The early transcriptomic response to interleukin 1β and interleukin 33 in rat neonatal cardiomyocytes

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The early transcriptomic response to interleukin 1β and interleukin 33 in rat neonatal cardiomyocytes

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ABSTRACT

In the heart, inflammatory cytokines including interleukin (IL) 1β are implicated in regulating adaptive and maladaptive changes, whereas IL33 negatively regulates cardiomyocyte hypertrophy and promotes cardioprotection. These agonists signal through a common co-receptor but, in cardiomyocytes, IL1β more potently activates mitogen-activated protein kinases and NFκB, pathways that regulate gene expression. We compared the effects of external application of IL1β and IL33 on the cardiomyocyte transcriptome. Neonatal rat cardiomyocytes were exposed to IL1β or IL33 (0.5, 1 or 2 h). Transcriptomic profiles were determined using Affymetrix rat genome 230 2.0 microarrays and data were validated by quantitative PCR. IL1β induced significant changes in more RNAs than IL33 and, generally, to a greater degree. It also had a significantly greater effect in downregulating mRNAs and in regulating mRNAs associated with selected pathways. IL33 had a greater effect on a small, select group of specific transcripts. Thus, differences in intensity of intracellular signals can deliver qualitatively different responses. Quantitatively different responses in production of receptor agonists and transcription factors may contribute to qualitative differences at later times resulting in different phenotypic cellular responses.

Abbreviations: ERK, extracellular signal-regulated kinase; IL, interleukin; MAPK, mitogen-activated protein kinase

1. Introduction

The interleukin 1 (IL1) family cytokines include IL1α/β which bind to IL1R1, and IL33 which binds to a transmembrane form of ST2 (gene symbol, IL1RL1) [1]. IL1R1 and ST2 interact with the same co-receptors, IL1R accessory proteins (IL1RAcPs), forming similar ternary complexes [2]. IL1RAcPs are the signaling components, recruiting adapter molecules (MyD88, IRAKs, TRAF6) that stimulate intracellular signaling pathways including mitogen-activated protein kinases (MAPKs) [extracellular signal-regulated kinases 1/2 (ERK1/2), c-Jun N-terminal kinases (JNKs), p38-MAPK(s)] and NFκB. NFκB becomes nuclear-localised where it stimulates transcription directly, whereas MAPKs regulate mRNA expression through phosphorylation of transcription factors to modulate their transactivating activities or via proteins that regulate mRNA stability [3].

IL1β is a major component of the innate immune response and is immensely important as an upstream initiator of inflammation [4]. IL33 is implicated in various systems, playing a particular role in initiating Th2-type responses (e.g. asthma and arthritis [5]). In heart, inflammatory cytokines including IL1β are associated with regulation of both adaptive and maladaptive changes [6] whereas IL33, despite having inflammatory effects, negatively regulates cardiomyocyte hypertrophy and promotes cardioprotection [7]. In many cells, IL33 is nuclear-localized and is released during apoptosis to initiate an inflammatory response [5, 8]. Alternatively, in fibroblasts, IL33 release may be instigated by mechanical strain [9]. Since cardiac fibroblasts are the major source of IL33 in the heart [10], increased biomechanical stretch/strain potentially stimulates release of IL33 from fibroblasts for paracrine stimulation of adjacent cardiomyocytes. Differences in receptor-based signaling alone could account for the differing effects of IL1β *vs* IL33 on cardiomyocytes and the heart. Although IL1β and IL33 stimulate similar temporal activation of MAPKs in cardiomyocytes

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(consistent with signaling through the same co-receptor), the degree of activation of these pathways and NFκB by IL1β is much greater than that induced by IL33 [10-12]. This probably reflects relative levels of expression of ligand binding receptors. Nevertheless, differential regulation of the same canonical signaling pathway can elicit different phenotypic outcomes. This is most well-established in PC12 cells in which differential regulation of ERK1/2 results in proliferation or differentiation [13]. A similar scenario may operate in cardiomyocytes exposed to IL1β *vs* IL33. Here, we used RNA expression profiling as a measure of the output from IL1β and IL33 signaling in cardiomyocytes. We demonstrate qualitative (rather than purely quantitative) differences in the profiles, propagation of which could contribute to the different effects of these cytokines on cardiac responses.

2. Methods

Neonatal rat ventricular cardiomyocytes were prepared from 2-4 day Sprague-Dawley rats as described [14]. Animals (maintained in accordance with Directive 2010/63/EU of the European Parliament) were culled by cervical dislocation. Cells were plated at 4×10^6 cells per 60 mm Primaria™ tissue culture dish in serum-containing medium (18 h). Cells were then cultured in serum-free medium (24 h) before addition of agonist. Recombinant rat IL1β (R & D Systems) and recombinant rat IL33 (Axxora) were prepared and diluted in PBS containing 0.1% (v/v) BSA.

