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Brief communication

Monoclonal antibody–assisted stimulation of adenosine A_{2A} receptors induces simultaneous downregulation of CXCR4 and CCR5 on $CD4^+$ T-cells

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ABSTRACT

Immunocompetent cells express various G-protein–coupled receptors that transduce extracellular signals across the plasma membrane. Among them, CXCR4 and CCR5 chemokines receptors and adenosine A_{2A} receptors ($A_{2A}R$) are involved in inflammatory processes. Considering that $A_{2A}R$ activation may have incidence on CXCR4 and CCR5 protein expression through heterologous desensitization process, we tested Adonis, an agonist-like monoclonal antibody to $A_{2A}R$ on $CD4^+$ CEM T-cells. We found that Adonis inhibited the CEM cell growth, upregulated $A_{2A}R$ and downregulated CXCR4 and CCR5 without modifying the CD4 expression. By reducing the expression of CXCR4 and CCR5 chemokines receptors utilized as entry co-receptors by HIV-1 during viral infection of CD4 expressing cells, Adonis stimulation of $A_{2A}R$ appears as a valuable means to treat infected cells.

The A_{2A} receptors ($A_{2A}R$) of adenosine are predominantly expressed on mouse [1] and human [2] T-cells, where they play a critical role in the protection of inflammatory damages and tumors [3,4]. Adenosine blocks the T-cell proliferation and chemokines secretion [5] as a function of the number of $A_{2A}R$ expressed on T-cells [6]. T-cells also express chemokine receptors CCR5 and CXCR4, which mediate several cellular functions such as development, cellular trafficking, angiogenesis and immune response [7]. $A_{2A}R$, CCR5, and CXCR4 are structurally related receptors belonging to the superfamily of the seven-transmembrane G-protein–coupled receptors [8]. Ligand binding to these receptors induces a conformational change of the receptor that links its cytoplasmic part to an intracellular G protein. This signal activates or inhibits intracellular enzymes involved in various pathways of cellular communication. Many of these pathways are dynamically regulated [9]. At the receptor level, regulation can occur via inhibition of receptor/G-protein coupling (desensitization), redistribution of cell surface receptors (trafficking), and receptor degradation (downregulation). Two protein families, G-protein–coupled receptor kinases and arrestins, play a critical role in regulating these processes. Kinases specifically phosphorylate the activated form of the receptor, which in turn promotes arrestin binding. Arrestin binding sterically inhibits coupling of the receptor to its respective G-protein and targets the receptor for internalization via clathrin-coated pits [10]. Receptors then are either recycled back to the plasma membrane or directed to the degradative pathway. $A_{2A}R$ was reported to induce heterologous desensitization of CCR5 [11]. As CXCR4 and CCR5 are co-recruited and may hetero-oligomerize on activated T-cells [12], we hypothesized that it was possible to induce heterologous desensitization and subsequent internalization of both co-receptors through stimulation of the adenosine $A_{2A}R$. Here, we took advantage of production of Adonis, a new agonist-like monoclonal antibody that binds to a linear epitope of the $A_{2A}R$ [13] to downregulate CXCR4 and CCR5 expression on a T-cell line.

CEM, a $CD4^+$ human T-lymphoma cell line endogenously expressing $A_{2A}R$, CXCR4, and CCR5, was obtained from American Type Culture Collection (Rockville, MD) and cultured at 37°C under 5% CO_2 in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mmol/l L-glutamine, and 100 IU/ml each penicillin-streptomycin. To establish the growth curve, CEM cells (1×10^5/ml) were seeded into 24-well culture plates, each well containing 1 ml culture medium with or without the presence of various doses of Adonis. During 6 days, the cells within the corresponding wells were day by day suspended and viable cells count was performed using the trypan blue dye exclusion method and a Malassez hemocytometer. Without Adonis, CEM cells exponentially grew until 5 days of culture to reach a plateau (Fig. 1A). Adonis inhibited the CEM cell growth depending on the dose to reach a plateau at day 4 for 0.2 and 0.4 μmol/l and at day 3 for 0.6 μmol/l (Fig. 1A). To further confirm the inhibition of the CEM cell growth by Adonis, we used the CytoX-Violet Cell Proliferation Assay according to the manufacturer's instructions (Epigentek,

