

Quantification of Human Chemokine Production in TLR-Stimulated and Antigen-Specific Recall Responses

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Abstract

Chemokines are primarily low molecular mass proteins that are produced and usually released by a wide variety of cell types. Differential chemokine responses can be excellent early markers of immune dysfunction, allowing clinical intervention prior to expression of full blown undesirable effector responses. Thus, assessment of the nature and intensity of Ag-dependent chemokine production provides a valuable tool for probing human immune regulation.

Here, we provide detailed instructions on approaches we have developed to assess the nature and intensity of recall responses to a wide variety of exogenous and endogenous antigens capable of consistently stimulating chemokine responses by PBMC from adult and pediatric populations. This chapter is divided into two sections. The first is focused on culture techniques for eliciting antigen-driven chemokine responses for a panel of chemokines that are relevant to immune function. The second section details assay systems for their quantitative analysis.

Key Words: Chemokine; quantitation; chemoattractant; human; immunoassay; ELISA; primary culture.

1. Introduction

During the 1970s, culture supernatants from a wide variety of stimulated leukocytes were shown to contain factors that acted as selective chemoattractants for monocytes, granulocytes, or lymphocytes. The cloning of these chemoattractants and their receptors now reveals in excess of 50 chemokines and 20 receptors. Through the late 1990s, our initial understanding of the activity of chemokines – selective leukocyte recruitment – was expanded as it became recognized that chemokines, like classical cytokines, play many other roles in

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biology. Thus, chemokines are primarily low molecular mass proteins that are produced and usually released by a wide variety of cell types during ontogeny, homeostasis, the initial phase of host response to injury, allergens, antigens, or invading microorganisms, and during ongoing maintenance of immune responses.

One area in which study of chemokine production and responsiveness has made major contributions has been in understanding immune regulation. Much as certain patterns of cytokine gene expression are frequently associated with particular effector responses and subsequent clinical outcomes (i.e., increased IL-4, IL-5, IL-13 synthesis with allergen-specific IgE responses and immediate hypersensitivity), there is much interest in how differential chemokine production or receptor expression relates to immune regulation. Differential chemokine responses can provide excellent early markers of immune dysfunction (i.e., in chronic renal allograft rejection), allowing clinical intervention prior to expression of full blown undesirable effector responses. Thus, assessment of the nature and intensity of *Ag-dependent* chemokine production provides a valuable tool for probing human immune regulation.

A major advantage of examining chemokine production for analysis of human immune responses is that they are usually expressed at levels one to several orders of magnitude more intense than is seen with typical (Th1, Th2, Treg) cytokines. This greatly simplifies the demands on the assays used, improves the ability to obtain detectable responses in virtually every individual examined, and strengthens assay-to-assay reproducibility as the responses seen are usually well above detection limits.

The great majority of studies of human chemokine responses have utilized potent pharmacologic stimuli (i.e., PMA plus calcium ionophore) or polyclonal activators (i.e., LPS, anti-CD3). These stimulate intense production of many chemokines but the relationship between this response and the response elicited on antigen or allergen exposure remains speculative. Here, we provide detailed instructions on approaches we have developed to assess the nature and intensity of recall responses to a wide variety of exogenous (i.e., grass pollen, streptokinase, cat Ag) and endogenous (reovirus) antigens capable of consistently stimulating chemokine responses by PBMC from adult and pediatric populations.

This chapter is divided into two sections. The first is focused on culture techniques for eliciting antigen-driven chemokine responses for a panel of chemokines that are relevant to immune function. The second section details assay systems for their quantitative analysis.

2. Materials

2.1. Primary Culture of Fresh Human PBMC

Following study approval by the local Ethics Committee and written informed consent from each individual, 10–50 mL fresh peripheral blood is taken into

EDTA- or citrate-containing tubes (typically 2 mL of 2.5% EDTA for 40–50 mL blood) by venipuncture.