Cardiomyocytes were unstimulated or exposed to IL1β or IL33 and total RNA prepared as previously described [14]. To minimise variation resulting from different cardiomyocyte preparations, equal amounts of RNA from three individual experiments were pooled to generate a single sample set. Samples (3 sets) were prepared and hybridized to Affymetrix rat genome 230 2.0 arrays as previously described [14]. The data are available through ArrayExpress (ArrayExpress ID: E-MEXP-1960 and E-MEXP-1912). Files (.CEL)

were imported into GeneSpring 11.5 (Agilent Technologies) and data were normalized using MAS5. Normalisation per gene was to the corresponding controls within each sample set. Probesets were selected for analysis if present or marginal in all of any condition, and filtered according to fold change (>1.5-fold) relative to controls. Probesets with statistically significant changes (false discovery rate, FDR<0.05) were identified by one-way ANOVA with Student-Newman-Keuls (SNK) post-test, using the Benjamini and Hochberg multiple testing correction. Data for IL1β and IL33 were analysed separately and lists combined for downstream analysis. Pathway analysis used Panther 7.2 and genes were classified according to Panther profiles [15].

qPCR was performed using an ABI Real-Time PCR 7500 system (Applied Biosystems) as previously described [11, 14]. Values for mRNAs (see Supplemental Table S1 for primer details) were normalized to glyceraldehyde 3-phosphate dehydrogenase (Gapdh) expression and then to control values.

3. Results and discussion

Neonatal rat ventricular myocytes were used for this study. As terminally-differentiated cells, they are highly synchronised and they can be rendered quiescent by serum starvation. Freshly isolated adult cardiomyocytes are stressed by the isolation procedure (MAPKs are activated with baseline changes in IEG expression, unpublished data) and cultured adult cardiomyocytes undergo apoptosis (25-50% loss of viability over 48 h) with dedifferentiation of surviving cells over time [16, 17], so they are less suitable. Cardiomyocytes were exposed to 100 ng/ml IL1β or IL33 (since 100 ng/ml IL1β is required for maximal activation of JNKs and 100 ng/ml IL33 gives maximal activation of MAPKs

without complications from sST2 [11, 12]), and we examined the early changes in RNA expression (0.5, 1 and 2 h) to focus on immediate early genes (IEGs). IEGs are regulated by pre-existing transcription factors immediately downstream of the protein kinase signaling events and are representative of receptor signaling.

Using Affymetrix microarrays, 609 RNAs were identified with significant changes in expression (>1.5-fold, FDR<0.05) in cardiomyocytes exposed to IL1β or IL33 (Supplemental Spreadsheet S1). The majority were selectively regulated by IL1β (247 RNAs upregulated; 229 RNAs downregulated) (Fig. 1A) and, even for transcripts that responded to either agonist (91 RNAs upregulated; 8 RNAs downregulated), most exhibited a greater response to IL1β than IL33 (Fig. 1B). A few transcripts (19 RNAs upregulated; 15 RNAs downregulated) were significantly changed only in response to IL33 (Fig. 1C). We did not detect IL5 or IL13 in cardiomyocytes exposed to IL33, although they are upregulated by IL33 in other cells [18]. This may be a cell-specific difference and/or reflect our focus on the early response to IL33 stimulation (in contrast to other studies that emphasise longer term changes over, for example, 48 h [18]).

To validate the microarray data by qPCR, we focused on inflammation-associated genes, selecting six that were upregulated by IL1β (Csf1, Csf2), both agonists (Csf3, Ptgs2) or IL33 (IL1α, IL1β) and examining their regulation over a more detailed time course (Fig. 1, D - I). The effect of IL33 on expression of IL1α/β mRNA suggests that some downstream, late effects of IL33 may be mediated via these cytokines. Other genes were chosen because of their association with cardiac disorders. Colony stimulating factor 1 (Csf1, also known as macrophage colony stimulating factor) is upregulated in heart in conditions of unstable angina and following myocardial infarction [19]. Csf2 (granulocyte and macrophage colony stimulating factor) and Csf3 (granulocyte colony stimulating factor) are both associated with cardiac remodelling following infarction [20]. Ptgs2 (also known as cyclooxygenase 2)

influences cardiac function, though whether or not it is beneficial is debated [21]. For all mRNAs, qPCR (left panels) and microarray data (right panels) were in accord. IL1β had a substantially greater effect than IL33 on expression of Csf1 and Csf2 mRNAs at all times studied (Fig. 1, D and E). Both agonists increased expression of Csf3 and Ptgs2 mRNAs, although IL1β still had a greater effect particularly at prolonged times (Fig. 1, F and G). IL33 (but not IL1β) increased expression of IL1α and IL1β mRNAs over 0.5 - 2 h (Fig. 1, H and I).

