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## LYMPHOCYTE SUBPOPULATIONS IN HUMAN EXPOSURE TO METALS

(IUPAC Technical Report)

*Prepared for publication by*

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# Lymphocyte subpopulations in human exposure to metals

## (IUPAC Technical Report)

*Abstract:* Numerous species of metal ions cause immunosensitization in humans. Possible approaches to determine those occupational and environmental exposures to metals that result in immunological changes include lymphocyte transformation assay, cytokine profiling, and measurement of lymphocyte subpopulations. In two previous papers, we considered lymphocyte transformation assay [1] and cytokine profiling [2]. Here we review the effects of exposures to metals on lymphocyte subpopulations. Specific consideration is given to beryllium, chromium, cobalt, nickel, palladium and platinum, cadmium, gold, mercury, and lead. Analysis of the scientific literature shows that immunosensitizing metals may have influences on the lymphocyte subset composition, but only in a few instances does exposure to metals cause reproducible shifts of lymphocyte subpopulations. If lymphocyte subpopulations are analyzed, each diagnostic step, including indication, sample handling, analytic procedure, and data interpretation, should adhere to good quality assurance and quality control.

*Keywords:* immunosensitization; lymphocyte; exposure to metals; immunosensitizing metals; lymphocyte subpopulations.

### 1. INTRODUCTION

Occupational and environmental illnesses are often associated with alterations in the immune system [3,4]. One way in which these alterations may be manifest is through changes in the relative proportions of subpopulations of lymphocytes, which are defined by the expression of specific surface markers [5]. They can be measured by flow cytometry, a useful additional method in some settings that provides complementary information to the lymphocyte transformation test (LTT) or the determination of cytokine profiles discussed earlier in this project [1,2]. For a brief description of flow cytometry, see Section 6.

The major subgroups of lymphocytes, namely T, B, and natural killer (NK) cells, can be distinguished by surface markers such as CD3, CD4, CD8, CD19, and CD56. The relative proportions of these subpopulations can change in various clinical situations, such as innate or acquired immune deficiencies (including HIV infections) [6,7], or autoimmune diseases and allergies [8–10]. Moreover, determination of the immunophenotype by means of further surface markers helps to differentiate between acute lymphatic and acute myeloid leukemia and is important in the classification of lymphomas [11,12]. This report discusses the importance of the determination of lymphocyte subpopulations in occupational and environmental medicine with special reference to immunological alterations caused by metals, and concludes that the utility is much more limited than in established clinical disease settings such as leukemia.

### 2. CLUSTER OF DIFFERENTIATION (CD) NOMENCLATURE

All cells express surface proteins that can be identified by use of specific antibodies and may reflect certain stages in the cell's development. Monoclonal antibodies that recognize specific surface structures have allowed assignment of these structures by international workshops to a "cluster of differen-

tiation" (CD) family. Thus, a common classification of surface molecules has arisen, although these have been given different primary names in various scientific disciplines. The first such workshop was held in 1982 and was intended to classify leukocyte antigens. The classification has now expanded to other cell types. The most recent (8<sup>th</sup>) Workshop and Conference on Human Leukocyte Differentiation Antigens (HLDA8, 2004) allocated 95 new CD designations. HLDA8 has now been succeeded by the "Human Cell Differentiation Molecules" (HCDM) as the HLDA Workshops have recognized for some time that leucocytes do not act alone, but rather markers on endothelial cells, stromal cells, and intracellular markers of differentiation, particularly for immunohistochemical studies, must be considered. The number of CD-designated molecules has now reached 350 [13].

A CD number can be assigned to a protein antigen when two specific monoclonal antibodies have been produced that recognize it. Some CD-designated proteins are highly lymphocyte-specific. For example, CD45 (type I leukocyte common antigen) is found on all leukocytes and is characteristic of them, whereas CD4 [a major histocompatibility complex (MHC) class II co-receptor] and CD8 (an MHC class I co-receptor) are characteristic of helper and suppressor T lymphocytes, respectively. Other proteins assigned a CD number have a much broader distribution and are better known by other names. Thus, for example, the transferrin receptor-1 is designated CD71, and CD54 is perhaps better known as the intercellular adhesion molecule ICAM-1. The various integrin subunits involved in matrix adhesion are also assigned CD numbers.