1. Polyclonal activators: anti-CD3 (30 ng/mL, BD Pharmingen) or TSST-1 (100 ng/mL, Sigma) provides excellent positive controls to assess the global capacity of an individual to generate the chemokine in question.
2. TLR ligands: TLR ligands act as excellent stimuli of chemokine production. Maximal responses by freshly obtained PBMC in primary culture are found using peptidoglycan (TLR2L) at 25 ng/mL, poly(I:C) (TLR3L) at 5 µg/mL, LPS (TLR4L) at 500 pg/mL, or TLR7 ligand 3M-011 at 50 ng/mL. In response to these stimuli, some chemokines are expressed at very high levels (i.e., MCP-1/CCL2, >500 ng/mL) whereas others are found at much lower levels (i.e., IP-10/CXCL10, ~2 ng/mL).
3. Antigen: In general we find that any antigen that elicits a detectable cytokine response in primary culture also elicits strong chemokine production. Relevant Ags we have used include streptokinase (Streptase; Aventis Behring), grass pollen (Bayer), and reovirus (American Type Culture Collection; atcc.org catalog # VR-824).
4. Miscellaneous reagents: Azide-free, low endotoxin anti-CD4, anti-CD8, anti-MHC class I or MHC class II, CTLA4-Ig fusion protein for cell culture can be obtained from multiple sources including BD Pharmingen.

2.2. Quantitation of Human Chemokine Responses

For antibodies and standards, we list three examples of suppliers below. Peprotech has a complete line, R&D and BD Pharmingen have many excellent reagents. We have obtained excellent results with several companies that supply similar materials. These reagents are available via the WWW from multiple sources including Peprotech (<http://www.peprotech.com>); BD Pharmingen (<http://www.bdbiosciences.com>), R&D Systems (<http://www.rndsystems.com>), and Serotec (<http://www.serotec.co.uk>). Prices vary considerably for products with very similar performance. Bear in mind that with polyclonal sources, the conditions we found to be optimal for these lots may vary somewhat from year to year as different animal pools are used. If you seek greater sensitivity (or lower cost), titrate the capture and development Abs twofold more concentrated/more dilute and extend the length of the color development phase to customize the assay for your purposes.

In some cases, hybridomas producing these Abs are available from the ATCC (<http://www.atcc.org>) providing a more cost-effective approach for labs which will make use of particular assays at high frequency. Here, we list combinations of clones and conditions that provide highly sensitive assays in our hands.

Establishing, validating, and optimizing one's own ELISA using commercial reagents takes a few days and is approximately 10 to 30-fold less expensive than buying kits. This is an important consideration if this technique is going to be commonly used in your laboratory.

Buffers: Coating, blocking, wash, and *p*-nitrophenyl phosphatase (PNPP) substrate buffers are prepared as detailed in Chapter 10.

2.2.1. Measurement of Human CCL2 (MCP-1) Levels

1. Capture antibody: mouse anti-human MCP-1 monoclonal (BD Pharmingen 23231D, clone 10F7.2, or Peprotech monoclonal or polyclonal).
2. Standard: recombinant human MCP-1 (BD Pharmingen or Peprotech).
3. Detection antibody: biotinylated mouse anti-human MCP-1 polyclonal (BD Pharmingen or Peprotech).

2.2.2. Measurement of Human CCL3 (MIP-1 α) Levels

1. Capture antibody: mouse anti-human MIP-1 α monoclonal (R&D Systems MAB670, clone 14215.41) or Peprotech monoclonal or polyclonal.
2. Standard: recombinant human MIP-1 α (PeproTech #300-08).
3. Detection antibody: biotinylated goat anti-human MIP-1 α polyclonal (R&D Systems BAF270 or Peprotech polyclonal).

2.2.3. Measurement of Human CCL17 (TARC) Levels

1. Capture antibody: mouse anti-human TARC monoclonal (R&D Systems MAB364, clone 54026.11) or Peprotech polyclonal.
2. Standard: recombinant human TARC (PeproTech #300-30).
3. Detection antibody: biotinylated goat anti-human TARC polyclonal (R&D Systems BAF364 or Peprotech polyclonal).

2.2.4. Measurement of Human CCL22 (MDC) Levels

1. Capture antibody: mouse anti-human MDC (R&D Systems MAB336, clone 57226.11) or Peprotech monoclonal or polyclonal.
2. Standard: recombinant human MDC (R&D Systems #336-MD).
3. Detection antibody: biotinylated chicken anti-human MDC polyclonal (R&D Systems BAF336 or Peprotech polyclonal).

2.2.5. Measurement of Human CXCL9 (MIG) Levels

1. Capture antibody: mouse anti-human MIG monoclonal (R&D Systems MAB392, clone 49106.11, or Peprotech monoclonal or polyclonal).
2. Standard: recombinant human MIG (PeproTech #300-26).
3. Detection antibody: biotinylated goat anti-human MIG polyclonal (R&D Systems BAF392 or Peprotech polyclonal).

