1. Aims

CD4 T cells are generally classified according to the cytokines they produce. As a consequence, different ‘T helper (Th)’ subsets were characterized with essential roles in autoimmune conditions such as rheumatoid arthritis (RA), inflammatory bowel diseases (IBD) and multiple sclerosis (MS). During an immune response, the local cytokine milieu triggers the expression of key transcription factors in naïve T cells which subsequently develop into a particular T helper cell class, i.e. they ‘commit’ to a certain Th profile. Three important Th subsets are Th1 (identified by predominant IFN-γ production), Th2 (IL-4, IL-10, IL-13) and Th17 (IL-17). These so-called ‘effector’ Th subsets are known to promote immune responses whereas regulatory T cells (Treg), another subset of CD4 T cells, counteract their function. The differentiation of these Th cell subsets is controlled by distinct transcription factors that were linked to Th1 (T-Bet), Th2 (GATA-3), Th17 (RORγt) or Treg (Foxp3).

In recent years, the long-held assumption that rheumatoid arthritis (RA) and its principal mouse model, collagen-induced arthritis (CIA), are T helper 1 type (Th1)-driven diseases has been challenged. Investigators were initially puzzled by the fact that IFN-γ, the key cytokine associated with Th1 immune responses, is not required for disease onset in the CIA model (1). The recent emergence of Th17 cells as a third CD4 T helper lineage with a pivotal pathogenic role in arthritis, we aim to directly influence structural joint damage.

The ultimate goal of treatment in RA is to halt the inflammatory process and the ensuing structural damage in the joints. The sole cell type responsible for bone resorption is the osteoclast, and as a consequence there is a profound interest in blocking its differentiation and activation in a therapeutic setting. There is now convincing evidence that Th17 cells constitute the principal immune subset governing osteoclastogenesis, whereas Th1, Th2 and Treg phenotypes appear to counteract this pathway (6, 7). Collectively, these findings support the notion that osteoclastic activity and bone destruction are critically linked to local autoimmunity giving rise to the new field of ‘osteoimmunology’.

CD1d-restricted invariant natural killer T cells (iNKT cells) are thought to bridge innate and adaptive immune responses at least in part by governing the development of CD4 T cells into preferential T helper subsets. Therefore, these cells have been proposed as suitable therapeutic targets in the prevention and treatment of autoimmune diseases. Our hypothesis was that targeted iNKT cell modulation has the potential to specifically skew the Th1/Th17 balance in autoimmune arthritis with direct implications on osteoclastogenesis. Thus, by exploiting the immunomodulatory properties of iNKT cells on CD4 T cell lineage differentiation in the context of arthritis, we aim to directly influence structural joint damage.

iNKT cells represent an attractive therapeutic target in arthritic disease, and their potent immunomodulatory properties have been successfully targeted using various glycolipid ligands (8). Whereas the primary focus until recently was on affecting the balance between Th1 and Th2-driven immunity, iNKT cells were also found to interfere with Th17 lineage development (9). Although iNKT cells themselves can be significant sources of IL-17 especially early after stimulation via their T cell receptor, we anticipated that downstream iNKT cell-mediated induction of Th17 cells constitutes the major source of this proinflammatory cytokine in vivo. Collectively, these data indicate that iNKT cells may have the potential to interfere both with early Th17 commitment and late conversion towards a Th1 phenotype. We set out to investigate the possibility of iNKT cell-mediated intervention in association with osteoclastogenesis in CIA as a potential therapeutic strategy for RA.
Our principal aims were to **improve our understanding of how different Th cell subsets influence osteoclastogenesis**. The aims, as outlined in the original application, are threefold:

**Aim 1**: Study the *in vitro* effect of Th17—Th1 balance on osteoclastogenesis  
**Aim 2**: Determine the Th1/Th17 ratio’s during development and treatment of arthritis  
**Aim 3**: Target Th balances via therapeutic NKT cell activation

2. **Methodology and results**

Aim 1: Study the *in vitro* effect of Th17—Th1 balance on osteoclastogenesis

*a) Introduction of osteoclastogenesis assays in the Elewaut lab*

We initially wanted to establish a solid *in vitro* system for the generation of osteoclasts for bone marrow precursors. Hereeto, bone marrow-derived cells were isolated from the femur and tibia and cultured in the presence of M-CSF and RANKL for 6 days. Several approaches were used to verify that our system yields a genuine osteoclast population.