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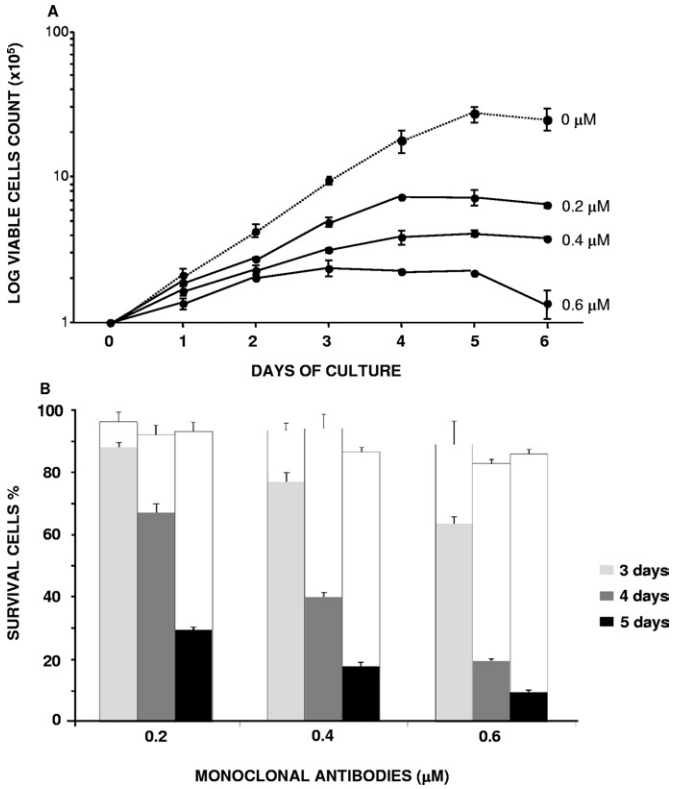

Fig. 1. Adonis effect on CEM cell growth. (A) Viable cells count was reported from day 0 to day 6. Standard curves in the absence of Adonis were given as control (dotted lines). Concentrations of Adonis in the culture medium were successively 0.2, 0.4, and 0.6 μmol/l. Results are mean values ± standard deviation (SD) of duplicates. (B) Percentage of survival cells in presence of Adonis (filled bars) and control (open bars) monoclonal antibodies was reported for 3, 4, and 5 days of culture. Concentrations of monoclonal antibodies in culture medium were successively 0.2, 0.4 and 0.6 μmol/l. Results are the mean ± SD of duplicates.

Brooklyn, NY). This kit is based on the fluorometric change of a special dye composition to measure cell viability that is proportional to the cellular deshydrogenase activity. CEM cells (1×10^5/ml) were mixed with Adonis in 96-microplate wells filled with 200 μl of culture medium. After 3–5 days' incubation at 37°C under 5% CO_2, 20 μl of indicator solution was added to each well. After 4 hours' incubation at 37°C, fluorescence was read at 560/590 nm. The anti-terpolymer (Glu–Ala–Tyr) monoclonal antibody, H56.406.48 [14] with the isotype (IgM, k) as Adonis, was used as control. Results were expressed as percentage of survival cells according to the formula: (fluorescent intensity with monoclonal antibody – blank/fluorescent intensity without monoclonal antibody – blank) × 100. We found that Adonis gradually decreased in a time- and dose-dependent fashion the CEM cells proliferation, whereas the irrelevant monoclonal antibody remained continuously negative (Fig. 1B). We previously found that Adonis stimulated the cAMP production and inhibited the cell proliferation of an A_{2A}R-expressing cell line [13]. The results obtained on the CEM cell line further agreed with the fact that A_{2A}R downregulate immune response *in vivo* by inducing elevation of intracellular cAMP which in turn inhibits the NF-κB pathway in lymphocytes [15].