   IL1β-responsive RNAs were clustered according to the time for maximal upregulation (Fig. 2A) or maximal downregulation (Fig. 2B). Each cluster was then interrogated for IL33-responsive RNAs (Supplemental Table S2). Cluster 1 (65 IL1β-responsive RNAs with maximal upregulation at 0.5 h) contained IL33-responsive RNAs with similar profiles (n=13) or with maximal upregulation at 1 h and a more sustained response (n=18). The degree of stimulation by IL33 was less than that induced by IL1β. Cluster 2 (64 IL1β-responsive RNAs with maximal upregulation at 1 h) contained 19 IL33-responsive RNAs with similar profiles and degrees of stimulation. Cluster 3 (199 IL1β-responsive RNAs with maximal upregulation at 2 h) contained IL33-responsive RNAs with either similar profiles and degrees of upregulation (n=51) or greater stimulation at 0.5-1 h (n=13). Cluster 4 (12 IL1β-responsive RNAs with maximal downregulation at 0.5 h) contained no IL33-responsive RNAs. Cluster 5 (34 IL1β-responsive RNAs with maximal downregulation at 1 h) contained 4 IL33-responsive RNAs with maximal downregulation at 2 h. Cluster 6 (195 IL1β-responsive RNAs with maximal downregulation at 2 h) contained 14 IL33 responsive RNAs with similar profiles and degrees of downregulation. We classified the genes according to the Panther system and profiles which considers the numbers in each category relative to the total number in the list and the total number in the database to generate a probability value for whether or not the pathway is over-represented [15]. Both agonists regulated genes

associated with inflammation-related pathways (Fig. 2C; Supplemental Table S3). However, IL1β also significantly affected genes associated with platelet-derived growth factor signaling, with a greater effect than IL33 on interleukin signaling and angiogenesis.

In conclusion, our transcriptomics data indicate that the changes in RNA expression profiles induced by extracellular application of IL1β and IL33 (i.e. focusing only on receptor-mediated effects) are qualitatively and quantitatively different. Generally, IL1β induced a greater change in the cardiomyocyte transcriptome than IL33 in terms of numbers of RNAs and degree of change of individual RNAs. Probably the most striking difference was in the greater number of downregulated RNAs in response to IL1β. The early qualitative differences could lead to different phenotypic outcomes in the heart. However, quantitative differences in regulation of downstream cytokines with the generation of different cytokine profiles are also likely to propagate a differential phenotypic outcome. Importantly, while a balanced inflammatory response as that induced by IL33 could induce tolerance and cardioprotection, excessive inflammation induced by IL1β could cause collateral damage and contribute to maladaptive changes in the heart.

Acknowledgements

References

Figure legends

Fig. 1. Regulation of the cardiomyocyte transcriptome by IL1β vs IL33. Cardiomyocytes were exposed to IL1β or IL33 and the transcriptome analysed with Affymetrix microarrays. Transcripts significantly changed (>1.5-fold; FDR<0.05) by either ligand were clustered according to regulation by only IL1β (A) or IL33 (C), or either agonist (B). Heatmaps are normalised to unstimulated controls (-2.5 (cyan) through 0 (black) to +2.5 (red); $\log_2$ scale) and are means for n=3. Clustering on entities used a Euclidean distance matrix and centroid linkage ratio. D-I, qPCR validation of microarray data for Csf1 (D), Csf2 (E), Csf3 (F), Ptgs2 (G), IL1α (H) and ILβ (I). Left panels, Cardiomyocytes were exposed to IL1β (solid circles, solid lines) or IL33 (open circles, dashed lines) for the times indicated and mRNA expressions analysed by qPCR. Results are means ± SEM (n=3 or 4). * p<0.05 for IL1β relative to zero time, # p<0.05 for IL33 relative to zero time (repeated measures one-way ANOVA with SNK post-test). Right panels, Microarray data for each mRNA studied are presented relative to unstimulated controls. Results are means for n=3.

Fig. 2. Classification of IL1β and IL33-responsive transcripts. IL1β-responsive mRNAs identified by microarray analysis were clustered according to the time to maximal upregulation (A) or downregulation (B). Clusters were interrogated for IL33-responsive RNAs. Results are means ± SEM for each group. * p<0.05 for IL1β relative to zero time, # p<0.05 for IL33 relative to zero time, † p<0.05 for IL33a (repeated measures one-way ANOVA with SNK post-test). C, Panther classification of responsive genes.

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