

Improvement of CXCR3 ligand CXCL11/I-TAC measurement in human plasma and serum

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SUMMARY

The chemokine receptor CXCR3 is involved in cell trafficking dysregulation associated with several inflammatory conditions, including autoimmune and viral diseases. Downregulation of CXCR3, through binding with its ligand CXCL11 (I-TAC), represents a key mechanism in lymphocyte recruitment. Determination of circulating I-TAC can provide useful information in the investigation of inflammatory/infectious conditions. The existing commercial kit does not measure CXCL11/I-TAC in complex matrices, such as human plasma and serum, as reliably as in *in vitro*-generated cell culture supernatants. We here describe means which lead to an improvement of CXCL11/I-TAC measurement in human plasma and serum.

KEY WORDS: CXCL11/I-TAC, CXCR3, ELISA, Chemokines, Chemokine receptors, Cell trafficking

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Chemokines and their receptors play an important role in cell trafficking during inflammatory and homeostatic conditions, due to their action in lymphocyte recruitment to inflammatory and homing sites and in T-cell activation. In particular, in the perspective of immune-based therapeutic interventions, chemokine and chemokine receptors are considered some of the most attractive drug targets (Charo and Ransohoff, 2006). Among chemokine receptors, CXCR3 is predicted to be involved in the induction and perpetuation of several human inflammatory disorders, including autoimmune diseases, atherosclerosis, transplant rejection, and viral infections. CXCR3 is a seven-transmembrane, G protein-coupled cell surface receptor preferentially expressed on activated Th1 cells (Liu *et al.*, 2005), often together

with CCR5. Three monocyte-derived inflammatory chemokines (ligands) are able to bind CXCR3: CXCL9/Mig (IFN- γ -induced monokine), CXCL10/IP-10 (IFN- γ -inducible 10-kD protein) and CXCL11/I-TAC (interferon-inducible T cell alpha chemoattractant) (Cole *et al.*, 1998). While CXCL9 and CXCL10 are key elements of CXCR3 action induction, CXCL11 appears to be more specific in inducing CXCR3 internalization, thus probably acting as a feed-back chemokine (Sauty *et al.*, 2001).

Because of the importance of CXCR3-induced pathways, measurement of its ligands in human samples is becoming important in order to find key features in its action related to pathology. CXCL11/I-TAC can be detected in human samples by molecular biology techniques (Northern blot, RT-PCR or microarray) aiming at quantifying the messenger RNA (mRNA) specific for CXCL11/I-TAC (Foley *et al.*, 2005; Pekarek *et al.*, 2007; Tensen *et al.*, 1999) or by directly measuring the CXCL11/I-TAC protein released in human blood-derived samples (Brainard *et al.*, 2007) mainly through enzyme-linked-immunosorbent assay (ELISA).

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The only commercially available source for detection of human CXCL11/I-TAC protein is provided by R&D Systems and is the Quantikine ELISA kit, which provides a ready-to-use-precoated plate (catalog number #DCX110) and the DuoSet ELISA Development kit (catalog number #DY672). The latter provides the capture and detection antibodies, the standard, horseradish peroxidase (HRP)-conjugated streptavidin and recipes for working buffers.

However, we ran into reproducibility problems using the DuoSet Kit with human plasma samples. The kit is indeed very reliable for cellular supernatants, but not designed specifically for a complex matrix, such as plasma and serum. Nevertheless, plasma and serum represent the most common biological specimens obtained and stored in clinical research settings. For these reasons, we investigated means to improve CXCL11/I-TAC detection in plasma/serum evaluating parameters such as the plastic of the microplates and the type of anti-coagulant.

We enrolled a total of 8 healthy donors in our study who gave their informed consent for blood withdrawal. Whole blood was collected in EDTA- or heparin-containing tubes (BD Vacutainer, #367864 and #367876, respectively). Plasma was obtained by centrifugation of blood at 200 g for 10 min, whereas serum was obtained by incubating plasma for 2 h in thrombin-containing tubes (BD #367817, 5 NIH units/each) followed by a centrifugation at 900 g.

