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Research Paper

Alteration in Ikaros expression promotes B-1 cell differentiation into phagocytes

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ABSTRACT

Ikaros is a broad transcription factor pointed as a critical regulator of lymphocyte development. Recent reports have emphasized that distinct isoforms of Ikaros control the dichotomy of the hematopoietic system into lymphoid and myeloid lineages. In addition, expression of dominant-negative isoforms of Ikaros is linked to abnormal hematopoiesis, which could culminate in hematological disorders due to loss of function of the protein. B-1 cells are an intriguing subtype of B-lymphocytes that preserves some myeloid characteristics. These cells are able to differentiate into phagocytes (B-1CDP – B-1 cell derived phagocytes) *in vitro* and *in vivo*. During such process, reprogramming of gene expression occurs: lymphoid genes are turned off, while expression of myeloid genes is increased. This study aims to investigate whether Ikaros could be related to the control of B-1 cell plasticity. Interestingly, Ikaros expression by B-1CDP cells was found to be relatively low, and the protein is abnormally localized in the cytoplasm. Moreover, the isoforms expressed by B-1 cells are different from those expressed by other lymphocytes, with expression of active isoforms being almost absent in B-1CDP. Based on these findings, Ikaros could be an important factor driving the differentiation and proliferation of B-1 cells.

1. Introduction

B-1 cells are a subpopulation of B-lymphocytes found preferentially in the peritoneal and pleural cavities, and constitute only a small fraction of B cells in the spleen (Deenen and Kroese, 1993). As B lymphocytes, these cells are able to endocytose antigens and present them to T-lymphocytes (Margry et al., 2013; Sato et al., 2004; Vigna et al., 2002; Wang and Rothstein, 2012; Zimecki and Kapp, 1994; Zimecki et al., 1994). In addition, B-1 cells are mainly involved in T-independent responses, producing most of the natural antibodies found in the serum (Choi and Baumgarth, 2008; Haas et al., 2005). The ability of B-1 cells to produce antibodies justifies their characterization as B-lymphocytes. However, several differences between conventional B-lymphocytes (B-2) and B-1 cells exist regarding their ontogeny, anatomical localization, antibody repertoire, antigen stimulus, and role in the immune response (Berland and Wortis, 2002; Hayakawa et al., 1983; Herzenberg, 1989; Montecino-Rodriguez and Dorshkind, 2006; Adolfsson et al., 2005). B-1 cells are characterized by expression of CD19, IgM^{high}, IgD^{low}, CD11b and absence of CD23 (Tung et al., 2006). Expression of CD5 divides them into two different subsets: B-1a cells ($CD5^+$), and B-1b cells ($CD5^-$) (Hayakawa et al., 1983; Tung et al., 2006).

In contrast to their lymphoid profile, B-1 cells retain several myeloid characteristics. It has been shown that B-1b cells proliferate in cultures of adherent mouse peritoneal cells and can become mononuclear phagocytic cells *in vitro* with no relation to blood-derived monocytes (Almeida et al., 2001). This so-called B-1 cell derived phagocyte (B-1CDP) is able to phagocytose and process solid particles, and also migrate to participate in inflammatory and infectious processes *in vitro* and *in vivo* (Almeida et al., 2001; Popi et al., 2012; Popi, 2015; Novo et al., 2015; Geraldo et al., 2016). Furthermore, it has been demonstrated that, upon LPS stimulation, peritoneal phagocytes appear in op/op$^{(-/-)}$ mice, which are devoid of monocyte-derived macrophages. The origin of these phagocytes from B-1 cells was confirmed based on detection of immunoglobulin gene rearrangement (VH11), often found in B-1 cells (Popi et al., 2012). It has also been shown that B-1 cells are more permissive to some infections and that they are less effective in killing pathogens than macrophages (reviewed by Popi, 2015; Nakashima et al., 2012). In spite of this, B-1CDP produce higher levels of nitric oxide (NO) than activated macrophages (Ghosn et al., 2006).