2.2.6. Measurement of Human CXCL10 (IP-10) Levels

1. Capture antibody: mouse anti-human IP-10 monoclonal (R&D Systems MAB266, clone 33036.211, or Peprotech polyclonal).
2. Standard: recombinant human IP-10 (PeproTech #300-12).
3. Detection antibody: biotinylated goat anti-human IP-10 polyclonal (R&D Systems BAF266 or Peprotech polyclonal).

2.2.7. Measurement of Human CCL11 (Eotaxin) Levels

1. Capture antibody: mouse anti-human Eotaxin monoclonal (R&D Systems MAB320, clone 43911.11).
2. Standard: recombinant human Eotaxin (PeproTech #300-21).
3. Detection antibody: biotinylated goat anti-human Eotaxin polyclonal (R&D Systems BAF320).

3. Methods

3.1. Culture Conditions for Generation of Antigen or TLR-Driven Chemokine Responses

Peripheral blood is collected by venipuncture into a tube containing 2 mL of 2.7% EDTA, mixed immediately, and diluted by 1:2 (i.e., by adding 20 mL saline to 40 mL blood to increase the yield of PBMC recovered for culture), then mixed immediately by inverting the tube. PBMC are isolated by standard Ficoll procedures (Sigma).

After washing isolated PBMC twice with saline, the cells are resuspended in complete media, typically at 3×10^6 cells/mL. A total of 300,000 cells/well (96-well FB culture plate) are cultured with optimized antigen concentrations (predetermined by the investigator in preliminary experiments), to yield a final volume of 200 μ L in round bottom plates. Cells are cultured for 16 h to 7 d depending on the chemokine analyzed and stimulus used. Typically, CCL2 has rapid kinetics, with peak polyclonal or Ag-driven responses evident at 12–18 h. CXCL9, CXCL10, CCL17, and CCL22 exhibit maximal polyclonally stimulated responses at 1–3 d and Ag-specific responses at ~5 d of culture (*see Note 1*).

Supernatants are harvested and stored at -20°C until chemokine concentrations are quantified as described below.

3.2. Analysis of Human Chemokine Levels

1. The ELISA plate is coated with 50 μ L/well capture antibody in a high pH coating buffer at a predetermined optimal concentration. This is generally between 50 ng/mL and 2 μ g/mL (*see Note 2*). For maximum sensitivity, this incubation is carried out overnight at 4°C in tightly wrapped plates that are kept in a plastic box lined with wet paper towels, to minimize evaporation that would lead to variability in edge wells (*see Note 3*).
2. Blocking, using 75 μ L/well, is carried out at 37°C for 1–3 h (*see Note 4*). We find no difference in background between these times. This time period is used to prepare samples for the next step. Wash four times manually or using plate washer. Afterwards, bang plate dry three times on paper towels.
3. The standard curve is generated on each assay plate using recombinant chemokine standard in dilution buffer (*see Note 5*). The titration range is determined by the anticipated intensity of one's experimental values. Commonly, titration curves run from 2000 to 15 pg/mL.

4. Each experimental sample should be titrated in dilution buffer from an empirically determined starting dilution (typically between 1:5 and 1:20) for a series of four twofold 50- μ L dilutions running down the plate (i.e., 1:2, 1:4, 1:8, 1:16) (*see Note 6*). This allows ~20 samples per plate. The initial dilutions should not sit in the starting well for more than 5 min before carrying out this titration or the resulting curves will not be linear.
5. Sample incubation can be 3 h at 37°C or overnight at 4°C. Maximal sensitivity is obtained following overnight incubation. Wash four times, then bang plates dry on paper towels.
6. Biotinylated development antibody in dilution buffer is added at a predetermined optimal concentration, generally between 50 ng/mL and 2 μ g/mL, using 50 μ L/well. Incubation can be 3 h at 37°C or overnight at 4°C. Maximal sensitivity is obtained following overnight incubation. Wash plates four times, then bang dry on paper towels.
7. Following washing, streptavidin–enzyme conjugate is added at a predetermined optimal concentration to maximize signal while minimizing background. In our experience, streptavidin-alkaline phosphatase used at 1:1000 to 1:5000 with 50 μ L/well is useful. This incubation (45 min at 37°C) is followed by washing four times, banging dry, then adding 50 μ L/well PNPP solution. This should be prepared no more than 30 min before use by adding one PNPP tablet to 5 mL of substrate buffer at room temperature (Sigma #S0942-200TAB).
8. Progress of the assay is monitored visually, with readings (405–690 nm) taken at intervals between 45 min and 6 h of assay development, depending on the sensitivity required. Running this phase of the assay at 37°C accelerates color development by ~40%. The standard curve should have an absorbance beginning from approximately 3.0 OD to obtain maximal sensitivity and precision.
9. A standard curve is prepared using the data obtained by serial dilution of the standard, with the chemokine concentration on the x axis (log) and the absorbance on the y axis (linear) to generate a sigmoidal curve.