**Figure 1** – Validation of the *in vitro* RANKL-M-CSF osteoclast differentiation system by quantitative real-time RT-PCR. Note that most osteoclast-specific genes become detectable on day 3 and are upregulated on day 6. Normalization was performed using the GAPDH, TBP and Rn18S housekeeping genes.

When samples were collected during the progression of osteoclastogenesis, it became evident that some of the signature osteoclast markers were upregulated around day 6 post culturing (Figure 1). Next, an unbiased real-time RT-PCR-based array for genes implicated in the TNF-signaling pathway was run on cell samples obtained before and after the culturing protocol (Figure 2). Here, certain TNF-members specific to osteoclasts, including RANK, APRIL and TWEAK were found to be differentially expressed.

**Figure 2** - Quantitative real-time RT-PCR-based profiling (PCR RT2 Profiler Array, SABiosciences) of TNF family members’ transcriptional activity in osteoclast cultures. This unbiased approach confirmed the osteoclast-specific differential regulation of markers such as RANK, TWEAK and APRIL, thus validating the quality of our culture system. Some genes such as LTb and LTbR were found to be downregulated and may in the future serve in spin-off projects stemming from the current BELSPO grant. Lines represent 4-fold difference barriers, red dots are upregulated genes, green dots downregulated ones.

Finally, the functionality of the generated osteoclast was tested by pit formation assay on calcium-coated plates, in parallel with TRAP staining (Figure 3). Cathepsin K staining was also performed (not shown). In conclusion, we can be confident that the cells generated using the RANKL-M-CSF system in our lab have all the features of genuine osteoclasts.

**Figure 3** – Pit formation (OsteoAssay®) as white spots on von Kossa-stained calcium surfaces (left). TRAP staining (Sigma TRAP staining kit) of a typical multinucleated cell in the osteoclast cultures (right).
b) Stability of Th17 cells in vitro

We questioned whether the cell types used by Sako et al. to demonstrate osteoclastogenicity of the Th17 subset were at all stable (6). We therefore introduced OT-II mice into the lab and isolated Th0 cells from the spleen, followed by defined Th1 and Th17 skewing protocols as indicated in Figure 4.

Figure 4 – Th1 and Th17 differentiation procedures. FlowCellect Mouse Th1 and Th17 kits were used (Millipore) were used for differentiation purposes. Intracellular cytokine staining was done with a FlowCellect Th1/Th17 ICCS kit.

These cultures were subsequently subjected to detailed mRNA (qRT-PCR) and protein (ELISA) analysis to confirm the success of the differentiation procedure (Figure 5).

Figure 5 – Detailed verification of Th1/Th17 skewing protocols. Th1-specific genes T-bet and IFN-γ were restricted to the Th1 cells, while IL-17 production is solely in the Th17 subset. In-house IFN-γ and IL-17 qPCR and ELISA assays were performed.

Next, it was asked whether these cells maintain their functional profile in culture over time (Figure 6). Intracellular cytokine staining (ICCS) data after culturing indicated that IL-17-producing cells over time become very rare and, moreover, that the Th1 lineage is the more stable subset.

Figure 6 – Instability of the Th1/17 lineage in vitro. ICCS (bottom) and ELISA (left) data showing IFN-γ and IL-17 production when cultured for 3 days. Data at transfer to the wells of the osteoclast experiment (see later), show degree of impurity of the Th17 set. Three days after culturing the IL-17 producing cells are barely detectable.