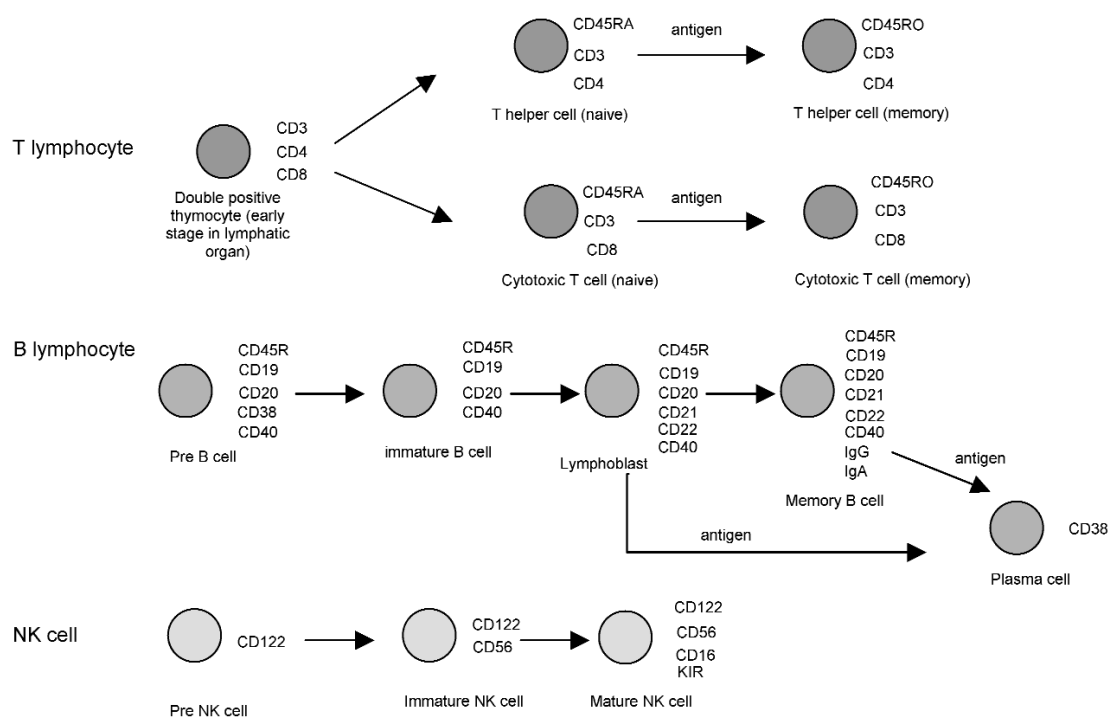
### 3. LYMPHOCYTE SUBPOPULATIONS

The various leukocyte subtypes were originally distinguished on the basis of histomorphology, but now they are more definitively identified by CD surface markers [14,15]. Appending + and – is generally used to indicate the presence or absence of a marker. Thus, in addition to being CD45+, granulocytes are CD15+, monocytes CD14+, and thrombocytes CD61+. With regard to the lymphocytes, the major distinction is that B lymphocytes are CD45+CD19+ and T lymphocytes are CD45+CD3+. CD19 serves as an accessory molecule in B cell signal transduction and is found in all B cell neoplasms. CD3 functions in T cell signaling and is positive in T cell lymphomas. T lymphocytes can be further subdivided into T helper (TH) cells (CD45+CD3+CD4+), cytotoxic T cells (CD45+CD3+CD8+), and activated T lymphocytes (CD45+CD3+CD25+). CD4 and CD8 serve in antigen recognition, and CD25 forms part of an interleukin-2 (IL-2) receptor. Surface markers are also used to characterize T cell production during hematopoiesis. CD3, CD4, CD8, CD19, and CD25 are all type I transmembrane proteins. Mature NK cells can be defined as CD16+CD56+CD3–. CD16 is a receptor for IgG involved in NK cell activation. CD56 functions in cell adhesion and is also known as neural cell adhesion molecule (NCAM). The major lymphocyte-associated markers are summarized in Fig. 1.

*T lymphocytes (T cells)* have T cell receptors (TCRs) that recognize peptides presented to them in conjunction with the MHC by antigen presenting cells. They also recognize other antigens such as glycolipids complexed with CD1 on NK cells [16]. The TCR forms a complex with the T cell marker CD3. T cells can be subdivided into functional subpopulations not only according to different surface (i.e., CD) molecules, but also on the basis of intracellular markers, and the secretion of cytokines. As noted above, however, the most important subpopulations are defined by the surface markers CD4 (TH cells) and CD8 (cytotoxic T cells). CD4 is the ligand associated with MHC-II, and CD8 with MHC-I.

Naive CD4+ T cells that have not yet been in contact with antigen can be differentiated from those "memory" T cells that have been exposed, based on CD45 isoforms. Namely, CD45RA is a marker for naive cells and CD45RO for memory cells, although additional markers such as CD27 and CD28 for naive cells [17] and CD29 for memory cells are required for more careful definition.