When carrying out sample data analysis, the mean of at least two points (and generally three or four) falling on the linear component of the standard curve of that plate is required. These calculated values should not differ from one another by more than ~15–20%, typically much less. Mean values + standard error should be reported. Interassay variability (the same samples titrated in an independent assay on another day) should be explicitly determined for a proportion of the samples to confirm reliability of the data/operator/assay. Sensitivity is defined in each assay as the beginning of the linear portion of the titration curve (*see Note 7*).

The relationship between expression of the chemokines discussed below and specific immune responses is a rapidly developing field and should be considered in light of recent literature. At present, CXCL9 and CXCL10 are considered associated with expression of Th1-like immunity (along with receptors CCR5 and CXCR3); CCL17 and CCL22 with Th2-like responses

(along with receptors CCR3, CCR4, CCR8); and CCL2, CCL17, and CCR21 with cells exhibiting regulatory T-cell-like activity. This list is not exhaustive, nor is expression of these ligands solely restricted to expression of that specific form of immunity.

Sample data for representative antigen-dependent chemokine responses to exogenous and endogenous Ags are provided in Fig. 1A,B respectively.

3.3. Detailed ELISA Protocols

3.3.1. CCL2 (MCP-1)

1. Following the protocol for ultrasensitive ELISA (*see* **Section 3.2.**), use the capture antibody, polyclonal anti-human MCP-1, at 1 µg/mL (either Peprotech or BD).
2. For the standard, use human rMCP-1 and titrate in eight twofold dilutions from 4000 to 31.3 pg/mL.
Carry out a titration curve of antigen or polyclonally stimulated samples four times starting from 1/50 (i.e., 1/50, 1/100, 1/200, 1/400).
3. Use the detection antibody, biotinylated mouse polyclonal anti-human MCP-1, 50 ng/mL (Peprotech) or 250 ng/mL (BD).
4. Read plate after 3 h with development substrate. Sensitivity should be at least 31 pg/mL.

3.3.2. CCL3 (MIP-1 α)

1. Following the protocol for ultrasensitive ELISA (*see* **Section 3.2.**), use the capture antibody, polyclonal anti-human MIP-1 α , at 350 ng/mL.
2. For the standard, use human recombinant MIP-1 α and titrate from 4000 to 31.25 pg/mL.
3. Dilute antigen and polyclonally driven samples 1/10.
4. Use the detection antibody, biotinylated goat anti-human MIP-1 α , at 15 ng/mL.
5. Read plate after 5–6 h with substrate for sensitivity of 31.3 pg/mL.

3.3.3. CCL17 (TARC)

1. Following the protocol for ultrasensitive ELISA (*see* **Section 3.2.**), use the capture antibody, polyclonal anti-human TARC, at 250 ng/mL (Peprotech or R&D Systems).
2. For the standard, use human recombinant TARC and titrate from 500 to 3.9 pg/mL.
3. Dilute antigen and polyclonally driven samples 1/10.
4. Use the detection antibody, biotinylated goat anti-human TARC, at 50 ng/mL (R&D Systems or Peprotech).
5. Read plate after 3 h with substrate for sensitivity of 3.9 pg/mL.

3.3.4. CCL22 (MDC)

1. Following the protocol for ultrasensitive ELISA (*see* **Section 3.2.**), use the capture antibody, polyclonal anti-human MDC, at 0.1 µg/mL (Peprotech) or 0.5 µg/mL (R&D Systems).