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Intriguingly, once a B-1 cell differentiates into a phagocyte, it assumes a myeloid profile in detriment to its lymphoid origin. It has been shown that lymphoid genes such as E2A, EBF and Pax5 are down regulated in B-1CDP cells, while expression of genes related to myeloid commitment are maintained (Popi, 2015; Popi et al., 2009). Concomitantly, the expression of sIgM and CD19 is abolished. During the establishment of hematopoietic lineages, key factors involved in gene expression regulation govern the cell differentiation process. Among them, Ikaros constitutes an important chromatin-remodeling complex that plays a role in the myeloid/lymphoid dichotomy (Westman et al., 2002; Winandy et al., 1999; Georgopoulos, 2002; Cortes et al., 1999; Tinsley et al., 2013; Payne et al., 2003).

Ikaros works in a multimeric complex in association with acetylases, methylases, deacytilases, NURD, and others (Kim et al., 1999; Georgopoulos, 2017). In this complex, different isoforms may interact and it is postulated that the diversity of isoforms contribute to drive the specification of hematopoietic cells. The N-terminal region of Ikaros mediates sequence-specific DNA binding, while the C-terminus is required for protein–protein interactions (Georgopoulos, 2017). The protein is typically localized in the cell nucleus where it exerts its normal function (Georgopoulos et al., 1994; Molnar and Georgopoulos, 1994). It is also known that alternative splicing generates dominant-negative isoforms unable to bind DNA (reviewed by Li et al., 2011). Such isoforms are involved hematopoietic disorders such as leukemia and are preferentially localized in cytoplasm (Capece et al., 2013; Nakase et al., 2000; Theocharides et al., 2015; Zhou et al., 2014).

Originally, Ikaros was regarded as a decisive factor during lymphoid lineage commitment (Georgopoulos et al., 1992). Later on, the discovery of alternative splicing-generated isoforms brought to light the role of Ikaros in myeloid differentiation as well (Payne et al., 2003; Georgopoulos et al., 1994; Molnar and Georgopoulos, 1994). Thus, Payne et al. (2003) demonstrated the selective expression of Ikaros isoform-x in cells with myeloid potential (Payne et al., 2003). Allman et al. (2006) demonstrated the requirement of Ikaros for dendritic cells differentiation. Macrophages also express a dominant-negative isoform of Ikaros which regulates important mechanisms of cell activation (Cho et al., 2008). Finally, B-1 cells were shown to express Ikaros as well (Oliveira et al., 2015). However, in these cells, Ikaros regulates different genes in comparison to B-2 cells, such as PU.1. In B-1 cells, PU.1 expression is negatively regulated by Ikaros, while in B-2 cells Ikaros is not able to interact with the promoter region of PU.1 (Oliveira et al., 2015; Scott et al., 1997). In the absence of Ikaros, B-1 cells loose their commitment to B cell lineage, suggesting that Ikaros is an important factor to assure the lymphoid side of B-1 cells (Oliveira et al., 2015).

Considerig the findings above described, the role of Ikaros in the differentiation of B-1 cells into phagocytes is the aim of this study. Results showed that, interestingly, Ikaros was weakly expressed in the cytoplasm of B-1CDP. Furthermore, silencing of Ikaros in B-1 cells promoted B-1CDP differentiation *in vitro*. These findings suggest that loss of expression or function of Ikaros drive B-1 cells to a myeloid commitment.

2. Materials and methods

2.1. Animals

BALB/c male mice (8–10 weeks old) were obtained from CEDEME (Centro de Desenvolvimento de Modelos Experimentais para Medicina e Biologia da Universidade Federal de São Paulo). All animals were maintained under specific pathogen free conditions. All experimental procedures were approved by the Ethical Committee at UNIFESP (protocol #2014/7916071113).

2.2. B-1 cells and B-1CDP differentiation

Peritoneal B-1 cells were obtained as described by Almeida et al. (2001), as follows. After 3 days, cells in the non-adherent fraction were harvested, labeled with biotin-conjugated rat anti-mouse CD19 (BD Biosciences) and the $CD19^+$ population enriched by using Anti-Biotin MicroBeads and MS Columns (Milteny Biotec) accordingly to the manufacturer instructions. An enrichment of ≥98% of $CD19^+$ cells was set as the minimum acceptable experimental condition. The percentage of B-1 cell enrichment was verified by addition of streptavidin-PE (BD Biosciences) and analysis in a FACSCantoII system (BD Biosciences).