Subpopulations of TH cells exist, and the best characterized are the TH1 and TH2 cells [18–20]. The best means of distinguishing these cells is probably by their cytokine production. The TH subpopulations differentiate from IL-2-secreting precursor TH0 cells. TH1 cells produce interferon-gamma



**Fig. 1** Some relevant surface markers for the determination of lymphocyte subsets.

(IFN- $\gamma$ ), IL-2, and tumor necrosis factor-beta (TNF- $\beta$ ), while TH2 cells secrete IL-4, IL-5, IL-10, and IL-13. TH1 cells initiate cytotoxic reactions in response to intracellular pathogens, whereas TH2 cells are involved in defense against extracellular pathogens and soluble antigens. Thus, TH2 cells activate B cells to produce IgE and other immunoglobulins, and they also activate eosinophils. A summary of cytokines secreted by various lymphocyte populations can be found in [2,21].

At present, then, the best way to distinguish TH1 from TH2 lymphocyte subpopulations is to measure their cytokine secretion into the supernatants by enzyme-linked immunosorbent assay (ELISA) or to detect the intracellular cytokines by flow cytometry. Moreover, flow cytometry can be used to detect the presence of defined TH1- or TH2-related chemokine receptors (CXCR3, CXCR5 on TH1 cells; CCR4, CCR3 on TH2 cells).

Recently, the existence of a further TH subtype has become apparent, the TH17 cells. These are characterized by the secretion of IL-17. Similarly to the TH1 cells, they seem to play an important role in several chronic inflammatory disorders [22]. Subpopulations of CD8+ cytotoxic T cells also occur with different patterns of cytokine expression [23,24], but these are less well defined.

Regulatory T cells have been shown in the last few years to be important cells with suppressive properties [26–30]. Three major types, CD4+CD25+ T cells, TH3 cells, and T regulatory type 1 (Tr1) cells have been described, but further subtypes may exist. The CD4+CD25+ T cells represent a naturally occurring population which seems to be involved in maintaining tolerance and preventing autoimmune reactions (e.g., diabetes mellitus in an animal model). They are characterized by the expression of Foxp3. The TH3 cells secrete TNF- $\beta$ , which has strongly suppressive functions, and Tr1 cells produce IL-10, which suppresses TH1 and TH2 activity.

*Natural killer cells (NK cells)* are cytotoxic cells that belong to the innate immune system. They carry the Fc receptor for IgG (CD16), the adhesion molecule CD56, the IL-2 and IL-15 receptor (CD 122) and “natural cytotoxicity receptors” (NCRs), and are able to kill tumor cells and virally infected

cells without requiring prior sensitization or further maturation [31,32]. Human NK cells express two structurally distinct types of inhibitory receptors with different specificities, namely “killer cell-inhibitory receptors” (KIRs) for MHC-I [33,34] and “killer cell lectin-like receptors” (KLRs) [35]. Genetically determined, activating isoforms of KIR sometimes occur and are termed “killer cell-activating receptors” (KARs) [33,34,36]. The levels of expression of activating and inhibitory receptors, together with the concentrations of their corresponding ligands, determine the cytolytic activity of NK cells. Predominance of an inhibitory profile suppresses NK cell activity, whereas a predominance of activating signals initiates the cytolytic program [37].

Subpopulations have been identified that express markers for both NK and T cells. Thus, CD3+CD56+ defines a T cell that is rare in peripheral blood (1–5 % of the total lymphocyte population) and is not MHC-restricted [38,39]. Another rare cell, the so-called NK-T cell, is characterized by coexpression of the NK-receptor NK1.1 (CD161 or NKR-P1) and a TCR with a diminished repertoire.

*B lymphocytes (B cells)* are CD45R+CD19+ cells that are further characterized by surface expression of CD20 and CD21 [40]. CD45R is a B cell-specific form of the CD45 protein originally known as the common leukocyte antigen. It is expressed throughout B cell development from pro-B cells up to the antibody-secreting plasma cells; it functions in B cell signaling. CD19 is expressed on all B cells from an early stage in their development and seems to contribute to signaling through the B cell receptor. CD20 is a type III transmembrane protein that forms a channel for Ca<sup>2+</sup> entry during B cell activation. CD21 is a type I transmembrane protein involved in signal transduction. CD22 is a B cell-restricted sialoglycoprotein present in the cytoplasm of virtually all B lineage cells but expressed on the B cell surface only at mature stages of differentiation. B cells are important for the specific humoral immune response and carry clonotypic, surface-bound immunoglobulins, which function as receptors for antigens. These are associated with the signal-transducing molecules Ig- $\alpha$  (CD79a) and Ig- $\beta$  (CD79b). Upon direct contact with the antigen, B cells can develop into either long-lived B memory cells or plasma blasts. Antigen-specific T cells may also participate in this process. The memory cells remain in circulation and in lymphatic organs, where they are prepared for a rapid defense (production and secretion of antibodies) upon re-exposure, whereas the plasma blasts migrate into bone marrow where they proliferate and undergo final differentiation into plasma cells. The stages of development and differentiation of B memory cells and plasma cells are characterized by the expression of different surface markers.