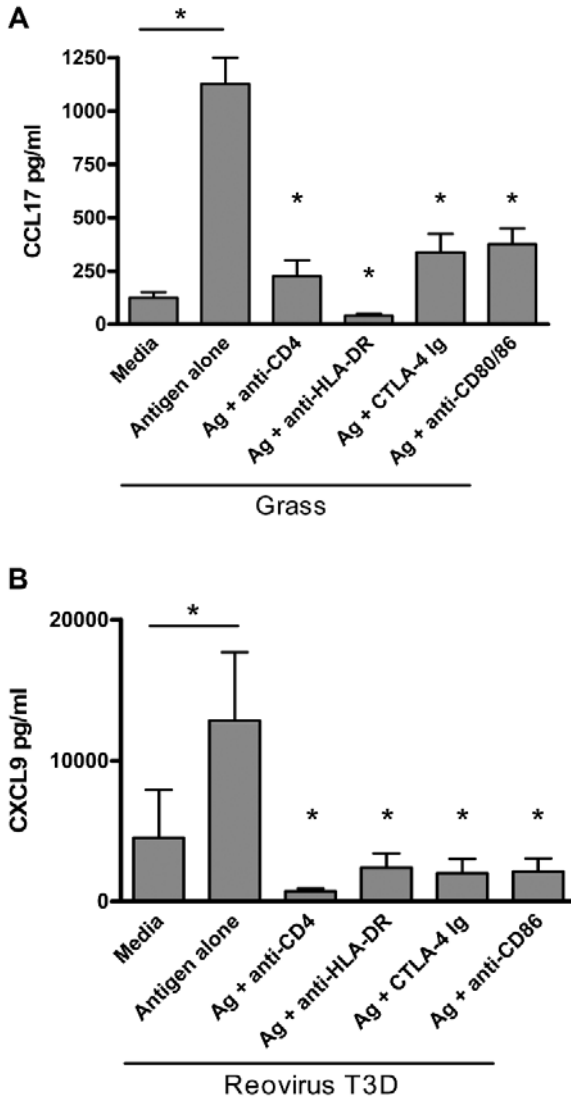


Fig. 1. (A) Antigen-driven chemokine production is CD4 T-cell dependent, requires CD28:CD80/86 costimulation and MHC class II dependent Ag-presentation. Freshly derived PBMC from 10 and (B) 5 adult volunteers were cultured 5 d as described, with grass pollen Ag at 50 $\mu\text{g/mL}$ (A) or reovirus T3D (2×10^6 pfu/well) either alone, or in the presence of anti-CD4 (BD Pharmingen 2 $\mu\text{g/mL}$), anti-HLA-DR(2 $\mu\text{g/mL}$), anti-CD80 or anti-CD86 (1 $\mu\text{g/mL}$) or CTLA4-Ig (5 $\mu\text{g/mL}$). Chemokine responses were assessed by ELISA as described above. Significance was determined using a paired *t*-test (**p* < 0.01).

2. For the standard, use human recombinant MDC and titrate from 1000 to 7.8 pg/mL.
3. Dilute antigen and polyclonally driven samples 1/10.
4. Use the detection antibody, biotinylated chicken anti-human MDC, at 20 ng/mL (R&D Systems or Peprotech).
5. Read plate after 1 h with substrate for sensitivity of 7.8 pg/mL.

3.3.5. CXCL9 (MIG)

1. Following the protocol for ultrasensitive ELISA (*see Section 3.2.*), use the capture antibody, polyclonal anti-human MIG, at 200 ng/mL (Peprotech) or 0.350 ng/mL (R&D Systems).
2. For the standard, use human recombinant MIG and titrate from 4000 to 31.25 pg/mL.
3. Dilute antigen and polyclonally driven samples 1/10.
4. Use the detection antibody, biotinylated goat anti-human MIG, at 15 ng/mL (R&D Systems or Peprotech).
5. Read plate after 3–5 h with substrate for sensitivity of 31.25 pg/mL.

3.3.6. CXCL10 (IP-10)

1. Following the protocol for ultrasensitive ELISA (*see Section 3.2.*), use the capture antibody, mouse anti-human IP-10, at 50 ng/mL (Peprotech) or 400 ng/mL (BD).
2. For the standard, use human recombinant IP-10 and titrate from 1000 to 7.8 pg/mL.
3. Dilute antigen and polyclonally driven samples 1/10.
4. Use the detection antibody, biotinylated goat anti-human IP-10, at 25 ng/mL (Peprotech) or 50 ng/mL (BD).
5. Read plate after 3–4 h with substrate for sensitivity of 7.8 pg/mL.