CXCL11/I-TAC concentration was measured using the DuoSet kit and following the manufac-

turer's directions. Briefly, plates were incubated overnight with the capture antibody (1 mg/ml, 100 μ l/well), washed 3 times with a multichannel pipet using washing buffer (300 μ l/well) and blocked 1h with blocking buffer (300 μ l/well). Samples and standards (100 μ l/well, each in triplicate) were incubated for 2h at room temperature.

After 3 washing steps, the detection antibody was added (200 ng/ml, 100 μ l/well) for 1h, 3 washing steps were performed, followed by incubation with streptavidin-HRP (100 μ l/well) for 20 min and with substrate solutions (R&D Systems, catalog number #DY999, 100 μ l/well) for another 20 min. The optical density of samples was read soon after adding the stop solution (50 μ l/well, 2N H₂SO₄) using a microplate reader set at 450 nm and with a wavelength correction set at 540 nm. The standard curve was determined by plotting the mean absorbance corresponding to the serial 1:2 dilutions of CXCL11/I-TAC on the y-axis against the concentration on the x-axis and by drawing the best curve fit through the points of the graph. The highest and lowest value of the standard curve were 1000 and 7.8 pg/ml, respectively.

Since the binding properties of different plastics can strongly affect the coating of the anti-CXCR3 antibody, four different plastic microplates with different binding properties were tested, all from Nunc (*Nunc-Immuno*TM 96 *MicroWell*TM Plates): maxisorp (#442404), multisorp (#467340), polisorp (#475094) and medisorp (#446470). Maxisorp and multisorp plates were dropped ear-

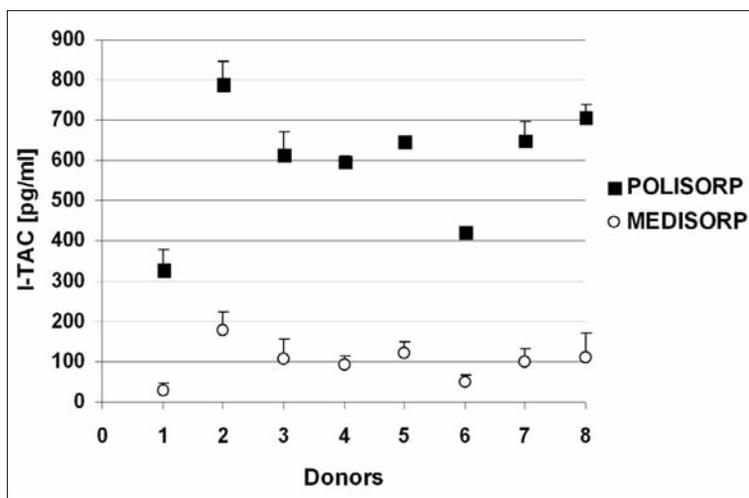


FIGURE 1 - I-TAC/CXCL11 concentration determined in plasma obtained from blood withdrawn in EDTA-containing tubes using both medisorp and polisorp microplates. Data represent the mean (\pm SD) for each of eight healthy donors. At least three evaluations were performed for each donor.

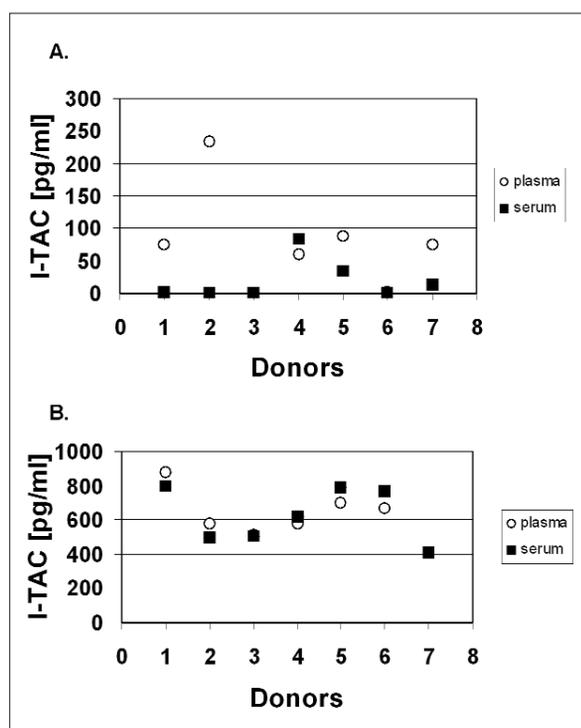


FIGURE 2 - I-TAC/CXCL11 concentration in human blood collected in tubes with EDTA (A) or heparin (B). One representative of three different measurements is shown.

ly because the results obtained were not reproducible, yielding inconsistent triplicates and over-scale values (data not shown).