The state of activation of T, B, or NK cells can be determined by the measurement of their specific activation markers [25]. These include CD69, CD25, CD71, and HLA-DR on CD4+ and CD8+ lymphocytes.

Typical ranges for reference values of the most common lymphocyte subpopulations are listed in Table 1.

**Table 1** Typical reference ranges of the major lymphocyte subpopulations in the blood of adults (according to [107]).

Cell type	Surface molecule	Percentage of total lymphocyte population
T cells	CD3	69–93
T helper cells	CD4	35–72
Cytotoxic T cells	CD8+	7–33
Naive T cells	CD4, CD45RA	14–43
Memory T cells	CD4, CD29, CD45RO	11–38
Activated T cells	CD3, CD25	1–7
	CD3, HLA-DR	1–11
Mature B cells	CD19	3–13
Immature B cells	CD19, CD5	0–2
	CD19, CD10	0–2
NK cells	CD16, CD56	3–31
	(lacking CD3)	

#### 4. CONSIDERATIONS WHEN MEASURING LYMPHOCYTE SUBPOPULATIONS

When studying lymphocyte subpopulations, only lymphocytes in peripheral blood are sampled. The whole blood volume contains about  $10^{10}$  lymphocytes, but these represent only about 2 % of the total lymphocyte pool in the human body, because most of the cells are in the lymphatic organs. Various physiological factors (stress, physical exertion, age, ethnicity, circadian rhythms, and medication) may affect the distribution of lymphocytes between blood and lymphatic tissue, as well as the pattern of lymphocyte subpopulations. Various disease states will also influence the subpopulation patterns. Therefore, a number of confounding factors may influence the interpretation of the measurement of lymphocyte subtypes.

Many basic studies concerning the effect of metals on lymphocyte subpopulations have been carried out with laboratory animals and cell culture systems. These results cannot be directly extrapolated to humans, because of the distinct species differences in the immune system, and the complexity of the human immune system with its responsiveness to exogenous and endogenous (endocrinological, neurological, emotional) influences. Therefore, extrapolation from effects in animals must be confirmed by human studies.

#### 5. EFFECT OF OCCUPATIONAL AND ENVIRONMENTAL METAL EXPOSURES ON LYMPHOCYTE SUBPOPULATIONS

In principle, environmental chemicals might influence lymphocyte subpopulations in one of two ways. They may act directly as antigens. Alternatively, they may be immunomodulatory, influencing the differentiation and/or activation of lymphocytes, and having either stimulatory or suppressive effects. Although these alternatives differ in their mechanisms and dose–response relationships, both can result in either a shift of subpopulations or in a change of markers of activation. Results of relevant studies are presented in Table 2 and discussed below.

**Table 2** Selected literature on the effect of metals on lymphocyte subpopulations (adapted from [4]).

Metal	Type of study	Type of exposure	Type of study group/investigated material	Effects on lymphocyte subpopulations	References
Beryllium	In vivo	Exposure	Patients with chronic Be disease	Detection of Be-specific CD4+ T cells in bronchial fluid, detection of circulating Be-specific CD4+ and CD8+ cells in blood	[41,45,46]
Chromium/chromate [heptaoxidodichromate]	In vivo	Exposure at workplace and smoking	Cr workers	Decrease of CD4+ and CD8+ T cells	[52,108]
		Dust exposure	Workers exposed to hexavalent Cr and "normal population", exposed to trivalent Cr	Exposure to trivalent Cr causes an increase of NK, B, and T cells in correlation with urine and blood levels of Cr	[109]
		Exposure at workplace	46 workers of a galvanic plant in Taiwan	Decrease of B cells	[110]
	In vitro	Chromium nitrate	Peripheral blood mononuclear cells (PBMCs) of healthy blood donors	No effect on NK cells	[111]
Nickel	In vivo/ in vitro		PBMCs of patients with Ni allergy	Ni-reactive T cells are CD4+CLA+ memory cells and express the chemokine receptors CXCR3, CCR4, CCR10, but not CCR6.	[60]
		In vitro	PBMCs of mouse	Ni salts destroy T lymphocytes of mouse in cell culture.	[112]
		24 h culture with Ni salts	PBMCs of healthy blood donors	Decrease of CD4+ T and NK cells, no effect on CD3, CD8, CD20, CD11a	[59]
Cadmium	In vivo	Workplace Environment	Exposed workers 1561 exposed, 480 nonexposed individuals	No effect on NK cells	[70]
				In adults with Cd levels in urine >1.5 µg/dL increase of B cells; combined effects of Pb and Cd	[69]
	In vitro	0–100 mg/L CdCl <sub>2</sub>	PBMCs from rats	Low dosage: (5–10 mg/L): decrease of B and T cells (CD4 and CD8); dosage >25: increase of B and T cells	[113]
		Cd sulfate and other Cd compounds Comparison of Cd with Zn	PBMCs of healthy blood donors	No effect on NK cells	[111]
			10 <sup>-3</sup> to 10 <sup>-8</sup> mol/L: increased expression of activity marker CD69; 10 <sup>-10</sup> mol/L strong immunosuppression	[68]	
Gold	In vivo	Chronic exposure	80 patients with rheumatic arthritis	No effect on CD4/CD8 ratio	[114]
	In vitro	Exposure in vitro	PBMCs of healthy donors	Inhibition of B cells at very low concentrations	[115,116]