3.3.7. CCL11 (Eotaxin)

1. Following the protocol for ultrasensitive ELISA (*see Section 3.2.*), use the capture antibody, mouse anti-human Eotaxin, at 350 ng/mL.
2. For the standard, use human recombinant Eotaxin and titrate from 500 to 3.9 pg/mL.
3. Dilute plasma samples 1/4.
4. Use the detection antibody, biotinylated goat anti-human Eotaxin, at 50 ng/mL.
5. Read plate after 3 h with substrate for sensitivity of 3.9 pg/mL.

4. Notes

1. It is important to assess the levels of endotoxin contamination in the antigen preparation used. Some have very low levels whereas others (i.e., some commercial preparations used clinically for skin testing) have levels sufficient to elicit strong Ag-nonspecific chemokine production from mononuclear cells. To determine the extent to which chemokine production reflects Ag-specific induction, parallel cultures should be carried out incorporating validated inhibitors of classical T-cell activation (i.e., anti-CD4, anti-CD8 mAb, CTLA4-Ig). In our hands, use of these reagents reduces chemokine production to <10% of that seen in Ag-stimulated controls, indicating that the chemokine production quantified is truly Ag dependent.

2. A variety of high-binding EIA plates are commercially available. We have found substantial differences in their ability to bind capture antibody or Ag. Costar 3369 plates work effectively.
3. Spurious values that are too high and do not agree with the rest of the titration curve result from evaporation in wells at the edge of the plate. The solution is to ensure that plates are kept tightly wrapped in a humidified chamber (plastic box with wet paper towels) for all incubations. Also, if samples are allowed to sit in plate for an extended period prior to carrying out the dilutions, substantial chemokine will be bound, resulting in a nonlinear titration curve.
4. A broad variety of blocking reagents (skimmed milk, bovine serum albumin (BSA), or gelatin) can be used. Recommended wash buffers sometimes utilize very high concentrations of BSA and Tween-20. In our experience, BSA at 0.17% (1.7 g/L) in PBS with 0.05% Tween-20 is sufficient to minimize backgrounds with less cost and pollution of the environment than buffers containing up to 5% BSA and 2% Tween-20.
5. Biological standards are calibrated in units of biological activity and usually established by consensus following exhaustive collaborative studies involving many laboratories. For cytokines, international standards should be used to provide a common currency against which all results can be evaluated. When estimating the biological activity of different preparations with different specific activities by bioassay, mass units are not useful as different commercial preparations vary widely in specific activity. Unfortunately, there are currently no international standards available for chemokines, so most laboratories report their data in mass units. Many laboratories choose to utilize recombinant standards from well-known manufacturers (i.e., Peprotech, BD Pharmingen, R&D Systems) and express their data in mass units as measured against that commercial preparation (and that lot). This makes possible comparison between experimental groups, but greatly complicates comparisons between laboratories.
6. If experimental values for a given sample do not agree throughout the titration range, it is possible that the dilution series selected does not fall within the linear range of the assay. The starting titration of the samples needs to be adjusted so that more of the points lie on the linear component of the standard curve. The most common reason for duplicates which do not agree is poor titration precision/operator error.
7. When assay sensitivity is not sufficient, the first step is to compare the sensitivity of the standard curve generated with those seen in the literature or those listed above. Assuming one obtains similar sensitivity, an estimate of the levels expected in your samples is required. Most chemokines are produced at substantial levels but some (i.e., Eotaxin) are not seen on stimulation of PBMC. A few additional strategies are useful to further enhance sensitivity. The assays described above are configured using minimum concentrations of these reagents in order to minimize cost. Use of higher concentrations of capture and development reagents often increases sensitivity by one or twofold. Similarly, there is a linear relationship between how long an ELISA is allowed to develop and the sensitivity of that assay.

Thus, chemokines such as CCL2 are rapidly produced at high concentrations (ng/mL levels) following stimulation with recall antigens, whereas chemokines such as CXCL10 or CCL17 are more tightly regulated. For chemokines produced at levels where sensitivity is a major issue, the assay is allowed to develop for up to 6 h. In all cases, the greater the difference between the absorbance of the maximum concentration of the standard and the background (all reagents except the recombinant chemokine standard), the better the precision of the assay.