We thus focused on the comparison of medisorp and polisorp plastics evaluating CXCL11/I-TAC concentration in plasma obtained from whole blood withdrawn in EDTA-containing tubes of healthy donors. The same standard curve and plasma samples were used simultaneously in the two different microplates. Both microplates gave good results and similar trends, as shown in Figure 1 where mean values (\pm SD) are depicted. Nevertheless, CXCL11/I-TAC concentrations were always higher in the polisorp microplate, with values often above the maximum detectable by the kit. For this reason we preferred the medisorp plastic plate which has a surface chemistry intermediate between polisorp (high affinity to molecules of a hydrophobic nature) and maxisorp (high affinity to molecules with mixed hydrophilic/hydrophobic domains). We cannot exclude that in particular inflammatory conditions which may lead to a consistent increase of I-

TAC levels appropriate dilutions of plasma/serum samples should be carried out.

The kind of anticoagulant used for sample preparation might also affect the results of CXCL11/I-TAC measurement, as in the detection of other chemokines (Diamandis, 2004). Thus, CXCL11/I-TAC content was measured with the medisorp plate and plasma obtained from blood treated with two commonly used anticoagulants, EDTA and heparin. Blood from the same donors was collected in tubes containing EDTA (1.8 mg/ml of whole blood) or heparin (17 I.U./ml) and processed to obtain plasma or serum. When blood was derived from EDTA-containing tubes, the plasma and serum samples of the same donor gave results that were statistically different ($p=0.049$) (Figure 2A). On the other hand, samples derived from blood collected in heparin-containing tubes showed almost coincident concentrations of CXCL11/I-TAC in plasma and serum ($p=0.466$) (Figure 2B). Previous studies have indeed reported that samples derived from blood treated with the anticoagulant EDTA showed interference with immunoassays that use horseradish phosphatase for detection, probably because EDTA can chelate the Ca^{2+} necessary for the activity of this enzyme (Bhattacharyya *et al.*, 1994). For this reason, heparin appears to be a better anticoagulant to collect blood to determine CXCL11/I-TAC concentration in both plasma and serum.

However, it should be noted that the values of CXCL11/I-TAC were consistently higher in plasma/sera derived from heparin-treated blood (Figure 2B). In this regard, it has been reported that systemic heparinization rapidly increased plasma levels of all three CXCR3 ligands, including CXCL11/I-TAC (Ranjbaran *et al.*, 2006). The mechanism involved was displacement of chemokines from low-affinity receptors (glycosaminoglycans) present on endothelial cells. Thus, it could be hypothesized that a similar mechanism may occur within the heparin-containing tubes also with blood leukocytes.

Last, by comparing values of CXCL11/I-TAC in plasma and serum (both from blood drawn in heparin-containing tubes) from the eight donors, the coefficient of variation was 0.24 and 0.25 respectively. Thus, both serum and plasma appear to be appropriate biologic specimens to measure CXCL11/I-TAC content, given that the amount of

variation is comparable between the different sets of data collected.

The aim of our characterizing effort was to measure CXCL11/I-TAC in plasma derived from heparinized blood of certain chronically HIV-1-infected individuals undergoing a particular form of immunotherapy, myeloid cell purging (Hasson *et al.*, 2007), as compared to healthy donors.

For this purpose the medisorp plate was used and a statistically significant increase of CXCL11/I-TAC was found in the former (Biswas *et al.*, manuscript in preparation), underscoring the usefulness of our present study.

We believe that our findings will contribute practical information to those who wish to have reliable results measuring CXCL11/I-TAC with the existing ELISA kit in complex matrices such as human plasma and serum deriving from anticoagulant-containing blood.

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