(continues on next page)

**Table 2** (Continued).

Metal	Type of study	Type of exposure	Type of study group/investigated material	Effects on lymphocyte subpopulations	References
Mercury	In vivo	Low doses, e.g., after removal of amalgam fillings	Man	Low doses have no effect on CD4+, CD8+, or B cells.	[74]
		Exposure	81–101 workers exposed to Hg vapor, 36 nonexposed workers	Increase of CD4+, CD8+ cells, no effect on NK cells	[75,117,118]
		Exposure	20 exposed workers, 20 nonexposed individuals	Decrease of CD4+ cells and NK cells	[76]
		Exposure	19 exposed, 25 nonexposed persons	Decrease of NK cells and monocytes	[77]
Lead	In vivo	Exposure, blood concentration 75 ± 18 µg/dL	Exposed industrial workers	Reduced TH cells, no influence on CD8+, B, and NK cells.	[80]
		Exposure due to contaminated soil	1561 exposed, 480 nonexposed	No difference with regard to immune markers, but in children <3 years and with Pb in blood >15 µg/dL increase of B cells	[69]
		Exposure, blood level 7–50 µg/ml	71 lead workers, 28 healthy blood donors	Decrease of CD3+CD45RO+ cells, decrease of NK cells, increase of CD8+ T cells at levels >20 µg/dL	[85,86]
		Exposure	Long-time workers in a battery manufacturing plant and teachers as controls	Decrease of B cells, monocytes, granulocytes, lymphocytes, CD8+ cells	[82]
	In vitro		PBMCs of healthy blood donors	No effect of NK cells	[111]

## Beryllium

Berylliosis is a beryllium-induced chronic granulomatous lung disorder that may develop after long-lasting inhalational exposure: it has considerable relevance in occupational medicine. CD4+ T cells play an essential role in berylliosis [41]. The proliferative capacity of lymphocytes from berylliosis patients correlates with the fraction of memory T cells in the T cell population [42]. Beryllium-specific CD4+ T cells can be detected in the granulomas, and beryllium-specific CD4+ and CD8+ T cells are found in the peripheral blood of the patients [43]. Berylliosis appears to be associated mainly with HLA-DP molecules, which exhibit a specific affinity to beryllium, probably due to a specific glutamate residue [44], and which present it to CD4+ T cells [45–48]. Thus lymphocyte subpopulations have been measured and beryllium-specific lymphocytes detected, but no meaningful population shifts have been documented.

## Chromium

Chromium acts as an unspecific irritant. It can induce immune reactions, resulting in allergic dermatitis of the delayed type. Sensitization and clinical signs are often observed in connection with the handling of cement and with tanned leather products. Immunostimulatory and immunoinhibitory features of chromium can depend on the chemical species; Cr<sup>VI</sup> penetrates the skin more readily than Cr<sup>III</sup>, and

hexavalent chromium compounds are generally stronger sensitizers than trivalent ones [49,50]. Urinary total chromium in an urban population of non-atopic women correlated with an increase of the NK, B, and T cells [51]. On the other hand, Tanigawa et al. [52] found a decrease of CD4+ and CD8+ T cells in chromate-exposed workers.