In particular when both Th types are co-cultured, secreted IL-17 levels are considerably lowered. These data indicate that, over time, the Th17 cells in our culturing system are expected to lose their IL-17 production while IFN-γ production maintains intact in the Th1 set. Whether true Th17-to-Th1 conversion takes place remains to be confirmed. The key lesson from this experiment is that the Th17 lineage as presented here represents an unstable mixture of IFN-γ and IL-17 producers that can be used to assess whether protective, anti-osteoclastogenic IFN-γ effects can overcome the driver capacity of IL-17.
c) Stability of Th1/Th17 cells in contact with osteoclast cultures and effect on osteoclastogenesis

An important knowledge gap exists on whether and how osteoclasts themselves regulate Th differentiation. Our data suggest that osteoclasts may accelerate the conversion of Th17 cells to Th1 cells as a negative feedback mechanism (Figure 7). Indeed, ICCS data indicate that IL-17 producers can only barely be detected after 3 days of culturing in the presence of osteoclast (precursors). ELISA data also show that the degree of IFN-γ accumulation eventually surpasses IL-17 levels in the Th17 subset. A new angle of investigation therefore could focus on the highly relevant search for osteoclast-derived factors that may provide protective feedback to T cells in the form of Th17-to-Th1 conversion.

Figure 7 – ICCS and ELISA data on Th subsets after 3 day culturing in the presence of developing osteoclasts.

The effect of these subsets and mixtures thereof was tested in the osteoclast culture system (Figure 8). Of note, not only Th1 cells but also the Th17 cells completely prevented full osteoclast maturation. From this series of experiments we can conclude that due to Th17>Th1 conversion and impurities inherent to the differentiation protocol, the osteoclastogenic effect of initial IL-17 production is potently counteracted by IFN-γ or other Th1-derived factors.

Figure 8 – Setup of a Th/osteoclast coculture system as in reference (6). Left inset shows representative TRAP+, multinucleated phenotype in control wells, right inset shows incompletely differentiated mononuclear TRAP+ cell of which some could be found in the Th-enriched cultures.

Thus, we conclude that RANKL-independent inhibition of osteoclastogenesis is exerted by both Th1 and Th17 differentiated cells and that protective Th1 immunity appears to prevail over Th17.

Finally, we tested whether analogous effects are achieved by the addition of the purified cytokines IFN-γ and/or IL-17. In order to achieve a better osteoclast differentiation rate, the RAW 264.7 macrophage cell line was purchased, which is a suitable osteoclast precursor that can be driven toward osteoclastogenesis by the addition of RANKL. Figures 9-10 show that the RAW cell system works well and consistently yields more osteoclasts in the control well (as compared to the BM differentiation system), while many show higher degrees of multinucleation. Addition of IFN-γ inhibited osteoclastogenesis as did IL-17, an effect that was noted previously (10). Importantly, the effect of IFN-γ clearly dominated over the effect of IL-17.

Figure 9 – Verification of osteoclast formation in the RAW264.7 system by confocal microscopy. (LEFT) RANK+ (green, Alexa 488) multinucleated (red, TOPRO-3) osteoclasts are clearly visible on day 5 after culturing. (RIGHT) these cells express Cathepsin K (pseudocolored green here, red is nucleus). For staining, the Magic Red Cathepsin K detection kit was used (Immuochemistry Technologies).
Aim 2: Determine the Th1/Th17 ratio’s during development and treatment of arthritis

We have determined the levels of IFN-γ and IL-17 over time in the serum of mice with collagen-induced arthritis. This type of analysis was not performed before in a similar longitudinal fashion and we aimed to correlate these cytokines with markers of bone resorption in order to investigate whether a correlation exists and whether these could be used as potential biomarkers of bone turnover. Thus, ELISA for IL-17, IFN-γ, osteoprotegerin (OPG) and RANKL was performed (Figure 9).

From these experiments it can be concluded that the upregulation of IFN-γ and IL-17 occurs simultaneously and that Th1 and Th17 subsets may thus interact very closely. Importantly, both cytokines are only upregulated during the late phase of CIA progression and roughly correspond with the upregulation of protective OPG expression.
measure of osteoclast activity. Unfortunately, we were unsuccessful in detecting IFN-γ or IL-17 on paraffin sections, even after antigen retrieval by trypsin digestion as described in the literature (12).

