochemistry of synovial tissue sections. The osteoclastogenic ligand RANKL is visualized by brown staining.

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Figure 7. Replenishing intracellular ROS in RA T-cells corrects ATM insufficiency, T-cell maldifferentiation and arthritogenic effector functions

CD4+CD45RO- T-cells from RA patients were stimulated as above. (A) On day 3, T-cells were treated with menadione or menadione plus KU-55933. Cell extracts were immunoblotted with anti-ATM, pATM, Chk2 and pChk2. (B) Amounts of dATM, mATM, p-dATM, p-mATM, Chk2 and pChk2 were quantified in 5 experiments. (C) Effect of menadione and 6-AN treatment on IFN- γ production under Th1-polarizing conditions. Representative dot plots (left) and results from 5 experiments (right). (D) CD45RO-depleted PBMC from RA patients were adoptively transferred into NSG mice engrafted with human synovium. To increase intracellular ROS levels, mice were treated with daily i.p. injections of menadione or BSO for 9 days. T-cell polarization and intensity of synovitis were analyzed as in Figure 5 and 6. Mean $\pm$ SEM from 8-13 synovial tissues. (E) Immunohistochemically analysis of synovial tissues for human CD3 (pink) and RANKL (brown). Double positive cells are marked by a white arrow head, CD3+RANKL- T-cells by a black star. (F) Effects of menadione and BSO on T-cell mobility measured in Transwell migration assays. Mean $\pm$ SEM from 9 experiments.