### **Cobalt**

Cobalt and chromium are the next most common metals, after nickel, to cause sensitization. The symptoms of cobalt allergy include allergic dermatitis after dermal contact and occupational asthma after long-lasting inhalational exposure. Workers in the cement industry were patch tested for sensitivity to cobalt and dichromate (i.e., heptaoxidodichromate) [53]. Upon retesting 2–6 years later, some had a diminished or absent response despite continuation of exposure. CD4+ lymphocytes reactive to the sensitizing metal were isolated from all patients. However, in patients who remained patch test-positive these were CD4+CD45RA+. Patients with failed hip prostheses and increased serum levels of cobalt and chromium had decreased counts of lymphocytes and CD16+ cells, but there was no correlation between cobalt levels and decreased lymphocytes [54]. On the other hand, only in patients with revision surgery and high chromium levels was there a decrease in CD4+CD16+ cells. T cell clones were isolated from synovial tissue of a patient with a failed cobalt alloy joint prosthesis [55]. Only cobalt-specific (but not nickel-specific) clones were identified, and they were all CD4+.

### **Nickel**

Nickel is the most common immunosensitizer of all metals [56]. In the past, nickel allergies were observed mainly in the workplace. However, allergies also occur in non-occupationally exposed persons, mainly in women. In part, this is due to the sensitizing effect of nickel in jewelry. Allergic reactions may also occur upon oral nickel exposure. The effects of nickel on the immune system have been studied in more detail than those of most other metals (reviewed in [57,58]). Nickel damages human and mouse lymphocytes *in vitro*, depending on dose and cell subtype [59], but it is not clear if these data can be extrapolated to clinical nickel allergy. Nickel-sensitive cells in the peripheral blood are mainly CD4+ TH1 cells [60]. Di Gioacchino et al. [61] studied patients with nickel allergic contact dermatitis with a 10-mg oral nickel challenge. Patients who experienced a flare-up of cutaneous symptoms after the oral challenge had CD45RO+ T cells in the intestinal mucosa, which were not observed in control patients. In a similar study, decreases in CD3+CD45RO+ and CD8CD45RO+ lymphocytes in blood were found in responsive patients, implying an increased migration of these cells to the periphery [62]. In addition, increased serum IL-5 suggested activation of TH2 cells. Among women living in an urban environment, urinary nickel was correlated with an increased ratio of CD4+CD45RO+ to CD4+CD45RO- cells, suggesting that nickel enhances maturation of memory cells [51].

### **Platinum and palladium**

Platinum and palladium are used as catalysts, and the environmental impact of their use and recycling in automobile catalytic converters has generated interest. Hexachloroplatinates are used as catalysts in the manufacture of silicone breast implants, where immunological effects have been debated. Chemotherapeutic platinum compounds expose not only the patient but also potentially health care professionals.

Sensitized subjects from a precious metals refinery with work-related asthma and a positive skin-prick test to sodium hexachloroplatinate were studied by Raulf-Heimsoth et al. [63]. After *in vitro* stimulation of peripheral blood lymphocytes with hexachloroplatinate, an increase in CD3+ cells was found with an increased frequency of TCRs V $\alpha$ 2a, V11, and V $\beta$ 21.3 in sensitized individuals compared to controls. Bamias et al. [64] studied lymphocytes in ascites fluid of ovarian cancer patients and found

increased accumulation of CD3+CD56+ cells in patients who had developed resistance to platinum chemotherapy. These data are too preliminary to allow firm conclusions. While palladium is also a sensitizing metal [65–67], we are unaware of any studies of its effects on lymphocyte subpopulations in humans.

### **Cadmium**

The primary target of cadmium toxicity is not the immune system, but rather organs such as kidney, lung, and bone. Nevertheless, the effects on the immune system deserve further attention. In both animal models and in human cell cultures, low doses ( $10^{-10}$  mol/L) of cadmium were immunosuppressive. At higher doses, cadmium was immunostimulatory, increasing expression of CD69 and activating B and T cells [68]. NK cells were not influenced by cadmium [69,70]. Human whole blood was treated with CdCl<sub>2</sub>, and flow cytometry was used to measure metallothionein, a cadmium-binding protein. CD4+ and CD8+ lymphocytes developed higher levels of metallothionein, than B lymphocytes and NK cells [71]. When primary mouse T lymphocytes were treated with cadmium, the CD4+/CD8+ ratio declined in a dose- and time-dependent manner, indicative of a greater susceptibility of CD4+ cells. Both populations were decreased in overall number [72].

### **Gold**

An important aspect of the immunology of gold is the development of allergy after therapeutic use of gold compounds in rheumatoid arthritis (chrysotherapy). Nickel or gold dental alloys were implanted in mice [73]. After one month, an increase in CD4+ and CD8+ T lymphocytes and Smig+ B lymphocytes was observed in peripheral blood of the nickel-implanted animals. Only a modest increase in CD4+ and CD8+ cells was noted in the gold-exposed animals. The authors concluded that the changes in lymphocyte profiles may have been due to the surgical procedure and not to the metals per se [73]. More data are needed before lymphocyte subpopulation profiling can be interpreted in the context of gold allergy.