**Aim 3: Target Th balances via therapeutic NKT cell activation**

Based on the previous data one could hypothesize that augmentation of the Th1 arm of immunity in vivo could curb inflammatory processes such as CIA. The Elewaut lab has designed a number of glycoside analogues of the prototypical NKT cell ligand alpha-galactosylceramide (aGC) with unique properties in terms of Th1 skewing(13). We therefore reasoned that therapeutic intervention with a Th1 skewing analogue could ameliorate CIA and selected a weak (BnNH-GSL-1’) and potent (NU) Th1 polarizing agent for treatment (Figure 10). This is a new approach because previous experiments in the literature always assumed that deviation from Th1 immunity was the favorable outcome. Unfortunately, no significant amelioration could be observed as compared to DMSO (vehicle control). If anything, the strong Th1 polarizer appears to aggravate CIA similar to aGC. The high clinical scores indicate functional deformation and thus bone and cartilage destruction, suggesting limited effect on osteoclast activity.

Since these ligands were unsuccessful at curbing autoimmunity in the CIA model, we chose to test our hypothesis in an alternative disease model, i.e. the NOD mouse model for type 1 diabetes. In this model, Th17 cells are also important drivers (14) and thus in vivo conversion toward Th1 or, perhaps indirectly, to Th2 via NKT cell ligation could theoretically result in altered disease penetration. NOD mice were administered xylo-α-GalCer (13) (5 microgram) at the time of diabetes development (blood glucose measurement > 300 mg/dl). In line with the data in the CIA model, a single shot of glycolipid ligand was unable to revert or otherwise alter the course of autoimmunity in this model.
References


3. Dissemination and valorisation

Publications since the start of the BELSPO mandate:


3. Coppieters, K. T. & von Herrath, M. G. Motifs for a deadly e


+Three invited book chapters:

‘Diabetes’, edited by Dr. Jeffrey A. Bluestone - Cold Spring Harbor Press
‘Virus Infections in type 1 diabetes’ by Ken T. Coppieters, Tobias Boettler and Matthias von Herrath (in press)


Conferences and seminars:

- Speaker at the ‘Réunion francophone de recherche en rhumatologie’, Annecy, France, June 1-2 2012 ‘Imaging of immune responses during autoimmune disease’
- Invited speaker at the University Medical Center Groningen, The Netherlands, July 3rd 2012 “Practical Course in Cellular Imaging: Intravital Imaging in Type 1 Diabetes”
- Invited speaker at the International Society for Pediatric and Adolescent Diabetes (ISPAD) meeting, being held from October 10-13, 2012, in Istanbul, Turkey “How anti-viral CTLs seek and destroy beta cells in type 1 diabetes”
- Invited speaker at the American Diabetes Association’s 72nd Scientific Sessions June 8-June 12 2012 in Philadelphia, PA, U.S.A. ‘Immunological Analysis of Type 1 Diabetic Islets’
- Invited seminar speaker at the Diabetes Research Center, Vrije Universiteit Brussel, February 17th 2012. ‘Specificity and dynamics of CD8 T cells in type 1 diabetes’
- Invited speaker at the 4th Annual meeting of the network for Pancreatic Organ Donors with Diabetes (nPOD), Miami, FL, U.S.A. to be held January 15-17, 2012 ‘In-situ Detection of Islet-Antigen specific CD8 T Cells in Insulitic Lesions of New-onset and Long-term Type 1 Diabetes’
- Annual Meeting of the International Society for Pediatric and Adolescent Diabetes (ISPAD), Miami Beach, FL, USA from October 19th – 22nd, 2011 ‘Persistence of Autoimmunity after Clinical Diagnosis of Type 1 Diabetes: Demonstration of Islet-autoreactive CD8 T Cells in Insulitic Lesions from Recent Onset and Long-term Type Diabetes Patients’
- Invited speaker and Faculty Member, Levine Symposium, Pasadena, CA (2011) ‘Live imaging of CD8 T cell-mediated beta cell destruction’
4. Conclusions and future perspectives

The conclusions from the partial fulfillment of the grant period can be summarized as follows, with respect to the three individual subaims:

1. Th17 cells are unstable functionally when cocultured with osteoclasts and therefore their net effect on the latter is difficult to establish. Rapidly after coculturing, the Th17 cells convert to Th1 cells, which our studies confirm are highly inhibitory in the context of osteoclastogenesis. Our data suggest that osteoclasts themselves can regulate conversion of Th17 to Th1 cells as a negative feedback mechanism. Importantly, the protective (inhibitory) role of Th1 immunity on osteoclasts dominates over the driver function of Th17. These effects are also seen to occur when purified cytokines are used. Thus, in vitro data suggest that therapeutic conversion of Th17 immunity to Th1 may offer protection against bone erosion in inflammatory disease by interfering with osteoclastogenesis.

2. The situation in vivo was monitored longitudinally in collagen-induced arthritis where it was found that serum levels of IFN-γ and IL-17 follow the same kinetics in terms of upregulation during disease progression. The kinetics also closely correspond to the onset of a protective osteoprotegrin feedback response. Whether IFN-γ/IL-17 balances are true biomarkers for bone erosion was not determined, although it is clear that the net result in terms of osteoclastogenesis and bone erosion will depend on the combined action of both cytokines as they are simultaneously present.

3. Targeting Th17/Th1 balances via therapeutic NKT cell activation appears challenging. In the collagen-induced diabetes model, elaborate in vivo testing showed no significant deviation of the arthritogenic response. Likewise, in the NOD mouse model for type 1 diabetes, no reversal was observed following administration of synthesis NKT cell ligands. It cannot be excluded that higher doses or alternative regimens could be successful or that different ligands are required to specifically alter ongoing T cell immunity. A future avenue worth investigating is whether osteoclasts play an active role in Th cell skewing as part of a protective feedback mechanism.

5. Career prospects after the Belspo mandate

Since I have determined that my principal interest is in translational medicine, I will join an industry-funded institute per 1 September 2012, focused on the development of tolerization therapies for autoimmune diseases. The Belspo mandate has enabled me to compete for various national and international grants during the past year. It could be anticipated that my academic career at the host institution would have been secured had I not chosen an alternative career path:

- Budgeted for 10% ZAP mandate in 2013
- FWO postdoctoral fellowship awarded starting October 2012
- Co-promoter on FWO project grant, compromoter project grant Stichting tegen Kanker
- Internally selected for Odysseus grant submission, result in November 2013
- Named Marie Curie Fellow, 4-year grant of 25,000 euros per annum
- Awarded $30.000 by the Juvenile Diabetes Research Foundation for diabetes research

6. Contribution to the host institute and Belgian science community

In addition to the financial contributions by grant writing as outlined above, I have represented our university at multiple international conferences and workshops. I had planned an outreach event for school children in September, was thesis promoter for Ms. Oonagh Paerewijck, guest lecturer 1st Master Biomedical Sciences, member of the reading commission of Yana Vandenbossche and wrote commentaries in leading scientific journals. I served as a reviewer for multiple manuscripts from leading journals such as Diabetes and grant agencies such as The Wellcome Trust, JDRF and NIH.
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In recent years, the long-held assumption that rheumatoid arthritis (RA) and its principal mouse model, collagen-induced arthritis (CIA), are T helper 1 type (Th1)-driven diseases has been challenged. The recent emergence of Th17 cells as a third CD4 T helper lineage with a pivotal pathogenic role in CIA and potentially RA provided a rationale for this apparent conundrum. It was long thought that once T cells differentiated into either of these functional subsets (e.g. Th17 or Th1), this was the end stage of development and conversion into other functional subsets was no longer achievable. However, Th17-committed cells were shown to exhibit the persistent potential to deviate towards an IFN-γ producing Th1 phenotype. The ultimate goal of treatment in RA is to halt the inflammatory process and the ensuing structural damage in the joints. The sole cell type responsible for bone resorption is the osteoclast, and as a consequence there is a profound interest in blocking its differentiation and activation in a therapeutic setting.

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Key words: osteoclasts, autoimmune disease, T cell immunity, arthritis, cytokines