Co-immunoprecipitation of Platelet Factor 4 and RANTES from human platelets

Rory Koenen
University Hospital RWTH Aachen
Christian Weber
University Hospital RWTH Aachen

Method Article

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Abstract

Introduction

Chemokines are small proteins that have an important function in the trafficking of immune cells. Platelets also contain chemokines, which are stored in the alpha-granula. Activated platelets are able to deposit chemokines onto inflamed endothelium via transient rolling interactions, thereby enhancing subsequent arrest of e.g. monocytes. The chemokines RANTES/CCL5 and platelet factor 4 \(PF4/CXCL4) are deposited by platelets and can form heteromers. These CCL5/CXCL4 heteromers are particularly potent as monocyte attractant. This protocol describes the precipitation of the CCL5/CXCL4 complex from platelets.

Reagents

Lysis buffer: 20 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.1 mM DTT, 1% NP-40, 1% CHAPSO containing protease inhibitors \(Roche, Mannheim, Germany). Krebs-Ringer buffer from Sigma-Aldrich at pH 5.5 \(platelet wash buffer). 20 mM Tris buffer pH 7.5. Washed human platelets. Rabbit anti-human CXCL4 \(Peprotech) or goat anti-human CCL5 polyclonal antibodies \(R&D Systems). Non-specific normal rabbit IgG or goat IgG \(Santa Cruz Biotechnology). Molecular MACS protein G affinity magnetic beads \(Miltenyi, Bergisch-Gladbach, Germany). Biotinylated anti-CXCL4 and anti-CCL5 antibodies \(R&D Systems). StreptABC HRP detection reagent \(Vector Labs, Burlingame, CA). Enhanced chemoluminescence reagent \(Pierce, Rockford, IL). 8-16% SDS-PAGE Gradient Ready gels \(Bio-Rad, Munich, Germany). Standard SDS sample buffer containing 5 mM Dithiothreitol. Roti Block solution \(Carl-Roth GmbH, Germany). Non-fat dried milk. Block buffer for Blots: TBS+0.1% Tween-20 with 3% milk and 1% Roti Block. Western blot stripping buffer \(Pierce).

Equipment

Molecular MACS micro columns and magnet \(Miltenyi, Bergisch-Gladbach, Germany). Branson sonifier with probe tip. Standard lab centrifuge and Eppendorf tube centrifuge. MACS pre separation filter. Fuji LAS-3000 Imager. We used Nitrocellulose Hybond-P from Amersham/GE Biosciences for Western blotting.

Procedure

1. Obtain fresh platelets from the local bloodbank or from healthy volunteers by venipuncture. Make sure to get informed consent from the volunteers. 2. Isolate and wash platelets using standard methods \(e.g. differential centrifugation; beyond the scope of this protocol). 3. Take an equivalent of platelets to 20 mL of blood as starting material. This corresponds to approx. $500x10^7$ platelets. 4. After washing, pellet the platelets \(10 minutes at 2000g) and suspend in 1 mL of lysis buffer. Keep on ice. 5. Sonicate the platelet lysate 3 times 1 second at 25% amplitude on ice. 6. Centrifuge the lysate for 10 minutes at 4,000g \(4°C)

and remove the floating lipid plug using a MACS pre separation filter. 7. Centrifuge the lysate for 20 minutes at 20,000g \(4°C) and keep the supernatant. 8. Add 100 µl of protein G magnetic beads and incubate on ice for 30 minutes. Pre-clearing. 9. Some of the magnetic beads may have formed aggregates due to the high protein content and may clog the columns. These may be removed by centrifuging 30 seconds at 20,000g. 10. Prepare a molecular MACS column by priming with 200 µl of lysis buffer. Pass the lysate over the column and collect the flowthrough as precleared lysate. 11. Split the precleared lysate in two equal portions. To one portion 2 µg of the specific anti-CCL5 or anti-CXCL4 antibody is added. To the other the corresponding non-specific control antibody is added. Mix very well, then add 50 µl of protein G magnetic beads to each portion. Mix again and incubate on ice for at least 30 minutes. 12. Prepare the molecular MACS columns by priming with 200 µl of lysis buffer. Discard the flowthrough. 13. Mix the antibody/bead-treated lysates again and pipet them into the molecular MACS columns. Use one column for each portion. Let the columns empty by gravity flow at ambient temperature. The flowthrough may be collected for analysis purposes. 14. Wash the columns 3 times with lysis buffer and one time with 20 mM Tris pH 7.5. Discard flowthrough. Warm the SDS sample buffer at 95 °C in the meantime. 15. To elute the CXCL4/CC5 complex first add 20 µl of hot SDS sample buffer and wait for 5 minutes. Collect any fluid that exits the column in a clean tube. 16. Add 50 µl of hot SDS sample buffer. Collect any fluid that exits the column in a clean tube. The samples can be stored at this point. 17. Analyze the samples on a ready gradient gel \(8-16%). Blot using your favorite standard methods onto nitrocellulose. Block with the mixture of polymer-based Roti Block and milk for at least 1 hour at room temperature. 18. In our hands, the biotinylated antibodies listed in the Reagents section above worked best, in combination with StreptABC complex. 19. Strip the blot using stripping buffer and analyse using the other biotinylated antibody. 20. Western Blotting can be performed using the standard techniques in the lab and we will not elaborate on the detailed procedure beyond the antibodies, developing and blocking reagents used.

Timing

3 hours

Critical Steps

In general, the antibodies used for a co-immunoprecipitation are critical. Use exactly the ones listed in this protocol for optimal results. The lysis buffer formulation is important too. Include CHAPSO and NP-40 in the buffer, these are detergents that greatly help dispersing lipid structures and can be considered critical components. Work quickly and with continuity. Do not stop the protocol at any point before step 16. Do not leave the experimental setup for longer times.

Troubleshooting

The MACS columns are somewhat prone to clogging. Centrifuging the samples briefly after incubation with protein G magnetic beads may improve the flow over the column. Diluting the lysates prior to

treatment with protein G magnetic beads can also reduce viscosity without negative effects on the results. After blotting it may turn out that the control antibody unspecifically captured some chemokine from the lysate. In this case, incorporate one or more wash steps with lysis buffer containing 300 mM NaCl in step 14 of the protocol.

Anticipated Results

If the protocol is followed strictly, the experimenter should get a band of both CCL5 and CXCL4 when the IP is performed with either anti-CCL5 and anti-CXCL4 antibodies.

References

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