### **Mercury**

Mercury has elicited much interest because of uncertainties surrounding the effects of amalgam fillings, and dental amalgam is the most important source of mercury exposure in many countries. Allergic reactions sometimes occur in the buccal mucosa near the amalgam fillings. The ability of mercury to induce autoimmune reactions has also been discussed. Transient low-dose increases in blood mercury occur during removal of dental fillings, but these do not appear to affect CD4+, CD8+, or B cell populations [74]. However, higher exposures, such as inhalation of metallic mercury fumes sufficient to increase urinary concentrations of mercury up to 12 µmol/L, increased the numbers of CD4+ and CD8+ cells [75]. In another study, where urine concentrations in the range 0.09–8.2 µmol/L were measured, CD4+ and NK cell numbers were decreased [76]. In a third study, no statistically significant correlation was found between urinary mercury concentration and NK cell numbers [77]. Thirty-three workers in a mercury plant, all with acceptable urinary mercury levels, had a decrease in the number of CD4+ lymphocytes and in CD4+/CD8+ ratio. These changes did not correlate with urinary mercury concentration or time of exposure [78]. Clearly, further studies are needed before measurement of subpopulations can be interpreted in cases of mercury exposure.

### **Lead**

Due to widespread environmental lead contamination in the past, possible effects of lead on human health have been studied intensively. These include effects on the immune system, although lead is not

considered a strong immunosensitizer. Studies on the immune effects of lead give contradictory results, as discussed in a detailed overview by Singh et al. [79]. Prolonged exposure of industrial workers to lead resulting in blood lead concentration of 1.2–3.6  $\mu\text{mol/L}$  induced a decrease of CD3+ and CD4+ T cells, monocytes, and granulocytes [80–82] resembling data from an animal model [83]. In contrast, Pinkerton et al. [84] did not observe a difference in CD3+, CD4+, or CD8+ T, B, or NK cells in lead-exposed workers with a mean blood lead level of 1.88  $\mu\text{mol/L}$ , compared to unexposed individuals. CD8+ cells were even increased at blood lead levels > 0.97  $\mu\text{mol/L}$ , and were accompanied by a decrease in NK cells and CD3+CD45RO+ (memory) T cells, as reported by Sata et al. [85,86]. Moreover, blood lead was correlated with CD4+CD45RO-, CD3+CD8+, and CD3-HLA-DR+ lymphocyte subsets in a population of non-atopic women in an urban environment [51]. Low doses of lead acetate given intraperitoneally to rats for 4 weeks resulted in a decrease in CD4+ cells and B cells and an increase in CD8+ cells [87].

## 6. METHODS FOR IDENTIFYING LYMPHOCYTE SUBPOPULATIONS

Identification of lymphocyte subpopulations in peripheral blood is based on recognition of their cell-specific surface markers by means of labeled monoclonal antibodies. Typically, the antibody is labeled with a fluorescent tag, and in medical diagnostics detection is mainly by flow cytometry. Additional methods such as chip technology and particle arrays are under development. In flow cytometry, a diluted cell suspension (about  $10^5$  cells) is forced through a nozzle that causes the cells to flow single file, one cell at a time, past a light beam [88]. Detectors are positioned to measure forward and side scattered light as well as any fluorescence emission that may be excited by the light beam. Analysis of the scattered light detects each cell and provides information on its volume and morphology, and the fluorescent detectors determine which tags, if any, were present on the cell. Most flow cytometers use multiple diodes or ion lasers as light sources with, for example, emission in the blue, green, and red, and thus permit the simultaneous determination of three to five different fluorescent tags on a single cell. The results can be visualized as histograms, or as dot plots where clusters of dots represent cells with the same set of characteristics.

Flow cytometry is not restricted to the detection of surface antigens (CD). It can also be used for functional characterization of subpopulations on the basis of intracellular cytokines and chemokines, DNA content, and other biomolecules (Table 3). This allows, for instance, the study of the extent of differentiation, the state of activation, and the ability to produce cytokines and chemokines [6,11,89]. Flow cytometers can also be equipped to break the cell stream into droplets containing single cells, and then impart a charge to the droplet based on the nature of the detected fluorescence. This facilitates electrostatic separation of homogeneous cell populations, and is termed fluorescence-activated cell sorting (FACS).