B-1-enriched cell fractions were submitted to the B-1CDP differentiation protocol described by Popi et al. (2009), with modifications. Briefly, B-1 cells were plated at 1×10^6 cells/mL in 24-well culture plates with R10 medium (RPMI + 10% of bovine serum) or with R10 medium supplemented with 50% of L929 fibroblast conditioned medium. Cells were maintained for 48 h at 37 °C and 5% CO_2.

2.3. RNA interference assays

Ikaros-specific siRNA (small interference RNA) from siRNA Accell SMART (*Thermo Scientific Dharmacon*) was used to silence the expression of Ikaros in B-1 cells, as previsously described (Oliveira et al., 2015). B-1 cells obtained as described above were cultured at 5×10^5 cells/mL in 96-well plates with 100 µl of siRNA in Accell medium supplemented with 3% of FBS (fetal bovine serum) for 48 h at 37 °C in 5% CO_2. After 48 h, R10 or L929 fibroblast conditioned was added and incubation continued for 3 additional days at 37 °C in 5% CO_2. Monitoring of transduction efficiency with fluorescein-labeled siRNA revealed that nearly 85% of cells were positive for GFP (green fluoresce protein) (*data not shown*).

2.4. Flow cytometry assay

Following silencing of Ikaros as above described, cells were submitted to flow cytometry analysis to quantify B-1 ($CD19^+CD23^-F4/80^-$) and B-1CDP cells ($CD19^-CD23^-F4/80^+$). Cells were pre-incubated with anti-CD16/CD32 monoclonal antibody (mAb) to block FcRII/III and labeled on ice for 15 min with the following fluorochrome conjugated mAbs: PE-conjugated rat anti-mouse CD19, FITC-conjugated rat anti-mouse CD23 and allophycocyanin (APC)-conjugated rat anti-mouse F4/80. Cells were analyzed on a FACSCantoII system (BD Biosciences). Post-acquisition analyses were performed with FlowJo software (version 9.7.6). For this analysis, two populations were determined based on expression of CD19, CD23 and F4/80. First of all, a dot plot with expression of CD19xCD23 was constructed, and only $CD19^+CD23^-$ cells were selected. From this gate, the expression of F4/80 was analyzed, considering that F4/80 negative cells are B-1 cells ($CD19^+CD23^-F4/80^-$) and $CD19^+CD23^-F4/80^+$ cells were phagocytes. The mean fluorescence intensity (MFI) of F4/80 from $CD19^+CD23^-F4/80^+$ cells was calculated to analyzed the level of expression of this marker in different culture conditions.

2.5. Gene expression analysis

RNA was isolated from B-1CDP and B-1 cells using Pure Link Kit RNA (Life Technologies) and used for cDNA synthesis with Superscript III cDNA Synthesis (Life Technologies). cDNA samples were used to analyze Ikaros (CCACAACGAGATGGCAGAAGAC; GGCATGTCTGACA GGCACTTGT), E2A (CCATGCTAGGTGACGGCTCTTC; GCGAGCCATTA ACCTCAGATCC), Pax-5 (TGACGCAGGTGTCATCGGTGAG, ATTCGGCA CTGGAGACTCCTGA), PU.1 (ATGTTACAGGCGTGCAAAATGG, TGATC GCTATGGCTTTCTCCA) and MCSFR (TGTCATCGAGCCTAGTGGC, CGG GAGATTCAGGGTCCAAG) expression levels by real-time PCR using a FAST Sybr Green Reagent (Applied Biosystems) on an Applied Biosystems 7500 Fast Real-Time PCR System. Amplification efficiencies were determined by comparing the dilution series of reference and target genes from a reference cDNA template. The amplification efficiency was calculated using the following equation: $E = 10^{(-1/}$