An early inflammatory response determines the onset of chondrogenesis

INTRODUCTION
Bone fracture healing by endochondral ossification critically depends on a haematoma-induced inflammatory response (1). The function of this inflammatory response is mainly studied in the context of the osteogenic phase of endochondral ossification, leaving a potential involvement of this inflammatory signaling in the chondrogenic phase poorly studied. We hypothesized that the early onset of chondrogenesis from mesenchymal progenitors is determined by inflammatory signaling. We therefore studied the involvement of inflammatory signaling during the early phases of chondrogenesis and its developmental contribution to late phase chondrogenic differentiation.

METHODS
The chondroprogenitor cell line ATDC5 was differentiated in the presence of NF-κB activating agents LPS (0.1 ng/ml) or TNFα (10 ng/ml) or with the NF-κB inhibitors TLCK (100 μM) or Parthenolide (10 μM). In addition a p65 knockdown was achieved by siRNA transfection and confirmed by immunoblotting. NF-κB activation was determined by analyzing nuclear translocation of NF-κB and expression of NF-κB targets (COX-2, IL-6, iNOS, TNFα) by RT-qPCR and immunoblotting. Chondrogenesis specific markers (Sox9, Col2A1, Col10A1) were also analyzed by RT-qPCR and immunoblotting. Similar experiments were performed with human bone marrow derived mesenchymal stem cells (hBMSCs). hBMSCs were obtained from iliac crest bone marrow aspirate from 5 young, genetically healthy individuals. Chondrogenesis of hBMSCs was performed in monolayer culture.

RESULTS
Involvement of inflammatory signaling during the very early onset of chondrogenesis was detected by a temporally upregulated expression of inflammatory mediators COX-2, iNOS, IL-6 and TNFα during the first 2-8 hours in differentiation. This inflammatory response is cell intrinsic and preceded by NF-κB nuclear translocation at 30-60 minutes in differentiation, indicating that the differentiation induced inflammatory response may be regulated by NF-κB activation. In the same time frame (2-8 hours) the key chondrogenic transcription factor and NF-κB target Sox9 was also temporally expressed, as confirmed on mRNA and protein level.

The association between NF-κB activation, inflammatory signaling and the early onset of chondrogenesis prompted us to test whether inhibition or stimulation of the early cell intrinsic inflammatory response was able to determine late chondrogenic cell fate. Inhibition of inflammatory signaling was achieved by pharmacologically inhibiting NF-κB activation or genetic knockdown of NF-κB subunit p65. Both experimental approaches resulted in reduced inflammatory signaling at the early onset of chondrogenesis as well as reduced early Sox9 expression. Pharmacologic inhibition of NF-κB activity resulted in a concentration dependent inhibition of Col2A1 and Col10A1 mRNA and protein expression during late chondrogenic phase. NF-κB signaling was enhanced by supplementing culture medium with low concentrations of LPS or TNFα. Subtle inflammatory provocation in the first 24 hours of differentiation resulted in increased upregulation of Sox9 at early time points and increased Col2A1 and Col10A1 expression in late phase chondrogenic differentiation.

To independently verify the results obtained with the ATDC5 system, chondrogenesis experiments were performed using hBMSCs. Relevantly, these cells also contribute to endochondral fracture healing in vivo. In concert with our findings in ATDC5 cells, early chondrogenesis of hBMSCs was accompanied by a similar temporally upregulation of inflammatory mediators as well as Sox9 expression at 2-8 hours in differentiation (Fig. 1A, B). NF-κB activation was detected at 1-4 hours in hBMSC chondrogenic differentiation. Furthermore, inhibition of NF-κB activation by TLCK prevented chondrogenic differentiation of hBMSCs (Fig. 1B, C). In contrast, stimulation of NF-κB signaling during early hBMSC chondrogenesis using low LPS concentrations resulted in increased Col2A1 protein levels (Fig. 1B, C). Independently of normal chondrogenic differentiation conditions, activation of NF-κB signaling in proliferating hBMSCs for 24 hours resulted in expression of Col2A1 at day 21.

DISCUSSION
Our data show that early chondrogenic programming and subsequent late phase chondrogenesis depends on a cell-intrinsic inflammatory signaling response. The key chondrogenic transcription factor Sox9 is temporally expressed during this short inflammatory phase. Although inhibition of early Sox9 expression by NF-κB inhibition affects late phase chondrogenesis, the role of this novel early Sox9 expression is currently unknown and might function in early chondrogenic chromatin priming.

The chondrogenesis-associated inflammatory response is cell intrinsic, however can be regulated or even initiated by exogenous NF-κB activating molecules. This finding implicates that extracellular inflammatory signaling cross-talks with the early cell intrinsic chondrogenic and inflammatory response and co-determines late chondrogenic fate. Relevantly, this situation can be imagined in an inflammatory haematoma environment and explain why haematoma formation is essential for endochondral ossification of bone fractures.

In summary, our data provide important insight into the timing of NF-κB function during chondrogenesis and show that it acts very early in differentiation as an essential transcription factor for Sox9. Our findings also demonstrate that inflammatory responses are not solely associated with cartilage degeneration and that the outcome of NF-κB signaling on chondrogenic cells depends on their differentiation status and magnitude, timing and duration of the inflammatory signal. These findings contribute to our understanding of chondrogenic developmental processes as skeletogenesis, bone fracture healing and optimization of adaptive treatment regimes in cartilage regenerative medicine (e.g. ACI (2) and in vivo bioreactor (IVB)(3)).

REFERENCES
1. Mountziaris et al. (2008), Tissue Eng Part B Rev.
3. Emans et al. (2010), Proc Natl Acad Sci USA

ACKNOWLEDGMENTS
This work is financially supported by the Dutch Arthritis Association (grant 08/42) and the Dutch Anna fonds foundation (grants 07/07 and 08/42).

Figure 1: Early inflammatory response determines chondrogenesis in hBMSCs. (A) mRNA expression at early time points in differentiation (0-24 hours) of COX-2, IL-6 and Sox9 (relative to t=0 and normalized to β-actin) as determined by RT-qPCR. Bars represent hBMSCs from 3 individuals. (B) hBMSCs were differentiated in the presence of LPS (0.1 ng/ml) or TLCK (100 μM) from onset of differentiation (LPS only during first 24 hours). Sox9 and COX-2 protein expression at 0, 2 and 4 hours in differentiation were determined by immunoblotting. (C) hBMSC day 21 samples were analyzed for Col2A1 protein expression.

Paper No. 153 • ORS 2011 Annual Meeting