**Table 3** Typical cellular properties that are measured by flow cytometry (adapted from [4]).

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Cell structure (surface molecules [CDs], size, granules, mitochondria)
Cell function (activation, phagocytosis, oxidative burst, cytotoxic activity, mitochondrial activity and membrane potential)
Cell status (viability, necrosis, apoptosis, intracellular pH, plasma membrane potential, $\text{Ca}^{2+}$ flux)
Macromolecules (DNA, RNA, proteins, cytokines)

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## 7. ANALYTICAL CONSIDERATIONS

The implementation of flow cytometry in routine clinical diagnostics has required increasingly demanding standardization of methods [90]. The variety of instrumentation and available protocols influences the results considerably and interlaboratory comparisons must be made with caution. There are several procedures for calibrating fluorescence intensities [91–96]. Universal standardization has not been achieved, making quality assurance schemes, and internal and external standards all the more important. Fluorescent calibration beads and control cell preparations are both commercially available for internal quality control. Different standardization procedures are used for absolute quantitation of a single cell type and for determining subpopulations relative to the total lymphocyte count. The National Committee for Clinical Laboratory Standards, the Center for Disease Control, and the College of American Pathologists have provided guidelines for many methods [97–100].

Routine diagnostics are performed on anticoagulated blood. Blood may be collected with EDTA, citrate, or heparin as the anticoagulant, although studies of  $\text{Ca}^{2+}$ -dependent phenomena require avoidance of citrate and EDTA. Fresh samples are desirable, and same-day analysis is advised for immunophenotyping. Blood should be transported and stored at room temperature.

Interlaboratory comparisons have shown good reproducibility in CD4+ measurement (<4 %), whereas CD8+ measurements show greater variability (ca. 33 %). Additional variation (even >1000 %) is introduced when combined parameters are measured (e.g., CD3+CD25+) [101]. More recently, Edwards et al. [102] conducted an interlaboratory comparison with 11 participants who were sent two standard protocols for staining plus gating strategy. There were no significant differences in lymphocyte counts between the two methods when results from the two assays and all participants were pooled. However, significant differences in results from the different participants were observed. Representative mean counts (geometric coefficient of variation) were 9 % for CD3, 12 % for CD4, and 13 % for CD8. The authors concluded that even experienced laboratories using protocols for the first time had some difficulties, and some reported outlying results, indicating the need for standardization of sample preparation and gating protocols.

## 8. INTERPRETATION

There are few clear and consistent published results concerning the frequency of changes in lymphocyte subpopulations as influenced by ambient exposures to metals in humans. A careful reading of the literature reveals that metal exposures are generally not accompanied by defined and reproducible alterations in these subpopulations. This may be, at least in part, because the reported ranges of reference values of each of the subpopulations are rather wide, due to both physiological fluctuations and analytical inaccuracies. Consequently, many observations reported as shifts in subpopulations are still clearly within the normal reference range, and must be viewed with appropriate skepticism. Moreover, even if statistically significant and consistent changes in individual immunological markers occur, there is not necessarily a correlation between such changes and pathophysiological mechanisms [103] or symptoms [104]. Values out of the normal range are, therefore, quite often not an indicator of disease, but rather the result of a successful adaptation of the immune system to the agent. The same comments can, of course, be made for many other immunological diagnostic tests (e.g., autoantibody levels, cytokine production, and lymphocyte proliferation indices).

It has been suggested that the analysis of lymphocyte subpopulations be included in the diagnosis of berylliosis [105], in the assessment of immunological effects of inhalation exposures in the workplace [106], and in other epidemiological studies, in order to improve knowledge of the relationship between immunological changes and pathomechanisms. However, even if patients exhibit shifts in lymphocyte subpopulations, these would not indicate that noxious exposures had taken place, nor would there be therapeutic consequences. There is, therefore, presently no scientific basis for the application

of the method in the diagnosis of individual patients with occupational or environmental exposures to metals.

## 9. SUMMARY AND RECOMMENDATIONS

Some 20 years ago, there were expectations that differentiation of lymphocyte subpopulations might become an important method for the diagnosis of environmental and occupational exposures to immunotoxic and immunosensitizing compounds. The major conclusion from the analysis of the scientific literature and from what has been said above is that these expectations have not yet been met. Diagnostic measurement of lymphocyte subpopulations should, therefore, only be considered, when well-founded hematological-immunological diagnosis shows suspicious values in need of further clarification, as for instance in leukemias, lymphomas, immune deficiencies or tumors,

Flow cytometry will continue to be an important tool in the determination of CD phenotypes and lymphocyte populations in experimental epidemiology and occupational health research, because of its unique capacity to uncover potential effects of metals on distinct immunocompetent cells. However, considering the lack of scientific evidence, consistent findings in current epidemiological studies, and specific response to exposure to individual agents, and recognizing the high physiological variability in lymphocyte populations, determination of lymphocyte subpopulations cannot be recommended for individual diagnosis in connection with environmental metal exposure.

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