To test the A_{2A}R, CXCR4, CCR5 and CD4 expression, CEM cells (1×10^5/ml) were seeded into 25-cm^2 tissue culture flasks containing 10 ml culture medium with or without the presence of various doses of Adonis. Considering the above results, CEM cells were incubated 4 days in the presence of 0.2 and 0.4 μmol/l of Adonis. As control, CEM cells were cultured without Adonis to measure the native expression of the cell receptors. Cells were washed with PBS and frozen at −70°C. Cells were then quickly thawed at 37°C and solubilized with 4% SDS aqueous solution by 30-minute sonication at 47 kHz. After protein quantification by microBCA (Pierce Biotechnology, Rockford, IL), 15 μg of cell solubilisate were diluted in 62.5 mmol/l Tris-HCl buffer, pH 8.3, containing 2% SDS, 10% glycerol, 0.01% bromophenol blue and 5% mercaptoethanol and subjected to standard electrophoresis procedure in Mini Protean II system (Bio-Rad, Hercules, CA). Separated proteins in 12% acrylamide minigel were electrotransfered onto a PVDF membrane. Blotted membrane was placed into the blot holder of the SNAP i.d. protein detection system (Millipore, Billerica, MA), saturated with nonfat dried milk and incubated 20 minutes with one of the appropriately diluted mouse monoclonal antibody, anti-A_{2A}R (Adonis), anti-CD4 (clone MEM-241, Immunotools, Friesoythe, Germany), anti-CCR5 (clone 12D1, Immunotools) and anti-CXCR4 (clone 1F8, Sigma-Aldrich, Saint Louis, MO). Antiglyceraldehyde 3-phosphate dehydrogenase (GAPDH) mouse monoclonal antibody (Clone GAPDH-71.1, Sigma-Aldrich) was used as loading control for normalizing blot results. Primary antibody was omitted to specifically reveal Adonis IgM κ light chain in the cell lysates. Blots were visualized by horse-radish peroxidase labeled anti-mouse IgG Fab specific antibodies and enhanced chemiluminescence substrate (SuperSignal West Femto, Pierce Biotechnology, Rockford, IL) using a Kodak Image Station 440CF (Eastman Kodak Compagny, Rochester, NY). The staining intensities of the bands were measured densitometrically on a Macintosh computer using the public domain NIH Image software developed at the US National Institutes of Health. Controls were reported for blots of CEM cells incubated with 0.2 μmol/l Adonis and revealed with only the anti-GAPDH monoclonal antibody or the whole set of monoclonal antibodies. In the first control, only the Adonis heavy-chain (65 kDa) and light-chain (25 kDa) bands were revealed in addition to the GAPDH (36 kDa) band and in the second control also appeared the four other bands specific for CD4 (51 kDa), A_{2A}R (45 kDa), CCR5 (41 kDa), and CXCR4 (40 kDa), respectively (Fig. 2A). Using this set of monoclonal antibodies, no crossing bands were evidenced in denaturing conditions and omitting one monoclonal antibody resulted in the loss of the corresponding band in the blot (not shown). We found that Adonis upregulated the A_{2A}R but downregulated the CCR5 and CXCR4 in a dose-dependent manner, whereas CD4 expression remained unchanged (Figs. 2A, 2B). We previously found that caffeine (1,3,7 trimethylxanthine) antagonized the Adonis-induced cAMP production in cells expressing A_{2A}R and was also able to upregulate the A_{2A}R cell-expression in a dose-dependent fashion [13]. Interestingly, Adonis remained linked to the A_{2A}R in a dose-dependent manner as shown by the presence of the 25-kDa IgM κ light chain of Adonis on the blots (Figs. 2A, 2B). The Adonis heavy chain gave faint bands and, consequently, was not chosen for the Adonis binding test. Probably, sustained binding of Adonis to A_{2A}R on the cell surface prevented the complexes made by the multivalent IgM and cross-linked receptors to be endocytosed. In contrast, CCR5 and CXCR4 decreased in net protein term, suggesting a possible internalization of the receptors not recycled back but directed to the degradative pathway [9].

It is likely that the A_{2A}R acts as homodimers at cell surface [16]. Because Adonis is a pentameric IgM antibody, it can stimulate A_{2A}R by stabilizing active homodimers at cell surface. This activating mechanism was already reported for another monoclonal antibody directed to the β_2-adrenergic receptors [17]. It was suggested for a long date that A_{2A}R desensitization is mediated by multiple, temporally distinct, agonist-dependent processes [18]. Agonist short-term desensitization of A_{2A}R was reported in numerous models [19] but, in most instances, long-term desensitization followed by internalization of A_{2A}R was not reported, A_{2A}R being rather considered as fairly resistant to agonist-induced internalization [20]. We found here that A_{2A}R prolonged (chronic) stimulation with Adonis did not downregulate but upregulated A_{2A}R expression. The extent